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ASSESSMENT OF THE NITRITATION AND ANAMMOX PROCESSES FOR MAINSTREAM WASTEWATER TREATMENT

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ESCOLA DE DOUTORAMENTO INTERNACIONAL

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DECLARACIÓN DO AUTOR/A DA TESE

ASSESSMENT OF THE NITRITATION AND ANAMMOX PROCESSES FOR MAINSTREAM WASTEWATER TREATMENT

Dna. Alba Pedrouso Fuentes

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En Santiago de Compostela., 7 de Outubro de 2019

Asdo. Alba Pedrouso Fuentes

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ASSESSMENT OF THE NITRITATION AND ANAMMOX PROCESSES FOR MAINTREAM WASTEWATER TREATMENT

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List of acronyms and symbols

AD	Anaerobic Digestion
aerHET	Aerobic HETerotrophic bacteria
aerHRT	Aerobic Hydraulic Retention Time
AMO	Ammonia MonoOxygenase
AMO-D	Aerobic Methane Oxidation process coupled to Denitrification
AMX	Anaerobic AMmonium Oxidation (anammox)
AnMBR	ANaerobic MemBrane Reactor
ANOVA	ANalysis Of VAriance
ANRE	Ammonium Nitrogen Removal Efficiency
AOA	Ammonium Oxidising Archaea
AOB	Ammonium Oxidising Bacteria
AOR	Ammonia Oxidation Ratio
ASM	Activated Sludge Model
ATU	AllylThioUrea
b	Specific endogenous decay rate
BBD	Box-Behnken Design
BOD_5	Biochemical Oxygen Demand
BOM	Biological Oxygen Monitor
CAS	Conventional Activated Sludge
CEPT	Chemically Enhanced Primary Treatment
CLD	ChemiLuminescence Detector
COD	Chemical Oxygen Demand
bCOD	Biodegradable Chemical Oxygen Demand
pCOD	Particulate Chemical Oxygen Demand
sCOD	Soluble Chemical Oxygen Demand
tCOD	Total Chemical Oxygen Demand
COD/N	Chemical Oxygen Demand to Nitrogen ratio
COD/TOC	Chemical Oxygen Demand to Total Organic Carbon ratio
Comammox	COMplete AMMonia OXidising

CSTR	Continuous Stirring Tank Reactor
CTAB	CetylTrimetylAmmonium Bromide
Cy3	Carbocyanine 3
DAPI	4,6-DiAmindino-2-PhenylIndole
DNA	DeoxyriboNucleic Acid
DO	Dissolved Oxygen
DO/TAN	Dissolved Oxygen to Total Ammonia Nitrogen ratio
Ea	Energy of Activation
EC	European Commission
EEA	European Environment Agency
ELAN®	*Eliminación Autótrofa de Nitrógeno*, in Spanish autotrophic nitrogen removal
EU	European Union
F	Formamide
F/M	Food to Microorganism ratio
FA	Free Ammonia
FAO	Food and Agriculture Organisation
FAS	Ferrous Ammonium Sulphate
FISH	Fluorescent *in situ* Hybridisation
FITC	Fluorescei-5-isocyanate
FNA	Free Nitrous Acid
GC	Gas Chromatography
GHG	GreenHouse Gas
H/D	Heigh to Diameter ratio
HAO	HydroxylAmine Oxidoreductase
HDN	Heterotrophic DeNitrifying bacteria
HRAS	High Rate Activated Sludge
HRT	Hydraulic Retention Time
IA	Intermediate Alkalinity
IAS	Individual and other Appropriate Systems
IC	Inorganic Carbon
IC/N	Inorganic carbon to Nitrogen ratio
IFAS	Integrated Fixed-biofilm Activated Sludge
IFPRI	International Food Policy Research Institute
K1	AnoxKaldness Type 1
MEC	Microbial Electrolysis Cell

MFC	Microbial Fuel Cell
N	Nitrogen
N-damo	Nitrite/nitrate dependent methane oxidation
NAR	Nitrite Accumulation Ratio
N.D	Not Detected
NDIR	Non-Dispersive InfraRed
NLR	Nitrogen Loading Rate
NOB	Nitrite Oxidising Bacteria
NRE	Nitrogen Removal Efficiency
NRR	Nitrogen Removal Rate
ORP	Oxidation Reduction Potential
OTU	Operational Taxonomic Unit
OUR	Oxygen Uptake Rate
p	Probability
P	Phosphorus
p.e.	Population Equivalent
PA	Partial Alkalinity
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PLC	Programmable Logic Controller
PN	Partial Nitritation
PN/AMX	Partial Nitritation and AnaMmoX
PPB	Phototrophic Purple Bacteria
qPCR	Quantitative Polymerase Chain Reaction
R	Ideal gas coefficient
rAOB	Ammonium Oxidising Bacteria rate
rNOB	Nitrite Oxidising Bacteria rate
rRNA	Ribosomal RiboNucleic Acid
RSM	Response Surface Methodology
SA	Specific Activity
SA_{aerHET}	Specific Activity of aerobic heterotrophic bacteria
SA_{AMX}	Specific Activity of AnaMmoX bacteria
SA_{AOB}	Specific Activity of Ammonium Oxidising Bacteria
SA_{HDN}	Specific Activity of Heterotrophic DeNitrifying bacteria
SA_{NOB}	Specific Activity of Nitrite Oxidising Bacteria
SBR	Sequencing Batch Reactor

SDS	SodiumDodecylSulfate
SHARON	Single reactor for High Activity Ammonia Removal over Nitrite
sNLR	Specific Nitrogen Loading Rate
sNRR	Specific Nitrogen Removal Rate
SOB	Sulphur Oxidising Bacteria
SRT	Sludge Retention Time
SRT_{min}	Minimum Sludge Retention Time
SVI	Sludge Volume Index
T	Temperature
TA	Total Alkalinity
TAN	Total Ammonia Nitrogen
TC	Total Carbon
TCD	Thermal Conductivity Detector
TH	Thermal Hydrolysis
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TOC	Total Organic Carbon
Tris	Tris(hydroxymethyl)aminomethane
TSS	Total Suspended Solids
UASB	Up-flow Anaerobic Sludge Blanket
UN	United Nations
USA	United States of America
UV	UltraViolet
UWTD	Urban Wastewater Treatment Directive
V	Volume
VER	Volume Exchange Ratio
VSS	Volatile Suspended Solids
WHO	World Health Organisation
WWTP	WasteWater Treatment Plant
X_N	Molar fraction of nitrogen in the biogas
μ	Specific growth rate
μ_{max}	Maximum Specific growth rate

Resumo

No eido da economía circular, as augas residuais xa non son consideradas residuos que teñen que ser tratados, senón unha fonte de auga para o reemprego, de enerxía, de nutrientes e de outros recursos que poden e deben ser valorizados. A agricultura é o sector cun maior consumo de auga representando o 69 % do total da auga doce extraída mundialmente. Cando a auga residual se reemprega, esta usase principalmente para fertilizar e irrigar neste sector, supoñendo un 32 % do total de auga reempregada. Ademais de constituír unha fonte alternativa de auga, as augas residuais proporcionan nutrientes (nitróxeno e fósforo) reducindo a necesidade de aplicar fertilizantes químicos. Sen embargo, a escorrentía de nitratos derivada da aplicación excesiva de nutrientes na agricultura constitúe un dos maiores problemas de calidade de auga a nivel mundial. De feito, a lexislación sobre o reemprego de augas establece que o seu contido de nutrientes debe avaliarse axeitadamente tendo en conta o equilibrio dos mesmos no solo. Este feito, xunto coa gran variabilidade estacional da demanda de auga na agricultura, fan que sexa necesario desenvolver alternativas para a eliminación de nitróxeno que permitan outros usos da auga ou, en última instancia, a súa descarga ao medio acuático.

Na actualidade, a eliminación de nitróxeno non se aplica aínda en todas as estacións depuradoras de augas residuais (EDAR) sendo unha práctica común nas EDAR de gran tamaño, pero apenas aplicada nas EDAR de pequenos núcleos de poboación (sistemas descentralizados). Na lexislación sobre tratamento de augas residuais de moitos países non se establecen límites para a eliminación de nitróxeno como é o caso da maioría dos países do Centro e Sur de América, mentres que en Europa tan só é obrigatorio nas EDAR que tratan cargas de máis de 10.000 habitantes equivalentes. Ademais da carencia de imposicións legais, a elevada demanda de enerxía asociada aos procesos convencionais de eliminación de nitróxeno limitou a súa implementación nas EDAR. O tratamento de augas residuais representa o 42 % do consumo enerxético do sector da auga nos países desenvolvidos. Con todo, as

augas residuais conteñen máis enerxía que a que potencialmente fai falta para o seu tratamento. O nitróxeno é habitualmente eliminado nos sistemas convencionais de lamas activas mediante a combinación dos procesos de nitrificación-desnitrificación que requiren unha elevada cantidade de enerxía para airear e materia orgánica que non se valoriza. A optimización dos procesos de eliminación de nitróxeno mediante procesos autótrofos podería converter ás EDAR en produtoras netas de enerxía e facilitar o reemprego da auga facendo un uso máis eficiente dos recursos presentes na auga residual.

Entre os diferentes procesos biolóxicos de eliminación de nitróxeno, a combinación dos procesos de nitritación parcial e anammox (NP/AMX) ten a vantaxe de reducir o consumo de enerxía un 60 %, xa que tan só é necesario oxidar a metade do contido amoniacal a nitrito e, ao ser procesos completamente autótrofos, producen menos lamas e non precisan materia orgánica. Polo tanto, a totalidade da materia orgánica pode transformarse en biogás maximizando a recuperación de enerxía.

O obxectivo principal de esta tese é avaliar a aplicación dos procesos de NP/AMX para a eliminación autótrofa do nitróxeno presente na liña principal das EDAR. Primeiro, estudouse a aplicación dos procesos NP/AMX nunha etapa para o tratamento das augas negras orixinadas nun sistema descentralizado con redes separativas coma unha alternativa axeitada para as EDAR de pequeno tamaño (Capítulo 3). Logo, avaliouse a viabilidade de aplicar os procesos baseados en NP/AMX para o tratamento de augas residuais municipais caracterizadas por ter concentracións de nitróxeno máis baixas (< 50 mg N/L), baixa temperatura (< 25 °C) e pola grande variabilidade nas súas características. Estudos previos sinalaron a supresión das bacterias oxidantes de nitrito (BON) como un dos principais retos para a aplicación dos procesos NP/AMX na liña principal das EDAR. A obtención do proceso de nitritación estudouse mediante dúas estratexias diferentes: diminuír o tempo de residencia hidráulico (TRH) nun quimiostato (Capítulo 4) ou inhibir as BON mediante a acumulación *in situ* de ácido nitroso libre (ANL) tratando tanto auga residual sintética (Capítulo 5) como auga residual municipal (Capítulo 6). A continuación, avaliouse a viabilidade e a estabilidade do proceso anammox operado a baixa temperatura (Capítulo 7). Ademais, explorouse a aplicación dos procesos NP/AMX alimentados con augas residuais municipais a escala piloto empregando dúas configuracións de reactor diferentes: un reactor híbrido de lamas activas de

leito fixo (IFAS, do inglés *Integrated Fixed-biofilm Activated Sludge*) (Capítulo 8), onde os procesos de NP/AMX ocorren simultaneamente, e nunha configuración de dúas etapas onde ambos procesos están separados en dous reactores distintos (Capítulo 9). Finalmente, discútense as conclusións máis relevantes obtidas así como as principais limitacións identificadas (Capítulo 10). Nas seguintes seccións, amósase un resumo dos contidos principais de cada un dos capítulos da presente tese.

Capítulo 1. Introdución

No Capítulo 1 amósase unha visión global do novo paradigma do tratamento de augas residuais nas que a escaseza de auga promove o reemprego das mesmas unha vez tratadas e unha mellor xestión dos recursos durante o seu tratamento para valorizar a enerxía. Ademais, inclúese unha revisión do estado actual da investigación e aplicación dos procesos de NP/AMX así como os factores principais que inflúen na selección das BON. Tamén se sinalan os principais desafíos para a aplicación dos procesos de NP/AMX tendo en conta as características principais das augas residuais na liña principal das EDAR. Para rematar, preséntanse os obxectivos principais e específicos da presente tese.

Capítulo 2. Materiais e Métodos

No Capítulo 2 descríbense con detalle os materiais e métodos empregados durante o traballo experimental descrito na presente tese. Estes inclúen as análises da fase líquida e sólida, así como os métodos para determinar as actividades específicas das diferentes poboacións bacterianas. Ademais, neste capítulo tamén se presenta a descrición minuciosa dos cálculos empregados nos diferentes capítulos para a determinación, entre outros, das velocidades de transformación e a eficiencia dos procesos biolóxicos. As descricións específicas dos reactores e montaxes experimentais amósanse nos capítulos correspondentes.

Capítulo 3. Robustez do sistema de NP/AMX baixo períodos repetidos de parada e reactivación para o tratamento das augas negras

No Capítulo 3, avaliouse a aplicación dun sistema en unha etapa cos procesos simultáneos de NP/AMX para tratar, a temperatura ambiente (14 - 21 °C), augas negras dixeridas anaeróbicamente procedentes dun sistema de tratamento de augas residuais descentralizado con redes separativas. As augas residuais procedían dun edificio de oficinas e a principal característica deste tipo de sistemas

descentralizados é que deben facer fronte á falta de dispoñibilidade de augas residuais para tratar de xeito continuado. Así, operouse un reactor secuencial (SBR, do inglés *Sequencing Batch Reactor*), de 4 L, aplicando períodos repetidos de parada, durante as noites e as fins de semana, e posterior reactivación. A pesar da baixa temperatura, as concentracións moderadas de nitróxeno total (NT, 120 mg NT/L) e as paradas regulares, lograrouse manter o proceso de eliminación de nitróxeno estable durante 100 días. Tras a implementación dunha fase anóxica para refinar a calidade do efluente utilizando a materia orgánica residual (100 mg/L como demanda química de osíxeno (DQO)), obtivéronse eficiencias de eliminación de nitróxeno do 95 % acadando velocidades de eliminación de nitróxeno (VEN) de aproximadamente 66 mg NT/(L·d). Ademais, o sistema NP/AMX recuperou inmediatamente (nun día) a súa capacidade de eliminación de nitróxeno despois de estar parado durante 15 días simulando un período de vacacións. O sistema mostrou unha excelente capacidade de retención de biomasa obtendo unha concentración de sólidos en suspensión volátiles (SSV) no efluente de entre 7 e 16 mg SSV/L. Durante todo o período de operación, a actividade das BON foi suprimida con éxito. Por outra banda, observouse a segregación das diferentes poboacións bacterianas sendo as bacterias oxidantes de amonio (BOA) e as heterótrofas (tanto aerobias como desnitrificantes) máis activas na biomasa floculenta mentres que a biomasa granular estaba enriquecida en bacterias anammox. Con respecto á calidade do efluente, este contiña baixas concentracións de nitróxeno (≤ 10 mg NT/L), de materia orgánica (≤ 30 mg DQO/L) e de sólidos (≤ 20 mg SST/L) cumprindo cos límites de descarga establecidos na Directiva europea de tratamento de augas residuais urbanas (91/271/CEE) e os requisitos mínimos de calidade para o reemprego da auga definidos pola Unión Europea (UE) en TA(2019)0071.

Capítulo 4. É a carga hidráulica un parámetro útil para desacoplar as actividades BOA e BON?

Neste capítulo, estudouse a selección con fins de enriquecemento das BOA sobre as BON de acordo coas súas velocidades de crecemento. Para iso, operouse un reactor continuo de tanque axitado (CSTR, do inglés *Continuos Stirred Tank Reactor*), de 7.15 L a 16 ± 1 °C, reducindo progresivamente o TRH dende 7,0 a 1,5 días durante 158 días e alimentado con auga residual sintética (50 mg NT/L). O reactor inoculouse con lamas activas nitrificantes. A acumulación de nitrito activouse a valores de TRH de 4,6 ± 0,2 días, pero o nitrato seguía a ser o principal produto de oxidación do

amonio representado o 80 %, mesmo cando o TRH se reduciu a 1,5 días. A pesar de que non se logrou establecer o proceso de nitritación con éxito, esta experiencia demostrou que a aplicación dunha elevada carga hidráulica podería brindar unha vantaxe competitiva das BOA sobre as BON. A relación entre as velocidades de reacción de ambos grupos de nitrificantes aumentou progresivamente alcanzando valores de 1,5 g $N\text{-}NH_4^+{}_{consumido}$/g $N\text{-}NO_2^-{}_{consumido}$. Por tanto, foi posible activar a acumulación de nitrito pero non obter un lavado eficaz das BON, que requiriría a combinación da aplicación da carga hidráulica con outros parámetros para acadar e manter con éxito o proceso de nitritación.

Capítulo 5. Supresión das bacterias oxidantes de nitrito baseada na produción *in situ* de ácido nitroso libre

No Capítulo 5 propúxose e avaliouse unha nova estratexia para establecer o proceso de nitritación. A maior sensibilidade das BON con respecto ás BOA fronte ó ANL permite promover o proceso de nitritación mediante a exposición das BON a concentracións de ANL superiores a 0,02 mg $N\text{-}HNO_2$/L. As concentracións inhibitorias de ANL xeráronse dentro do reactor mediante o proceso de oxidación de amonio por si mesmo, aproveitando a produción de nitrito e a diminución do pH asociada ao consumo de alcalinidade durante a nitritación. Esta alternativa simple explorouse nas condicións da liña principal de augas, tratando auga residual simulada (50 mg NT/L) a 16 ° C, nun reactor SBR de 2 L, durante 690 días. Así, se obtivo e mantivo estable a longo prazo o proceso de nitritación acadando concentracións de ANL de ata 0,06 mg $N\text{-}HNO_2$/L. Dado que o lodo inoculado tiña actividades BOA e BON similares, probáronse dous estimuladores de acumulación de nitrito: azida de sodio e nitrito. A análise microbiolóxica revelou que as poboacións de BON (sendo dominante o xénero *Nitrospira*) foron eliminadas do reactor. O éxito da estratexia de acumulación de ANL *in situ* baséase na relación entre as concentracións de nitróxeno e carbono inorgánico (CI) das augas residuais. Por iso, avaliáronse diferentes proporcións desta relación (entre 0,5 - 1,0 g N/g CI), observando que cando é menor a 0,6 g N/g CI, as concentracións de ANL non son suficientes para inhibir as NOB. Baixo estas condicións non inhibitorias, as BON demoraron aproximadamente 40 días en desenvolver unha actividade significativa. Este longo período amosa a robustez da estratexia baseada no ANL en canto ao eficaz lavado de BON. Durante a operación do reactor de nitritación, non se observou ningún efecto negativo sobre a actividade BOA asociado ás concentracións de ANL

acadadas ou do baixo pH, obtendo valores de actividade de ata 400 mg $N\text{-}NH_4^+$/(g SSV·d).

Capítulo 6. Comportamento dos procesos de nitritación e oxidación de materia orgánica tratando augas residuais municipais

No Capítulo 6, inoculouse un segundo SBR (de 2 L) para levar a cabo o proceso de nitritación empregando un lodo enriquecido en BOA e con actividade insignificante de BON. O proceso de nitritación estableceuse e mantivose con éxito mediante a estratexia de acumulación *in situ* de ANL tratando auga sintética (50 mg NT/L) a 15 ± 1 °C. Logo, a operación continuouse pero tratando augas residuais municipais despois do tratamento primario (que contiñan 18 - 55 mg $N\text{-}NH_4^+$/L e unha concentración de carbono orgánico total (COT) de 20 - 50 mg COT/L), para estudar a viabilidade do proceso de nitritación baseado na estratexia do ANL. A acumulación de nitrito mantívose en valores de practicamente o 100 % do amonio oxidado e a estabilidade do proceso de nitritación sostívose a longo prazo (354 días dos cales 191 tratou auga municipal), mediante a acumulación *in situ* de ANL en concentracións entre 0,02 e 0,20 mg $N\text{-}HNO_2$/L. Ademais, aínda que se alcanzaron valores mínimos de pH de 5,6, estes non produciron ningún efecto adverso sobre o proceso de nitritación nin sobre a actividade específica das BOA que se mantivo ao redor de 200 mg $N\text{-}NH_4^+$/(g SSV·d). Independentemente da presenza de materia orgánica, que tamén fora sinalada previamente como un reto para a aplicación dos procesos de NP/AMX en condicións da liña principal de augas, o proceso de nitritación permaneceu estable e o 80 % da materia orgánica foi eliminada no mesmo reactor. Este feito confire a esta estratexia unha gran vantaxe, xa que permite ser máis flexible coas eficiencias de eliminación requiridas nas unidades anteriores para a eliminación de materia orgánica. Por outra banda, observouse que para facer fronte ás flutuacións das concentracións de nitróxeno e materia orgánica das augas residuais, requírese a optimización da duración do ciclo de operación do SBR. Por tanto, a duración variable do ciclo proporcionaría unha operación máis axeitada evitando períodos inactivos cando o amonio se consume por completo ao tratar auga residual máis diluída.

Capítulo 7. Avaliación do comportamento do proceso anammox

O Capítulo 7 baséase no estudo da viabilidade da aplicación do proceso anammox en condicións da liña principal de augas das EDAR así como o seu

comportamento a baixa temperatura a longo prazo. Con este obxectivo, inoculouse un SBR (cun volume de 5 L) con lodo granular procedente dun reactor ELAN® (ELiminación Autótrofa de Nitróxeno) que trata augas residuais con concentracións elevadas de nitróxeno en condicións mesófilas e expúxose directamente ás concentracións baixas de nitróxeno (50 mg NT/L) e baixa temperatura (15 °C) características da liña principal das EDAR. Os resultados demostraron que o período de aclimatación progresiva ás novas condicións non sería necesario, acurtando os períodos de arranque, xa que se obtiveron resultados de eficacias de eliminación similares aos publicados por outros autores que realizaron unha adaptación lenta da biomasa ás novas condicións. Ademais, demostrouse a estabilidade do proceso anammox a longo prazo (485 días) tratando tanto augas residuais sintéticas con alcalinidades decrecentes así como con auga residual municipal nitritificada. Esta última consistía no efluente do reactor de nitritación descrito no Capítulo 6 mesturado con auga municipal sen tratar no devandito reactor, para axustar a relación nitrito/amonio necesaria para o proceso anammox en 1,2 g $N\text{-}NO_2^-$/g $N\text{-}NH_4^+$. A pesar de que no caso da operación con auga real, a alimentación do reactor anammox tiña concentracións de alcalinidade baixas (2 ± 1 mg CI/L) e valores de pH ao redor de 6,2, estes non afectaron á estabilidade do proceso. Por outra banda, a presenza de concentracións residuais de materia orgánica da auga residual municipal tras o tratamento primario contribuíu a un aumento da eficiencia de eliminación de nitróxeno dende valores en torno ao 80 % a 90 %, acadando concentracións de NT no efluente inferiores a 10 mg NT/ L (límite de vertedura na Unión Europea nas áreas sensibles para grandes depuradoras). A biomasa anammox retívose satisfactoriamente no sistema e a súa actividade específica aumentou ao longo da operación dende 53 ± 11 mg N/(g SSV·d), no inóculo, ata valores máximos de 78 ± 8 mg N/(g SSV·d) ao final da operación con auga sintética. Con todo, durante o período de operación con auga municipal observouse un descenso da actividade a valores medios de 58 ± 6 mg N/(g SSV·d), probablemente debido ao desenvolvemento de bacterias heterótrofas desnitrificantes. Considerando a actividade específica anammox e a concentración de biomasa no reactor, o sistema tiña unha capacidade de eliminación potencial maior á VEN tratada (40 mg NT/(L·d)) indicando que esta estaba limitada pola carga aplicada. Por outra banda, estudouse o efecto do pH (6 - 8), temperatura (15 - 30 °C) e concentración COT (0 - 75 mg COT/ L) sobre a actividade específica anammox en ensaios en descontinuo. Os resultados indican

que o pH é o parámetro ensaiado con maior efecto sobre a actividade e que o rango de pH óptimo varía coa temperatura.

Capítulo 8. Comportamento dos procesos de nitritación parcial-anammox nunha etapa nun sistema IFAS

No Capítulo 8 preséntase o reactor IFAS como unha configuración prometedora para levar a cabo os procesos de NP/AMX nunha etapa dado á súa capacidade de segregar as diferentes poboacións bacterianas en distintas fraccións de biomasa (biomasa en suspensión e biopelícula). O reactor IFAS a escala piloto (200 L) alimentouse de xeito continuo con auga residual municipal pretratada anaeróbicamente e operouse a temperaturas decrecentes dende 21 a 15 ° C. A pesar de que a alimentación contiña unha DQO relativamente alta con relacións DQO/N de 2.5 ± 0.3 g DQO/g N, os balances de materia indican que o proceso anammox é o maior responsable da eliminación de nitróxeno obtida. Durante a operación obtivéronse eficiencias de eliminación de nitróxeno de 72 ± 11 % e valores medios de VEN de 37 ± 3 mg NT/(L·d) a 15 °C. Con respecto á distribución das poboacións principais, observouse unha segregación sendo as BOA as máis abundantes na biomasa en suspensión mentres que as bacterias anammox se atopaban principalmente na biopelícula formada sobre os soportes. Con todo, a abundancia das BON era similar en ambas fraccións de biomasa e, a pesar de que a súa actividade específica diminuíu durante a operación do reactor, non foron eliminadas por completo do sistema. Adicionalmente, o patrón de aireación intermitente empregado no reactor IFAS permitiu a limitación da actividade das BON dentro do sistema observándose unha actividade no reactor de en torno ao 10 - 20 % da actividade máxima. Con respecto á calidade do efluente, obtivéronse concentracións de nitróxeno de 12 ± 5 mg NT/ L, o que demostra que se require unha maior optimización do sistema para cumprir cos límites de vertedura establecidos pola lexislación europea para as áreas sensibles: 15 mg NT/L para as EDAR que tratan cargas de entre 10.000 e 100.000 habitantes equivalentes e 10 mg NT/ L para as de maior tamaño.

Capítulo 9. Aplicación dun sistema de nitritación parcial e anammox en dúas etapas a escala piloto

Os prometedores resultados obtidos a escala laboratorio coa configuración de NP/AMX en dúas etapas conduciron a probar esta configuración por primeira vez a

escala piloto (600 L cada unidade) como se describe no Capítulo 9. Neste caso, a planta piloto instalouse nunha EDAR municipal onde operaba un reactor aerobio de lodos activos de alta carga para a eliminación de materia orgánica (sen afectar á concentración de nitróxeno). O sistema NP/AMX operouse sen control de temperatura (variando entre 11 e 28 °C) e facendo fronte ás mesmas flutuacións nas características da auga residual ás que estaba sometida a EDAR municipal. O efluente do reactor aerobio de alta carga caracterizábase por ter concentracións de amonio entre 22 e 63 mg $N\text{-}NH_4^+$/L, que se oxidou parcialmente a nitrito na unidade de nitritación parcial. Durante toda a operación non se observou actividade das BON que estaban inhibidas mediante a acumulación de ANL *in situ* (0,015 - 0,200 mg $N\text{-}HNO_2$/L). Para obter unha relación nitrito/amonio no efluente de 1,2 g $N\text{-}NO_2^-$/ g $N\text{-}NH_4^+$, axeitada para alimentar ao reactor anammox en serie, implementouse unha estratexia de control para a etapa de aireación baseándose no valor de pH no interior do reactor de nitritación. Deste xeito, o reactor puido soportar mellor as flutuacións nas características das augas residuais. Por outra banda, no reactor anammox alcanzouse unha eficiencia de eliminación de nitróxeno do 80 % cunha VEN de 99 ± 26 mg NT/(L·d), limitada principalmente polo TRH imposto previamente na unidade de NP para lograr unha adecuada retención de biomasa. De feito, a principal limitación atopada nesta experiencia foi a retención de biomasa nesta unidade. Con respecto á calidade do efluente, obtívose unha concentración de 12 ± 3 mg NT/ L. Con todo, é necesario sinalar que o efluente do reactor aerobio de alta carga da EDAR alimentado ao sistema NP/AMX xa contiña aproximadamente 5 mg $N\text{-}NO_3^-$/L de nitrato. El nitrato non é eliminado no sistema NP/AMX, limitando por tanto a súa eficacia total de eliminación de nitróxeno. A optimización do sistema aerobio de alta carga da EDAR, mediante a redución do tempo de retención celular para limitar o proceso de nitrificación nesta unidade e evitar a formación de nitrato, fomentaría a obtención dun efluente de maior calidade na saída do sistema NP/AMX, cumprindo cos límites de vertedura establecidos comunmente na lexislación. Finalmente, realizouse un estudo dos potenciais aforros comparando a combinación do sistema aerobio de alta carga e a eliminación de nitróxeno mediante NP/AMX cun sistema convencional de lodos activos (a tecnoloxía máis comunmente empregada para a eliminación de nitróxeno nas EDAR). Os resultados amosaron que a implementación dos procesos de eliminación autótrofa de nitróxeno permite duplicar a recuperación de enerxía da planta xa que máis materia orgánica é tratada no dixestor anaerobio de lodos producindo máis biogás. Ademais, a eficiencia enerxética da planta

aumenta do 21 % cando se aplica o sistema convencional de lodos activos ao 57 % coa aplicación dos procesos de NP/AMX, e os custos de operación tamén se reducen á metade. Con todo, a produción de lamas que deben ser xestionadas como residuo é menor no escenario convencional xa que o reactor aerobio de alta carga ten unha produción elevada de lamas incrementando os custos asociados á súa xestión.

Capítulo 10. Conclusións xerais e futuros desafíos para a eliminación autótrofa de nitróxeno na liña principal de augas

No capítulo 10 preséntase unha discusión xeral co obxectivo de integrar os principais resultados e conclusións máis relevantes obtidos durante a realización desta tese. En xeral, os resultados desta tese contribúen a incrementar a comprensión do proceso de NP/AMX e proporcionan información interesante sobre a viabilidade de aplicar os procesos de NP/AMX para eliminar de xeito autótrofo o nitróxeno a baixa temperatura. Así téntase responder a algunhas das principais cuestións que fican abertas neste eido:

i) É posible aplicar sistemas baseados no proceso anammox en sistemas de tratamento de augas descentralizados?
ii) É posible suprimir a actividade das BON en condicións da liña principal de augas?
iii) Son capaces as bacterias anammox de tratar cargas suficientemente altas de xeito estable para a eliminación de nitróxeno na liña de augas?
iv) Como afecta a composición da auga residual municipal ao comportamento dos procesos NP/AMX?
v) Cal é a configuración de reactor máis eficaz para a implementación dos procesos de NP/AMX para o tratamento de augas domésticas?

Demostrouse que o tratamento das augas negras mediante un sistema NP/AMX nunha etapa é viable, probablemente porque a súa composición é relativamente máis alta e estable en comparación coas augas residuais municipais, unha vantaxe para a estabilidade do proceso de NP/AMX. Os sistemas descentralizados, especialmente os de pequena escala, poderían enfrontarse a períodos de ausencia de augas residuais para tratar, pero comprobouse que as paradas que se ocasionarían no sistema de tratamento non impiden a aplicación dos procesos de NP/AMX. Así, poderíase incrementar o número de pequenas instalacións con sistemas de eliminación de nitróxeno para promover o reemprego

da auga. Por outra banda, o sistema IFAS permite segregar as diferentes poboacións microbianas nas fraccións de biomasa en función da súa velocidade de crecemento. As BON poderían eliminarse do sistema mediante un control axeitado do tempo de retención celular incrementado a eficacia de eliminación de nitróxeno. Ademais, obtivéronse resultados especialmente relevantes e novidosos no marco da aplicación dos procesos de NP/AMX en dúas etapas. Primeiro propúxose unha nova estratexia operativa baseada na acumulación de ANL *in situ* a escala laboratorio e tratando auga residual sintética. Unha vez entendido o mecanismo de inhibición das BON, validouse a estratexia tratando auga residual urbana con presenza de materia orgánica. Logo, eliminouse o nitróxeno en reactores anammox nos que se maximiza a eliminación autótrofa de nitróxeno ao ser un reactor exclusivamente anóxico con ausencia de materia orgánica, xa que é oxidada na unidade NP previa. Obtivéronse eficacias de eliminación entre 80 e 90 % obtendo efluentes de alta calidade. Así, o sistema de NP/AMX en dúas etapas permite acadar eficacias de eliminación con estratexias de operación simples que non requiren sistemas de control complexos o que favorece a súa implementación. Por último, neste capítulo discútense as principais limitacións para a aplicación dos procesos de NP/AMX na liña principal das EDAR a baixa temperatura prestando especial atención ás propostas de traballo futuro.

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Resumen

En el ámbito de la economía circular, las aguas residuales ya no son consideradas residuos que tienen que ser tratados sino una fuente de agua para reutilizar, de energía, de nutrientes y de otros recursos que pueden y deben ser valorizados. La agricultura es el sector con mayor consumo de agua representando el 69 % del agua dulce extraída mundialmente. Cuando el agua residual es reutilizada, esta se emplea principalmente para fertilizar e irrigar en este sector suponiendo un 32 % del total de agua reutilizada. Además de constituir una fuente alternativa de agua, las aguas residuales proporcionan nutrientes (nitrógeno y fósforo) disminuyendo la necesidad de aplicar fertilizantes químicos. Sin embargo, la escorrentía de nitratos derivada de un exceso de nutrientes aplicados en la agricultura es uno de los mayores problemas de calidad del agua a nivel mundial. De hecho, la legislación sobre la reutilización de aguas establece que el contenido de nutrientes del agua reutilizada debe evaluarse adecuadamente teniendo en cuenta el equilibrio de los mismos en el suelo. Este hecho junto con la gran variación estacional de la demanda de agua en la agricultura, hacen que sea necesario desarrollar alternativas para la eliminación de nitrógeno que permitan otros usos del agua o, en última instancia, su descarga al medio acuático.

En la actualidad, la eliminación de nitrógeno no está todavía implementada en todas las estaciones depuradoras de aguas residuales (EDAR) siendo una práctica habitual en las EDAR de gran tamaño, pero apenas aplicada en las EDAR de pequeños núcleos de población (sistemas descentralizados). En la legislación de tratamiento de aguas residuales de muchos países no se establecen límites para la eliminación de nitrógeno como es el caso de la mayoría de los países de Centro y Sur de América mientras que en Europa solo es obligatorio para EDAR que tratan cargas de más de 10.000 habitantes equivalentes. Además de la ausencia de imposiciones legales, la elevada demanda de energía asociada a los procesos convencionales de eliminación de nitrógeno limitó su implementación en las EDAR. El tratamiento de aguas residuales representa el 42 % del consumo de energía del sector del agua en los países desarrollados. Sin embargo, las aguas residuales contienen más energía que

la que potencialmente hace falta para su tratamiento. El nitrógeno es habitualmente eliminado en los sistemas convencionales de lodos activos mediante la combinación de los procesos de nitrificación-desnitrificación que requieren una elevada cantidad de energía para airear y materia orgánica que no se valoriza. La optimización de los procesos de eliminación de nitrógeno mediante procesos autótrofos podría convertir a las EDAR en productoras netas de energía y facilitar la reutilización del agua haciendo un uso más eficiente de los recursos del agua residual.

Entre los diferentes procesos biológicos de eliminación de nitrógeno, la combinación de los procesos de nitritación parcial y anammox (NP/AMX) tiene la ventaja de reducir el consumo de energía un 60 %, ya que tan solo es necesario oxidar la mitad del contenido amoniacal a nitrito y al ser procesos completamente autótrofos producen menos lodo y no requieren materia orgánica. Por tanto, la totalidad de la materia orgánica puede transformarse en biogás maximizando la recuperación de energía.

El objetivo principal de esta tesis es evaluar la aplicación de los procesos de NP/AMX para la eliminación autótrofa del nitrógeno presente en la línea principal de las EDAR. Primero, se estudió la implementación de los procesos de NP/AMX en una etapa para el tratamiento de aguas negras originadas en un sistema descentralizado con redes separativas como una buena alternativa para las EDAR de pequeño tamaño (Capítulo 3). Luego, se evaluó la viabilidad de aplicar los procesos basados en NP/AMX para el tratamiento de aguas residuales municipales caracterizadas por tener concentraciones más bajas de nitrógeno (< 50 mg N/L), baja temperatura (< 25 °C) y por la gran variabilidad de sus características. Estudios previos señalaron la supresión de las bacterias oxidantes de nitrito (BON) como uno de los principales problemas para su implementación en las condiciones de la línea principal de la EDAR. La obtención del proceso de nitritación se estudió mediante dos estrategias diferentes: disminuir el tiempo de residencia hidráulico (TRH) en un quimiostato (Capítulo 4) o inhibir las BON mediante la acumulación *in situ* de ácido nitroso libre (ANL) tratando tanto agua residual sintética (Capítulo 5) como agua residual municipal (Capítulo 6). A continuación, se evaluó la viabilidad y la estabilidad del proceso anammox operado a baja temperatura (Capítulo 7). Además, se exploró la implementación de los procesos NP/AMX alimentados con aguas residuales municipales a escala piloto empleando dos configuraciones de reactor diferentes: un reactor híbrido de lodos activos de lecho fijo (IFAS, del inglés *Integrated Fixed-biofilm*

Activated Sludge) (Capítulo 8), donde los procesos de NP/AMX tienen lugar simultáneamente, y en una configuración de dos etapas donde ambos procesos están separados en dos reactores distintos (Capítulo 9). Finalmente, se discuten las conclusiones más relevantes obtenidas y las principales limitaciones identificadas (Capítulo 10). En las siguientes secciones, se muestra un resumen de los contenidos principales de cada uno de los capítulos de la presente tesis.

Capítulo 1. Introducción

En el Capítulo 1 se muestra una visión global del nuevo paradigma del tratamiento de aguas residuales en el que la escasez de agua promueve la reutilización de éstas una vez tratadas y una mejor gestión de los recursos durante su tratamiento para valorizar la energía. Además, se incluye una revisión del estado actual de la investigación y aplicación de los procesos de NP/AMX así como los factores principales que influyen en la selección de las BON. También se señalan los principales desafíos para la implementación de los procesos NP/AMX teniendo en cuenta las características principales de las aguas residuales en la línea principal de las EDAR. Finalmente, se presentan los objetivos principales y específicos de la presente tesis.

Capítulo 2. Materiales y Métodos

En el Capítulo 2 se describen con detalle los materiales y métodos empleados durante el trabajo experimental descrito en la presente tesis. Estos incluyen los análisis de la fase líquida y sólida, así como los métodos para determinar las actividades específicas de las diferentes poblaciones bacterianas. Además, en este capítulo también se presenta la descripción minuciosa de los cálculos empleados en los diferentes capítulos para la determinación, entre otros, de las velocidades de transformación y la eficiencia de los procesos biológicos. Las descripciones específicas de los reactores y montajes experimentales se muestran en cada uno de los capítulos correspondientes.

Capítulo 3. Robustez del sistema NP/AMX bajo períodos repetidos de parada y reactivación para el tratamiento de las aguas negras

En el Capítulo 3, se evaluó la aplicación de un sistema en una etapa con los procesos simultáneos de NP/AMX para tratar, a temperatura ambiente (14 - 21 °C), aguas negras digeridas anaeróbicamente procedentes de un sistema de tratamiento

de aguas residuales descentralizado con redes separativas. Las aguas residuales procedían de un edificio de oficinas y la principal característica de este tipo de sistemas descentralizados es que deben hacer frente a la fata de disponibilidad continuada de aguas residuales para tratar. Por lo tanto, se operó un reactor secuencial (SBR, del inglés *Sequencing Batch Reactor)*, de 4 L, aplicando períodos repetidos de parada, durante las noches y los fines de semana, y posterior reactivación. A pesar de la baja temperatura, las concentraciones moderadas de nitrógeno total (NT, 120 mg NT/L) y las paradas regulares, se logró mantener el proceso de eliminación de nitrógeno estable durante 100 días. Tras la implementación de una fase anóxica para refinar la calidad del efluente utilizando la materia orgánica residual (100 mg/L como demanda química de oxígeno (DQO)), se obtuvieron eficiencias de eliminación de nitrógeno del 95 % alcanzando velocidades de eliminación de nitrógeno (VEN) de aproximadamente 66 mg NT/(L·d). Además, el sistema NP/AMX recuperó inmediatamente (1 día) su capacidad de eliminación de nitrógeno después de estar parado durante 15 días simulando un período de vacaciones. El sistema mostró una buena capacidad de retención de biomasa obteniendo una concentración de sólidos en suspensión volátiles (SSV) en el efluente de entre 7 y 16 mg SSV/L. Durante todo el período de operación, la actividad de las BON fue suprimida con éxito. Por otra parte, se observó la segregación de las diferentes poblaciones bacterianas siendo las bacterias oxidantes de amonio (BOA) y las heterótrofas (tanto aerobias como desnitrificantes) más activas en la biomasa floculenta mientras que la biomasa granular estaba enriquecida en bacterias anammox. Con respecto a la calidad del efluente, este contenía una baja concentración de nitrógeno (≤ 10 mg NT/L), de materia orgánica (≤ 30 mg DQO/L) y sólidos (≤ 20 mg SST/L) cumpliendo con los límites de descarga establecidos en la Directiva europea de tratamiento de aguas residuales urbanas (91/271/CEE) y los requisitos mínimos de calidad para la reutilización del agua definidos por la Unión Europea (UE) en TA(2019)0071.

Capítulo 4. ¿Es la carga hidráulica un parámetro útil para desacoplar las actividades BOA y BON?

En este capítulo, se estudió la selección con fines de enriquecimiento de las BOA sobre las BON de acuerdo con sus velocidades de crecimiento. Para ello, se operó un reactor continuo de tanque agitado (CSTR, del inglés *Continuos Stirred Tank Reactor*) con un volumen de 7.15 L a 16 ± 1 °C, reduciendo progresivamente el TRH

desde 7,0 a 1,5 días durante 158 días alimentado con agua residual sintética (50 mg NT/L). El reactor se inoculó con lodos activos nitrificante. La acumulación de nitrito se activó cuando el TRH se estableció en valores de 4,6 ± 0,2 días, pero el nitrato seguía siendo el principal producto de oxidación del amonio representado el 80 % incluso cuando el TRH se redujo a 1,5 días. A pesar de que no se logró establecer el proceso de nitritación con éxito, se demostró que la aplicación de un elevado estrés hidráulico podría brindar una ventaja competitiva de las BOA sobre las BON. La relación entre las velocidades de reacción de ambos grupos de nitrificantes aumentó progresivamente alcanzando valores de 1,5 g $N\text{-}NH_4^+{}_{consumido}$/g $N\text{-}NO_2^-{}_{consumido}$. Por lo tanto, fue posible activar la acumulación de nitrito pero no obtener un lavado de las BON eficaz, que requeriría la combinación de la aplicación del estrés hidráulico con otros parámetros para lograr y mantener con éxito el proceso de nitritación.

Capítulo 5. Supresión de las bacterias oxidantes de nitrito basada en la producción *in situ* de ácido nitroso libre

En el Capítulo 5 se propuso y evaluó una nueva estrategia para establecer el proceso de nitritación. El hecho de que las BON sean más sensibles que las BOA al ANL permite promover el proceso de nitritación mediante la exposición de las BON a concentraciones de ANL superiores a 0,02 mg $N\text{-}HNO_2$/L. Las concentraciones inhibitorias de ANL se generaron dentro del reactor mediante el proceso de oxidación de amonio por sí mismo, aprovechando la producción de nitrito y la disminución del pH asociada al consumo de alcalinidad durante la nitritación. Esta alternativa simple se exploró en las condiciones de la línea principal de aguas, tratando agua residual simulada (50 mg NT/L) a 16 °C en un reactor SBR de 2 L durante 690 días. Así, se obtuvo y mantuvo estable a largo plazo el proceso de nitritación alcanzado concentraciones de ANL de hasta 0,06 mg $N\text{-}HNO_2$/L. Debido a que el lodo inoculado tenía actividades BOA y BON similares, se probaron dos estimuladores de acumulación de nitrito: azida de sodio y nitrito. El análisis microbiológico reveló que las poblaciones de BON (siendo el género *Nitrospira* el dominante) fueron eliminadas del reactor. El éxito de la estrategia de acumulación de ANL *in situ* se basa en la relación entre las concentraciones de nitrógeno y carbono inorgánico (CI) de las aguas residuales. Por eso se evaluaron diferentes proporciones de esta relación (0,5 - 1,0 g N/g CI), observando que cuando es menor a 0,6 g N/g CI, las concentraciones de ANL no son suficientes para inhibir las NOB. Bajo estas condiciones no inhibitorias, las BON demoraron aproximadamente 40 días en

desarrollar una actividad significativa. Este largo período muestra la robustez de la estrategia basada en el ANL en cuanto al eficaz lavado de BON. Durante la operación del reactor de nitritación, no se observó ningún efecto negativo asociado a las concentraciones de ANL alcanzadas o del bajo pH sobre la activad BOA, obteniendo valores de actividad de hasta 400 mg $N\text{-}NH_4^+$/(g SSV·d).

Capítulo 6. Comportamiento de los procesos de nitritación y oxidación de materia orgánica tratando aguas residuales municipales

En el Capítulo 6, se inoculó un segundo SBR (de 2 L) para llevar a cabo el proceso de nitritación utilizando un lodo enriquecido en BOA y con actividad insignificante de BON. El proceso de nitritación se estableció y mantuvo con éxito mediante la estrategia de acumulación *in situ* de ANL tratando agua sintética (50 mg NT/L) a 15 ± 1 °C. Luego, la operación continuó pero tratando aguas residuales municipales después del tratamiento primario (que contenían 18 - 55 mg $N\text{-}NH_4^+$/L y concentración de carbono orgánico total (COT) de 20 - 50 mg COT/L), para estudiar la viabilidad del proceso de nitritación basado en la estrategia del ANL. Se observó una acumulación de nitrito de prácticamente el 100 % del amonio oxidado y la estabilidad del proceso de nitritación se mantuvo a largo plazo (354 días de los cuales 191 trató agua municipal), mediante la acumulación de ANL en concentraciones variando entre 0,02 y 0,20 mg $N\text{-}HNO_2$/L. Además, aunque se alcanzaron valores mínimos de pH de 5,6, estos no produjeron ningún efecto adverso sobre el proceso de nitritación ni sobre la actividad específica de las BOA que se mantuvo alrededor de 200 mg $N\text{-}NH_4^+$/(g SSV·d). Independientemente de la presencia de materia orgánica, que también había sido señalada como un reto para la implementación de los procesos de NP/AMX en condiciones de la línea principal de aguas, el proceso de nitritación se mantuvo estable y el 80 % de la materia orgánica fue eliminada en el mismo reactor. Este hecho confiere a esta estrategia una gran ventaja, ya que permite ser más flexible con las eficiencias de eliminación requeridas en las unidades anteriores para la eliminación de materia orgánica. Se observó que, para hacer frente a las fluctuaciones de las concentraciones de nitrógeno y materia orgánica de las aguas residuales, se requiere la optimización de la duración del ciclo de operación del SBR. Por lo tanto, la duración variable del ciclo proporcionaría una operación más adecuada evitando períodos inactivos cuando el amonio es consumido por completo al tratar agua residual diluida.

Capítulo 7. Evaluación del comportamiento del proceso anammox

El Capítulo 7 se basa en el estudio de la viabilidad de aplicar el proceso anammox en condiciones de la línea principal de aguas de las EDAR, así como su comportamiento a baja temperatura a largo plazo. Con este objetivo, se inoculó un SBR (con un volumen de 5 L) con lodo granular procedente de un reactor ELAN® (ELiminación de Nitrógeno Autótrofa) que trata aguas residuales con concentraciones elevadas de nitrógeno en condiciones mesofílicas y se expuso directamente a las concentraciones bajas de nitrógeno (50 mg NT/L) y baja temperatura (15 °C) características de la línea principal de las EDAR. Los resultados demostraron que el período de aclimatación progresiva a las nuevas condiciones no sería necesario, acortando los períodos de arranque, ya que se obtuvieron eficacias de eliminación similares a los publicadas por otros autores realizando una adaptación lenta de la biomasa a las nuevas condiciones. Además, se demostró la estabilidad del proceso anammox a largo plazo (485 días) tratando tanto aguas residuales sintéticas con alcalinidades decrecientes, así como con agua residual municipal nitritificada. Esta última consistía en el efluente del reactor de nitritación descrito en el Capítulo 6 mezclado con agua municipal sin tratar en dicho reactor, para ajustar la relación nitrito/amonio necesaria para el proceso anammox en 1,2 g $N\text{-}NO_2^-$/g $N\text{-}NH_4^+$. A pesar de que, en el caso de la operación con agua real, la alimentación del reactor anammox tenía concentraciones de alcalinidad bajas (2 ± 1 mg CI/L) y valores de pH alreador de 6,2, estos no afectaron a la estabilidad del proceso. Por otra parte, la presencia de concentraciones residuales de materia orgánica del agua residual municipal tras el tratamiento primario contribuyó a un aumento de la eficiencia de eliminación de nitrógeno desde valores alrededor del 80 % a 90 %, alcanzando concentraciones de NT en el efluente inferiores a 10 mg NT/L (límite de vertido en la Unión Europea en las áreas sensibles para grandes depuradoras). La biomasa anammox se retuvo satisfactoriamente en el sistema y su actividad específica aumentó a lo largo de la operación desde 53 ± 11 mg N/(g SSV·d), en el inóculo, hasta valores máximos de 78 ± 8 mg N/(g SSV·d) al final de la operación con agua sintética. Sin embargo, durante el período de operación con agua municipal se observó un descenso de la actividad a valores medios de 58 ± 6 mg N/(g SSV·d), probablemente debido al desarrollo de bacterias heterótrofas desnitrificantes. Considerando la actividad específica anammox y la concentración de biomasa en el reactor, el sistema tenía una capacidad de eliminación potencial mayor a la VEN

tratada (40 mg NT/(L·d) indicando que ésta estaba limitada por la carga aplicada. Finalmente, se estudió el efecto del pH (6 - 8), temperatura (15 - 30 °C) y concentración de materia orgánica (0 - 75 mg COT/L) sobre la actividad específica anammox en ensayos en discontinuo. Los resultados indican que el pH es el parámetro ensayado con mayor efecto sobre la actividad y que el rango de pH óptimo varía con la temperatura.

Capítulo 8. Comportamiento de los procesos de nitritación parcial-anammox en una etapa en un sistema IFAS

En el Capítulo 8 se presenta el reactor IFAS como una configuración prometedora para llevar a cabo los procesos de NP/AMX en una etapa debido a su capacidad de segregar las diferentes poblaciones bacterianas en distintas fracciones de biomasa (biomasa en suspensión y biopelícula). El reactor IFAS a escala piloto (200 L) se alimentó continuamente con agua residual municipal pretratada anaeróbicamente y se operó a temperaturas decrecientes desde 21 a 15 °C. A pesar de que la alimentación contenía una DQO relativamente alta con relaciones DQO/N de 2.5 ± 0.3 g DQO/g N, los balances de materia indican que el proceso anammox es el mayor responsable de la eliminación de nitrógeno obtenida. Durante la operación se obtuvieron eficiencias de eliminación de nitrógeno de 72 ± 11 % y valores medios de VEN de 37 ± 3 mg NT/(L·d) a 15 °C. Con respecto a la distribución de las poblaciones principales, se observó una segregación siendo las BOA más abundantes en la biomasa en suspensión mientras que las bacterias anammox se encontraban principalmente en la biopelícula formada sobre los soportes. Sin embargo, la abundancia de las BON era similar en ambas fracciones de biomasa y, a pesar de que su actividad específica disminuyó durante la operación del reactor, no fueron eliminadas por completo del sistema. Adicionalmente, el patrón de aireación intermitente empleado en el reactor IFAS permitió la limitación de la actividad de las BON dentro del sistema observándose una actividad en el reactor de en torno al 10 - 20 % de la actividad máxima. Con respecto a la calidad del efluente, se obtuvieron concentraciones de nitrógeno de 12 ± 5 mg NT/L, lo que demuestra que se requiere una mayor optimización del sistema para cumplir con los límites de vertido establecidos por la legislación europea para las áreas sensibles: 15 mg NT/L para las EDAR que tratan cargas de entre 10.000 y 100.000 habitantes equivalentes y 10 mg NT/L para las de mayor tamaño.

Capítulo 9. Implementación de un sistema de nitritación parcial y anammox en dos etapas a escala piloto

Los prometedores resultados obtenidos a escala laboratorio con la configuración de NP/AMX en dos etapas condujeron a probar esta configuración por primera vez a escala piloto (600 L cada unidad) como se describe en el Capítulo 9. En este caso, la planta piloto se instaló en una EDAR municipal donde operaba un reactor aerobio de lodos activos de alta carga para la eliminación de materia orgánica (sin afectar a la concentración de nitrógeno). El sistema NP/AMX se operó sin control de temperatura (variando entre 11 y 28 °C) y haciendo frente a las mismas fluctuaciones en las características del agua residual a las que estaba sometida la EDAR municipal. El efluente del reactor aerobio de alta carga se caracterizaba por tener concentraciones de amonio entre 22 y 63 mg $N\text{-}NH_4^+$/L, que se oxidó parcialmente a nitrito en la unidad de nitritación parcial. Durante toda la operación no se observó actividad de las BON que estaban inhibidas mediante la acumulación de ANL *in situ* (0,015 - 0,200 mg $N\text{-}HNO_2$/L). Para obtener una relación nitrito/amonio en el efluente de 1,2 g $N\text{-}NO_2^-$/g $N\text{-}NH_4^+$, adecuada para alimentar al reactor anammox en serie, se implementó una estrategia de control para terminar la etapa de aireación basándose en el valor de pH en el interior del rector de nitritación. De este modo, el reactor pudo soportar mejor las fluctuaciones en las características de las aguas residuales. Por otra parte, en el reactor anammox se alcanzó una eficiencia de eliminación de nitrógeno del 80 % con una VEN de 99 ± 26 mg NT/(L·d), limitada principalmente por el TRH impuesto previamente en la unidad de NP para obtener una adecuada retención de biomasa. De hecho, la principal limitación encontrada en esta experiencia fue la retención de biomasa en esta unidad. Con respecto a la calidad del efluente, se obtuvo una concentración de 12 ± 3 mg NT/L. Sin embargo, es necesario señalar que el efluente del reactor aerobio de alta carga de la EDAR alimentado al sistema NP/AMX ya contenía aproximadamente 5 mg $N\text{-}NO_3^-$/L de nitrato. El nitrato no es eliminado en el sistema NP/AMX, limitando por tanto su eficacia total de eliminación de nitrógeno. La optimización del sistema aerobio de alta carga de la EDAR, mediante la reducción del tiempo de retención celular para limitar el proceso de nitrificación en esta unidad, fomentaría la obtención de un efluente de mayor calidad cumpliendo con los límites de vertido establecidos comúnmente en la legislación. Finalmente, se realizó un estudio de los potenciales ahorros comparando la combinación del sistema aerobio de alta carga y la

eliminación de nitrógeno mediante NP/AMX con un sistema convencional de lodos activos (la tecnología más comúnmente empleada para la eliminación de nitrógeno en las EDAR). Los resultados demuestran que la implementación de los procesos de eliminación autótrofa de nitrógeno permite duplicar la recuperación de energía de la planta ya que más materia orgánica es tratada en el digestor anaerobio de lodos produciendo más biogás. Además, la eficiencia energética de la planta aumenta del 21 % con el sistema convencional de lodos activos al 57 % con la aplicación de los procesos de NP/AMX, y los costes operacionales también se reducen a la mitad. Sin embargo, la producción de lodos que deben ser gestionados como residuo es menor en el escenario convencional ya que el reactor aerobio de alta carga tiene una producción elevada de lodos incrementando los costos asociados a su gestión.

Capítulo 10. Conclusiones generales y futuros desafíos para la eliminación autótrofa de nitrógeno en la línea principal de aguas

En el capítulo 10 se presenta una discusión general con el objetivo de integrar los principales resultados y conclusiones más relevantes obtenidos durante la realización de esta tesis. En general, los resultados de esta tesis contribuyen a incrementar la comprensión del proceso de NP/AMX y proporcionan información interesante sobre la viabilidad de aplicar los procesos de NP/AMX para eliminar autotróficamente el nitrógeno a baja temperatura. Así se intenta responder a algunas de las principales preguntas que permanecen abiertas en este campo:

i) ¿Es posible aplicar los sistemas basados en el proceso anammox en sistemas de tratamiento de aguas descentralizado?

ii) ¿Es posible suprimir la actividad de las BON en condiciones de la línea principal de agua?

iii) ¿Son capaces las bacterias anammox de tratar cargas suficientemente altas de forma estable para la eliminación de nitrógeno en la línea de aguas?

iv) ¿Cómo afecta la composición del agua residual municipal al comportamiento de los procesos de NP/AMX?

v) ¿Cuál es la configuración de reactor más eficaz para la implementación de los procesos de NP/AMX para el tratamiento de aguas domésticas?

Se demostró que el tratamiento de las aguas negras mediante un sistema NP/AMX en una etapa es viable, probablemente porque su composición es

relativamente más alta y estable en comparación con las aguas residuales municipales, una ventaja para la estabilidad del proceso de NP/AMX. Los sistemas descentralizados, especialmente los de pequeña escala, podrían enfrentarse a períodos de ausencia de aguas residuales para tratar, pero se ha comprobado que las paradas que se ocasionarían en el sistema de tratamiento no impiden la aplicación de los procesos de NP/AMX. Así, se podría incrementar el número de pequeñas instalaciones con sistemas de eliminación de nitrógeno para promover su reutilización. Por otra parte, el sistema IFAS permite segregar las diferentes poblaciones microbianas en las fracciones de biomasa en función de su velocidad de crecimiento. Las BON podrían eliminarse del sistema mediante un control adecuado del tiempo de retención celular incrementado la eficacia de eliminación de nitrógeno. Además, se han obtenido resultados especialmente relevantes y novedosos en el marco de la aplicación de los procesos de NP/AMX en dos etapas. Primero se propuso una nueva estrategia operativa basada en la acumulación de ANL *in situ* a escala laboratorio y tratando agua residual sintética. Una vez entendido el mecanismo de inhibición de las BON, se validó la estrategia tratando agua residual urbana con presencia de materia orgánica. Luego, se eliminó el nitrógeno en reactores anammox en los que se maximiza la eliminación autótrofa de nitrógeno al ser un reactor exclusivamente anóxico con ausencia de materia orgánica, porque es oxidada en la unidad NP previa. Se obtuvieron eficacias de eliminación entre 80 y 90 % obteniendo efluentes de alta calidad. Así, el sistema de NP/AMX en dos etapas permite alcanzar eficacias de eliminación obteniendo efluentes de alta calidad con estrategias de operación simples que no requieren sistemas de control complejos lo que favorece su implementación. Por último, en este capítulo se discuten las principales limitaciones para la implementación de los procesos de NP/AMX en la línea principal de las EDAR a baja temperatura prestando especial atención a las propuestas de trabajo futuro.

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Chapter 1

Introduction

SUMMARY

Efficient nitrogen removal from sewage is crucial since wastewater treatment plants (WWTPs) are facing more and more stringent effluent quality requirements. Nitrogen is conventionally removed via biological nitrification-denitrification processes. These processes are large energy-consumers and organic matter-demanding as aeration and organic carbon need to be provided for nitrification and denitrification, respectively. For these reasons, new technologies are under development and study to improve nitrogen removal from wastewater without compromising the WWTP energy efficiency. Among them the nitritation-denitritation, partial nitritation-anammox (PN/AMX) or those processes based on microorganisms that use methane as electron donor stand out.

In particular, the implementation of the PN/AMX processes in the mainstream is expected to transform the WWTP into an energy producer facility instead of the current energy sink, while the produced effluent fulfils the nitrogen discharge limits fixed by the European Union.

In the present chapter, the scope and motivations of the thesis are thoroughly explained and the existent alternatives to biologically remove nitrogen from municipal wastewater analysed. Special attention is paid to the application of the PN/AMX processes for the treatment of low-strength wastewater at low temperature. Finally, the main objectives pursued in this thesis are presented.

OUTLINE

1.1. Shift from wastewater treatment to water reuse and resource recovery

1.1.1. Water scarcity

Water demand is steadily increasing as a function of population growth, economic development and change in lifestyle (AQUASTAT, 2019). Indeed, the predicted global water requirements by 2030 will exceed the current and reliable water supplies by 40% (International Food Policy Research Institute, 2019).

Agriculture (including irrigation, livestock and aquaculture) water consumption accounts for 69 % of global freshwater withdrawal, whereas industry uses 19 % and municipalities the remaining 12 %. The Food and Agriculture Organisation (FAO) estimates that food production must increase by 70 % by 2050 to meet the growing population demand, increasing agriculture water pressure. The water withdrawal due to the industrial sector is dominated by energy generation and it is also expected to increase by 48 % by 2040 (over 2012 levels) (UN-Water, 2018).

Thus, water scarcity, defined as a gap between water available supply and demand, is evident and it is already affecting 40 % of the global population (UN-Water, 2018), at least 11 % of the European Union (EU) population and 17 % of the EU territory. It will also affect 50 % of EU river basins by 2030 (European Comission, 2016). Water stress is distributed differently worldwide, and it can be expressed as water intensity use index in percentage (Figure 1.1). Within Europe, water stress is more acute in Southern-Mediterranean countries such as Cyprus and Italy (30 %), Spain (43 %) or Malta (83 %). The Middle East and Northern Africa countries are already in critical water scarcity levels with 17 countries experiencing water stress values above 100 % like Saudi Arabia and the United Arab Emirates where it reaches values up to 870 % and 2,000 %, respectively. In these countries, the demand for freshwater is largely met by desalination (AQUASTAT, 2019, UN-Water, 2018). Sub-Saharan African countries are not under physical water scarcity (< 10 %) but economical water scarcity since their water limitation comes from the deficient water extraction, which does not cover the population demands. One limitation of this water stress indicator is the fact that it is calculated using annual averages and therefore if water is abundant in one period of the year (raining season) it can arise a low value but being a country with water limitations (UN-Water, 2018).

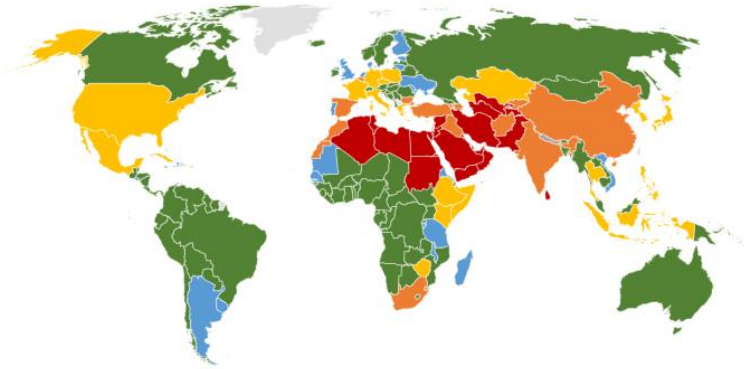

Figure 1.1. Water intensity use index by country: ■ low (< 10 %), ■ low to medium (10 - 20 %), ■ medium to high (20 - 40 %), ■ high (40 - 80 %), ■ extremely high (> 80 %) and ■ no data. Elaborated from AQUASTAT database.

Water scarcity does not solely relate to water quantity but water quality (UN-Water, 2018). Approximately 80 % of wastewater is released to the environment without any treatment resulting in a deterioration of water resources quality, reducing the freshwater availability and degrading the ecosystems. Globally, nutrient release due to agriculture irrigation is the primary source of discharging nitrogen (N) and phosphorus (P) to the environment being one of the most prevalent water quality-improvement challenges. Indeed, it is estimated that 10 % of all river basins in Latin America, Africa and Asia are already at or above the level of "increasing restriction" for use in irrigation, and nutrient emissions to surface water are projected to increase (UN-Water, 2018). In Europe, still almost 18 % of total groundwater exceeds the standard of nitrates established by the World Health Organisation (WHO) and only 38 % of monitored lakes, rivers and other surface water bodies are in good chemical status (European Environment Agency, 2017).

Rapidly growing cities, particularly in developing countries, are projected to become significant sources of nutrient emissions. Nowadays, 54 % of the total population lives in urban areas and it is expected to rise to 66 % by 2050 (European Environment Agency, 2017, UN-Water, 2018). Collecting, treating and reusing wastewater to reduce diffuse pollution and improve water quality are significant challenges for the water sector to overcome both environmental impacts and water scarcity (UN-Water, 2018).

1.1.2. Domestic wastewater reuse as a pool of resources

Nowadays, wastewater remains as an undervalued resource that is usually transported and treated in a wastewater treatment plant (WWTP) throughout energy-demanding processes. Nevertheless, wastewater should not be seen as a "waste" anymore but as a pool of reclaimed water, energy, nutrients and other useful by-products (European Comission, 2016). Improvement wastewater management fostering the wastewater resources recovery and water reuse has gained great attention as it is essential in the frame of circular economy and to ensure water security (Scherson and Criddle 2014, UN-Water, 2018).

1.1.2.1. Reclaimed wastewater reuse

The treatment level applied to wastewater is defined by the water quality requirements of the intended use according to the "fit-for-purpose" water reuse principle. In this way, reclaimed water presents potential for different applications (Figure 1.2). Globally, agriculture irrigation represents the primary use consuming 32 % of the total reclaimed water, followed by landscape irrigation accounting a 20 % while 19 % goes for industrial purposes (UN-Water, 2018). The water reuse percentage and uses vary along with the geography and for example, in Spain, 71 % of wastewater reused is for agriculture irrigation purposes (Sato et al. 2013).

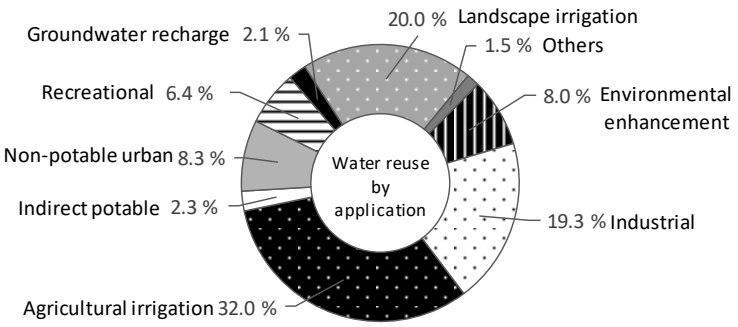

Figure 1.2. Global water reuse after advanced treatment. Adapted from Global Water.

In developed countries, reclaimed water is submitted until completing its secondary treatment and then it is often used to irrigate non-agricultural land such as parks or golf courses or for non-potable urban uses. As an example, Spain reuses

about 11 % of the total volume of generated wastewater especially in the Mediterranean area where reuse can be as high as 50 - 60 % as in the case of Segura basin and the Balearic Islands (AQUASTAT, 2019). In countries where water is scarcer, water reuse percentage rises to 99 % in the case of Cyprus. Israel reuses more than 87 % of the WWTPs effluent for agriculture irrigation accounting almost half of the water use by farmers (AQUASTAT, 2019, European Commission, 2016). In the United States of America (USA), the reuse of partially treated wastewater for non-potable uses is widely established and it is delivered in dual distribution systems through a parallel network (Environmental Protection Agency, 2012). Mexico is the country leading the water reuse in volume representing 70 % of the total wastewater produced, from which 80 % is reused without any treatment (AQUASTAT, 2019).

The use of reclaimed water in substitution of potable water is not common (Figure 1.2), and it is strongly limited by public perception. However, this use is well established in countries such as Australia, Namibia, Singapore and some regions in the USA such as California, Virginia or New Mexico. Reclaimed water is used for augmentation of raw freshwater supply followed by an environmental buffer (Environmental Protection Agency, 2012).

New regulations in an international context are expected to promote the use of reclaimed water broadening its final application. Water reuse criteria are principally oriented to public health protection, although the maximum allowable concentrations of nutrients (N and P) are considered to prevent eutrophication as well. Distinct threshold values are fixed in the different regulations in force nowadays (Table 1.1). Since agriculture is by far the largest water consumer, an increase of irrigation with reclaimed water would have a positive impact on alleviating water stress in other sectors. Moreover, as wastewater is a nutrient-rich stream, its use for irrigation reduces the need for chemical fertilisers. However, the United Nations (UN) reported that irrigation with untreated wastewater is a widespread critical practice in developing countries that might cause environmental and health issues (UN-Water, 2018). In addition, N and P content in the reclaimed water needs to be accurately assessed to benefit the receiving soil nutrient balance, which also depends on the crop uptake rate (and how this varies across a season), the nature of the soil and variations among seasons (Food and Agriculture Organisation, 2019). Water demand for irrigation also changes with the season of

the year being barely needed during autumn (Figure 1.3). Therefore, alternatives for N removal are required enabling other uses or the ultimate discharge.

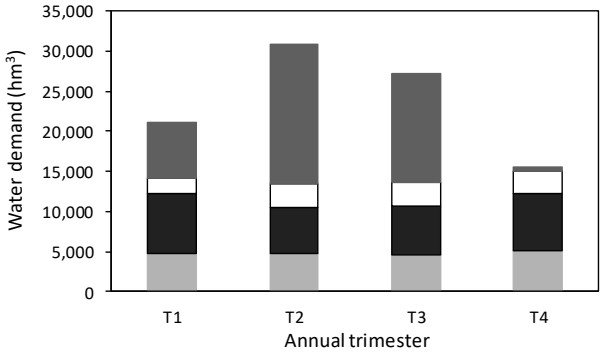

Figure 1.3. Annual water use in the European Union by economic sectors and trimesters: T1 (January, February and March), T2 (April, May and June), T3 (July, August and September) and T4 (October, November and December). Industry ((■), energy production (■), households (□) and agriculture (■). Adapted data from European Environment Agency (2017).

The lack of policies in large world areas slows the water reuse application (Table 1.1). For example, the reclaimed water was already highlighted within EU water policy almost 20 years ago (Water Framework Directive 2000/60/EC) as an alternative water source to consider within water-scarcity planning. However, until this year, the legal regime for the reuse of treated wastewater in the EU was the responsibility of each country and only six of them in Southern Europe developed their particular normative (Table 1.1). In 2019, the European Commission (EC) has regulated the minimum quality requirements for the reuse of treated wastewater for agricultural irrigation and aquifers recharge (P8_TA(2019)0071). This regulation established organic matter removal and disinfection as necessary treatments, but it does not include nitrogen standard limits. It recalls that reclaimed water must comply with the Nitrates Directive (91/676/EEC) and the Urban Wastewater Treatment Directive (UWTD, 91/271/EEC).

Table 1.1. Example of reference documents and regulations in force of water reuse worldwide and corresponding established nitrogen limits (elaborated from Paranychianakis et al. (2015) and cited legal documents).

Territory	Uses					
	Agriculture	Urban	Industrial	Environmental	Groundwater recharge (non potable)	Indirect potable reuse
USA [1]*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
California [1]	10 mg N/L					
Florida [1]	Soil balance	N.S	N.S	6 mg N/L, 4 mg NH_4/L	< 12 mg N/L	10 mg N/L
Arizona [1]	10 mg N/L	10 mg N/L	N.S.	N.R.	N.S.	10 mg N/L
North Carolina [1]	6 mg NH_3-N/L	N.S.	N.S.	N.S	N.R.	N.R.
Australia [2]*	Soil balance	N.S.	N.S.	N.S.	0.5 mg NH_3/L, 50 mg NO_3/L and 3 mg NO_2/L	
Israel [3]	< 25 mg N/L and 10 mg NH_3-N/L					
European Union [4]	N.S.	N.R.	N.R.	N.R.	N.S.	N.R.
Italy [5]	35 mg N/L	N.R.	N.R.	N.R.	15 mg N/L; 2 mg NH_4^+/L	N.R.
Cyprus [6]	15 mg N/L					
Portugal [7]	N.S.	N.R.	N.R.	N.R.	N.R.	N.R.
Spain [8]	10 mg N/L	N.S.	N.S.	10 mg N/L and 25 mg N/L		
France [9]	N.S.	N.R.	N.R.	N.R.	N.R.	N.R.
Greece [6]	30 mg N/L				15 mg N/L, 2 mg NH_4^+/L	N.R.

N.S.: not specified values for nitrogen; N.R.: non-regulated use; USA: United States of America.

*National reference document for reuse guidance but not legally binding as actual regulation occurs at state level.

References: [1] EPA/600/R-12/618; [2] NRMMC-EPHC, 2006 and 2008; [3] Israel Ministry of Environmental Protection (2010); [4] P8_TA(2019)0071; [5] D152/2006; [6] Paranychianakis et al. (2015); [7] NP 4434/2005 and ReciRAR 2/2007; [8] RD 1620/2007; [9] JORF 0201/31.08.10/n 34 page 15828.

The Nitrates Directive aims to protect water sources which are or may become exposed to nitrogen pollution from agricultural activities. This directive considers the treated wastewater containing nitrogen as fertiliser and limits its disposal in the so-called nitrate vulnerable zones (a specific type of eutrophic sensitive area) that are those at risk of exceeding 50 mg NO_3^-/L (Figure 1.4). Moreover, 16 member States apply a more stringent treatment than the secondary one in their whole territory which is considered a sensitive area (article 3.5 of UWTD). In total sensitive areas amount to 75 % of the entire EU territory (Figure 1.4).

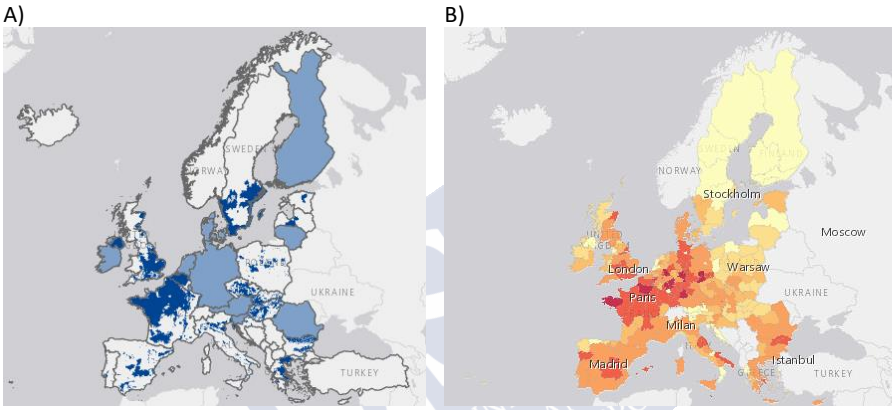

Figure 1.4. A) Nitrate vulnerable zones (■) and areas applying the article 3.5 of the Urban Wastewater Treatment Directive (i.e. more stringent treatment in the whole territory) (■). B) Percentage of monitoring points with annual average nitrate concentrations above 25 mg/L: ■ 75 - 100 %, ■ 50 - 75 %, ■ 25 - 50 %, ■ 15 - 25 %, ■ 5 - 15 % and ■ 0 - 5 %. Reprinted from Water portal, European Commission.

Despite the benefits of recycling water, there are several concerns related to its associated environmental and health risks. If it is not properly managed, irrigation induces nitrate run-off and ultimate soil acidification. Moreover, the presence of inorganic compounds such as metals or salts might limit the acceptability of reclaimed water for several reuse applications like irrigation, industrial uses as salts might damage the machinery or groundwater recharge varying the composition of the receiving water body (Rahman et al. 2016, UN-Water, 2018). The main problem due to salinity, when the desired use is irrigation, is the loss of soil structure accelerating the salinisation within the root zone. According to the FAO, 20 % of the

irrigated land is salt affected, mainly located in the USA, Australia, China, India, Argentina and Central Asia (UN-Water, 2018).

Over the last decade, salinisation of water bodies increased due to the leaching of salts accumulated in solids, production of brackish drainage, industrial activity, intrusion of seawater in aquifers (due to the excessive water extraction) and the salts added by urban uses. Different tertiary treatment steps, including desalinisation and reverse osmosis, are required to enable the water reuse or discharge. Other concern is the presence of complex toxic compounds (more frequent if wastewater from some industries is collected), heavy metals, or substances of emerging concern that are not removed in the WWTPs and therefore, they could be accumulated in reclaimed water (UN-Water, 2018).

1.1.2.2. Energy recovery

Wastewater plays a significant role in the water-energy nexus. Water cycle needs approximately 4 % of the total energy consumed worldwide (UN-Water, 2018). Wastewater is a source of chemical, thermal and hydraulic energy that can be recovered as biogas, heating and electricity. In developed countries, 42 % of electricity consumption in the water sector is used for wastewater treatment. By 2040 it is estimated that water collection and treatment will require 60 % more electricity. If energy is properly valorised, wastewater treatment could produce 30 - 100 TWh by 2040 (UN-Water, 2018) converting the WWTPs into net energy-producing facilities (Garrido et al. 2013).

Among the possible options, the chemical energy recovery as biogas is the most popular alternative since organic matter removal takes place simultaneously. Anaerobic wastewater digestion is only applied in temperate climates, but in many cases, the produced biogas is not recovered as energy but released to the environment (Chernicharo et al. 2015, Noyola et al. 2012, Stazi and Tomei 2018).

In cold climates, the conventional activated sludge (CAS) is the most widely applied technology. In these WWTPs primary and secondary sludge are anaerobically digested to produce biogas. Nevertheless, energy recovery is limited to 10 - 50 % (Burton et al. 2014, Longo et al. 2016). For this reason, sewage sludge pretreatments are applied to improve anaerobic biodegradability such as the thermal hydrolysis (TH), mechanical disintegration, application of acidic or alkaline reagents (Carrère et

al. 2010, Zhen et al. 2017). The TH is a well-established technology with commercial solutions (e.g., CambiTHP™ and Biothelys®) applied worldwide to enhance the methane yield from approximately 30 to 80 % depending on the operating conditions (Nordin et al. 2018, Sapkaite et al. 2017, Zhen et al. 2017).

Another alternative to further increase energy generation in WWTPs is to use co-digestion that consists in mixing the sewage sludge with other organic-rich wastes (e.g., animal manure, food-processing waste, grease, microalgae) increasing the biogas production (Elalami et al. 2019, Guven et al. 2019). The addition of new wastes must be appropriately assessed considering its composition since besides organic matter other substances such as nitrogen would be supplied too (Rodriguez-Verde et al. 2018, Taboada-Santos et al. 2019a). Moreover, the digested solids might be also valorised as a soil conditioner and fertiliser for agriculture or further treated off-site in centralised plants through incineration for energy recovery (Guven et al. 2019, UN-Water, 2018).

Innovative technologies such as microbial fuel cell (MFC) or microbial electrolysis cell (MEC) allow converting the wastewater organic matter content either directly into electrical energy or indirectly through hydrogen gas production, respectively. Combined with anaerobic digestion (AD) or constructed wetlands they are also expected to increase their efficiency. These technologies are being tested at pilot-scale and face limitations related to large investment costs or upscale technical issues (Guadarrama-Pérez et al. 2019, Katuri et al. 2019, Pham et al. 2006, Zhen et al. 2017). Other alternatives like the use of algae or phototrophic purple bacteria (PPB) are also under consideration. The phototrophic systems use energy from sunlight reducing the chemical oxygen demand (COD) required for biological nitrogen removal. Moreover, PPB assimilates approximately 16 g COD/g N and produces hydrogen from organic matter or other value-added products such as polyhydroxyalkanoates or proteins (Vasiliadou et al. 2018). Algae are used to harvest the organic matter and nutrients on their biomass obtaining a concentrated stream. Algae biomass is then digested producing biogas.

1.1.2.3. Nutrients recovery

One of the main identified problems for resource recovery from domestic wastewater is the fact that it is a diluted stream which involves the treatment of high-water volumes with low concentrations of valorising compounds (Liu et al.

2018b, Winkler and Straka 2019). Thus, diluted wastewater streams containing ammonium and phosphorus concentrations below 100 mg NH_4^+-N/L and 40 mg P/L, respectively, are preferable submitted to biological nutrient removal treatments for their better cost-effectiveness compared to resource recovery processes (Mulder 2003). Although there are other alternatives, for example, P chemical precipitation is technically feasible and produces a phosphorus-rich sludge that might be used as a soil amendment (Zhou et al. 2018). However, the N recovery technologies are not competitive with those used in the chemical industry for nitrogen-based fertiliser production (Winkler and Straka 2019). Among the proposed technologies, other examples are the promising results obtained by MEC or the adsorption of ammonium and phosphorus onto zeolite surface obtaining a solid that might be used as soil fertiliser (Winkler and Straka 2019, You et al. 2019).

1.1.3. Nitrogen removal in municipal wastewater treatment: current status

Wastewater treatment level and regulations concerning N compounds highly vary within countries (Figure 1.5). With this in mind, the UN analysed 275 national standards from 100 countries and almost 80 % established discharge limits for total nitrogen (TN). Nitrogen discharge limits varied from 80 mg TN/L (mainly in Latin America) to values as low as 2 mg NH_4^+-N/L allowed in Switzerland. Nevertheless, in African or Asian countries standards are often not accomplished (UN-Water, 2018).

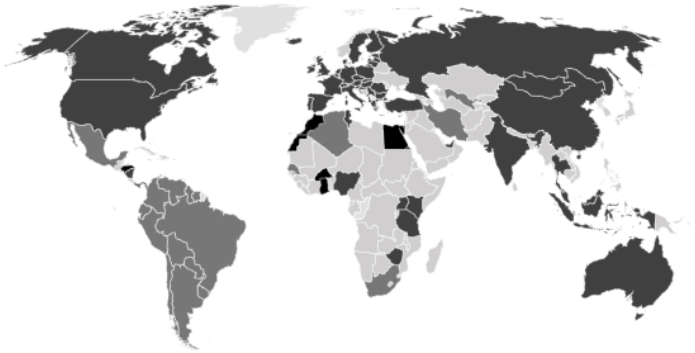

Figure 1.5. Total nitrogen (TN) standard limits for treated wastewater discharge in different countries: limit lower than 15 mg TN/L (■), regulated but at values between 20 and 80 mg TN/L (■), no regulated (■) and no data (■). Compilation based on the revision of country policies.

In Latin America, by 2015, less than 60 % of the population was connected to sewer systems and nearly all urban wastewater was discharged without any treatment (UN-Water, 2018). The principal applied treatment technologies are the stabilisation pounds (80 % in number of facilities and treated flow), CAS and up-flow anaerobic sludge blanket (UASB) reactors (Noyola et al. 2012). Nowadays, in Chile, Brazil, Mexico and Uruguay at least 50 % of the used water is treated being wastewater treatment less likely in other countries (UN-Water, 2018). Nevertheless, regulations established high TN concentrations limits (40 - 80 mg TN/L) to be reached before discharge into the environment (Figure 1.5).

In the EU, an average 83 % of the total wastewater is treated in WWTPs whereas 5 % is treated in individual and other appropriate systems (IAS) and the remaining 12 % is not treated (European Environment Agency, 2017). In small agglomerations (< 2,000 p.e., population equivalent) 34 % of the water remains untreated being clear that this is a niche for decentralised wastewater treatment systems. The big agglomerations (> 100,000 p.e) account for only 2 % of the total municipal WWTPs but these facilities treat 51 % of the total load (as p.e.) (Figure 1.6).

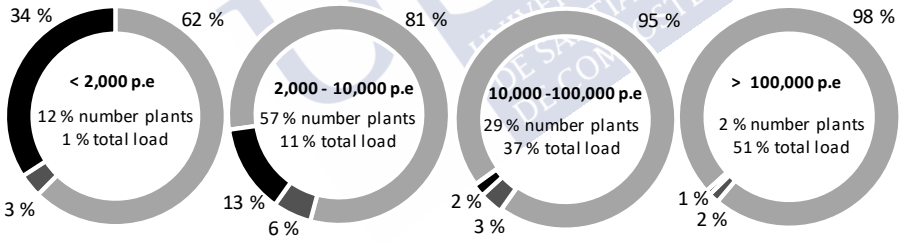

Figure 1.6. Distribution of the percentage of wastewater treated in municipal WWTPs (■), individual and other appropriate systems (IAS, ■) and untreated (■) according to the agglomeration population size in EU. Elaborated with data from European Environment Agency (2017).

The wastewater treatment level depends on the size of the WWTPs, the larger the WWTPs the more intensive the applied treatment (Figure 1.6 and Figure 1.7.A). The UWTD regulated the wastewater treatment of agglomerations from 2,000 p.e or more that should be subjected wastewater to secondary treatment. This directive established TN discharge limits to sensitive areas for WWTPs with capacities of

treatment over 10,000 p.e.: between 10,000 and 100,000 p.e. the discharge standard is 15 mg TN/L while it is reduced to 10 mg TN/L for loads above 100,000 p.e. Alternatively a reduction of the 70 - 80 % of the applied nitrogen load might be requested.

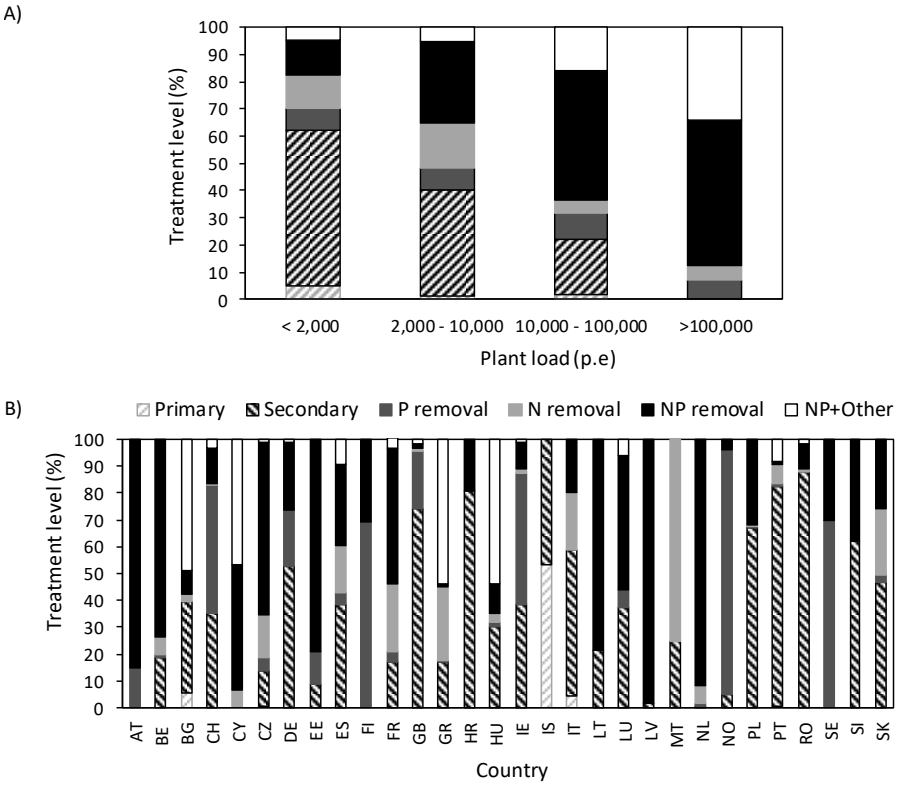

Figure 1.7. Wastewater maximum treatment level: primary treatment, secondary treatment, phosphorus (P) removal, nitrogen (N) removal, N+P removal and N+P removal plus more stringent treatment. A) Depending on the wastewater treatment plant size in population equivalent (p.e.) and B) In different countries of which the names are coded according to the ISO 3166-1. Note that to obtain the total percentage of nitrogen removal the three last categories are considered. Elaborated from data from European Environment Agency (2017).

Regarding nutrients, N is only removed in 30 % of the small WWTPs while 97 % of the biggest ones already remove it. It is worth to note that advanced treatments (e.g., ozonation, ultraviolet) were considered combined with nutrients removal in Figure 1.7, but the use of tertiary treatments occurs in other WWTPs with lighter

treatment. Moreover, the treatment level differs also depending on the geographical location and while N is removed in the 98 % of the WWTP in The Netherlands and Latvia, its removal amounts to less than 10 % in countries such as Iceland, Norway and United Kingdom (Figure 1.7.B). Thus, improvements for the treatment level applied are still required.

In addition, effluent discharge limits worldwide are expected to become more stringent and will drive future innovations due to the increasing relevance of indirect water reuse (UN-Water, 2018). For this reason, most of the recent studies focus on the development of new technologies producing effluents with improved quality, which help to increase the economic and environmental sustainability of WWTPs where they are implemented.

1.1.3.1. Conventional activated sludge systems

In CAS systems the COD contained in wastewater is converted to sludge and CO_2 in a similar approximate ratio. For removing nitrogen, CAS systems are operated at long sludge retention times (SRTs), diminishing the sludge biodegradability. When the nitrification-denitrification processes take place the energy and resource demand, together with the operational costs and WWTP carbon footprint, augmented (Siegrist et al. 2008).

The high energy consumption for aeration and the excessive produced waste activated sludge, due to the heterotrophic bacteria growth, are the main drawbacks of the CAS systems. Indeed, the aeration in the biological reactors and the sludge handling units (dewatering, thickening…) are the major energy consumers using 50 - 60 % and 15 - 25 % of the total energy consumption, respectively (Liu et al. 2018b, Wang et al. 2019). Thus, new alternatives are under study to improve the WWTPs from an economical and environmental sustainable point of view.

1.1.4. Innovative wastewater treatment plants

The simultaneous increase of the energy recovery and the reduction of aeration energy consumption in large WWTPs is faced nowadays by augmenting the COD directed to AD. With this objective, a stage for organic matter removal (A-stage) followed by another stage for N removal (B-stage) are proposed to replace the CAS systems.

When dealing with small agglomerations decentralised wastewater treatment systems become an attractive alternative to be applied diminishing the pumping costs, and enabling the energy and nutrient recovery (WWAP, 2017) since raining water is not collected (or in a lesser extent) (Verstraete and Vlaeminck 2011).

1.1.4.1. Organic matter removal (A-Stage)

The energy contained in wastewater can be recovered either by direct anaerobic wastewater digestion (Chernicharo et al. 2015) or by pre-concentrating the COD in the sludge, facilitating the energy recovery in anaerobic sludge digesters, requiring less energy for aeration and reducing the sludge production (Guven et al. 2019). COD capture from municipal wastewater is possible by applying chemically enhanced primary treatment (CEPT) or high rate activated sludge (HRAS) processes. In the A-Stage, only 10 to 30 % of the N is removed by microbial growth (De Graaff et al. 2016, Guven et al. 2017, He et al. 2016a) and it varies with the influent biological oxygen demand (BOD_5) to nitrogen ratio(BOD_5/N), SRT and the amount of N in recycling streams from solids dewatering after anaerobic digestion (Burton et al. 2014).

- <u>High Rate Activated Sludge</u>

The aerobic HRAS process consists in applying high loading rates of 2 g BOD_5/(g VSS·d) at short SRT values of 0.2 - 4.0 days and short hydraulic retention time (HRT) of 0.5 - 4.0 h. In the HRAS process, COD is adsorbed onto the activated sludge flocs resulting in COD removal efficiencies of 50 - 70 %, minimising its conversion to CO_2 and consequently reducing the energy consumption for aeration (Jimenez et al. 2015, Meerburg et al. 2015). The SRT and the concentration of the COD fractions are the main parameters determining COD biosorption. Besides soluble COD (sCOD), particulate COD (pCOD) is also removed and reached efficiency increases with the lengthening of SRT (Jimenez et al. 2015). In HRAS, a COD-rich sludge is produced with a volatile fraction of 78 %, higher than the one from the sludge generated in the CAS systems (58 %). Thus, methane production yield from the former is expected to be larger than that of the latter. Nevertheless, the higher COD diversion to sludge in HRAS systems and the high water content in sludge may complicate its dewatering process (Cagnetta et al. 2019).

- Chemically enhanced primary treatment

During the CEPT coagulants are added to enhance the removal efficiency of primary settling tanks from 50 % of total suspended solids (TSS) and 30 % of pCOD to up to 90 % TSS and 75 % COD (Guven et al. 2019). Besides pCOD, up to 55 % of sCOD can also be removed (Guven et al. 2019, Taboada-Santos et al. 2019b). Additionally, when aluminium or ferric salts are used, up to 80 % of the phosphorus is chemically removed (Burton et al. 2014, Guven et al. 2019). CEPT is characterised by low energy requirements, simple operation and maintenance, high treatment capacity and removal efficiencies, and the ability to cope with overloads better than CAS systems (Murugesan et al. 2014). The used salts exert no adverse effect on methane production during anaerobic sludge digestion but provoke the increase in sludge production and consequently in the operational costs (Guven et al. 2019). An additional issue is the alkalinity depletion (Equation 1.1). Large amounts of coagulant dosages may result in acidic pH, which can damage downstream processes (Burton et al. 2014) limiting, for example, the nitrification process efficiency.

$$2\ FeCl_3 + 3\ Ca(HCO_3)_2 \rightleftarrows 2\ Fe(OH)_3 + 3\ CaCl_2 + 6\ CO_2 \qquad \text{Eq. 1.1}$$

- Anaerobic digestion (AD) of wastewater

Direct anaerobic treatment of diluted municipal wastewater leads to removal efficiencies ranging from 65 to 80 % of COD and 75 to 95 % of BOD_5 at HRT values of 6 - 10 hours (Chernicharo et al. 2015). Anaerobic treatments are more efficient than aerobic ones when treating municipal wastewater characterised by medium to high organic matter content (> 1 g COD/L). The effluent quality from anaerobic wastewater digesters is worse than those from aerobic treatments, in terms of COD and suspended solids (Stazi and Tomei 2018). Nevertheless, compactness of the technologies, minimal energy requirements and low sludge production make AD attractive and competitive in climate regions with temperatures above 20 °C (Chernicharo et al. 2015). AD feasibility to treat low strength wastewater at psychrophilic conditions is still under evaluation (Arias et al. 2018, Silva-Teira et al. 2017, Stazi and Tomei 2018, Zhou et al. 2018). Besides methane (CH_4) and CO_2, mineralised compounds such as ammonium, phosphate and hydrogen sulphide (H_2S) are produced. A primary concern towards its applicability is the potential stripping of H_2S and CH_4 dissolved in the anaerobic digester effluent contributing to bad

odours and the greenhouse gases (GHGs) emissions, respectively (Kampman et al. 2014). The economic biogas recovery in AD for the treatment of low-strength wastewater constitutes an additional challenge. To tackle this situation alternatives such as the gas stripping in a flash chamber by increasing the liquid turbulence (Souza et al. 2012) or the CH_4 and H_2S use as additional electron donors for N removal were proposed (Arias et al. 2018, Chernicharo et al. 2015).

1.1.4.2. Nitrogen removal (B-Stage)

In CAS systems, the nitrogen removal efficiency (NRE) depends on organic matter availability (for the denitrification process). If COD is driven for biogas production in A-Stage, alternative nitrogen removal process requiring less organic matter need to be considered (B-stage). Different options for N removal are presented within Section 1.2.

The AB-process layout is not only interesting for the design of new WWTPs or the improvement of the energy-efficient of the already existing facilities but also for upgrading those WWTPs that were not constructed to remove nitrogen. In Europe, there are still 50 % of the total WWTPs, where N is not removed that can also benefit from this development (European Environment Agency, 2017).

1.1.4.3. Decentralised systems

More concentrated streams are obtained in source-separation systems, like those used in decentralised wastewater treatment, allowing to segregate wastewater in different fractions depending on their characteristics and their final use (WWAP, 2017). Domestic wastewater can be divided into blackwater (i.e., toilet water) and greywater. In some cases, yellow water is also distinguished comprising the urine, but this stream in practice is difficult to separate from blackwater in the already existing installations (Malila et al. 2019). If only blackwater and greywater are considered, the former is an organic matter and nutrients concentrated stream containing approximately 92 % of TN, 75 % of P and 52 % of COD of the mixed municipal wastewater (Gottardo Morandi et al. 2018). From this stream, nutrients and energy might be more easily recovered whereas the greywater might be reused after a simple aerobic treatment (Eshetu Moges et al. 2018, Gao et al. 2019, Malila et al. 2019). Source-separation systems are a good solution to apply, especially in

small agglomerations, where wastewater treatment implementation is not complete enabling the wastewater valorisation.

1.2. Nitrogen cycle and opportunities for municipal wastewater treatment

N is an essential and abundant element present in the environment at different oxidation states from -III to +V that serves as an electron donor or acceptor in microbial transformations (Figure 1.8).

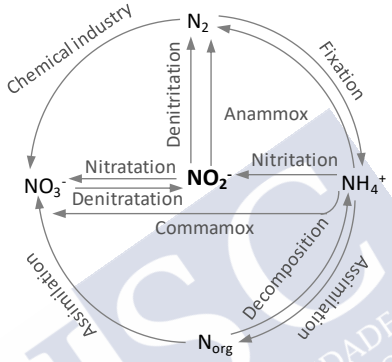

Figure 1.8. Schematic representation of the main processes of the nitrogen cycle and main biological processes involved. Adapted from Daims et al. (2016).

In natural conditions, the N fixed is balanced by the amount converted to dinitrogen gas (N_2). However, the N fluxes in the cycle are already dominated by the human action, as anthropogenic nitrogen fixation (e.g., fossil fuel combustion, industrial ammonia production) reached values like the natural ones doubling the global cycling of N over the last century (Fowler et al. 2013, Galloway et al. 2003). The inclusion of fertilisers production in the cycle increases the N losses. It is estimated that 15 % of the N applied to agriculture ended up in the wastewater (European Commission, 2016).

The release of nitrogenous compounds into the aquatic environment leads to serious environmental problems such as oxygen depletion, eutrophication, soil acidification or toxicity. Indeed, ammonia and nitrite concentrations as low as 0.02 mg NH_3-N/L and 2 mg NO_2^--N/L, respectively, cause chronic fish toxicity (Bregnballe 2015). Other intermediate products such as NO_x (NO_2, NO) and N_2O might be released provoking the stratospheric ozone depletion (Fowler et al. 2013). N_2O

attracted particular attention since it is a harmful GHG with a global warming effect 300 times stronger than CO_2 (Stocker et al. 2013) and toxic to living organisms (Fowler et al. 2013).

In wastewater, the nitrogen is usually as ammonium or organic nitrogen (that is easily converted to ammonium in the biological reactor), therefore its conversion to N_2 gas is required to maintain the balance of the N compounds in the environment. In the following subsection, the basis of the process for N removal involved in the natural N cycle and relevant in engineered systems are described. Special focus is given to the processes which differ shortcuts to the N cycle (based on nitrite as an intermediate product) since they make more sustainable use of wastewater resources.

1.2.1. Nitrification (nitritation and nitratation)

Nitrification is a two-step biological oxidation process performed by autotrophic bacteria. First, ammonia is oxidised to nitrite (nitritation process) by ammonia oxidising bacteria (AOB) according Equation 1.2. Then, nitrite is oxidised to nitrate (nitratation process) by nitrite oxidising bacteria (NOB) following Equation 1.3.

$$NH_4^+ + 1.985\ HCO_3^- + 0.074\ CO_2 + 1.403\ O_2 \rightarrow 0.985\ NO_2^- + 1.985\ CO_2 + 0.015\ C_5H_7O_2N + 2.941\ H_2O \quad \text{Eq. 1.2}$$

$$NO_2^- + 0.005\ NH_4^+ + 0.005\ HCO_3^- + 0.020\ H_2CO_3 + 0.471\ O_2 \rightarrow NO_3^- + 0.005\ C_5H_7O_2N + 0.008\ H_2O \quad \text{Eq. 1.3}$$

From the stoichiometry of Equations 1.2 and 1.3 it can be inferred that nitritation consumes higher alkalinity, as inorganic carbon (IC), (1.7 g IC/g N) and higher oxygen (3.2 g O_2/g N) than nitratation process with values of 0.02 g IC/g N and 1.1 g O_2/g N, respectively. Alkalinity is consumed due to the release of protons during the N oxidation, that are neutralised by the buffer capacity of the carbonate system through CO_2 stripping. When the system has not enough buffering capacity, the pH of the medium drops (Burton et al. 2014). Moreover, nitrification process was identified as the main contributor in the release of nitric oxide (NO) and nitrous oxide (N_2O) emissions originated in the WWTPs that are intermediates in the nitritation process (Kampschreur et al. 2009). The AOB denitrification was pointed out as the main pathway for N_2O production in WWTPs (Campos et al. 2016, Duan et al. 2018).

The AOB and NOB are phylogenetically independent groups with different growth rates, but they usually are present together in environment and reactor systems. Both AOB and NOB are chemolithotrophs that fix IC for growth. The energy generated in these reactions is minimal and, therefore, their growth yields are meagre. The nitritation process is performed by AOB genera such as *Nitrosomonas, Nitrosospira, Nitrosovibrio, Nitrosolobus* and a cluster of *Nitrosococcus* genus, but also by ammonium oxidising archaea (AOA) belonging to *Candidatus "*Nitrosopumilus maritimus" specie. However, limited information is available on the kinetics of AOA to understand their competition with AOB. In CAS systems *Nitrosomonas* is usually the most abundant AOB genus (Saunders et al. 2016). The nitratation process is only performed by bacteria belonging to the *Nitrospira, Nitrobacter* and *Candidatus* "Nitrotoga" genera (Saunders et al. 2016). In municipal WWTPs the most widespread NOB is *Nitrospira* spp. that are k-strategist with high affinity for their substrate. *Nitrobacter* spp. are abundant in reactors treating N-rich wastewater streams such as the supernatant of anaerobic sludge digesters (Nogueira and Melo 2006). The niche differentiation for C. *Nitrotoga* spp. is still unclear but it can dominate low-temperature systems (Lucker et al. 2015).

To apply the nitrite shortcut to wastewater N removal, the nitritation process must take place preferentially and, in this way, organic carbon requirements for N removal are significantly reduced or eliminated. For this purpose, AOB and NOB growth rates have to be decoupled. This aspect is further discussed in section 1.4.1.

1.2.2. Complete ammonia oxidising (comammox) bacteria

The complete ammonia oxidising (comammox) bacteria are members of the genus *Nitrospira* (traditionally considered as NOB) that possess the ammonia monooxygenase (AMO) and the hydroxylamine oxidoreductase (HAO) enzymes necessary for ammonia oxidation to hydroxylamine and then to nitrite. Therefore, comammox bacteria can perform the complete oxidation of ammonia to nitrate (Daims et al. 2016, van Kessel et al. 2015). They were already theoretically described in 2006 by Costa et al. (2006) but they were not experimentally discovered until 2015 (Daims et al. 2016, van Kessel et al. 2015). Comammox cannot be distinguished by 16S rRNA-based methods requiring more sophisticated molecular tools such as metagenomics and functional gene-based polymerase chain reaction (PCR) (Koch et al. 2019, Pjevac et al. 2017).

Comammox bacteria have been identified in different engineered, natural freshwater and terrestrial ecosystems but their role and interaction with other microorganisms from the N cycle and niche separation are still poorly understood (Koch et al. 2019).

Different comammox species were isolated like *Nitrospira nitrosa*, *Nitrospira nitrificans* and *Nitrospira inopinata* (Koch et al. 2019). The kinetic and physiological analysis of *N. inopinata* sp. demonstrated that, compared with AOB, they have a higher affinity for ammonia, slower growth rate, lower maximum ammonia oxidation rate and higher yield (Kits et al. 2017). Surprisingly the nitrite affinity of *N. inopinata* sp. is approximately 50-fold lower than for canonical NOB (Kits et al. 2017). *N. nitrosa* sp. were highly enriched in a system operated under low dissolved oxygen (DO) concentrations indicating that they might outcompete AOB under limited oxygen conditions (Camejo et al. 2017). Another advantage of comammox over AOB is the potential lower N_2O production during nitrification (Kits et al. 2019)

Results suggest that comammox might oxidise the ammonia in a more efficient way competing with AOB and they might represent an additional challenge to limit the nitrate production generally ascribed to NOB (Roots et al. 2019). Nevertheless, under substrate limiting conditions, they only oxidise the ammonia into nitrite, as this is the most favourable step from a thermodynamic point of view.

1.2.3. Heterotrophic denitrification (denitratation and denitritation)

The denitrification process is the reduction of nitrate and/or nitrite into dinitrogen gas, under anoxic conditions, and consuming organic matter as electron donor. In WWTPs, the common electron donor is sCOD (Eq. 1.4).

$$C_{18}H_{19}O_9N + 7.5\ NO_3^- + 7.5\ H^+ \rightarrow 3.5\ N_2 + 10.3\ CO_2 + 1.5\ C_5H_7O_2N + 7.9\ H_2O \qquad \text{Eq. 1.4}$$

The denitrification process increases alkalinity 0.9 g IC/g NO_3^--N and produces high amounts of sludge with 1.6 g VSS/g NO_3^--N, as volatile suspended solids (VSS).

In some WWTPs, the sCOD contained in municipal wastewater is not enough to achieve high-efficient N removal and an external carbon source is added increasing both the operational costs and the sludge production (Burton et al. 2014). Methanol is the most commonly used electron donor due to its larger denitrification efficiency,

lower cost and higher availability in the market when it is compared with ethanol or acetate (Pelaz et al. 2018b).

The denitrification process is carried out in sequential steps through the different oxidation states of N: NO_2^-, NO, N_2O and N_2. The stoichiometric reactions using methanol as electron donor are presented in Equations 1.5 to 1.8.

$$2\,CH_3OH + 6\,NO_3^- \rightarrow 6\,NO_2^- + 2\,CO_2 + 4\,H_2O \quad \text{Eq. 1.5}$$

$$CH_3OH + 6\,NO_2^- + 2\,H_2O \rightarrow 6\,NO + CO_2 + 4\,OH^- \quad \text{Eq. 1.6}$$

$$CH_3OH + 6\,NO \rightarrow 3\,N_2O + CO_2 + 2\,H_2O \quad \text{Eq. 1.7}$$

$$CH_3OH + 3\,N_2O \rightarrow 3\,N_2 + CO_2 + 2\,H_2O \quad \text{Eq. 1.8}$$

From the previous stoichiometry equations, it can be inferred that 40 % of the sCOD is consumed for the reduction of nitrate into nitrite (denitratation process) that might be avoid if nitrite shortcut N removal is applied.

The denitrification process is performed by a wide range of organisms (bacteria, archaea and eukaryotes) that are widely distributed in the environment and engineered ecosystems. In WWTPs the most common heterotrophic denitrifying bacteria genera are *Pseudomonas*, *Bacillus* and *Paracoccus.* Most of these bacteria are facultative aerobic organisms with the ability to use oxygen as well as nitrate or nitrite as electron acceptor (Burton et al. 2014). Nevertheless, many bacteria and archaea are not able to encode all the enzymes and they can release intermediates such as NO and N_2O well-known for being GHGs. Environmental conditions like low pH values or limited sCOD concentrations are also proposed as probable causes for this behaviour but further research about the GHGs emission in WWTPs is needed (Campos et al. 2016).

Nitrite accumulation has been frequently observed due to limited COD/N ratios, different pH values, biofilm structures or low HRT among other factors (Cui et al. 2017, Du et al. 2016, Ge et al. 2012). Some denitrifying bacteria are known by being only capable of reducing the nitrate into nitrite but not to N_2 (denitritation process, Eq. 1.5). In the denitratation systems, the reported dominant genera are *Thauera, Halomonas* and *Candidatus* "Saccharibacteria" and they are useful when nitrate contaminated waters are treated, polishing effluents or when nitrite supplied to other process is required (Du et al. 2019).

1.2.4. Anaerobic ammonia oxidation: anammox

The anaerobic ammonium oxidation (anammox; AMX) bacteria are chemolithoautotrophic microorganisms which anoxically oxidise ammonium, using nitrite as electron acceptor, to nitrogen gas, producing residual amounts of nitrate in the anabolism. The anammox process was thermodynamically predicted by Broda (1977) but it was in 1990s when Mulder et al. (1995) experimentally proved its existence in a denitrifying pilot plant. Anammox stoichiometry was first proposed by Strous et al. (1999) (Equation 1.9) and recalculated later by Lotti et al. (2014a) (Equation 1.10).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad \text{Eq. 1.9}$$

$$NH_4^+ + 1.146\ NO_2^- + 0.071\ HCO_3^- + 0.057\ H^+ \rightarrow 0.986\ N_2 + 0.161\ NO_3^- + 0.071\ CH_{1.74}O_{0.31}N_{0.20} + 2.002\ H_2O \quad \text{Eq. 1.10}$$

The amount of nitrate produced represents 11.2 % (Strous et al. 1999) or 7.5 % (Lotti et al. 2014a) of the TN converted depending on the stoichiometry used, limiting the NRE of the system. The anammox process has a low impact on wastewater alkalinity and the low biomass yield, ranging from 0.07 to 0.13 g VSS/g NH_4^+-N, is an advantage from the sludge handling point of view but it requires to operate the anammox systems at long SRT values and long start-up periods.

One of the main advantages of the anammox process is that COD is not needed for N removal. Furthermore, hydrazine (N_2H_4) and nitric oxide (NO) are metabolic intermediates of the process but N_2O is not produced potentially decreasing the GHGs emissions originated in WWTPs (Campos et al. 2016, Castro-Barros et al. 2015, Kampschreur et al. 2008). Anammox bacteria can reduce nitrate first to nitrite and then to ammonia using formate, acetate or propionate as electron donors (Kartal et al. 2007). However, the presence of COD usually promotes the heterotrophic denitrifying bacteria overgrowth (Castro-Barros et al. 2017, Xu et al. 2015).

Anammox bacteria are ubiquitous in WWTPs and natural environments such as marine sediments. Anammox bacteria belong to the phylum *Planctomycetes* and overall ten anammox species have been described within five different genera: *Candidatus* "Kuenenia" (represented by K. stuttgartiensis sp.), *Candidatus* "Brocadia" (with the species B. anammoxidans, B. fulgida and B. sinica), *Candidatus*

"Anammoxoglobus" (A. propionicus sp.), *Candidatus* "Jettenia" (J. asiatica sp.), *Candidatus* "Scalindua" (with the species S. brodae, S. sorokinii, S. wogneri and S. profunda). All of them were enriched from WWTP sludge samples except *C.* "Scalindua" that comes from marine sediments. *C.* "Kuenenia" and *C.* "Brocadia" are the most commonly found genera in enrichments from WWTPs. The average optimal temperature and pH values have been found to be 35 °C and 8, respectively. The anammox bacteria are characterised by low productivity with typical doubling times as long as 11 days (Strous et al. 2002). More recently, a faster-growing anammox culture with doubling times of 3 days was also obtained at the optimal conditions (Lotti et al. 2015a) but in practical applications, they are still considered as slow-growing microorganism.

One of the most relevant features of anammox bacteria is its high affinity for both substrates, ammonium and nitrite, being the affinity coefficients under 0.10 mg N/L (Strous et al. 1999). On the contrary, anammox bacteria sensitiveness is widely reported (Jin et al. 2012) being affected by numerous compounds such as DO concentration (Seuntjens et al. 2018), COD (Giustinianovich et al. 2016), nitrite (or its protonated form, free nitrous acid (FNA)) (Fernández et al. 2012, Lotti et al. 2012, Puyol et al. 2014b), ammonium concentration (or it unionized form free ammonia (FA)) (Fernández et al. 2012, Puyol et al. 2014a), pH (Daverey et al. 2015), temperature (Dosta et al. 2008, Lotti et al. 2015c) or salts (Dapena-Mora et al. 2007, Fajardo et al. 2014). Considerable differences were found in the inhibition thresholds probably due to the anammox different enrichment degree of the performed studies. Thus, an assessment of the wastewater composition is recommended before the anammox implementation.

1.2.5. Other autotrophic denitrification processes

Other appealing autotrophic denitrification processes might occur using the CH_4 or H_2S (produced during the AD) as alternative inexpensive electron donors (Chernicharo et al. 2015).

1.2.5.1. Methane autotrophic denitrification

Methane oxidation linked to denitrification can occur either through aerobic or anaerobic processes. In the aerobic methane oxidation coupled to denitrification (AMO-D) process, aerobic methanotrophs oxidise CH_4 into products such as

methanol, acetate and carbohydrates, that can be used by the heterotrophic denitrifiers to reduce nitrite (Equation 1.11) and/or nitrate (Equation 1.12) to dinitrogen gas (Burton et al. 2014). Methanotrophs are strictly aerobic bacteria and use IC for microbial growth whereas CH_4 is used as carbon and energy source.

$$3\,CH_4 + 3\,O_2 + 4\,NO_2^- + 4\,H^+ \rightarrow 2\,N_2 + 3\,CO_2 + 8\,H_2O \quad \text{Eq. 1.11}$$

$$5\,CH_4 + 5\,O_2 + 4\,NO_3^- + 4\,H^+ \rightarrow 2\,N_2 + 5\,CO_2 + 5\,H_2O \quad \text{Eq. 1.12}$$

In anoxic conditions, Raghoebarsing et al. (2006) reported the first enriched culture of nitrite/nitrate dependent anaerobic methane oxidation (N-damo) microorganisms and since then, it became a hot topic in wastewater treatment research. During the N-damo process, CH_4 is oxidised to CO_2 coupled to both nitrate (performed by archaea, Equation 1.13) and nitrite (performed by bacteria, Equation 1.14) reduction and using IC for biosynthesis. The most studied N-damo archaea is *Candidatus* "Methanoperedens nitroreducens" sp. (Haroon et al. 2013) while N-damo bacteria belong to the NC10 phylum being *C.* "Methylomirabilis oxyfera" and *C.* "Methylomirabilis sinica" the most researched species (Ettwig et al. 2010, He et al. 2016b).

$$2\,CH_4 + 8\,NO_3^- \rightarrow 8\,NO_2^- + 2\,CO_2 + 4\,H_2O \quad \text{Eq. 1.13}$$

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \rightarrow 4\,N_2 + 3\,CO_2 + 10\,H_2O \quad \text{Eq. 1.14}$$

As N-damo bacteria have long doubling times of up to two weeks, the existence of efficient biomass retention systems is crucial (Ettwig et al. 2009). Moreover, N-damo bacteria have the advantage of avoiding the production of N_2O since this is not an intermediate in their metabolic pathway (Ettwig et al. 2010). The anoxic N-damo processes make more efficient use of the methane for N removal requiring 0.7 g CH_4/g NO_3^--N whereas AMO-D process uses 1.4 g CH_4/ g NO_3^--N and requires energy for aeration. Municipal wastewater has approximately 50 mg TN/L, thus, a concentration of 35.5 mg CH_4/L would be required for N removal. However, CH_4 concentrations in the anaerobic municipal wastewater digester ranged from 12 to 20 mg CH_4/L limiting the NRE.

Despite the high interest attracted by N-damo, few is known about them and its prevalence in natural environments. Kampman et al. (2012) incorporated the N-damo process as an UASB digester system and a nitritation reactor to treat municipal wastewater and managed to remove 38 mg TN/(L·d), although the contribution of

the N-damo to the TN removal is unclear. Different co-cultures were obtained, mostly combined with anammox bacteria where relatively high nitrogen removal rates (NRRs) were obtained like: 1,030 mg TN/(L·d) at 35 °C (Xie et al. 2017) and 275 mg TN/(L·d) at 21 °C (Xie et al. 2018) in membrane biofilm reactors or 126 mg TN/(L·d) obtained by Allegue et al. (2018) at 28 °C in a membrane bioreactor. Moreover, in cultures where anammox and N-damo bacteria and archaea coexist, anammox bacteria tend to outcompete the N-damo bacteria that progressively disappear (Hu et al. 2015). This fact also happens under nitrite limiting conditions, since anammox bacteria present affinities for nitrite higher than N-damo microorganisms (van Kessel et al. 2018). Thus, most of the studies ended up with the use of a combined N-damo and anammox culture to achieve an excellent effluent quality (Hu et al. 2015, Liu et al. 2019, Stultiens et al. 2019, van Kessel et al. 2018, Xie et al. 2018).

1.2.5.2. Sulphur autotrophic denitrification

The sulphur oxidising bacteria (SOB) reduced the nitrate and/or nitrite to dinitrogen gas using sulphur compounds ($S_2O_3^{2-}$, S^{2-}, S^0, $S_4O_6^{2-}$, SO_3^{2-}) as electron donors and IC as carbon source for bacterial biosynthesis.

In the case of sulphide, the dissolved form abundances changes according to the pH value varying from hydrogen sulphide (H_2S; pka=7.0), bisulphide (HS^-; pka=12.9) and sulphide (S_2^-) ions. The total sulphide concentration depends mainly on the sulphate content of the raw wastewater that varies according to the geographical location (Sánchez-Ramírez et al. 2015) and can reach values up to 87 mg S/L when municipal wastewater is treated (Delgado Vela et al. 2015). This compound was found inhibitory for the biological processes but SOB have high tolerance (Fajardo et al. 2012, Lu et al. 2018). Thus, SOB might improve other biological removal process performances by removing the toxic sulphide concentrations from the media (Cui et al. 2019b, Di Capua et al. 2019).

Stoichiometric reactions are complex since different sulphur compounds can be used and produced from these reactions. As an example, Equations 1.15 and 1.16 show the complete oxidation of HS^- to produce sulphate (SO_4^{2-}) and in Equations 1.17 and 1.18 the HS^- is oxidised to elemental sulfur (S^0), using in both cases either nitrate or nitrite as electron acceptor (Burton et al. 2014, Di Capua et al. 2019). During this process, intermediate compounds are usually detected and mixed final products are

obtained (Cai et al. 2008, Campos et al. 2019, Di Capua et al. 2019). In fact, different HS^- oxidation degrees are expected depending on the relative S/N molar ratio (Dolejs et al. 2015, Liu et al. 2017a). It was also reported that under sulphur limiting conditions (S/N ratio < 6.51 g S/g N), nitrate reduction will be only carried out to nitrite instead to N_2 (Oh et al. 2000).

$$5\,HS^- + 8\,NO_3^- + 3\,H^+ \rightarrow 5\,SO_4^{2-} + 4\,N_2 + 4\,H_2O \quad \text{Eq. 1.15}$$

$$3\,HS^- + 8\,NO_2^- + 5\,H^+ \rightarrow 3\,SO_4^{2-} + 4\,N_2 + 4\,H_2O \quad \text{Eq. 1.16}$$

$$5\,HS^- + 2\,NO_3^- + 7\,H^+ \rightarrow 5\,S^0 + N_2 + 6\,H_2O \quad \text{Eq. 1.17}$$

$$3\,HS^- + 2\,NO_2^- + 5\,H^+ \rightarrow 3\,S^0 + N_2 + 4\,H_2O \quad \text{Eq. 1.18}$$

SOB populations are widespread (Lu et al. 2018). Some of these bacteria only use sulphur compounds as electron donor such as *Thiobacill*us *denitrifican*s sp., *Thiobacillus thiophilus* sp. or *Sulfurimonas denitficans* sp. whereas others can use either organic matter or sulphur compounds like *Paracoccus* sp. (with *P. denitrificans, P. ferrooxidans* and *P. pantotrophus* species*)* (Di Capua et al. 2019).

Other advantages of this process are the SOB low biomass yield that ranged from 0.15 to 0.6 g VSS/g N (Cui et al. 2019b, Di Capua et al. 2019) and the significant lower N_2O production compared with the heterotrophic denitrification process (Campos et al. 2019, Cui et al. 2019b, Fajardo et al. 2014). However, the associated high alkalinity consumption and the possible release of sulphate or other intermediate compounds to the environment difficult its implementation, especially in acidic wastewater streams (Cui et al. 2019b, Di Capua et al. 2019).

1.3. Towards efficient wastewater treatment: nutrient removal alternatives

New technologies, to achieve economic and environmentally sustainable wastewater treatment, are developed which minimise energy consumption, are reliable and cheap. Figure 1.9 shows the combination of different biological processes to optimise the nitrogen removal from wastewater.

Traditional nitrification-denitrification (N-HDN) is an energy-intensive nitrogen removal pathway mainly due to electricity consumption for aeration (4.57 kg O_2/kg N) and organic matter use as electron donor (2.86 kg COD/kg N) that cannot be valorised as biogas. The combined nitritation and denitritation processes (N-HDN via

nitrite) reduce by 40 % the required COD and by 25 % the energy for aeration. However, 1.71 g COD/g N is still required for N removal (Figure 1.10).

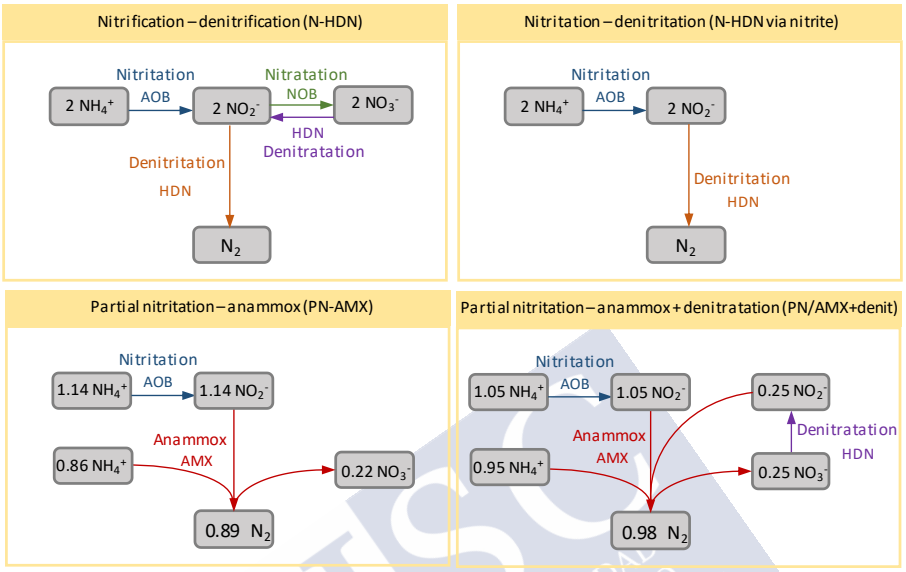

Figure 1.9. Comparison of different nitrogen removal processes combinations involving ammonium oxidising bacteria (AOB), nitrite oxidising bacteria (NOB), heterotrophic denitrifying bacteria (HDN) and anammox (AMX) bacteria.

The application of autotrophic N removal process enables more sustainable use of wastewater resources maximising the energy recovery from the wastewater COD and reducing the sludge production (Kartal et al. 2010). The combination of the PN/AMX processes requires 40 % of the aeration energy (only half of the incoming N is oxidized to nitrite by AOB), produces less than 89 % of the sludge and it allows to remove the N by whole autotrophically process (Figure 1.10) (Morales et al. 2015).Thus, all the COD contained in the wastewater is driven for biogas.

The SOB and N-damo based processes are interesting since methane and sulphur dissolved in the liquid media are removed improving the wastewater reuse possibilities and decreasing the operational costs (reducing the consumption of COD). However, the limited concentrations of CH_4 and H_2S in the anaerobically digested municipal wastewater define their potential contribution to the removal of N by these routes and the predominance of the occurring anammox process. N-damo

and SOB processes might contribute to polish the effluent from anammox based systems by reducing the produced nitrate into nitrite that could be consumed by the anammox process increasing the nitrogen removal efficiency (PN/AMX+denit in Figure 1.9) (Hu et al. 2015, van Kessel et al. 2018, Xie et al. 2018). For example, Stultiens et al. (2019) observed that all the nitrate produced by anammox bacteria was reduced by N-damo archaea and consequently 70 % of nitrite was removed by anammox. Indeed, the coexistence and interaction between the anammox bacteria, N-damo bacteria and archaea and SOB were reported in several studies (Chen et al. 2016, Langone et al. 2014, Pelaz et al. 2018a, Xie et al. 2018). The denitratation process for anammox effluents polishing might be also performed using residual COD requiring approximately 5 % of the total COD present in the wastewater (Figure 1.9 and Figure 1.10).

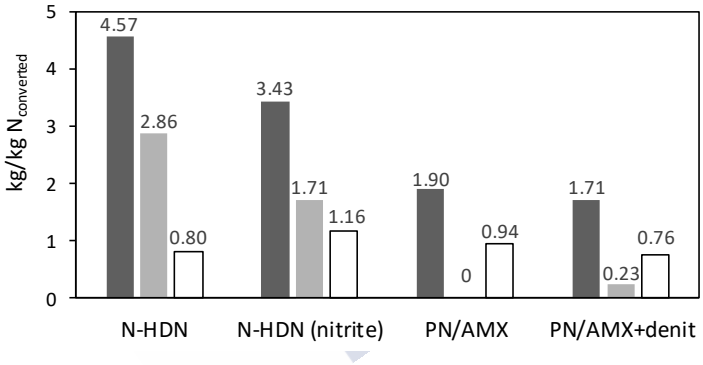

Figure 1.10. Comparison of oxygen consumption for the biological reaction (■, in kg O_2/kg N), organic matter use as electron donor (■, in kg COD/kg N) and alkalinity as inorganic carbon (IC) (□, in kg IC/kg N) in the different nitrogen removal combined processes: complete nitrification-dentirification (H-DN), nitritation-denitritation (H-DN nitrite), partial nitritation-anammox (PN/AMX) and PN/AMX plus denitratation (PN/AMX+denit). Note that for PN/AMX+denit, hetetrotrophic denitration was considered to estimate the consumptions.

In general, nitrite production from AOB is the limiting step to implement the PN/AMX processes. Some authors also proposed the combination of nitrification-denitratation-anammox processes instead of nitritation (Du et al. 2019), but from an energetic point of view this combination is less favourable than PN/AMX requiring 2.51 kg O_2/kg N (to oxidise 55 % of ammonium to nitrate) and 0.63 kg COD/N (for nitrate reduction into nitrite). These features reduce its attractive but it might be a

solution when the complete NOB suppression is not feasible since the denitratation process is presumably easier to control (Du et al. 2019).

1.3.1. Partial nitritation and anammox processes application in WWTPs

The successful PN/AMX implementation relies on favouring the AOB and anammox bacteria growth while outcompeting the undesired NOB. The application of the PN/AMX processes can be performed in two different system configurations: (a) two reactors located in series, where the partial nitritation (PN) takes place in the first aerobic unit and the anammox (AMX) process in the second anoxic unit; (b) a single reactor where both processes occur simultaneously. In the two-reactor configuration, respective optimal operational conditions of both processes are imposed in the corresponding unit. In the one-stage configuration, both processes co-occur by controlling parameters like the DO concentration, reaction time (aerobic and anoxic alternative periods) and/or the distribution of the microorganisms in the reactor by the use of biomass that grows as biofilm. Inside the biofilms, different environments exist, in such a way that AOB grow in the outer layers consuming the oxygen and creating anoxic conditions in the deeper zones of the biofilm for the anammox bacteria (Agrawal et al. 2018).

The single reactor configuration is the most frequently applied at full-scale as it requires less complex control systems and lower investment costs (Lackner et al. 2014). The most common application of PN/AMX systems is for the treatment of the supernatant of the anaerobic sludge digester in municipal WWTPs (i.e., sidestream) (Figure 1.11) representing the 75 % of the total plants (Cao et al. 2017, Lackner et al. 2014). This stream amounts to 10 - 20 % of the TN load entering into the WWTPs. The implementation of the PN/AMX processes in the sidestream allows for reducing by approximately 26 % the energy requirements for aeration, producing 18 % more biogas since less organic matter is consumed for denitrification, and increasing by 17 % the solids derived from the primary settling (Morales et al. 2015).

Nowadays, the challenge for the PN/AMX processes application at sidestream conditions is related to the implementation of sludge pre-treatments to increase methane production, being the TH the most common method. When a TH process is applied, the obtained digestate is more concentrated in ammonia, but it also contained other compounds such as recalcitrant organic matter, volatile fatty acids

or others still undefined that might affect the PN/AMX processes performance (Figdore et al. 2011, Han et al. 2017, Zhang et al. 2016). Information about the applicability of the PN/AMX processes treating the TH-pretreated AD digestate lacks and more research about this topic is needed.

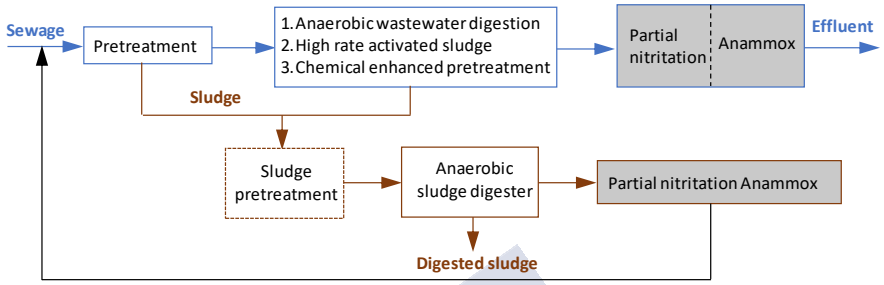

Figure 1.11. Scheme for efficient WWTPs: in the mainstream (—) with different alternatives (1, 2 and 3) for the A-stage and with a two-stage or hybrid PN/AMX configuration for the B-stage; while in the sidestream (—) a single-stage PN/AMX is proposed.

Moreover, 80 - 90 % of the TN load entering the WWTPs is still treated in the CAS system. For the implementation of the PN/AMX processes in the mainstream of municipal WWTPs, the COD needs to be removed in a previous A-stage as it was discussed previously in Section 1.1.4 (Figure 1.11). For source-separation decentralised systems, the anaerobic wastewater digestion is highly recommended. In these installations, wastewater is expected to be more concentrated and the anaerobic sludge digester implementation would be not feasible due to the high investment cost due to the relatively low sludge production to be valorised.

1.4. Application of PN/AMX processes at mainstream conditions

The application of anammox based process for the treatment of the mainstream in the WWTPs has been revealed as one of the most promising alternatives for improving the municipal WWTPs energy efficiency enabling to reach the WWTPs self-sufficiency (Garrido et al. 2013, Kartal et al. 2010). The mainstream of a WWTP is characterised by low nitrogen concentration (< 50 mg TN/L), low temperature (< 25 °C) and high variable composition (Cao et al. 2017). Although the application of the mainstream PN/AMX processes has been proposed almost 20 years ago (Kartal et al. 2010, Siegrist et al. 2008), the challenge of attaining stable nitrite pathway performances has hindered its implementation (Agrawal et al. 2018).

1.4.1. Factors affecting NOB suppression

NOB suppression is crucial to obtain stable PN/AMX processes performances as they compete with AOB for oxygen and with anammox bacteria for nitrite. In the following section, factors that selectively inhibit or limit NOB growth are summarised. A combination of different factors is usually applied in order to guarantee the NOB suppression (Agrawal et al. 2018).

1.4.1.1. Aerobic sludge retention time

At high temperature, AOB grow faster than NOB and therefore if short SRT values are applied NOB are washout from the system. However, at low temperature, NOB growth rates are higher than those from AOB being unfeasible to achieve the nitritation process by solely shortening the SRT (Figure 1.12.A, Jubany et al. 2008, Regmi et al. 2014).

1.4.1.2. Dissolved oxygen concentration

Traditionally the application of low DO concentration (< 1.5 mg O_2/L) was considered crucial for establishing the nitritation process since AOB have higher oxygen affinity than NOB at sidestream conditions (Blackburne et al. 2008). However, this behaviour is not observed at low temperature since the dominant genus of NOB shifts from *Nitrobacter* to *Nitrospira*. *Nitrospira spp.*, as k-strategist, present oxygen affinities higher than AOB (Figure 1.12.B, Ma et al. 2016, Regmi et al. 2014). Even though, under DO limiting concentrations (< 0.2 mg O_2/L) stable NOB suppression was obtained but probably due to the combination with other factors (Akaboci et al. 2018, Laureni et al. 2019, Yang et al. 2017). The suitable DO concentration and the oxygen affinities highly vary within systems (Cao et al. 2017) due to the oxygen mass transfer resistance, enrichment degree, existence of biofilm biomass and operational conditions.

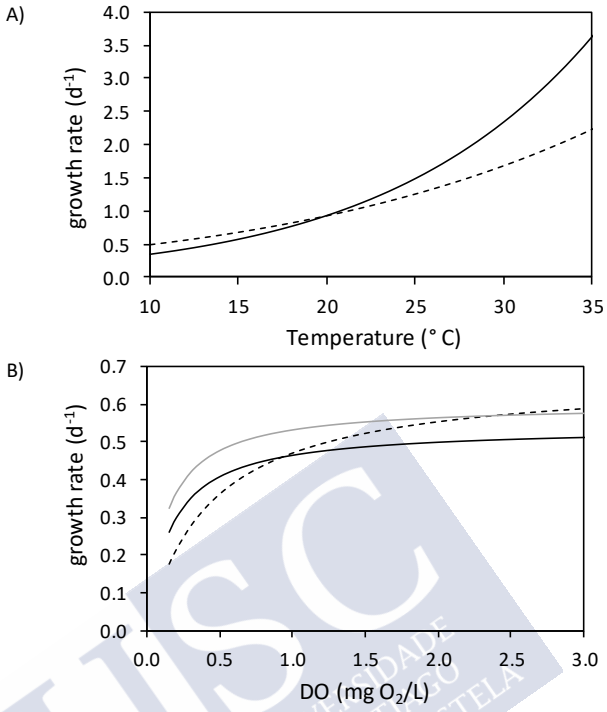

Figure 1.12. A) Evolution of the maximum specific growth rate of ammonium oxidising bacteria (AOB, —) and nitrite oxidising bacteria (NOB, --) with temperature for a pH value of 7.0. Obtained using data from Jubany et al. (2008). B) Evolution of the maximum growth rate with dissolved oxygen (DO) concentration for AOB (——) and NOB distinguishing *Nitrobacter* spp. (--) and *Nitrospira* spp. (——). The used constant oxygen affinities were: 0.16, 0.43 and 0.13 mg O_2/L for AOB, *Nitrobacter* spp. and *Nitrospira* spp., respectively (Cao et al. 2017, Soler-Jofra et al. 2019).

1.4.1.3. Alternating anoxic and aerobic conditions

The application of transient anoxia conditions was proposed as another alternative to suppress NOB activity since the delay time (lag phase) once oxygen addition is restored is longer for nitrate production than for ammonium oxidation (Gilbert et al. 2014, Ma et al. 2015, Malovanyy et al. 2015). Different factors such as reactor configuration, airflow rate and biomass characteristics affect the length of the NOB lag phase. Although alternating anoxic (minimum 15 - 30 min) and aerobic conditions succeeded on the NOB suppression when nitrogen-rich wastewater was treated, complete NOB suppression at mainstream conditions was not achieved

using this strategy (Akaboci et al. 2018, Gilbert et al. 2014, Malovanyy et al. 2015). Thus, this fact indicates that NOB might presumably adapt to transient anoxia conditions. Moreover, applying intermittent aeration would reduce the ammonium oxidation rates as AOB would be inactive during the anoxic periods.

1.4.1.4. Aeration time control

As NOB depends on the nitrite produced by AOB, they can be gradually limited by turning off the aeration when ammonium oxidation is completed (Blackburne et al. 2008, Regmi et al. 2014, Yang et al. 2007). As no DO would be present, some nitrite would remain in the effluent decreasing the NOB growth. When ammonium is completely depleted, DO concentration sharply rises (nitratation requires only 25 % of the oxygen consumed via nitritation) and pH that during the nitrification process decreases, began to increase due to CO_2 stripping. The control of the aeration length is widely used and can be controlled indirectly by measuring the oxidation-reduction potential (ORP), DO concentration or pH value (Yang et al. 2007), or directly using ammonium probes (Regmi et al. 2014). This strategy is also named “ammonium valley” (Gu et al. 2012).

1.4.1.5. Dissolved oxygen to total ammonium nitrogen concentration ratio

In biofilm systems, AOB usually grow on the outer layers while NOB in the deeper ones. The dissolved oxygen to total ammonium nitrogen concentration (DO/TAN) ratio control relies on suppressing NOB by limiting the DO supplied (Pérez et al. 2014). Boundary levels would depend on the operational temperature, biofilm thickness and growth kinetics of the involved microorganisms, among others (Cao et al. 2017, Isanta et al. 2015, Pérez et al. 2014). Typical values of this ratio ranged from 0.02 to 0.40 g DO/g TAN. This strategy requires maintaining a residual ammonium concentration in the effluent and therefore, further effluent polishing might be required. Indeed, maintaining residual ammonium concentration (2 - 5 mg NH_4^+-N/L) itself is widely reported to help the NOB suppression (Cao et al. 2017, Ma et al. 2016). At lower ammonium concentrations, the growth rate of AOB slowed down consuming less oxygen and giving the opportunity of NOB to convert nitrite into nitrate (Soler-Jofra et al. 2019).

1.4.1.6. pH value with free nitrous acid and free ammonia concentrations

The influence of pH on the nitritation process is not only related to its direct effect but also to the changes on the FNA and FA concentrations. During nitritation 2 moles of alkalinity are consumed per mol of nitrogen (Equation 1.2). Alkalinity availability might either limit ammonium oxidation or decrease the pH to such low values that AOB activities are negatively affected (Burton et al. 2014). Nevertheless, the pH diminishment could be also an advantage since NOB are more sensitive to pH changes and the FNA. Indeed, AOB are generally more tolerant to FNA and FA than NOB (Vadivelu et al. 2006). These compounds not only affect the bacterial activity but their anabolism, impeding their growth (Ma et al. 2016).

The pH medium during nitritation process would be affected by the carbonate-bicarbonate-CO_2 equilibria as well as the FNA and FA ones (Equations 1.19 to 1.22).

$$H_2CO_3 \rightleftarrows HCO_3^- + H^+, pKa = 6.35 \quad \text{Eq. 1.19}$$

$$HCO_3^- \rightleftarrows CO_3^{2-} + H^+, pKa = 10.33 \quad \text{Eq. 1.20}$$

$$NH_4^+ \rightleftarrows NH_3 + H^+, pK_a = 9.24 \quad \text{Eq. 1.21}$$

$$HNO_2 \rightleftarrows NO_2^- + H^+, pK_a = 3.25 \quad \text{Eq. 1.22}$$

Concentrations of FNA and FA vary with the pH of the medium, temperature and nitrite or ammonium concentrations (Anthonisen et al. 1976). The FNA and FA thresholds for AOB and NOB inhibition are shown in Table 1.2.

Table 1.2. Free nitrous acid and free ammonia inhibition thresholds for nitrifying bacteria.

	AOB	NOB
FNA (mg HNO_2-N/L)	0.40-0.63 [a] [1]	0.022 - 0.22 [2]
FA (mg NH_3-N/L)	10 -150 [3]	0.1 -1.0 [3]

[a] Values for 50 % inhibition instead of 100 %.

References: [1] Vadivelu et al. (2007); [2] Zhou et al. (2011); [3] Anthonisen et al. (1976).

To achieve inhibitory concentrations of FNA and FA at mainstream conditions is difficult (Figure 1.13). In the case of FNA, low pH values (< 7) are required. As nitrite concentration increases the FNA one, to oxidise all the ammonium to nitrite is an advantage (Figure 1.13.A). Regarding FA, high pH values (> 7.5 and usually higher than 8.0) are needed when ammonium concentrations are low. Moreover, as ammonium is oxidised the FA inhibitory concentration decreases (Figure 1.13.B). The FA based strategies require the maintenance of a residual ammonium concentration

in the bulk liquid, thus, in this case, partial nitritation (oxidation of only half of the wastewater ammonium) is recommended.

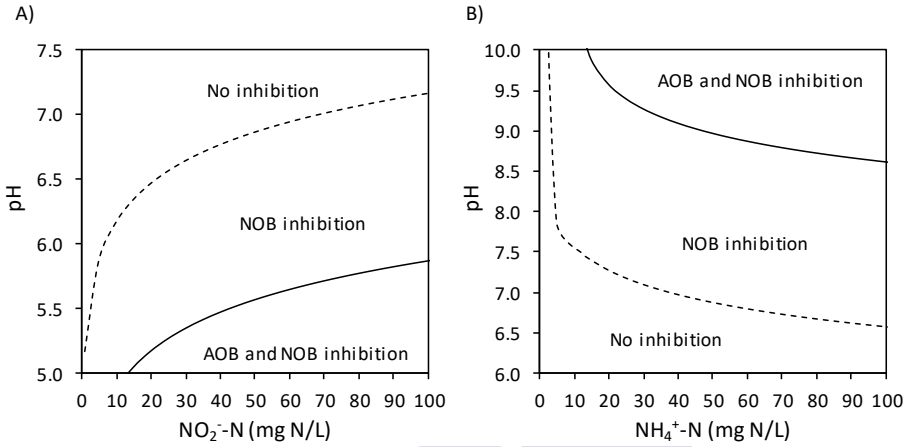

Figure 1.13. A) Prediction of free nitrous acid (FNA) inhibitory effect on nitrifying populations activity as a function of pH and nitrite concentration in the liquid medium: FNA equal to 0.02 mg HNO_2-N/L (---) and 0.4 mg HNO_2-N/L (——). B) Prediction of free ammonia (FA) inhibitory effect on nitrifying populations as a function of pH and ammonium concentration in the liquid medium: FA equal to 0.1 mg NH_3-N/L (---) and equal to 10 NH_3-N/L (——). Calculations were performed according to the equations proposed by Anthonisen et al. (1976) considering a temperature of 15 °C.

1.4.2. One-stage PN/AMX configuration at mainstream conditions

Initially, PN/AMX mainstream studies were focused on the use of one-stage systems due to the successful results obtained at sidestream conditions and due to their lower investment costs (Agrawal et al. 2018, Cao et al. 2017, Lackner et al. 2014). Promising results regarding the operation of PN/AMX systems are reported in laboratory-scale reactors but scarce information about the operation of PN/AMX processes treating municipal wastewater at psychrophilic temperature (< 25 °C) is available (Table 1.3). Moreover, few studies evaluate the system performance under realistic variable ambient temperature, nitrogen concentrations and flows.

Table 1.3. Review of studies performed in one-stage PN/AMX systems at pilot scale treating municipal wastewater at low temperature (≤ 25 °C)

Reactor			**Feeding**				**Process performance**		
Type	Volume (m^3)	T (°C)	$_T$COD (mg/L)	$_S$COD (mg/L)	Nitrogen (mg NH_4^+-N/L)	A-Stage	NLR (mg TN/(L·d))	NRE (%)	**Reference**
CSTR	4.0	23 ± 1[a] 13 ± 1	-	-	33 ± 7 [b] 14 ± 4	HRAS	200 -400 [d] 100 - 400	40 - 60[d] 20 - 50	[1]
SBR	0.6	12 - 18[a]	16 - 197	-	6 - 25 [c]	None	67	50	[2]
Plug flow-IFAS	0.2	19 - 31[a]	41 ± 10	32 ± 7	26 ± 4 [c]	CEPT + HRAS	140	70	[3]
SBR	0.2	15	44 - 71	-	46	UASB	40	40	[4]
CSTR-IFAS	7.0	20	120	-	34 - 41 [c]	HRAS	100	51	[5]
CSTR-IFAS	0.2	25	49 - 106	44 - 88	35 - 50	UASB	100	52	[6]
Plug flow	4.0	19	-	62 ± 17	27 ± 5	Aerobic	400	39	[7]

CEPT: Chemically enhanced primary treatment; $_S$COD: Soluble chemical oxygen demand; $_T$COD: Total chemical oxygen demand; CSTR: Continuous stirred tank reactor; HRAS: High rate activated sludge; IFAS: Integrated fixed activated sludge; NLR: Nitrogen loading rate; NRE: Nitrogen removal efficiency; SBR: Sequencing batch reactor; T: Temperature; TN: Total Nitrogen; UASB: Upflow anaerobic sludge blanket.

[a] Temperature not controlled.

[b] Biodegradable oxygen demand to nitrogen ratio ranging from 0.3 to 4 g BOD_5/g N.

[c] Expressed as total nitrogen (TN).

[d] values estimated throughout figures from the cited research work.

References: [1] Hoekstra et al. (2019); [2] Pedrouso et al. (2018); [3] Han et al. (2016); [4] Trojanowicz et al. (2016); [5] Seuntjens et al. (2016); [6] Malovanyy et al. (2015); [7] Lotti et al. (2015b).

Han et al. (2016) achieved a NRE up to 70 % treating a nitrogen loading rate (NLR) of 140 mg TN/(L·d) without temperature control which ranged from 19 to 31 °C. At lower temperatures (12 - 18 °C), Pedrouso et al. (2018) obtained a NRE of 50 % treating primary settled municipal wastewater with a NLR of 67 mg TN/(L·d). Despite the COD presence, a high composition variability and the low temperature, mass balances indicated that the PN/AMX processes constituted the main N removal pathway. The critical effect of the temperature decrease was also reported by Hoekstra et al. (2019) who observed a diminishing of NRR from 223 ± 29 mg TN/(L·d), at 23.2 ± 1.3 °C, to 97 ± 16 mg TN/(L·d) at winter temperatures 13.4 ± 1.1 °C (Table 1.3).

Lotti et al. (2015b) evaluated the PN/AMX processes in a one-stage plug flow granular system operated at 19 °C treating aerobically pretreated wastewater (with a sCOD/N of approximately 2.3 g/g) and obtained NRE of approximately 39 ± 8 % at NLR higher than 400 mg TN/L. These authors observed difficulties to maintain the appropriated balance of microbial populations and detected the development of NOB whereas heterotrophic denitrifiers did not outcompete the anammox bacteria. On the contrary, Seuntjens et al. (2016) applied a NLR of 90 - 100 mg TN/(L·d), at 20 °C, with a COD/N ratio higher than 3.5 g COD/g N and achieved NRE of 51 ± 23 % but the nitrite nitrogen removal ranged from 62 to 49 % whereas the rest of N was removed by conventional heterotrophic denitrification (Table 1.3).

In many of these pilot-scale research works, PN/AMX systems were fed with municipal wastewater artificially supplemented with ammonium increasing the NLR and softening the wastewater composition fluctuations (Hoekstra et al. 2019, Seuntjens et al. 2016). Moreover, chemicals (NaOH or Na_2HCO_3) were supplied to control the pH in the range of 7.0 to 7.5 (Hoekstra et al. 2019, Lotti et al. 2015b, Seuntjens et al. 2016). However, this addition of chemicals is not recommended due to its high costs associated with the mainstream elevated water flows.

Overall, the NOB suppression was identified as one of the most challenging aspects for the one-stage PN/AMX system implementation at mainstream conditions. The fact that long SRT are applied in these systems challenges the NOB washout. Indeed, the nitrate produced by NOB activity limited the NRE and effluent quality in many research works at both laboratory (De Clippeleir et al. 2013, Hu et al.

2013, Morales et al. 2016) and pilot-scale (Hoekstra et al. 2019, Lotti et al. 2015b, Pedrouso et al. 2018, Seuntjens et al. 2016).

The ability to decouple the SRT of different populations (i.e., hybrid systems) arose as an exciting approach to improve the process stability by selectively washing out the NOB. Malovanyy et al. (2015) obtained a NRE of 52 % treating a NLR of 100 mg TN/(L·d) in an integrated fixed-bed activated sludge (IFAS) system at 25 °C fed with anaerobically pretreated wastewater. However, nitrate was still observed in the pilot-scale IFAS systems (Han et al. 2016, Malovanyy et al. 2015).

More recently and posterior to the research study performed in this thesis, Laureni et al. (2019) operated a laboratory-scale unit (12 L) and achieved stable PN/AMX processes performance with a NRR of 80 mg TN/(L·d) in a sequencing batch reactor (SBR) IFAS system. This system treated aerobically pretreated municipal wastewater at 15 °C by controlling the SRT of the flocculent sludge and suppressing the NOB activity in the biofilm by limiting the DO concentration. The achieved NRE of 88 ± 5 % and residual TN concentrations of 3 mg TN/L constitute the best performance and produced effluent quality reported so far. The process performances at pilot-scale facing the wastewater composition fluctuations should be assessed. Moreover, Li et al. (2019) reported in a PN/AMX IFAS system (at 30 °C), where granules and flocculent biomass coexisted, the significant reduction of NOB activity but also AOB one by reducing the SRT from 30 to 20 days. Thus, further optimisation of the applied SRT-control strategy is required.

1.4.3. Two-stage configuration

Due to the observed difficulties in implementing the PN/AMX processes at mainstream conditions with the one-stage configuration, some research studies were recently focused on splitting the system into two stages optimising both processes separately (Cao et al. 2017). With this configuration, in the aerobic nitritation unit, the remaining organic matter coming from the A-stage will be aerobically oxidised decreasing the potential for heterotrophic denitrifying bacteria development and fostering the autotrophic N removal. Then, in the anoxic unit, the anammox biomass retention can be maximised since no biomass out selection is required. Furthermore, the anammox activity is expected to be promoted as neither COD nor DO will be present in the liquid media. However, scarce information is

available, in general, for the operation of mainstream PN/AMX processes, and particularly in the two-stage configuration, treating municipal wastewater (Agrawal et al. 2018, Cao et al. 2017, Pedrouso et al. 2018). In the following sections, a brief description of the main reported studies (still at laboratory scale) are presented.

1.4.3.1. Nitritation process

Different strategies for the achievement of the nitritation process were tested but in most of the studies treating municipal wastewater, the long-term stable nitrite accumulation was an issue.

The major part of the reported successful strategies relies on the treatment of the nitritation sludge in an external unit by exposing it to inhibitory conditions for NOB. Cui et al. (2019a) proposed to treat the sludge with nitrite. This strategy was characterised by long start-up periods (30 days after 32 days of nitrite exposure). They treated municipal wastewater, but the operational period in the nitritation unit lasted only for 50 more days. Another common strategy is the achievement of the nitritation process by subjecting it to FNA inhibitory concentrations (Wang et al. 2016, Wang et al. 2014). For successful NOB suppression, the combination of DO control and FNA treatment is required (Wang et al. 2016). Indeed, nitrate production was observed when the DO concentration increased from 0.5 to 3 mg O_2/L and the stable nitritation process completely disappeared at DO concentrations of 5 mg O_2/L (Jiang et al. 2018). Moreover, NOB can ultimately adapt to the relatively high FNA concentration (Duan et al. 2019, Ma et al. 2017). Duan et al. (2019) observed that this adaptation phenomenon could be overcome by alternating FNA and FA treatment of the biomass. The use of the FNA-strategy to treat the sludge requires another nitritation reactor in operation at sidestream, that has associated potential high N_2O emissions due to the vast accumulated nitrite concentrations (Jiang et al. 2019, Kampschreur et al. 2009). Optimization of the sludge treatment in terms of concentration of toxic (FNA or FA), ratio of sludge treated and frequency of exposure is required (Duan et al. 2018, Jiang et al. 2018).

Other authors controlled the DO/TAN ratio for establishing the nitritation process (Isanta et al. 2015, Reino et al. 2016). These authors operated granular nitritation systems, treating synthetic media, and obtained ammonium oxidation rates of 630 - 700 mg TN/(L·d) at temperatures as low as 10.0 - 12.5 °C. Nevertheless, this strategy seems to be only feasible for granular biomass. Liu et al. (2017b) did not

manage to suppress the NOB in flocculent sludge based on DO/TAN ratio control although higher temperature was applied (22 °C).

Zhang et al. (2018) obtained promising results treating municipal wastewater with 91 - 232 mg COD/L and 39 - 79 mg NH_4^+-N/L at 12 - 17 °C. These authors obtained a nitrite accumulation ratio (NAR) of 97.3 % and COD removals ranging from 60 to 85 % by alternating anaerobic-aerobic conditions for 150 days. The control of the aeration time based on the ammonium valley also produced excellent results of nitrite accumulation when municipal wastewater (containing also organic matter) is treated at low temperatures (Yang et al. 2007). Indeed, (Gu et al. 2012) obtained, at 11 - 16 °C, a stable NAR of 90 % in a pilot-scale reactor (7 m^3) treating municipal wastewater by controlling the frequency of the blower supplying air to the system and the pH of operation.

1.4.3.2. Anammox process

Few studies addressed the study of single anammox reactors at mainstream conditions (De Cocker et al. 2018, Hendrickx et al. 2014, Laureni et al. 2015, Lotti et al. 2014b, Ma et al. 2013, Reino et al. 2018, Sánchez Guillén et al. 2016). Their main feature is the specific anammox activity (SA_{AMX}) temperature dependence (Dosta et al. 2008, Lotti et al. 2015c, Tomaszewski et al. 2017). It was reported an SA_{AMX} decrease of approximately 10-fold when the temperature diminished from 30 °C to 10 °C (De Cocker et al. 2018). This SA_{AMX} loss might be overcome if sufficient biomass is accumulated in the system. Long SRT are required since doubling time increased from 35 to 77 days with the temperature decrease from 20 to 15 °C (Lotti et al. 2014b). Reino et al. (2018) successfully operated an upflow anammox sludge bed reactor at decreasing temperatures. Despite these authors reported an adverse effect on the anammox process performance after shifting from artificial feeding to municipal wastewater, they achieved a NRR of 1,200 ± 500 mg TN/(L·d) at 11 °C.

Less information is available about the performance of the coupled nitritation and anammox processes to treat municipal wastewater in two-stage configuration systems. Liu et al. (2018a) operated a two-stage system treating anaerobically pretreated municipal wastewater at 7 - 15 °C achieving NAR of 98 % in the aerobic reactor by applying the DO/TAN ratio control strategy. In the anammox reactor, a NRE of 80 % and a NRR of 70 - 280 mg TN/(L·d) were reached. Nevertheless, the operational period was limited to less than 60 days. Jin et al. (2019) obtained high

NRE ranging from 78 to 90 % by controlling the nitritation process by the ammonium valley based strategy. Nevertheless, the operational temperature was higher than the ones commonly used at mainstream conditions with values of 23 - 25 ° C in the nitritation reactor and 29 - 30 °C in the anammox reactor.

1.4.4. Main challenges of autotrophic nitrogen removal in mainstream

Apart from the previously explained conditions required to operate the PN/AMX systems in stable conditions, several challenges still need to be addressed before their practical application at mainstream. Besides NOB suppression, other remaining issues are the control of the fast-growing heterotrophic bacteria overgrowth, the maintenance of good AOB and anammox activities balance, the achievement of good biomass retention and to fulfil the N discharge limits while treating the nitrogen loads (Cao et al. 2017, Hoekstra et al. 2019, Ma et al. 2016). The mainstream municipal wastewater characteristics that slow down the PN/AMX processes implementation are discussed below.

1.4.4.1. Low temperature

Although mainstream is characterised by low temperatures that vary throughout the year ranging from 10 to 25 °C (Lackner et al. 2015), most of the studies both at laboratory scale and pilot-scale were performed with temperature control. The study of PN/AMX processes at low temperature and/or without temperature control needs to be further explored.

The low temperature causes a decrease in the bacterial activities and growth rates of all microorganisms involved decreasing the potential treated loads (Agrawal et al. 2018). Therefore, to ensure enough biomass retention to cope with the present loads (above 50 mg TN/(L·d) (Burton et al. 2014) is a requisite, in particular for anammox bacteria. This aspect is more challenging in the one-stage configuration than in the two-stage one since anammox bacteria are more sensitive to temperature changes (especially at low temperature) than the nitritation process hindering the maintenance of AOB and anammox bacteria balance (Lotti et al. 2015c, Ma et al. 2016). Moreover, the low temperatures limit the efficiency of the strategy based on controlling the SRT to suppress NOB activity (Cao et al. 2017).

1.4.4.2. Low concentrations and high flows

The treatment of diluted wastewater is a challenge due to the low net biomass production associated to the low N concentrations (20 - 60 mg TN/L) treated and to the biomass washout provoked by the high flowrates, which lead to relatively high applied hydraulic loads. To optimise the biomass retention and define the optimum operational HRT maintaining the PN/AMX processes reliability is of great interest. Moreover, the low N concentrations difficult the accumulation of FA and FNA inhibitory concentrations needed to suppress NOB activity compared to the operation with N-rich wastewater streams (Agrawal et al. 2018, Duan et al. 2019).

1.4.4.3. Presence of organic matter

The required COD/N ratio (usually below 2 - 3 g COD/g N, Agrawal et al. (2018)) to achieve stable PN/AMX processes performance is difficult to be found at mainstream conditions and hinders its application as it triggers the heterotrophic bacteria proliferation (Leal et al. 2016, Liu et al. 2018b, Xu et al. 2015). The fact that heterotrophic bacteria easily overgrow anammox bacteria is widely reported for one-stage systems (Hoekstra et al. 2019, Lotti et al. 2015b, Seuntjens et al. 2016). Moreover, if COD is present in the influent to the PN/AMX stage its oxidation will increase the aeration requirements and worsen the energy balance of the WWTPs. In the case of two-stage systems, most of the available studies are performed with synthetic wastewater without organic matter. Thus, this aspect should be further investigated to determine whether the residual COD coming from the A-stage could deteriorate the anammox process stability (by its inhibitory effect or heterotrophic bacteria overgrowth) or promote the NRE by polishing the anammox effluent (Giustinianovich et al. 2016, Jin et al. 2019). Moreover, the optimisation of a robust A-stage for organic matter removal and the definition of the COD/N limits that the PN/AMX system can tolerate at mainstream conditions is crucial.

1.4.4.4. Wastewater alkalinity limitations

Ammonium oxidation consumes alkalinity. However, when combined with the anammox process, wastewater cannot compensate for this loss as in the case of the denitrification. Therefore, in these conditions, the wastewater buffering capacity could limit the feasibility of the PN/AMX processes due to both the pH decrease or the insufficient IC for the growth of autotrophic bacteria. This effect was investigated

during the treatment of industrial wastewater at moderate temperature (Sun et al. 2016) or in batch tests (Kimura et al. 2011, Torà et al. 2010). Although no information is available for the treatment of mainstream in municipal WWTPs.

1.4.4.5. Fluctuation of wastewater characteristics

The impact of the fluctuations of the inlet nitrogen concentrations and temperature need to be considered in the future as a possible factor that can compromise the fulfilment of the discharge limits (Pedrouso et al. 2018). Wastewater characteristics vary seasonally (mainly due to the rainfalls and temperature changes) and daily due to the population habits (Burton et al. 2014). The frequent variation in feeding composition can cause over aeration, resulting in a failed suppression of NOB activity, or under aeration, provoking a limitation of AOB activity restricting the nitrite supplied for anammox bacteria. Severe deterioration of NRE would subsequently occur due to the imbalance between control parameters in time (Jin et al. 2019). More straightforward and dynamic control systems are required to cope with these wastewater fluctuations. However, most of the studies are performed under a controlled environment (mainly with artificial feeding and temperature control) without needing to cope with the wastewater characteristics fluctuation that usually WWTPs have to face (Agrawal et al. 2018, Pedrouso et al. 2018). The system resilience to high wastewater variability in terms of temperature and composition need to be further researched to improve the PN/AMX process control and to select the controlled and modifiable parameters under realistic mainstream conditions.

1.4.4.6. Source separation wastewater management

Finally, despite source-separation on-site wastewater treatment systems arose as an efficient wastewater management approach, information about the nitrogen removal in these systems is limited in the literature (de Graaff et al. 2011, Vlaeminck et al. 2009). The digested blackwater is characterised by higher and more stable N concentrations and temperatures than municipal wastewater (Eshetu Moges et al. 2018, Gao et al. 2019). Therefore, the implementation of the PN/AMX processes should be more natural than with more diluted streams such as the mainstream of municipal wastewater (Gonzalez-Martinez et al. 2016, Morales et al. 2016). Nevertheless, in small WWTPs the daily pattern of wastewater fluctuations is more acute and wastewater availability might be an issue especially in those systems

treating waster from office buildings, shops or recreational areas where the lack of water in no-working periods might stop the treatment train.

1.5. Aims and scope

The overall objective of the present doctoral thesis is to research the feasibility of implementing the autotrophic nitrogen removal PN/AMX processes in conditions of low temperature and low nitrogen concentrations (i.e., mainstream conditions). Special attention is paid to assessing the performance of two-stage PN/AMX systems, while one-stage system feasibility is also evaluated for decentralised wastewater treatment.

To accomplish the main aim of this thesis, the following specific goals were defined:

- To demonstrate the feasibility of the application of the PN/AMX processes to treat, at ambient temperature, anaerobically digested blackwater originated in decentralised wastewater treatment systems where wastewater availability is discontinuous (Chapter 3).
- To stimulate the AOB growth over NOB to operate the nitritation process stable at mainstream conditions (Chapters 4 to 6). More specifically:
    - To promote the NOB washout while retaining the AOB inside a continuous reactor to accumulate nitrite through a progressive increase of the applied hydraulic load by progressively decreasing the HRT (Chapter 4).
    - To develop and asses a strategy based on the *in situ* FNA production to achieve the nitritation process and to maintain its long-term stability. The effect of the influent N/IC ratio over the NOB suppression based on this strategy will be studied as well (Chapter 5).
    - To prove the feasibility of achieving and maintaining the stable nitritation process treating primary settled municipal wastewater containing organic matter. The operational strategy will be optimised to perform in the same unit the long-term nitritation and organic matter oxidation processes in stable conditions (Chapter 6).

- To evaluate the feasibility of applying the anammox process at mainstream conditions using anammox granular sludge without being previously acclimated to low nitrogen, neither low temperature. Firstly, to study the long-term process stability, the reactor will treat mimicked wastewater to have a better understanding of the process performance under different N/IC ratios. Then, the effect of treating partially nitritified municipal wastewater will be evaluated mainly focusing on the effect of pH, low temperature and the presence of organic matter that might lead to the heterotrophic bacteria competition (Chapter 7).
- To test the implementation of the combination of the partial nitritation and anammox processes fed with municipal wastewater at pilot-scale (Chapters 8 and 9). Two different configurations will be evaluated treating different pre-treated wastewaters:
    - To analyse the performance and stability of an innovative hybrid one-stage PN/AMX reactor (IFAS system) to remove the nitrogen from the effluent of an UASB reactor. The effect of the low temperatures typically found at mainstream conditions will be evaluated by step-wise decreasing the temperature from 21 to 15 °C. The microbial population segregation between suspended and biofilm fractions will be evaluated to understand the role of the reactor configuration for the NOB suppression (Chapter 8).
    - To explore the feasibility of implementing the two-stage PN/AMX processes treating the effluent of an HRAS full-scale plant. The two-stage system will be operated without temperature control and coping with the same wastewater characteristic fluctuations that faced the municipal WWTP where it was located (Chapter 9).
- To identify the main outcomes of the thesis and the main gaps that deserve further exploration before the process implementation (Chapter 10).

1.6. References

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Chapter 2

Materials and methods

SUMMARY

The general analytical methods used during the experimental work performed in this thesis are summarised within this chapter. A description of the methodology applied to measure the conventional physicochemical parameters used for the wastewater (e.g., organic matter, alkalinity, nitrogen compounds, pH) and biomass characterisation is presented.

The biomass was characterised by means of parameters such as sludge volume index, granule size and the specific bacterial activities. A description of the molecular techniques applied for the identification of the microbial populations involved in the biological processes is also included in this chapter.

Finally, the descriptions of the calculations, based on mass balances and process stoichiometry, used in different chapters of this thesis are also provided. The specific analytical methods or the calculations used exclusively in a single study and the corresponding experimental setups are described in the corresponding chapters.

OUTLINE

2.1. Analysis of the liquid phase

This section describes the analytical methods employed for the determination of the conventional parameters to characterise wastewater. Samples were filtered through a mixed cellulose and ester filter of 0.45 µm pore size (Advantec, Japan) to remove suspended solids and colloids for soluble fraction analysis. When unfiltered samples were used, it was indicated.

2.1.1. Carbon compounds

2.1.1.1. Chemical oxygen demand

The Chemical Oxygen Demand (COD) is defined as the amount of oxygen required to oxidise all the organic matter present in a sample (wastewater) using a strong chemical oxidant (potassium dichromate) in an acid medium. A catalyst (silver sulphate) is used to improve the oxidation of some organic compounds during the digestion of the samples. The occurring global reaction is:

$$Cr_2O_7^{2-} + Organic\ Matter\ (6\ e^-) + 14\ H^+ \xrightarrow{Ag_2SO_4} 2\ Cr^{3+} + CO_2 + 7\ H_2O$$

The COD determination is performed based on the method 5220-D described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 2012) and measuring the quantity of unreduced oxidant that remains after the digestion. In general, the modification proposed by Soto et al. (1989) is used to measure the quantity of remaining $K_2Cr_2O_7$ by titration of the digestion solution with ferrous ammonium sulphate (FAS) solution and using a ferroin solution as indicator. In Chapters 8 and 9, the COD concentration is analysed by following a commercial colourimetric method using Hach Lange Kits and Dr. Lange Spectrophotomer (Hach Lange, Germany) based on the measurement of the green colour of the reduced Cr^{3+}. The amount of oxidable organic matter is expressed in terms of its oxygen equivalents. The soluble and total COD (sCOD and tCOD) are determined depending whether the sample is filtrated or not.

In the following sections, the reagents and procedure applied for the standard method are described. The interferences described are common for both methods.

Reagents

- Concentrated potassium dichromate digestion solution: 10.23 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved into 500 mL of distilled water. Afterwards, 167 mL of concentrated H_2SO_4 are added. Finally, distilled water is supplied to obtain a final volume of 1 L of solution, which is then cooled down to room temperature before using it.
- Diluted potassium dichromate digestion solution: 2.44 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved into 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 are added, and afterwards distilled water is supplied to reach a total volume of 1 L.
- Sulphuric acid catalytic solution: 10.7 g of Ag_2SO_4 are dissolved into 1 L of concentrated H_2SO_4. The solution needs to be prepared at least two days before being used.
- Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2{\cdot}H_2O$ (phenanthroline monohydrate) and 0.695 g of $FeSO_4{\cdot}7\ H_2O$ are dissolved in 100 mL of distilled water.
- Standard potassium dichromate solution (0.05 N): 1.226 g of $K_2Cr_2O_7$, previously dried at 105 °C for 2 hours, are dissolved in 500 mL of distilled water.
- Concentrated FAS titrant solution (0.035 N): 13.72 g of $Fe(NH_4)_2(SO_4)_2{\cdot}6\ H_2O$ are dissolved in distilled water. Then, 20 mL of concentrated H_2SO_4 are added, and finally, the solution is cooled down and diluted to 1 L.
- Diluted FAS titrant solution (0.016 N): 6.28 g $Fe(NH_4)_2(SO_4)_2{\cdot}6\ H_2O$ are dissolved in distilled water and 20 mL of concentrated H_2SO_4 are added. Finally, distilled water is added to reach a total volume of 1 L.

Concentrated FAS and digestion solution of potassium dichromate are used for samples with a COD concentration ranging from 90 to 900 mg COD/L whereas the diluted ones are used for samples with a COD concentration below 90 mg COD/L.

Determination procedure

A volume of 2.5 mL of each wastewater sample (diluted if it is necessary) is placed into a 10 mL Pyrex® glass tubes. Then, 1.5 mL of the digestion solution (concentrated or diluted according to the organic matter content of the sample) are

added, and 3.5 mL of sulphuric acid reagent is slowly poured on the inner wall of the tube, which must be slightly inclined to avoid any possible mixing and consequent beginning of the oxidation reaction. A blank sample using distilled water is prepared in the same way, and it acts as "reference" value. Later, the Pyrex® glass tubes are tightly sealed with Teflon® and covered with Bakelite® caps to avoid the leakage of produced gases. Then, tubes are mixed by inverting them three times to achieve the homogenous mixture, and finally, tubes are placed in the block thermodigester (VELP ECO16) preheated at 150 °C. After 2 hours of digestion, the tubes are cooled down to room temperature, and the content is transferred to a beaker, and once 1 or 2 drops of ferroin indicator are added, the solution is titrated under rapid stirring with the corresponding FAS solution.

The COD concentration (in mg COD/L) of the different wastewater samples is calculated with the Equation 2.1:

$$\text{COD (mg/L)} = \frac{(A - B) \cdot 8{,}000 \cdot N_{FAS}}{V} \qquad \text{Eq. 2.1}$$

where:

A: is the volume of FAS consumed by the blank (mL).

B: is the volume of FAS consumed by the sample (mL).

8,000: is the milliequivalent weight of oxygen in 1,000 mL/L.

N_{FAS}: is the normality of the FAS solution (N).

V: is the volume of sample used in the titration procedure, equal to 2.5 mL.

The concentration of the FAS solutions as normality is measured together with the samples. For this purpose, 3.5 mL of H_2SO_4 are mixed with 5 mL of distilled water, cooled down, and then 5 mL of standard potassium dichromate solution are supplied. Then this mixture is titrated after the addition of 3 drops of ferroin solution, to visualise the titration end-point by the change of colour from blue-green to reddish-brown. The normality of the FAS solution is calculated using Equation 2.2:

$$N_{FAS} = \frac{V_{K_2Cr_2O_7} \cdot N_{K_2Cr_2O_7} \cdot}{V_{FAS}} \qquad \text{Eq. 2.2}$$

where:

$V_{K_2Cr_2O_7}$: is the added volume of the standard $K_2Cr_2O_7$ solution, equal to 5 mL.

$N_{K_2Cr_2O_7}$: is the normality of the standard $K_2Cr_2O_7$ solution, equal to 0.05 N.

V_{FAS}: is the volume of FAS solution consumed in the titration procedure.

Interferences

The most common interference is the chloride anion that reacts with silver cation precipitating silver chloride and inhibiting the catalytic activity of silver. The used Hach Lange kits allow measuring COD in the presence of maximum chloride concentrations of 1,500 mg/L (kits for salty samples are also available), whereas Soto et al. (1989) proposed an adaptation of the method to be applied to high salinity water samples (up to 30 g Cl^-/g COD). Bromide, iodide and any other reagent that inactivates the silver ion can interfere similarly. These interferences can be mainly overcome, though not wholly, by complexing these ions with mercuric sulfate. Ammonia and its derivates are not oxidised. However, elemental chlorine reacts with these compounds. Hence, corrections for chloride interferences are difficult. Nitrite exerts a COD of 1.1 mg COD/mg NO_2^--N but this interference can be eliminated by the addition of 10 mg of sulfamic acid per mg of nitrite present in the sample. Note that sulfamic acid should also be added to the blank sample. Reduced inorganic species (e.g., ferrous iron or sulphide) are oxidised quantitatively under the test condition. For samples containing significant concentrations of these species in a known concentration, stochiometric oxidation can be assumed and COD correction can be made to the obtained COD value.

2.1.1.2. Dissolved total, organic and inorganic carbon

The organic carbon in water and wastewater samples include a variety of organic compounds in different oxidation states. Some of these carbonaceous compounds can be further oxidised by biological or chemical processes and the biochemical oxygen demand (BOD_5) and COD may be used to characterise these fractions. Unlike COD, total organic carbon (TOC) is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganic compounds that can contribute to the oxygen demand measured by COD (APHA-AWWA-WEF, 2012). The measure of TOC is performed according to the method 5310 of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 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 is determined by a Shimadzu analyser (TOC-L CSN) as the difference between the total carbon (TC) and the inorganic carbon (IC)

concentrations. The instrument is connected to an automated sampler (Shimadzu, ASI-L). Dissolved carbon forms are measured as the equipment only accepts filtered samples.

The TC concentrations are determined from the amount of CO_2 produced during the combustion of the sample at 720 °C by using platinum immobilised over alumina spheres as a catalyst. High purity air is used as carrier gas supplied at a flow rate of 150 mL/min. The combustion products are carried to an electronic dehumidifier where the gas is cooled and dehydrated. Then, the gas carries the sample combustion products through a halogen scrubber to remove chlorine and other halogens. Finally, the produced CO_2 is detected with a non-dispersive infrared (NDIR) analyser, which generates a peak with an area related to the concentration of the compounds.

The measured IC consists of carbon derived from carbonates, hydrogen carbonates and dissolved carbon oxide. The IC concentration is obtained from the amount of CO_2 produced by acidifying the sample with hydrochloric acid (HCl) 1 N at room temperature to obtain a pH below 3. Then, the produced CO_2 is detected with the NDIR analyser.

A calibration curve in the range of 0.5 to 200 mg C/L is used for the quantification of the carbon compounds. Commercial standard solutions of total inorganic carbon and total organic carbon of 1,000 ± 5 mg C/L are used as standards to obtain an equation that mathematically expresses the relationship between peak area and TC, TOC and IC concentrations. The detection limit of the equipment is 50 µg/L and 4 µg/L for TC and IC, respectively.

2.1.2. Nitrogen compounds

In water and wastewater samples, the forms of nitrogen of greater interest are, in order of decreasing oxidation state: nitrate, nitrite, ammonia and organic nitrogen. All these forms, as well as dinitrogen gas (N_2), are biochemically inter-convertible following the processes of the nitrogen cycle.

2.1.2.1. Total nitrogen

Total Nitrogen (TN) is the sum of organic and inorganic nitrogen. The TN is determined in the same Shimadzu analyser (TOC-L CSN) as the TOC concentration

coupled to a TNM-L Unit (Shimadzu). The sample is injected in the combustion tube where the oxidative pyrolysis occurs at 720 ˚C, to convert all nitrogenous compounds into nitric oxide gas (NO). Dinitrogen gas does not become NO under these conditions. The carrier gas together with the produced NO are cooled by an electronic dehumidifier to eliminate any possible condensation. The obtained NO is forced to react with in-situ produced ozone (O_3) obtaining nitrogen dioxide in an unstable excited state (NO_2*). The NO_2* reached its fundamental state by releasing a proton while emitting a photon in the range of 590 - 299 nm, which is detected by a chemiluminescence detector (CLD). The calibration curve is carried out using a standard commercial solution of 1,000 mg NH_4^+/L (Merck) in the range of 0.5 - 777 mg TN/L. The analyser detection limit is 20 µg TN/L.

2.1.2.2. Ammonium

Ammonium concentration is determined spectrophotometrically using two different methods based on the formation of the indophenol blue: 1) the Hach Lange Kits and Dr. Lange Spectrophometer (Hach Lange, Germany) and 2) the method proposed by Bower and Holm-Hansen (1980). In the second method, the indophenol blue is produced by the reaction of the ammonium ions, at pH 12.6, with salicylate and hypochlorite ions, in the presence of sodium nitroprusside as catalyst (Bower and Holm-Hansen 1980). The characteristic blue colour produced by increasing concentrations of ammonia makes the assay useful for the direct, visual estimation of ammonia in culture systems. In Chapters 8 and 9, the Dr. Lange cuvettes instructions are followed, whereas for the Bower and Holm-Hansen (1980) reagents preparation and procedure are detailed below.

Reagents

- Reagent A: solution of 0.28 g/L of sodium nitroprusside ($C_5FeN_6Na_2O$) and 440 g/L of sodium salicylate ($C_7H_5NaO_3$).
- Reagent B: solution of 18.5 g/L of sodium hydroxide (NaOH) and 120 g/L of trisodium citrate ($Na_3C_6H_5O_7$).
- Reagent C: standard commercial solution of sodium hypochlorite (NaOCl) with 4.00 to 4.99 % active chlorine.
- Reagent D: solution prepared by mixing 7 parts of reagent B and 1 part of reagent C. Reagent D is stable for 1 hour after preparation.

- Standard commercial solution of 1,000 ± 4 mg NH_4^+/L (Sigma-Aldrich).

Determination procedure

The maximum detection limit of the method is 0.9 mg NH_4^+-N/L; thus, the filtered sample will be diluted if necessary. Then, reagent A (600 µL) and reagent D (1 mL) are added to 5 mL of sample. Samples with reagents are gently shaken and stored, protected from light, to react for more than 2 hours but less than 3 hours. The measurements of the coloured samples are done with a spectrophotometer (Shimadzu, UV-1800) at a wavelength of 640 nm. The quantification is done with a calibration curve in the range of 0 – 0.9 mg NH_4^+-N/L using a commercial solution of NH_4Cl as standard solution and obtaining a regression equation that correlates the measured absorbances with the corresponding ammonium concentration.

Interferences

Residual chlorine reacts with ammonia and should be removed by sample pretreatment. The determination should be promptly made on fresh samples in order to avoid bacterial conversion. At least filtration of the samples should be done immediately after collection.

2.1.2.3. Nitrite

Nitrite concentration is determined following the method 4500-NO_2^--B (Colourimetric Method) described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 2012). The standard procedure is used in all studies except in Chapters 8 and 9 where Hach Lange cuvette kits are employed. Nitrite reacts with primary aromatic amines in acidic solutions to form diazonium salts. These salts combine with aromatic compounds that contain an amino group or a hydroxyl group to form intensively coloured azo dyes. According to the standard method 4500-NO_2^--B, nitrite is determined through the formation of a reddish-purple azo dye produced at pH 2.0 - 2.5 by coupling diazotised sulphanilamide ($C_6H_8N_2O_2S$) with N-(1-napththyl)-ethylenediamine dihydrochloride (NED dihydrochloride). The reagents preparation and the determination procedure for this method are described below.

Reagents

- Sulphanilamide: 10 g of sulphanilamide ($C_6H_8N_2O_2S$) are dissolved in 100 mL of concentrated HCl and 600 mL of distilled water. After cooling, the volume is filled up to 1 L with distilled water.
- NED solution: 0.5 g of NED are dissolved in 500 mL of distilled water.
- Standard commercial solution of 1,000 ± 4 mg/L of NO_2^- (Sigma-Aldrich).

Determination procedure

The applicable range of the method is from 0 to 0.3 mg NO_2^--N/L, therefore samples might be diluted to fit within this range if it is necessary. Then, add 100 µL of each reagent (sulphanilamide and NED) to a volume of 5 mL of sample. After waiting 20 minutes (in the dark) for colour stabilisation, the coloured samples (pink) are measured in a spectrophotometer (Shimadzu, UV-1800) at a wavelength of 543 nm. The quantification is done with a calibration curve in the range of 0 - 0.3 mg NO_2^--N/L, using $NaNO_2$ as standard.

Interferences

Nitrite is highly unstable; therefore, the determination should be promptly made on fresh samples in order to avoid bacterial conversion of nitrite. At least filtration of the samples should be conducted immediately after collection. Chemical incompatibility makes it unlikely that nitrite, free chlorine and nitrogen trichloride (NCl_3) coexist in the same sample. NCl_3 imparts a false red colour when the colour reagent is added. The following ions interfere because of precipitation under test conditions and should be absent: Sb^{3+}, Au^{3+}, Bi^{3+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^+, chloroplatine and metavanadate. Moreover, cupric ion may cause low results by catalysing the decomposition of the diazonium salt.

2.1.2.4. Nitrate

Nitrate concentration is determined according to the method 4500-NO_3^--B (Ultraviolet (UV) Spectrophotometric Screening Method) described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 2012) or by the Dr. Lange cuvette kits (Hach Lange) in Chapters 8 and 9. The presence of nitrite in the sample causes interferences on the nitrate concentration measured value (determined by both methods). For this reason, a spatula-tipfull of sulfamic

acid is added to the sample to remove the associated nitrite interferences. The colourimetric method using Hach Lange kits is based on the reaction of nitrate ions in solutions containing sulphuric and phosphoric acids with 2,6-dimethylphenol forming 4-nitro-2,6-dimethylphenol. Then, following the Dr. Lange Hach instructions nitrate concentration is directly obtained in a spectrophotometer.

The method 4500-NO_3^--B used is based on the direct measurement of the nitrate in acid media. Measurement of UV absorption at 220 nm enables the rapid determination of NO_3^- ions. A second measurement at 275 nm is used to correct the NO_3^- value since the dissolved organic matter can absorb at 220 nm and NO_3^- does not absorb at 275 nm. This method is described below.

Reagents

- Hydrochloric acid: a solution of HCl 1 N.
- Sulfamic acid (H_3NSO_3) powder.
- Standard commercial solution of 1,000 ± 4 mg/L of NO_3^- (Sigma-Aldrich).

Determination procedure

In a volume of 5 mL of sample (diluted if necessary, to fit the concentration range of the method, up to 3 mg NO_3^--N/L), 100 µL of HCl (1 N) and a spoon of sulfamic acid are added (approximately 1 - 2 mg). After being thoroughly mixed, the absorbance at 220 and 275 nm is measured in a spectrophotometer (Shimadzu, UV-1800) using rectangular quartz cells. The absorbance related to nitrate is obtained by subtracting two times the absorbance read at 275 nm from that read at 220 nm. If the correction value is more than 10 % of the reading at 220 nm, this method should be substituted. The quantification is done with a calibration curve in the range of 0 - 3.0 mg NO_3^--N/L, using a KNO_3 solution as standard.

Interferences

Dissolved organic matter, surfactants, nitrite and Cr^{6+} interfere with nitrate determination. Moreover, various inorganic ions such as chlorite and chlorate may interfere. The determination should be promptly made on fresh samples in order to avoid bacterial conversion of nitrite. At least the filtration of the samples should be performed immediately after the samples collection. For more extended storage periods of unchlorinated samples (more than two days), preserve with 2 mL of

concentrated H_2SO_4 (98 %) and store at 4 °C. It is worth to note that when the sample is preserved with acid, nitrate and nitrite cannot be determined as single species.

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-}), thiosulphate ($S_2O_3^{2-}$) and the cations sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) are determined by ion chromatography using a 861 Advanced Compact ion chromatography system equipped with a CO_2 suppressor (MCS 853, Metrohm) and a 838 Advanced Sample Processor (Metrohm, Switzerland). Table 2.1 shows the calibration ranges for the different inorganic ion concentrations. Therefore, if it is necessary samples with a higher concentration of one compound should be diluted with distilled water to fit these ranges.

Table 2.1. Calibration range of concentrations for the different inorganic ions (mg/L).

Anion	Lower limit	Upper limit	Cation	Lower limit	Upper limit
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
$S_2O_3^{2-}$	1.5	150			

The analysed sample goes through the column and the ions (cations or anions depending on the column used) are separated by retention along the resin column (Metrohm, Switzerland). Anions are determined with a Metrosep A column (250 x 4.0 mm) and a carbonate-bicarbonate mobile phase (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 Metrosep C3 resin column (250 x 4.0 mm) and nitric acid 3.5 mM is used as mobile phase. Then, the sample passes through a conductimetric detector after the separation, where the obtained signal corresponding to each retention time is registered. The resulting chromatograms identify each measured ion by its retention time position together with the amount of ion, which is related to the area. Standard commercial solutions

(Sigma-Aldrich) of each anion and cation are used to correlate the peak area with the ion concentration. The injection volume of the sample is 20 µL and data collection is done by using the Processor software IC Net 2.3.

2.1.4. Alkalinity

The alkalinity is an indicator of the acid-neutralising capacity of a wastewater sample. The alkalinity is related to the presence of buffering agents like salts and weak acids. Since the alkalinity of many surface water is primarily a function of carbonate, bicarbonate and hydroxide content, it is taken as an indication of the concentration of these components (APHA-AWWA-WEF, 2012). However, it may also include contributions from borates, phosphates, silicates and/or other bases. Hydroxyl ions present in a sample as a result of dissociation or hydrolysis of solutes react with additions of standard acid. Alkalinity thus depends on the end-point pH used. The pH values are suggested as the equivalence points for the corresponding alkalinity concentration as mg $CaCO_3$/L.

The total alkalinity (TA) is determined at pH 4.3. It can be considered as the sum of the alkalinity due to bicarbonate together with that corresponding to the volatile fatty acids and its end-point corresponds to a pH of 4.3. The partial alkalinity (PA), measured by the titration until pH 5.75, corresponds to bicarbonate while the intermediate alkalinity (IA) is defined as the difference between TA and PA, and corresponds approximately to the alkalinity related to the volatile fatty acid content. In urban wastewater, TA and PA alkalinity are approximately equal.

The alkalinity concentration is determined using colourimetric Hach Lange kits (Chapter 8), indirectly utilising the IC concentration (Chapter 3 - 7) or following the method 2320 described in the *Standard Methods for the examination of Water and Wastewater* (APHA-AWWA-WEF, 2012) in Chapter 9. The colourimetric method using Hach Lange cuvette kits is based on the reaction of calcium and magnesium ions with metal phthalein generating a violet dye. The method 2320 consists in a titration of a sample volume (usually 25 mL) at room temperature with a standard acid (H_2SO_4 standardised against Na_2CO_3) to reach the desired pH (pH 5.75 for PA and pH 4.3 for TA). The alkalinity value (mg $CaCO_3$/L) is calculated from Equation 2.3:

$$\text{Alkalinity} = \frac{A \cdot N \cdot 50{,}000}{V} \qquad \text{Eq. 2.3}$$

where:

A: volume of H_2SO_4 used to decrease the pH to 5.75 (PA) or to 4.3 (TA) (mL).

N: normality of the H_2SO_4 in equivalents/L. This concentration depends on the expected alkalinity of the water and usually varies from 0.05 to 0.10 N.

V: sample volume (mL).

2.1.5. Other control parameters

2.1.5.1. pH

The pH is one of the key parameters measured in wastewater biological treatment systems since its control is essential to maintain the activity of the microorganisms involved in the different treatment processes and indicate which process is taking place (e.g., nitrification or denitrification). The pH value, of the unfiltered samples, is measured using different instruments. The specific device is indicated in each chapter. The electrodes are calibrated at room temperature with two standard buffer solutions of 7.0 and 4.01.

2.1.5.2. Dissolved oxygen

The dissolved oxygen (DO) concentration in aerobic reactors is measured using different instruments in the laboratory and pilot-scale reactors. The specific device used is specified in the corresponding chapter. DO probes are calibrated monthly.

2.1.5.3. Temperature

In the aerobic reactors, the used DO probes mentioned in the previous section are equipped with a thermopar that measures the temperature. In the anaerobic reactors, provided with a chamber in the walls, the temperature is controlled by a thermostatic bath which circulated water at the desired temperature.

2.2. Biomass characterisation

In this section, the analytical methods to characterise the solid phase (sludge) are described. Both conventional sludge physical properties and more specific parameters are included, as the performance of different types of biomass is studied in this thesis: suspended (Chapters 4, 5, 6 and 9), granular (Chapters 3, 7 and 9), and attached to carriers (Chapters 8).

2.2.1. Solid concentration

Solids present in water and wastewater treatment units can be organic or inorganic. The total suspended solids (TSS) and the volatile suspended solids (VSS) concentrations are determined according to the methods 2540-D (Total Suspended Solids Dried at 103 – 105 °C) and 2540-E (Fixed and Volatile Solids Ignited at 550 °C) described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 2012). The determination of VSS concentration is of high interest in the control of wastewater treatment process as it offers a rough approximation of the amount of organic matter present in the solid fraction of wastewater or activated sludge samples. Indeed, the VSS inside a reactor is assumed as the biomass concentration.

Determination procedure

First, a fibreglass filter (Merck Millipore Ltd., APFC04700, 47 mm of diameter, 1.2 µm of pore size) is placed in a muffle furnace (J.P Selecta, Select-Horn-TFT) at 550 °C for 30 minutes to remove the humidity and possible organics and get a constant weight. Then, it is located inside a desiccator to achieve room temperature and after that, it is weighed to obtain the value of F_0 (g).

For the determination of the TSS, a known well-mixed sample volume (V_0 in L) is collected to obtain a residue between 2.5 and 200 mg and filtered through the dried fibreglass filter. The residue retained on the filter is dried for at least 2 hours (and generally for 24 h) at 105 °C in an oven (J.P Selecta 2000210), until reaching a constant weight. After that, it is located inside a desiccator until it achieves room temperature. Finally, it is weighed to obtain the value of F_1 (g). The increase in the weight of the filter represents the TSS. The concentration of TSS, in g/L, is determined according to Equation 2.4:

$$\text{TSS (g/L)} = \frac{F_1 - F_0}{V_0} \qquad \text{Eq. 2.4}$$

Finally, for the determination of the VSS, the filter previously dried to obtain the TSS concentration is burnt inside a muffle furnace at 550 °C for half an hour. Then, it is located inside the desiccator to reach room temperature and weighed to obtain the value of F_2. The weight loss during ignition corresponds to the VSS content and its concentration (in g/L) is determined according to Equation 2.5:

$$\text{VSS (g/L)} = \frac{F_1 - F_2}{V_0} \qquad \text{Eq. 2.5}$$

Interferences

The exclusion of large floating particles or submerged agglomerates of non-homogenous materials from the sample is recommended if it is determined that their inclusion is not representative. Highly mineralised water with a significant concentration of calcium, magnesium, chloride and/or sulfate may be hygroscopic and require prolonged drying, desiccation and rapid weighing. Some inorganic salts such as hydroxides, carbonates or ammonium salts are decomposed and volatilized at 550 °C and, therefore, they can give a higher value than the real one for the volatile content in the sample. Excessive residue in the filter may form a water-trapping crust, thus limiting the sample to a maximum of 200 mg of residue is recommended.

2.2.2. Solid concentration as biofilm

The type of used carrier (in Chapter 8) was K1 AnoxKaldness (Veolia Water Technologies, AB) characterised by a specific surface area of 500 $m^2/m^3_{carrier}$. A gravimetry method to estimate the concentration of solids attached to the supporting material is developed and described here. A randomly collected known number of carriers are dried in the oven at 105 °C for at least 12 h. After dried and cooled down to room temperature in a desiccator, the biomass is mechanically detached and weighted. To determine the volatile fraction the detached biomass is burnt inside a muffle furnace at 550 °C.

2.2.3. Sludge volume index

The sludge volume index (SVI) determination is defined in the *Standards Methods for the Examination of Water and Wastewater* (APHA-AWWA-WEF, 2012) as the volume in millilitres occupied by 1 g of TSS after 30 min settling. The SVI is typically used to monitor settling characteristics of the activated sludge and other biological suspensions. However, as suggested at the "1st IWAS-Workshop Aerobic Granular Sludge" (Munich, 2004), the SVI_{10} (SVI after 10 minutes of settling) is more convenient for determining the SVI of granular sludge. Low SVI_{30} value does not necessarily imply sludge granulation and vice versa. Nevertheless, a granular sludge bed does consolidate much faster, i.e., the terminal SVI_{30} is already reached after 10

minutes of settling. For this reason, both SVI_{30} and SVI_{10} are measured in this thesis. The SVI (in mL/g TSS) is determined according to Equation 2.6:

$$SVI = \frac{V_{settled\ sludge}}{TSS} \quad \text{Eq. 2.6}$$

where:

$V_{settled\ sludge}$: is the volume, in mL, occupied by the sludge bed in a litre of reactor mixed liquid after 30 or 10 minutes.

TSS: the total suspended solids concentration (g TSS/L) in the sludge sample.

2.2.4. Density of the granules

The biomass density (as the mass of granules per volume of granules) was determined using the method described by Beun et al. (2002) and modified in the laboratory of the Environmental Biotechnology. First, an amount of a homogenous biomass sample is taken from the reactor and weighed (W_1) in a tare weighed graduated cylinder (W_2). Then, an amount of liquid is removed from the sample (W_3). A volume of a dextran blue solution (1 g/L) is added to the granular sludge sample, in a volume ratio of about 1:1. The mixture is gently mixed, and subsequently, the granules are allowed to settle (W_4). An amount of the liquid above the settled granules is removed, and a sample is taken from it. This fraction (Abs_1) and the original dextran blue solution (Abs_0) are analysed by a spectrophotometer at 620 nm. Subsequently, the volume occupied by the biomass in the reactor sample is calculated, since dextran blue only binds water and not the biomass. By measuring the VSS concentration, the density of the granules ($\rho_{granule}$) can be calculated as gram of biomass per litre of granule (g VSS/$L_{granule}$) using Equation 2.7:

$$\rho_{granule} = \frac{W_1 - W_2}{W_4 - W_1 - \left(\frac{Abs_0}{Abs_1} \cdot (W_4 - W_3)\right)} \quad \text{Eq. 2.7}$$

being:

VSS: volatile suspended solids concentration (g VSS/L).

W_1: weight of the cylinder with sample (g).

W_2: weight of the empty cylinder (g).

W_3: weight of the cylinder with sample after removal of supernatant (g).

W_4: weight of the cylinder with sample after dextran blue addition (g).

Abs_0: absorbance of the dextran blue solution (1 g/L).

Abs_1: absorbance of the sample.

2.2.5. Average diameter of the granules

Changes in the morphology of the granules are followed by image analysis. A representative sample is taken from the reactor and washed to remove the liquid colour. Images of the granular sludge are taken (at least 200 granules per sample resulting in approximately 20 images) with a digital camera (Coolsnap, Roper Scientific Photometrics) combined to a stereomicroscope (Stemi 2000-C, Zeiss), with a zoom of 0.65X, using the RS Image software. For digital image analysis, the software Image ProPlus® is used (Figure 2.1).

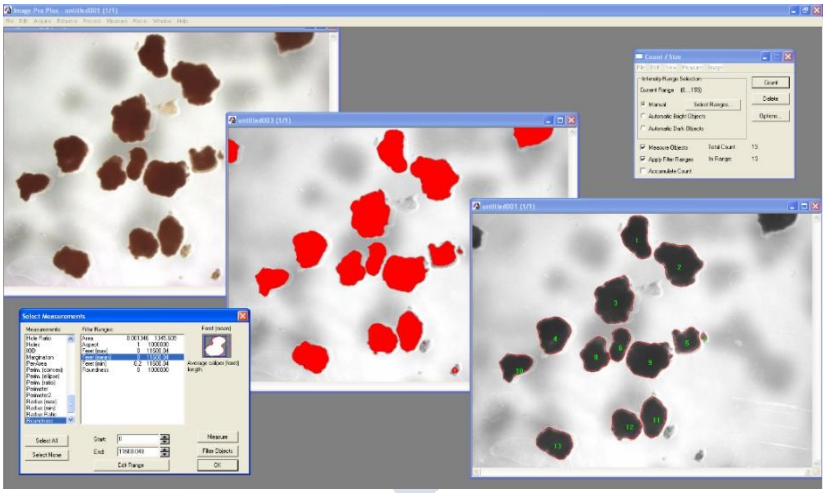

Figure 2.1. Example of the use of Image ProPlus® software. From left to right: original granule image, area recognised by the software (in red), and granule definition and counting.

The procedure of average diameter determination consists in:

1. Converting the original image of granules to black and white mode simplifying the image processing.

2. Defining the range of colours corresponding to the area of interest in the image, i.e., the granules (Manual or Automatic). Images might be manually edited to delete cut objects and split granules.

3. Selecting the measurements of interest with the software (e.g., area, aspect, feret min, feret max and roundness). The average diameter obtained from the software corresponds to the mean feret diameter of the granules. The minimum

feret to be considered a granule in the image analysis is fixed at 0.2 mm. The feret diameter is calculated as an average value from the shortest and the longest measured segments of each granule.

4. Exporting the data of interest selected with the software to an Excel worksheet.

5. Utilising the histogram tool (data analysis tool pack) to calculate the frequency and construction of the histogram. The average diameter can be calculated from a frequency, surface or volumetric distribution (Figure 2.2).

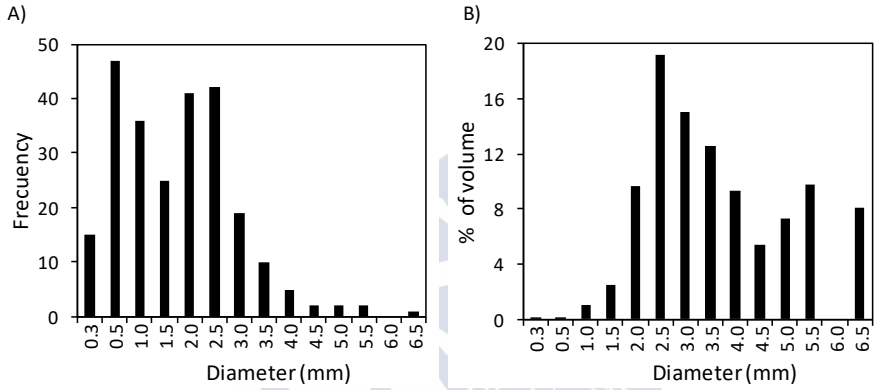

Figure 2.2. Example of histograms corresponding to the average granule diameter in: A) frequency and B) volume distribution.

2.2.6. Specific bacterial activities by batch tests

2.2.6.1. Specific anammox activity assays

The specific activity of anammox bacteria (SA_{AMX}) is determined in manometric batch tests according to the methodology described by Dapena-Mora et al. (2007). It is based on the measurement throughout the time of the overpressure generated by the dinitrogen gas produced in completely closed vials with a total volume of 38 mL and 25 mL of liquid volume. First, biomass samples are washed at least three times with phosphate buffer (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4/L) removing any substrate or inhibitory compound that may be present in the liquid media and fixing the pH value at 7.8. Biomass concentration at the beginning of the experiment is generally fixed at approximately 1.0 g VSS/L. This value is increased when a too low activity is expected or when the effect of inhibitory compounds is assessed. Then,

vials are closed with a septum lid and the vial headspace is purged with Helium gas to remove the oxygen from the bulk liquid and headspace. Later vials are placed in a thermostatic shaker, fixing the agitation speed at 150 rpm and the temperature (T) at the desired value, until stable conditions are reached. At this point, substrate concentrated solutions (3.5 g N/L of ammonium and nitrite) are added (0.5 mL of each one) with a syringe throughout the septum, to have initial concentrations of 70 mg N/L of each ammonium and nitrite. If the inhibitory effect of a compound is being evaluated, it is added together with the substrates. After substrate addition, and pressure equalisation to the atmospheric one, the N_2 gas production is determined in the gas phase as the increment of the pressure in the headspace of the vials, measured using a differential pressure transducer (0 – 5 psi, linearity 0.5 % of full-scale) manufactured by Centerpoint Electronics. All the batch tests are performed in triplicate. The maximum SA_{AMX} is estimated from the maximum slope of the curve described by the cumulative N_2 production throughout time (Figure 2.3) and related to the biomass concentration (VSS) contained in the vials. When standard conditions (30 °C and pH 7.8) are used the measured overpressure corresponded to the N_2 gas production (composition higher than 99 %) (Dapena-Mora et al. 2007). To check that, the gas samples collected from each vial at the end of the SA_{AMX} test are analysed to determine the gas composition and therefore the molar percentage of nitrogen in the produced gas (X_N).

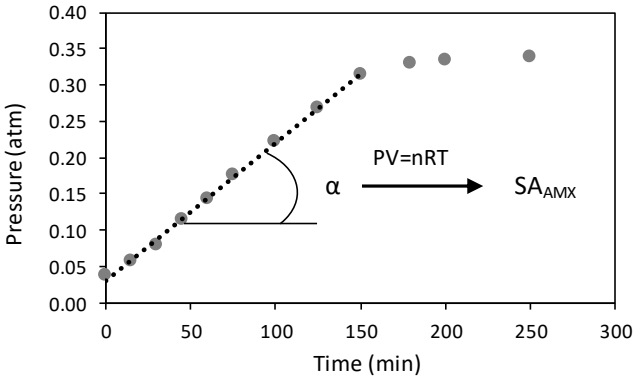

Figure 2.3. Pressure increase in the vial headspace during the manometric SA_{AMX} batch test.

The N_2 gas production rate (dN_2/dt, moles N_2/min) is calculated with the ideal gas equation from the maximum slope of the curve describing the pressure increase in the vial throughout time (α, atm/min) multiplied by X_N using the Equation 2.8:

$$\frac{dN_2}{dt} = \alpha \cdot \frac{V_G}{R \cdot T} \cdot X_N \qquad \text{Eq. 2.8}$$

being V_G the volume of the gaseous phase (L), R the ideal gas coefficient ((atm·L)/(mol·K)) and T the temperature (K).

Thus, the SA_{AMX} in g N_2-N/(g VSS·d) is calculated from the gas production rate and the biomass concentration in the vial (g VSS/L) according to Equation 2.9, where V_L is the volume of the liquid vial phase (L):

$$SA_{AMX} = \frac{\frac{dN_2}{dt}}{VSS \cdot V_L} \cdot \frac{28\ g\ N}{mol\ N_2} \cdot \frac{1{,}440\ min}{d} \qquad \text{Eq. 2.9}$$

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 ammonium and nitrite concentrations used.

In Chapter 8, a modified version of this method is employed by using 250 mL bottle filled with a mixture of phosphate buffer and biomass. The bottle content is purged with nitrogen gas and, after temperature acclimation, substrates are added resulting in an ammonium and nitrite concentrations of 70 mg N/L, respectively. The increase of the pressure is continuously measured (mm Hg) and logged with a manometric Greisinger GMH 5150 device and it is later transferred to the computer through the GSOFT3050 software. Then, data handling like that previously described (Equations 2.8 and 2.9) is performed.

Biogas composition

The biogas samples (1 mL) collected from the headspace of the vials are analysed by gas chromatography (GC) to determine the nitrogen molar fraction. Biogas composition (N_2, CH_4, CO_2, N_2O and H_2S) is determined in a GC system (Hewlett Packard 5890 Series II instrument) equipped with a Thermal Conductivity Detector (TCD) and an 80/100 Porapak Q column (2x1/8”, Supelco). The mobile phase consists of Helium gas supplied at a flow rate of 16 mL/min and oven, detector and injector temperatures are fixed at 35, 110 and 110 °C, respectively. The

calibration is performed with a standard mixture of gases (29.0 % N_2, 49.7 % CH_4, 17.5 % CO_2, 2.4 % N_2O and 1.4 % H_2S in molar fractions), by using a response factor method.

2.2.6.2. Specific heterotrophic denitrifying activity assays

The specific activity of heterotrophic denitrifying microorganisms (SA_{HDN}) is also determined in a manometric batch test by a modified version of the SA_{AMX} test described by Dapena-Mora et al. (2007) but adding 50 mg NO_3^--N/L or 50 mg NO_2^--N/L and 200 mg COD/L as substrates.

2.2.6.3. Specific aerobic activities by respirometric assays

Respirometric assays were conducted to determine specific aerobic activities. The aerobic heterotrophic activity and the nitrifying capability of the biomass are quantified by measuring the oxygen uptake rate (OUR).

The methodology is adapted from that previously described by Lopez-Fiuza et al. (2002). Respirometric batch experiments are performed using a biological oxygen monitor (BOM, YSI Inc. model 5300) equipped with oxygen selective probes (YSI 5331) connected to a data acquisition system (Labtech) and provided with a thermostatic control chamber for the vials. The batch experiments are performed in hermetically closed vials of 15 mL with a useful volume of 10 mL. First, biomass is washed three times with a phosphate buffer solution (0.143 g KH_2PO_4/L and 0.747 g K_2HPO_4/L) to remove residual substrate or inhibitory compounds present in the biomass liquid matrix. Then, the re-suspended biomass is temperature acclimated by means of a thermostatic bath until the established assay temperature is achieved and during at least 30 min. The liquid and biomass mixture are added in the vials, which are placed in the thermostability control chamber, provided with a magnetic stirring system. Later, they are gently bubbled with air for 15 minutes to reach the oxygen saturation. At this moment, 100 µL of the concentrated solution of the specific inhibitory compound needed to ensure that only the microbial population of interest is active is added (Table 2.2). Allylthiourea (ATU) is added when the specific activity of aerobic heterotrophs (SA_{aerHET}) is determined as this compound inhibits the nitrifying bacteria activity. Whereas sodium azide (NaN_3) is added when the specific activity of ammonia oxidising bacteria (SA_{AOB}) is determined as NaN_3 is a nitrite oxidising bacteria (NOB) selective inhibitory compound. No inhibitory

compound is added for the determination of the specific activity of NOB (SA_{NOB}). Meanwhile, the two electrodes for oxygen measurement are calibrated to 100 % oxygen saturation. To begin the experiment, aeration is removed, and the oxygen probes are carefully introduced in the vessels avoiding the presence of bubbles and the data acquisition software is initialised.

Table 2.2. Substrate and specific inhibitory compounds added during the respirometric activity tests according to Lopez-Fiuza et al (2002) and Mosquera-Corral et al. (2005).

Activity	Substrate	Inhibitory compound added	Stoichiometric coefficient (g O_2/g N)
SA_{aerHET}	CH_3COONa (100 mg COD/L)	ATU (5 mg/L)	-
SA_{AOB}	NH_4Cl (35 mg N/L)	Sodium azide (5 mg/L)	3.42
SA_{NOB}	$NaNO_2$ (35 mg N/L)	None	1.14

The oxygen depletion is monitored during the time through the connection of the oxygen electrode to the data acquisition system (Figure 2.4). The endogenous respiration is measured at the beginning of the assay during enough time to obtain the slope (α_1) of the consumed oxygen (g O_2/(L·d)). Then, the substrate was injected into the vial (10 µL) and the new slope of the oxygen consumption was determined (α_2) in g O_2/(L·d). The oxygen consumption due to the biomass activity is determined by subtracting the endogenous consumption rate to the total oxygen consumption rate (α_2 - α_1).

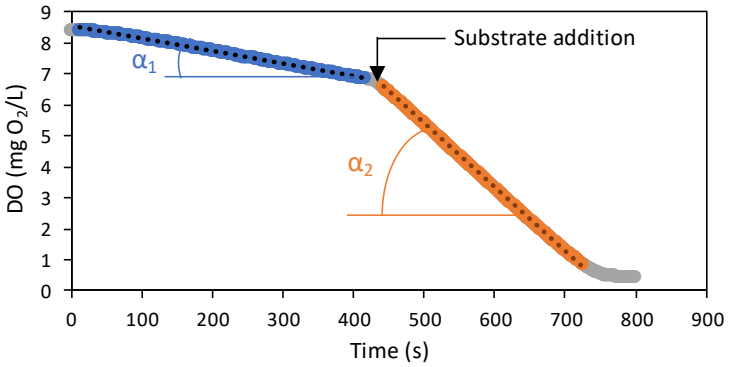

Figure 2.4. Profile of dissolved oxygen (DO) concentration decrease in the respirometric assay: oxygen depletion during endogenous respiration (blue) and endogenous respiration plus substrate consumption (orange).

After the experiment, the solid concentration in each of the vials was determined according to *Standard Methods* (APHA-AWWA-WEF, 2012). Finally, the specific activity (SA) of the biomass is determined by dividing the oxygen consumption rate by the solids content in the vial, which can be referred to the specific substrate using the stoichiometric coefficient (Table 2.2).

In Chapter 8, the aerobic activities are determined by an adaptation of the method described by Surmacz-Gorska et al. (1996). This method is also based on the OUR but the SA_{aerHET}, SA_{AOB} and SA_{NOB} are consecutively determined in a single test. This methodology separates the DO consumption of the different microorganisms by adding specific metabolic inhibitors of NOB and AOB during the time of the assay. The biomass is washed, and temperature acclimated as it is described for the previous method. The biomass in phosphate buffer is introduced in a 1.2 L glass bottle and placed on a magnetic stirrer in a water bath where the liquid media is aerated. Once the temperature acclimation and the DO liquid saturation are reached, the test is started by adding the substrates (COD, ammonium and nitrite simultaneously according to Table 2.3) and introducing the DO meter (Hach Lange LDO meter, HQ30D) to the bottle turning off the aeration.

Table 2.3. Substrate and specific inhibitory compounds added and calculations to obtain the specific bacterial activities according to Surmacz-Gorska et al. (1996).

Activity	Substrate	Inhibitory compound	Calculation
SA_{aerHET}	CH_3COONa (100 mg COD/L)	none	α_3
SA_{AOB}	NH_4Cl (50 mg N/L)	ATU (5 mg/L)	$\alpha_2 - \alpha_3$
SA_{NOB}	$NaNO_2$ (15 mg N/L)	$NaClO_3$ (1.8 g/L)	$\alpha_1 - \alpha_2$

Then, the DO concentration during the time is continuously measured and recorded obtaining a curve like the one shown in Figure 2.5. First, the total OUR is measured obtaining the slope (α_1) of the consumed oxygen (g O_2/(L·d)). Once the DO concentration decreased approximately 0.7 mg O_2/L (or after 10 min), $NaClO_3$ is added inhibiting the NOB activity. The OUR is determined to obtain a slope α_2. The difference between the total OUR and the OUR measured in the presence of $NaClO_3$ is ascribed to the oxygen uptake due to the nitrite oxidation (Figure 2.5). Finally, after 8 minutes or a DO concentration decrease of approximately 0.5 mg O_2/L, ATU solution is added to inhibit all the nitrifying bacteria activity. The slope of the oxygen

depletion is determined (α_3) corresponding to the aerobic heterotrophic bacteria activity. The AOB activity is determined by the difference between α_2 and α_3 in g O_2/(L·d) (Figure 2.5). With this method, endogenous respiration is not determined but total activities (the sum of the DO consumption by the substrate uptake and the endogenous respiration).

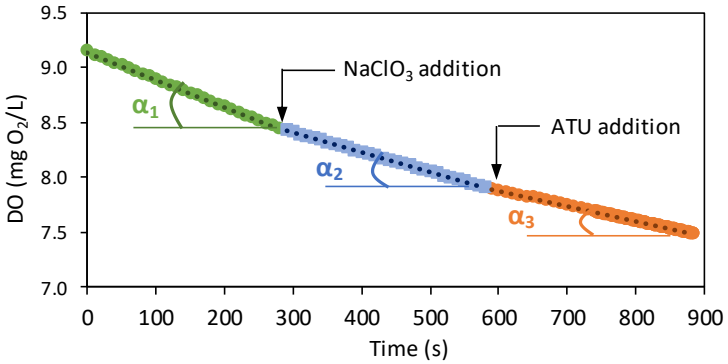

Figure 2.5. Oxygen depletion during the respirometric test: total oxygen consumption (green), total-NOB oxygen consumption (blue) and total- NOB-AOB oxygen consumption (orange).

2.3. Microbial population identification

Molecular techniques based on ribosomal ribonucleic acid (rRNA) are presented in the next section. The fluorescence *in situ* hybridisation (FISH) technique makes the identification of active microorganisms at any desired taxonomical level possible, depending on the specificity of the used probe. The technique based on 16S rRNA gene amplicon libraries (sequenced by Illumina®) allows to quantitatively identify all the present microorganisms.

2.3.1. Fluorescence *in situ* hybridisation

The main active bacterial populations present in the sludge samples are identified by applying FISH molecular technique. Specific regions of the 23S or 16S rRNA are detected by the hybridisation of fluorescent-labelled probes. These probes hybridise with the targeted sequence of any microorganism and they can be later identified microscopically. According to Amann et al. (1990), the FISH protocol includes four steps (Figure 2.6): sample fixation and permeabilisation; hybridisation

of the targeted sequence with the specific probe; washing step to remove the unbound probe; and finally, the detection of labelled cells by microscopy.

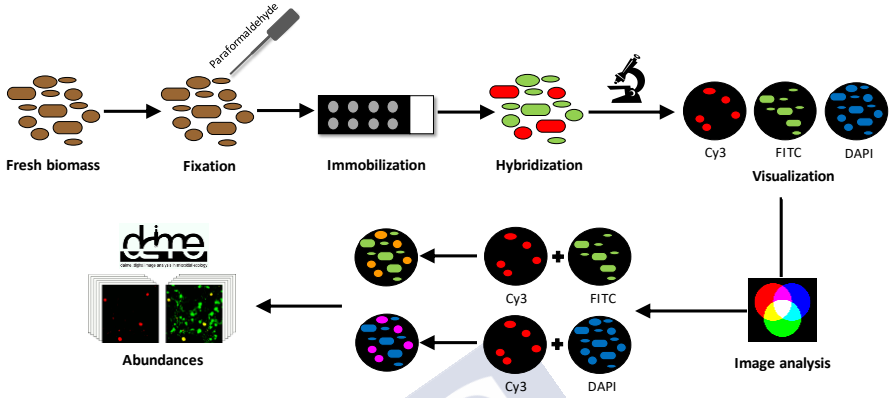

Figure 2.6. Schematic description of the FISH technique methodology.

This protocol must be applied to disrupted biomass; thus, the biofilm biomass disintegration is needed before starting the procedure. To achieve the biomass breakage, it is sonicated for 1 minute at 65 % of amplitude and frequency of 0.5 s^{-1} using an ultrasonic probe device (UP200s, Dr. Hielscher). The time of sonication is selected to achieve the breakage of the granules but not of the cells. Biomass attached to the surface of the K1 carriers (Chapter 8) is manually detached to avoid bacterial deactivation.

The hybridisation of the samples is carried out simultaneously for the general bacteria probe and for the specific probes that target microorganisms belonging to the main groups of interest AOB, NOB and anammox bacteria (Table 2.4). The used probes are 5'-labelled with the fluorochromes FITC (fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). Details about the oligonucleotide probes are available at probeBase (Greuter et al. 2016). DAPI (4,6-diamindino-2-phenylindole) is used as a universal dye for all deoxyribonucleic acid (DNA).

Reagents

- *Phosphate Buffer Solution (PBS) (3x)*: 0.49 g of KH_2PO_4 are dissolved in 80 mL of Milli-Q water, 2.3 g of NaCl are added and pH is adjusted to 7.2. Finally, the volume is adjusted to 100 mL.

Table 2.4. List of 16S rRNA-targeted oligonucleotide probes used for biomass fluorescent *in situ* analysis.

Probe	Target organism	Probe sequence (5'→ 3')	% F [**]
	Bacteria domain		
EUB338I[*]	Most Bacteria	GCT GCC TCC CGT AGG AGT	0-50
EUB338II[*]	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	0-50
EUB338III[*]	*Verrucomicrobiales*	GCT GCC ACC CGT AGG TGT	0-50
	AOB group		
Nso190	Betaproteobacterial ammonia-oxidizing bacteria	CGA TCC CCT GCT TTT CTC C	55
Nso1225	Betaproteobacterial ammonia-oxidizing bacteria	CGC CAT TGR ATT ACG TGT GA	
	NOB group		
NIT3	*Nitrobacter* spp.	CCT GTG CTC CAT GCT CCG	40
	Competitor for NIT3	CCT GTG CTC CAG GCT CCG	
Ntspa712	Most members of the phylum *Nitrospirae*	CGC CTT CGC CAC CGG CCT TCC	35
	Competitor for Ntspa 712	CGC CTT CGC CAC CGG TGT TCC	
NTG840	*Nitrotoga arctica sp.*	CTA AGG AGG TCT CCT CCC	
	AMX group		
Amx368	All anammox bacteria	CCT TTC GGG CAT TGC GAA	
Amx820	*Candidatus* Brocadia anammoxidans and Kuenenia stuttgartiens	AAA ACC CCT CTA CTT AGT GCC C	35
BAN162	*Candidatus* Brocadia anammoxidans	CGG TAG CCC CAA TTG CTT	40

[*] EUB338I, EUB338II and EUB338III are equimolarly mixed and applied as EUB338mix.

[**] Percentage of formamide (F) used in the hybridization.

- *PBS (1x)*: 1:3 dilution of PBS (3x) in Milli-Q water.
- *Fixative solution:* 6.5 mL of Milli-Q water are heated to 60 °C and 0.4 g of paraformaldehyde are added. One drop of 1 M NaOH is added and the solution is vigorously shaken until it becomes nearly clarified (1 - 2 min). Then, 3.3 mL of PBS (3x) are added and the pH is adjusted to 7.2 with HCl 1 M (one drop). Finally, the solution is filtered through a 0.2 µm membrane filter.
- *Hybridisation buffer:* it is prepared in a 2 mL Eppendorf tube by mixing: 360 µL of NaCl (5 M), 40 µL of Tris/HCl (1 M, pH 8.0) and 4 µL of sodiumdodecylsulfate (SDS, 10 % wt/vol). Formamide (F) and Milli-Q water are added to the mixture, according to the fluorescence probe used (Table 2.5). The hybridisation buffer is kept at room temperature.

Table 2.5. Volumes of formamide and water added to the hybridisation buffer.

% Formamide (v/v)	Formamide (µL)	Milli-Q water (µL)
0	0	1,600
5	100	1,500
10	200	1,400
15	300	1,300
20	400	1,200
25	500	1,100
30	600	1,000
35	700	900
40	800	800
45	900	700
50	1,000	600

- *Washing buffer:* the buffer is prepared in a 50 mL Falcon tube by mixing: 1 mL of Tris/HCl 1 M (pH 8.0) and the required volumes of NaCl (5 M) and ethylenediamine-tetraacetic acid disodium salt (Na_2EDTA, 0.5 M at pH 8.0), according to the used probe (Table 2.6). Then, the Falcon tube is filled up to 50 mL with Milli-Q water. The washing buffer is preheated at 48 °C before the washing step.

Table 2.6. Volumes of NaCl (5 M) and EDTA (0.5 M) added to the washing buffer.

% Formamide (v/v)	NaCl 5 M (µL)	EDTA 0.5 M (µL)
0	9,000	-
5	6,300	-
10	4,500	-
15	3,180	-
20	2,250	500
25	1,590	500
30	1,120	500
35	800	500
40	560	500
45	400	500
50	280	500

Determination procedure

1. Cell fixation: biomass samples are collected from the reactors and washed in PBS (1x) three times. Then, three volumes of the fixative solution are added to one volume of biomass suspension. The mixture is kept at 4 °C for 2 - 3 h. Afterwards, the biomass sample is washed again, and the cells are re-suspended in PBS (1x). Finally, 1.25 volumes of ethanol 98 % (at -20 °C) are added to one volume of biomass suspension and samples are stored at -20 °C.

2. Immobilisation: a volume of 10 µL of a fixed biomass sample are spread on a well of a coated Teflon/glass microscope slide. The sample is dried at 46 °C for 10 minutes. Afterwards, cells are dehydrated by successive passage through 50, 80 and 98 % (v/v) ethanol (3 minutes each), and dried.

3. Hybridisation: a volume of 10 µL of hybridisation buffer is pipetted into each well of the microscope slide with the immobilised biomass. Then, FISH probes are added into these wells (1 µL of stock solution with a final concentration of 30 ng/µL and 50 ng/µL for Cy3 and FITC labelled-probes, respectively). The solution is mixed without scratching the slide and cell layer. A 50 mL Falcon hybridisation tube is prepared by folding a tissue inside and pouring the rest of the hybridisation buffer onto the tissue. The slide is immediately transferred into the hybridisation tube and it is incubated for 1.5 - 2.0 hour in an oven (Memmert) at 46 °C. In the meantime, the washing buffer is prepared and preheated in a water bath at 48 °C.

4. Washing: This step must be performed rapidly. The slides are quickly transferred into the Falcon tube containing the washing buffer by immersing the whole slide and incubating it for 15 minutes at 48 °C. Then, the slides are removed from the washing buffer and dipped into cold Milli-Q water for few seconds. Finally, the slide is dried.

5. Microscopy and slide image acquisition: Slide wells are embedded with Vectashield H-1200 (which amplifies the fluorescence, avoids fading and contains DAPI dye), and a coverslip is set on them. Then, fluorescence signals are recorded with an acquisition system (Coolsnap, Roper Scientific Photometrics) coupled with an epifluorescence microscope (Axioskop 2 plus, Zeiss). Images are acquired with RS Image software (v 1.7.3, Roper Scientific, Inc.). The semi-quantitative counting of the bacterial populations bases on the ratio of their specific biovolume to the total bacterial (or the more general probe applied) bacterial biovolume and it is determined using the DAIME software (Daims et al. 2006). Paired images (FITC, Cy3, or DAPI) of each field of view are stored and they are merged with the Serif Photo plus software (Figure 2.6).

2.3.2. 16S rRNA gene-based amplicon analysis (Illumina®)

The 16S rRNA gene-based amplicon analysis (Base Space, Illumina®) is performed, according to the procedure described by Caporaso et al. (2011), to identify the microorganisms present in the biomass samples from Chapter 5. The homogenous biomass samples are directly collected from the reactor and immediately frozen at - 20 °C. The sequence of the V3 - V4 region of the 16S rRNA gene is used as the taxonomic basis to initially identify the bacterial populations present in the samples by the Illumina technique (Caporaso et al. 2011).

First, total genomic DNA is extracted according to the phenol-chloroform protocol (Alonso-Gutierrez et al. 2009). The 16S rRNA gene region is amplified through two consecutive rounds of polymerase chain reaction (PCR) technique analysis. The first round of PCR amplifies the targeted region, while the second round of PCR attaches the sample barcode and sequencing adapters. Samples of extracted DNA are amplified using specific primers with the following sequences: 806R and 515F. Total DNA concentrations are quantified in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA) and the size integrity is tested by gel electrophoresis. The

second PCR of 15 cycles is applied to add the individual barcode to each sample, as well as to incorporate specific sequences in the amplicon libraries. The adapter primers include the specific sequences, the unique barcodes and the universal fusion sequences CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGG TCT). Individual libraries are analysed using a Bioanalyser 2100 (Agilent) to estimate the concentration of the specific PCR products. After library preparation, samples are pooled at equimolar ratios, cleaned and quantified by real-time PCR using specific primers (Kapa Biosystems). Finally, samples are denatured and prepared at 12 pM to be seeded into a Miseq flowcell (Illumina) and run under a 2x250 paired-end sequencing procedure (Parque Científico de Madrid, Spain).

A total amount of > 100,000 reads are obtained for each of the analysed samples. After quality filtering and demultiplexing, data are analysed using the 16S rRNA gene-based amplicon sequencing (Base Space, Illumina®). MSR software is used for the analysis. Bioinformatic assays are performed with predominant operational taxonomic units (OTUs). OTUS were considered abundant if the relative abundance was larger than 0.5 % in at least one sample. Each OTU is assigned taxonomic information using the lowest common taxonomic level. After resolving the number of sequences per OTU, the percentage of each organism is individually calculated for each sample. Relative abundances of reads are calculated by taxonomic level for each library. Values represent the percentage of reads of sequences obtained at each taxonomic identity (according to a degree of similarity) within the total set of reading from the library. OTUs that receive no matches against the sequences are identified as “Unclassified.”

DNA extraction

Reagents:

- Sodium phosphate buffer (0.2 M, pH 8.0): 5.3 mL of a stock solution of 2.76 g NaH_2PO_4/L are mixed with 84.7 mL of a 28.4 g Na_2HPO_4/L solution. Both solutions prepared with Milli-Q water.
- Cetyltrimetylammonium bromide (CTAB, 10 % wt/vol): dissolve 4.1 g of NaCl in 80 mL of Milli-Q water. Slowly add 10 g of CTAB and heat up to 65 °C to dissolve. Finally, the volume is adjusted to 100 mL with Milli-Q water. The solution must be heated at 65 °C before use.

- Extraction buffer: it is prepared in a volume of 120 mL by mixing 12 mL of Tris/HCl 1 M (pH 8.0), 24 mL of Na_2EDTA 0.5 M, 60 mL of sodium phosphate buffer (0.2 M, pH 8.0), 1 mL of proteinase K (10 mg/mL) and 1.8 mL of lysozyme (100 mg/mL).

Determination procedure

1. The freeze biomass samples are introduced in a 2 mL Eppendorf tube and centrifuged at 12,900 rpm for 10 min.
2. The supernatant is discharged, and the pellet is suspended in 600 µL of extraction buffer. This mixture is horizontally shaken for 1 hour at 200 rpm.
3. The DNA is incubated at 65 °C for 15 min after the addition of 10.5 µL of Triton X, 150 µL of SDS (10 % wt/vol), 225 µL of NaCl 5 M and 75 µL of CTAB (10 %).
4. Finally, the DNA is washed:
    a. The extracted DNA is divided into two Eppendorf tubes (520 µL) and 1 volume of chloroform (520 µL) is added to each. Then, they are mixed and centrifuged for 5 min at 12,900 rpm.
    b. 300 µL of each supernatant are collected mixed in one Eppendorf tube (600 µL). After the addition of 1 volume of a commercial solution of Phenol:Chloroform:Isoamyl alcohol (25:24:1) (600 µL) they are mixed and centrifuged for 5 min at 12,900 rpm.
    c. Then, get 400 µL of supernatant and add 1 volume of a commercial solution of Chloroform: Isoamyl alcohol 24:1 (400 µL). Mix and centrifuge for 5 min at 12,900 rpm.
    d. Collect 300 µL of supernatant and add 0.7 volumes of isopropanol (210 µL) and 0.3 volumes of ammonium acetate (10 M, pH 7). Mix and centrifuge for 20 min at 12,900 rpm.
    e. The supernatant is discharged and the pellet re-suspended in 500 µL of ethanol (70%) and the sample is centrifuged for 15 min at 12,900 rpm.
    f. Then, the supernatant is discharged and the pellet is dried.
    g. Finally, the dried pellet is suspended 50 µL of sterile Milli-Q water and the extracted DNA is measured with Nanodrop (Biometra). The extracted DNA is kept at -20 °C.

2.4. Calculations

2.4.1. Free ammonia and free nitrous acid calculation

The free ammonia (FA) and free nitrous acid (FNA) concentrations are calculated according to Equations 2.10 and 2.11 as proposed by Anthonisen et al. (1976). They are in equilibrium with ammonium and nitrite, respectively, and the abundancy of the ionised of the non-ionized form depends on the operating temperature and the pH in the bulk liquid.

$$\mathrm{FA\ (mg\ NH_3\text{-}N/L)} = \frac{\mathrm{NH_4^+\text{-}N}}{\left(\frac{e^{(6344/(T+273))}}{10^{pH}} + 1\right)} \qquad \text{Eq. 2.10}$$

$$\mathrm{FNA(mg\ HNO_2 - N/L)} = \frac{\mathrm{NO_2^- - N}}{10^{pH} \cdot e^{(-2300/(T+273))}} \qquad \text{Eq. 2.11}$$

where: NH_3-N, NH_4^+-N, HNO_2-N, NO_2^--N are the concentrations of FA, ammonium, FNA and nitrite inside the reactor, T is the temperature in K, and pH is the pH value in the reactor liquid media.

2.4.2. Statistical analysis

Statistical differences between the results obtained in the different operational stages are tested by one-factor analysis of variance (ANOVA) using the statistical software R version 3.5.2 (The R Foundation Statistical Computing). First, variance homogeneity is confirmed by Levene's test and normal distribution by the Shaphiro's test. Then, if data set meets the homogeneity and normal distribution prerequisites, ANOVA is carried out to determine if the values obtained were different at the 95 % of confidence level ($p < 0.05$). A post hoc analysis (Tukey's HSD) is applied every time that ANOVA resulted in a significant difference, to finde between which values the difference is significant, considering a level of significance of 0.05. If data variance homogeneity and/or normal distribution requirements are not fulfilled, the non-parametric Kruskal-Wallis analysis is applied and afterwards the Wilcoxon post hoc one.

2.4.3. Nitrogen transformation ratios and rates in the nitritation process

During the nitritation process, ammonium is consumed to produce nitrite according to equation 2.12:

$$NH_4^+ + 1.985\ HCO_3^- + 0.074\ CO_2 + 1.403\ O_2 \rightarrow 0.985\ NO_2^- + 1.985\ CO_2 + 0.0148\ C_5H_7O_2N + 2.941\ H_2O \quad \text{Eq. 2.12}$$

The degrees of ammonium oxidation and nitrite accumulation are defined as ammonium oxidation ratio (AOR) and nitrite accumulation ratio (NAR), in percentage. The AOR and NAR are the main parameters assessing the nitritation process performance and are calculated according to Equations 2.13 and 2.14, respectively.

$$\text{AOR}\ (\%) = \frac{(\text{NH}_4^+ - \text{N})_{\text{inf}} - (\text{NH}_4^+ - \text{N})_{\text{eff}}}{(\text{NH}_4^+ - \text{N})_{\text{inf}}} \cdot 100 \quad \text{Eq. 2.13}$$

$$\text{NAR}\ (\%) = \frac{(\text{NO}_2^- - \text{N})_{\text{eff}}}{(\text{NO}_2^- - \text{N})_{\text{eff}} + (\text{NO}_3^- - \text{N})_{\text{eff}}} \cdot 100 \quad \text{Eq. 2.14}$$

where: NH_4^+-N, NO_2^--N, NO_3^--N are the concentrations of ammonium, nitrite and nitrate nitrogen in the influent (inf) and effluent (eff) of the reactor

Moreover, the ammonium oxidising and nitrite oxidising bacteria rates (rAOB and rNOB, respectively) are estimated based on the nitrogen mass balances considering the hydraulic retention time (HRT, d) and are calculated according to Equations 2.15 and 2.16.

$$\text{rAOB}\ \left(\frac{\text{mg N}}{\text{L} \cdot \text{d}}\right) = \frac{(\text{NH}_4^+ - \text{N})_{\text{inf}} - (\text{NH}_4^+ - \text{N})_{\text{eff}}}{\text{HRT}} \quad \text{Eq. 2.15}$$

$$\text{rNOB}\ \left(\frac{\text{mg N}}{\text{L} \cdot \text{d}}\right) = \frac{(\text{NO}_3^- - \text{N})_{\text{eff}} - (\text{NO}_3^- - \text{N})_{\text{inf}}}{\text{HRT}} \quad \text{Eq. 2.16}$$

2.4.4. Nitrogen removal rates in the anammox process

The anammox process transforms ammonium and nitrite, in anoxic conditions, to produce nitrogen gas and residual amounts of nitrate (Equation 2.17).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad \text{Eq. 2.17}$$

The nitrogen removal rate (NRR), in mg N/(L·d), and the nitrogen removal efficiency (NRE), in %, are calculated according to Equations from 2.18 to 2.20.

$$\Delta N \left(\frac{\text{mg N}}{\text{L}}\right) = (NH_4^+ - N + NO_2^- - N + NO_3^- - N)_{inf} - (NH_4^+ - N + NO_2^- - N + NO_3^- - N)_{eff} \quad \text{Eq. 2.18}$$

$$NRR \left(\frac{\text{mg N}}{\text{L} \cdot \text{d}}\right) = \frac{\Delta N}{HRT} \quad \text{Eq. 2.19}$$

$$NRE\ (\%) = \frac{\Delta N}{(NH_4^+ - N + NO_2^- - N + NO_3^- - N)_{inf}} \cdot 100 \quad \text{Eq. 2.20}$$

2.4.5. Mass balances in one-stage partial nitritation-anammox systems

In the one-stage partial nitritation and anammox (PN/AMX) systems, at least the nitritation (Equation 2.12) and the anammox processes (Equation 2.17) occur simultaneously. If organic matter is also present, its conversion, either by aerobic oxidation (Equation 2.21) or during the denitrification process (Equation 2.22), needs to be also considered. Moreover, the nitratation process (Equation 2.23) might also occur if the NOB are not completely inhibited. Therefore, nitrogen mass balances are calculated considering the stoichiometries corresponding to these five different biological processes.

$$C_{18}H_{19}O_9N + 0.74\ NH_4^+ + 8.8\ O_2 \rightarrow 1.74\ C_5H_7O_2N + 9.3\ CO_2 + 0.74\ H^+ + 4.52\ H_2O \quad \text{Eq. 2.21}$$

$$7\ NO_3^- + 7.5\ H^+ + 0.5\ NH_4^+ + C_{18}H_{19}O_9N \rightarrow 3.5\ N_2 + 1.5\ C_5H_7O_2N + 7.9\ H_2O + 10.3\ CO_2 \quad \text{Eq. 2.22}$$

$$NO_2^- + 0.005\ NH_4^+ + 0.005\ HCO_3^- + 0.020\ H_2CO_3 + 0.471\ O_2 \rightarrow NO_3^- + 0.005\ C_5H_7O_2N + 0.008\ H_2O \quad \text{Eq. 2.23}$$

As those systems are complex and it is difficult to calculate the contribution of each specific microbial group, the following assumptions are considered to simplify the calculations:

a) The anammox process is the main pathway over the denitrification process.

b) Denitrification over nitrite is not considered.

c) Nitratation and denitrification processes do not occur simultaneously.

d) If the denitrification process occurred, the organic matter is preferentially removed by this route over the aerobic oxidation.

Calculations are then carried out as follows. First, the TN removed is estimated according to Equation 2.24.

$$\Delta \mathrm{TN} = (\mathrm{NH_4^+\text{-}N} + \mathrm{NO_2^-\text{-}N} + \mathrm{NO_3^-\text{-}N})_{\mathrm{inf}} - (\mathrm{NH_4^+\text{-}N} + \mathrm{NO_2^-\text{-}N} + \mathrm{NO_3^-\text{-}N})_{\mathrm{eff}} \quad \text{Eq. 2.24}$$

Then, it is assumed that all the TN is removed by the anammox bacteria according to the stoichiometry shown in Equation 2.17. Thus, the expected ammonium (Eq. 2.25) and nitrite consumptions (Eq. 2.26), the nitrate production (Eq. 2.27) and the ammonium used for anammox growth ($(\Delta NH_4^+\text{-}N)_{AMXgrowth}$) (Eq. 2.28) are determined by the respective equations.

$$(\Delta N H_4^+\text{-}N)_{AMX} = \frac{\Delta TN}{2.04} \quad \text{Eq. 2.25}$$

$$(\Delta NO_2^-\text{-}N)_{AMX} = 1.32\ (\Delta N H_4^+\text{-}N)_{AMX} \quad \text{Eq. 2.26}$$

$$(\Delta NO_3^-\text{-}N)_{AMX} = 0.26\ (\Delta N H_4^+\text{-}N)_{AMX} \quad \text{Eq. 2.27}$$

$$(\Delta N H_4^+\text{-}N)_{AMXgrowth} = 0.066 \cdot 0.15 \cdot (\Delta N H_4^+\text{-}N)_{AMX} \quad \text{Eq. 2.28}$$

When the measured nitrate production (as the difference between effluent and influent nitrate concentrations) is higher than the estimated nitrate production by anammox bacteria (Eq. 2.27), it is considered that the nitratation process (Eq. 2.23) occurs and that the heterotrophic denitrification activity is negligible. If the measured nitrate production is lower than the expected one, heterotrophic denitrification (Eq. 2.22) is considered to occur while NOB activity is assumed zero.

The ammonium consumption for NOB assimilation ($(\Delta NH_4^+\text{-}N)_{NOBgrowth}$) is calculated according to Equation 2.29.

$$(\Delta N H_4^+\text{-}N)_{NOBgrowth} = 0.005\ (NO_3^-\text{-}N_{eff} - (\Delta NO_3^-\text{-}N)_{AMX}) \quad \text{Eq. 2.29}$$

Then, ammonium consumption by AOB is estimated using Equation 2.30. As only ammonium is provided in the feeding, the nitrite consumed by anammox (Eq. 2.26) would be produced by AOB, as well as the nitrite present in the effluent and the excess of nitrate after subtracting the production by anammox. In fact, when nitratation activity occurred, the nitrite oxidised to nitrate is necessarily produced by AOB. Moreover, the ammonium consumption for AOB growth ($(\Delta NH_4^+\text{-}N)_{AOBgrowth}$) is calculated according to Equation 2.31.

$$(\Delta N H_4^+\text{-}N)_{AOB} = \frac{(\Delta NO_2^-\text{-}N)_{AMX} + NO_2^-\text{-}N_{eff} + NO_3^-\text{-}N_{eff} - (\Delta NO_3^-\text{-}N)_{AMX}}{0.985} \quad \text{Eq. 2.30}$$

$$(\Delta N H_4^+\text{-N})_{\text{AOBgrowth}} = 0.0148\ (\Delta N H_4^+\text{-N})_{\text{AOB}} \quad \text{Eq. 2.31}$$

The ammonium assimilated by heterotrophic denitrifying bacteria ($(\Delta NH_4^+\text{-N})_{HDNgrowth}$), the organic matter used (as TOC) and its contribution to the total nitrogen removal is estimated according to Equations 2.32 to 2.34, respectively.

$$(\Delta N H_4^+\text{-N})_{\text{HDNgrowth}} = \frac{7}{0.5}\ ((\Delta NO_3^-\text{-N})_{\text{AMX}} - NO_3^-\text{-N}_{eff}) \quad \text{Eq. 2.32}$$

$$(\Delta\text{TOC})_{\text{HDN}} = \frac{18 \cdot 12}{7 \cdot 14}\ ((\Delta NO_3^-\text{-N})_{\text{AMX}} - NO_3^-\text{-N}_{eff}) \quad \text{Eq. 2.33}$$

$$\%\ \Delta N_{\text{HDN}} = \frac{((\Delta NO_3^-\text{-N})_{\text{AMX}} - NO_3^-\text{-N}_{\text{eff}})}{\Delta\text{TN}} \cdot 100 \quad \text{Eq. 2.34}$$

Finally, the contribution of the aerobic heterotrophic bacteria to organic matter and ammonium removal is calculated according to Equations 2.35 and 2.36.

$$(\Delta\text{TOC})_{aerHET} = TOC_{inf} - TOC_{eff} - (\Delta\text{TOC})_{\text{HDN}} \quad \text{Eq.2.35}$$

$$(\Delta N H_4^+\text{-N})_{aerHETgrowth} = \frac{0.74 \cdot 14}{18 \cdot 12}\ (TOC_{inf} - TOC_{eff} - (\Delta\text{TOC})_{\text{HDN}}) \quad \text{Eq. 2.36}$$

Thus, the overall ammonium uptake due to biomass growth ($\Delta NH_4^+\text{-N})_{growth}$ is calculated as the sum of each contribution of: anammox (2.28), NOB (Eq. 2.29), AOB (Eq. 2.31), heterotrophic denitrification (Eq. 2.32) and aerobic heterotrophic oxidation (Eq. 2.36). The combination of these Equations results in Equation 2.37.

$$(\Delta N H_4^+\text{-N})_{\text{growth}} = 1.7829\ \Delta\text{TN} - 13.8743\ NO_3^-\text{-N}_{eff} + 0.0150\ NO_2^-\text{-N}_{eff} + 0.048\ (TOC_{inf} - TOC_{eff}) \quad \text{Eq. 2.37}$$

Since it is assumed that all the nitrogen removed is converted into dinitrogen gas by anammox (no growth), although biomass production and heterotrophic denitrification occur, the iterative calculation is used to estimate the actual contribution of each metabolic pathway to nitrogen removal. Thus, Equation 2.27 is modified by Equation 2.38 and everything is automatically recalculated. Iteration is stopped when the difference from initial and final values is < 0.01 mg N/L.

$$(\Delta NO_3^-\text{-N})_{\text{AMX}} = 0.26 \cdot \frac{\Delta\text{TN} - (\Delta N H_4^+\text{-N})_{\text{growth}} - (NO_3^-\text{-N}_{eff} - (\Delta NO_3^-\text{-N})_{\text{AMX}})}{2.04} \quad \text{Eq. 2.38}$$

2.4.6. Nitrogen removal rates in one-stage PN/AMX system

The global NRR and NRE are calculated using Equations 2.19 and 2.20, respectively. Besides these parameters, ammonium nitrogen removal efficiency (ANRE), in %, and the ammonium and nitrite oxidation rates (rAOB and r NOB, respectively) are estimated based on nitrogen mass balances explained in the previous Section as shown in Equations 2.39, 2.40 and 2.41.

$$\text{ANRE}\ (\%) = \frac{\Delta\text{N} - (\Delta\text{NH}_4^+\text{-N})_{\text{growth}}}{(\text{NH}_4^+ - \text{N} + \text{NO}_2^- - \text{N} + \text{NO}_3^- - \text{N})_{\text{inf}}} \quad \text{Eq. 2.39}$$

$$\text{rAOB}\ \left(\frac{\text{mg N}}{\text{L} \cdot \text{d}}\right) = \frac{(\Delta\text{NH}_4^+\text{-N})_{\text{AOB}}}{\text{HRT}} \quad \text{Eq. 2.40}$$

$$\text{rNOB}\ \left(\frac{\text{mg N}}{\text{L} \cdot \text{d}}\right) = \frac{\text{NO}_3^-\text{-N}_{\text{inf}} - (\text{NO}_3^-\text{-N}_{\text{eff}} - (\Delta\text{NO}_3^-\text{-N})_{\text{AMX}})}{\text{HRT}} \quad \text{Eq. 2.41}$$

2.5. References

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Chapter 3

PN/AMX system robustness under repeated starvation and reactivation periods for blackwater treatment

SUMMARY

Wastewater source-separation and on-site treatment systems frequently need to cope with periods of wastewater unavailability. After its separation in source, the black fraction can be treated by anaerobic digestion followed by a partial nitritation-anammox (PN/AMX) systems. In this chapter, the effect of repeated short-term starvation and reactivation periods on a PN/AMX processes was assessed at room temperature (ranging from 14 to 21 °C) for the treatment of digested blackwater. The PN/AMX sequencing batch reactor, of 4 L, was fed during working hours with anaerobically digested blackwater from an office building. Repeated stops were applied during night-time (12 h) and weekends (2 days) simulating the discontinuous wastewater production. Despite the low temperature, moderate total nitrogen (TN) concentrations (120 mg TN/L) and regular stops, nitrogen removal efficiencies up to 95 % with nitrogen removal rates of approximately 66 mg TN/(L·d) were obtained. Moreover, the PN/AMX processes performance was immediately recovered after a long starvation period of 15 days (simulating holidays). During the whole operation period, nitrite oxidising bacteria (NOB) activity was suppressed and no significant differences were measured in the specific activities of the involved bacterial populations. The produced effluent presented a good quality in terms of TN concentration that was below 10 mg TN/L, the discharge limit in sensitive areas in the European Union. Results proved for the first time the feasibility of applying the PN/AMX processes for the treatment (and potential reuse) of blackwater produced in a decentralised system where wastewater is not continuously available.

OUTLINE

3.1. Introduction

The increasing water scarcity and resources depletion have triggered efforts on the implementation of sustainable water management approaches (European Commission, 2016, WWAP, 2017). Decentralised wastewater treatment systems become an attractive alternative to be applied in small agglomerations enabling the energy and nutrients recovery, ensuring the local water availability by reusing the treated water and decreasing both investment and operational costs (WWAP, 2017). Source-separation systems allow segregating the different streams for a more intensive treatment, depending on their characteristics and final use, promoting the water reuse (WWAP, 2017). Blackwater (i.e., toilet water) is an organic matter and nutrients concentrated stream contributing to approximately 92 % of total nitrogen (TN), 75 % of phosphorus and 52 % of the organic matter contained in mixed domestic sewage (Gottardo Morandi et al. 2018). Moreover, blackwater composition considerably varies according to its origin, infrastructure, toilet flushing systems and user habits (Gao et al. 2019, Ren et al. 2018), as it is summarised in Table 3.1 blackwater is more concentrated in residential areas whereas the one deriving from workplaces or touristic installations is generally more diluted.

The anaerobic digestion of blackwater allows recovering its energy content as biogas (Gao et al. 2019, Moges et al. 2018). When anaerobic membrane reactors (AnMBR) are used, the high quality and disinfected nitrogen-rich permeate, after the ammonium oxidation to nitrate, may be used as fertiliser while irrigating. However, the irrigation water requirements (volume and nutrients concentration) vary throughout the year and the type of crop growing (European Commission, 2016). Thus, a nitrogen removal system needs to be also considered to obtain a clean effluent suitable for irrigation only, other reuse purposes (e.g., street cleaning, recharge aquifers or industrials uses), or ultimately, for discharge, to reduce the environmental impact of the anaerobic digestion process.

Nitrogen removal can be accomplished autotrophically by the combination of the partial nitritation and anammox (PN/AMX) processes which allows the recovery of the wastewater energy contained in the organic matter. Moreover, the anaerobic biodegradability of the blackwater ranges from 40 to 80 %, meaning that residual organic matter is present in the effluent of the anaerobic digester (De Graaff et al. 2010, Gao et al. 2019).

Table 3.1. Literature review about blackwater composition depending on the origin and toilets flushing system.

Origin	tCOD (g/L)	sCOD (g/L)	pH	TN. (mg N/L)	NH_4^+-N (mg N/L)	Toilet flushing (L/flush)	Reference
Office building	2.0 ± 0.2	0.67 ± 0.16	8.0	145 ± 24 [a]	102 ±8	5 toilet + 1.5 urinal	[1]
Office building	0.4 ± 0.1	0.31 ± 0.03	-	74 ± 6	66 ± 7	-	[2]
32 houses	19 ± 3.4	3.2 ± 0.6	8.6	-	1400 ± 300	1	[3]
32 houses	7.7 ± 2.5	2.3 ± 0.8	8.6 ± 0.5	1200 ± 180	850 ± 150	1 (7.8 L/(p·d)	[4]
44 houses	0.8 - 3.1	-	8.9 - 9.1	130 - 180 [a]	-	-	[5]
15 inhabitants residential	2.9 ± 0.8	-	9.0 ± 0.1	273 ± 39 [a]	202 ± 32	9	[6]
2 houses	0.7 ± 0.1	0.40 ± 0.06	-	149 ± 19	139 ± 20	-	[2]
University	9.5 ± 6.5	1.4 ± 0.5	8.8 ± 0.2	1000 ± 130 [a]	710 ± 10	1	[3]
Student dormitory (48 hab.)	5.5 ± 1.3	1.2 ± 0.3	9.0 ± 0.3	-	900 ± 200	1.2	[7]
Campus lodges	1.1 ± 0.6	0.4 ±1.2	8.0 ± 0.3	180 ± 28 [a]	147 ± 18	9	[8]
Student dormitory (48 hab.)	8.9 - 11.4	-	-	1400 - 1700[a]		1.2	[9]
Tourist park	-	2.2 ± 1.0	8.5 ± 0.6	-	810 ± 240	0.8	[10]
Hotel	-	1.1 ± 0.3	7.2 ± 0.1	194 ± 24 [a]	164 ±28	4	[11]
Clinic		6.1 ± 0.8	7.0 ± 0.2	703 ± 267 [a]	161 ± 20	4	[11]
Residential school	1.7 ± 0.2	0.9 ± 0.2	8.1 ± 0.2	117 ± 28	88 ± 19	5	[12]
Fire Station	0.2 ± 0.1	0.13 ± 0.01	-	62 ± 7	54 ± 9	-	[2]
2 Hotels	0.7 ± 0.2	0.45 ± 0.12	-	69 ± 16	61 ± 14	-	[2]

tCOD: Total chemical oxygen demand; sCOD: soluble chemical oxygen demand; TN: Total nitrogen; [a]TKN: Total Kjeldahl Nitrogen.

References: [1] Gallagher and Sharvelle (2011); [2] Ren et al. (2018); [3] Zeeman et al. (2008); [4] De Graaff et al. (2010); [5] Palmquist and Hanæus (2005); [6] Knerr et al. (2011); [7] Moges et al. (2018); [8] Murat Hocaoglu et al. (2010); [9] Todt et al. (2015); [10] Oarga-Mulec et al. (2017); [11] Lansing et al. (2017); [12] Sharma et al. (2016)

Nevertheless, previous studies indicate that the PN/AMX processes operate stably at moderate nitrogen concentrations and temperature (i.e. the conditions of the anaerobically digested blackwater) when moderate organic matter concentrations are present in the wastewater (Hoekstra et al. 2019, Pedrouso et al. 2018). Despite the number of research studies about blackwater treatment exponentially rose, scarce information is available about the performance of the anammox based processes to treat this type of stream.

When dealing with decentralised systems, the large variations of wastewater flows and concentrations must be considered when defining the operation of the treatment systems. Decentralised systems would have to deal with even more significant fluctuations in both the flow and composition of wastewaters than municipal wastewater treatment plants (European Commission, 2016). In the case of a single office building wastewater, wastewater is not produced during night-time, weekends and holidays. Thus, the biological systems are frequently exposed to famine conditions affecting the process robustness (Wang et al. 2018). Furthermore, in the periods when treated wastewater is used for irrigation nitrogen removal is not required and the corresponding treatment stopped. As anammox bacteria are traditionally considered sensitive to environmental changes, the study of the stability of the anammox process to short-term starvation conditions, mainly focussed on the biomass storage, is available (Wang et al. 2018, Ye et al. 2018). However, limited information exists about the response of the simultaneous PN/AMX processes under oxygen and nitrogen absence (Reeve et al. 2016). In addition, information is missing on the influence of repeated short-term starvation periods on the biomass from a reactor operating in transient conditions, as it can occur in a decentralised system treating blackwater.

3.2. Objectives

The main goal of this chapter is to evaluate the performance of a PN/AMX based process, treating anaerobically digested blackwater at room temperature, and to assess the impact of regular supply and absence of fed wastewater.

Additionally, the effect of the starvation/reactivation periods on the specific activity of the microbial populations present in the sludge (anammox, ammonium and nitrite oxidisers and heterotrophs) is evaluated.

3.3. Materials and Methods

3.3.1. Reactor setup and operation

A sequencing batch reactor (SBR) reactor, with a working volume of 4 L and a volume exchange ratio (VER) of 20 % was operated to perform the PN/AMX processes in one-stage (Figure 3.1). The reactor was inoculated with biomass from a full-scale ELAN® reactor (from the Spanish, *ELiminación Autótrofa de Nitrógeno,* one-stage PN/AMX technology with granular sludge) treating the supernatant from an anaerobic sludge digester of a municipal wastewater treatment plant located in Guillarei (Tui, NW Spain) (Morales et al. 2018). Neither temperature nor pH were controlled while the airflow rate (ranging from 1.0 - 1.5 L/min) was manually adjusted by means of a gas flow meter valve (P model, Aalborg). Mechanical stirring (with a rotational speed of 40 - 50 rpm) was provided to guarantee the reactor mixture.

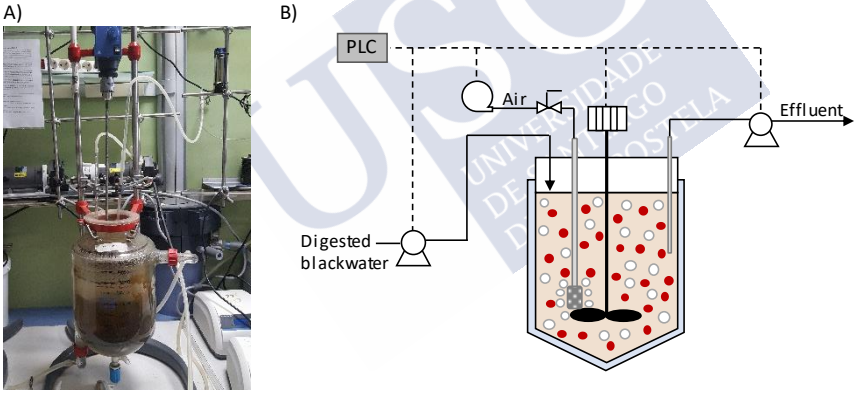

Figure 3.1. A) PN/AMX SBR image and B) Scheme of the PN/AMX reactor set-up.

The SBR was fed with anaerobically digested blackwater (see composition in Table 3.2) collected in an office building (nearly 200 employees) located in Porto do Molle Business Center (Nigrán, NW Spain). The used blackwater was less concentrated than the one used in other studies (Table 3.1), as it was mainly composed by urine and diluted using regular flushing toilets (3.0 - 4.5 L/flush). Raw blackwater (Table 3.2) was digested in an AnMBR comprising an anaerobic stirred reactor (2.8 m^3) coupled to a membrane tank (1 m^3) equipped with an ultrafiltration flat-sheet membrane module (6.25 m^2). The AnMBR was operated at room

temperature (18 - 26 °C) achieving a 90 % of chemical oxygen demand (COD) and producing an effluent with a COD to nitrogen ratio (COD/N) of 0.9 g COD/g NH_4^+-N.

Table 3.2. Characterisation of the raw and anaerobically digested blackwater from Porto do Molle Business Center.

Parameter	Raw	Anaerobically digested
tCOD (mg/L)	2,325 ± 58	98 ± 3
sCOD (mg/L)	553 ± 3	98 ± 3
TOC (mg/L)	117 ± 12	32 ± 6
IC (mg/L)	159 ± 10	124 ± 13
TN (mg/L)	115 ± 6	121 ± 5
NH_4^+-N (mg/L)	95 ± 5	120 ± 12
NO_2^--N (mg/L)	0.02 ± 0.01	0.02 ± 0.01
NO_3^--N (mg/L)	0.3 ± 0.1	0.5 ± 0.1
pH	7.50 ± 0.05	7.25 ± 0.15
Conductivity (mS/cm)	-	1.5 ± 0.2

IC: inorganic carbon**;** TN: total nitrogen**;** TOC: Total organic carbon; tCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand.

The SBR operated in 3-hour cycles distributed as depicted in Table 3.3. It was stopped during the night-time (12 hours) and weekends (2 days) simulating the lack of wastewater produced in a decentralised treatment system from an office building. Therefore, it was only fed 4 cycles/day and 5 days/week resulting in hydraulic retention time (HRT) of 1.75 days.

Table 3.3. Configuration of the Sequencing Batch Reactor operational cycles.

Cycle 1

Anoxic mixed feeding	■			
Aerated reaction		■		
Settling			■	
Withdrawal				■
Time (min)	**5**	**160**	**10**	**5**

Cycle 2

Anoxic mixed feeding	■				
Anoxic reaction		■			
Aerated reaction			■		
Settling				■	
Withdrawal					■
Time (min)	**5**	**20**	**140**	**10**	**5**

The SBR was operated for 100 days in three operational stages depending on the cycle configuration and the regime of stops (Table 3.4). It was started-up and operated for 40 days (Stage I) with the cycle configuration named Cycle 1 (Table 3.3). Then an anoxic reaction phase (20 min) was implemented after the feeding (Stage II-Cycle 2, days 41 - 58). Thus, the aerobic reaction phase was shortened to 140 min (Table 3.3). The reactor was stopped from day 59 to 74 when no monitoring was done. Finally, it was re-started and operated from day 75 to 100 (Stage III) (Table 3.4).

Table 3.4. Summary of the SBR operational conditions during the different stages.

Stages (days)	Temperature (°C)[a]	Cycle[b]	Stops
Stage I (0 - 40)	14.0 - 21.3 (19 ± 2)	Cycle 1	Nights and weekends
Stage II (41 - 58)	14.0 - 20.0 (17 ± 2)	Cycle 2	Nights and weekends
Starvation (59 - 74)	12.8 - 15.6 (15 ± 1)[c]	No	15 days
Stage III (75 - 100)	14.2 - 20.3 (19 ± 2)	Cycle 2	Nights and weekends

[a] As temperature was not controlled, the range indicates the minimum and maximum values measured in the period, while the average value with the standard deviation for the corresponding operational period is reported in brackets.

[b] See in Table 3.3 the definition of each cycle.

[c] Low average values due to winter holidays, with no central heating in the laboratory building.

3.3.2. Ex-situ specific activity tests in batch mode

Ex-situ specific activity (SA) tests were performed collecting the biomass from the reactor in different operational days and following the corresponding protocol in batch mode. The maximum specific anammox activity (SA_{AMX}) was determined according to the manometric method described by Dapena-Mora et al. (2007) and employing 70 mg N/L of both nitrite and ammonium as substrates. The specific heterotrophic denitrification activity (SA_{HDN}) was assessed by the same procedure but using 200 mg COD/L (as acetate) and 25 mg NO_3^--N/L as substrates. Respirometric assays were conducted to determine the specific aerobic heterotrophic activity (SA_{aerHET}), as well as specific ammonium and nitrite oxidising activities (SA_{AOB} and SA_{NOB}, respectively) (Lopez-Fiuza et al. 2002) using a biological oxygen monitor (BOM, Ysi Inc. model 5300) equipped with oxygen selective probes (YSI 5331). All these activity tests were performed in triplicate at 20 °C, and SA_{AMX}

tests were also carried out at 30 °C (temperature of reference). Detailed protocols are provided in Chapter 2, section 2.2.6.

3.3.3. Analytical methods

Influent and effluent SBR streams were periodically sampled to follow the process performance. All samples were filtered using a 0.45 µm pore size filters prior to analysis. Spectrophotometric methods were applied to determine the ammonium (Bower and Holm-Hansen 1980), nitrite and nitrate (APHA-AWWA-WEF, 2012) concentrations. Total chemical oxygen demand (tCOD) in raw samples and soluble COD (sCOD) in filtered samples were also determined according to Standards Methods (APHA-AWWA-WEF, 2012). Dissolved total organic and inorganic carbon concentrations (TOC and IC, respectively) were measured with a Shimadzu analyser (TOC-L-CSN). Total nitrogen (TN) concentration was measured in the same Shimadzu analyser with a TNM-L Unit. The dissolved oxygen (DO) concentration and temperature in the bulk liquid were on-line measured using a luminescent DO probe (LDO, Hach Lange). The pH and conductivity values were determined with electrodes (pH1 and EC5, respectively) connected to a Hach Sension+ meter. The concentration of the total suspended solids (TSS), volatile suspended solids (VSS) and sludge volume index at 30 min (SVI_{30}) and at 10 min (SVI_{10}) were determined according to Standard Methods (APHA-AWWA-WEF, 2012). The average diameter of the granules and size distribution was determined with a stereomicroscope (Stemi 2000-C, Zeiss) incorporating a digital camera (Coolsnap, Roper Scientific Photometrics) for image acquisition and then these images were processed using the Image ProPlus® software. The separation of both granular and suspended sludge fractions to further characterise the biomass from the SBR was performed by means of a 200 µm sieve. The granular biomass density was determined as the mass of VSS per granule volume using the blue dextran method (Beun et al. 2002). Full description of the analytical methods is provided in Chapter 2.

3.3.4. Calculations

Mass balances and calculations were done considering the stoichiometric reactions and equations described in Chapter 2 “Materials and Methods” Section 2.4.5. Statistical analysis was conducted with the software R (version 3.5.2, R Core Team 2015). Details are provided in Chapter 2, section 2.4.2.

3.4. Results and discussion

3.4.1. Performance of the PN/AMX processes

The SBR was inoculated with biomass acclimated to mesophilic temperatures (around 30 °C) and large nitrogen concentrations (> 500 mg TN/L), as well as to lower COD/N ratios (< 0.5 g sCOD/g TN). However, from the start-up, the PN/AMX processes satisfactory took place (Figure 3.2) treating the anaerobically digested blackwater under regular starvation and reactivation periods.

During Stage I, with an applied nitrogen loading rate (NLR) of 70 ± 6 mg TN/L(L·d), the average ammonium removal efficiency was 88 ± 6 % and the average nitrogen removal efficiency (NRE) 79 ± 7 %, with an effluent TN concentration of 24 ± 7 mg TN/L. The organic matter removal efficiency was approximately 46 % with an effluent concentration of 17 ± 6 mg TOC/L. From day 20 onward, when the processes operated stable, nitrite concentration in the effluent was negligible and ammonium and nitrate concentrations close to 10 mg N/L each were measured (Figure 3.2). Moreover, the observed nitrate production to ammonium consumption ratio (0.08 ± 0.01 g NO_3^--N/ g NH_4^+-N, days 20 - 40) was lower than the expected according to the PN/AMX processes stoichiometry (0.11 g NO_3^--N/g NH_4^+-N) (Strous et al. 1999), suggesting the presence of heterotrophic denitrifying activity (Figure 3.2). Mass balance calculations indicated that more than 90 % of the nitrogen removed was due to the anammox process. Organic matter removal occurred by both aerobic (mineralisation) and anoxic routes (nitrate heterotrophic denitrification). The predominant TOC removal pathway highly depended on the DO concentration in the system.

Then, in Stage II an anoxic phase was implemented after the feeding (Cycle 2, Table 3.3) to promote the effluent polishing by using the COD to denitrify the nitrate produced by the PN/AMX processes. Consequently, the NRE significantly increased up to 91 ± 4 % ($p < 0.05$) due to the occurrence and enhancement of the denitrification process. The nitrate production to ammonium consumption ratio decreased from 0.08 ± 0.01 to 0.02 ± 0.01 g NO_3^--N/g NH_4^+-N ($p=0.03$) (Figure 3.2), and the contribution of the denitrification process to the overall nitrogen removal increased from 5 % to 9 % ($p=0.015$). At the end of this period, the TN concentration

in the effluent was lower than 10 mg TN/L (more strict discharge limit in the EU for sensitive areas).

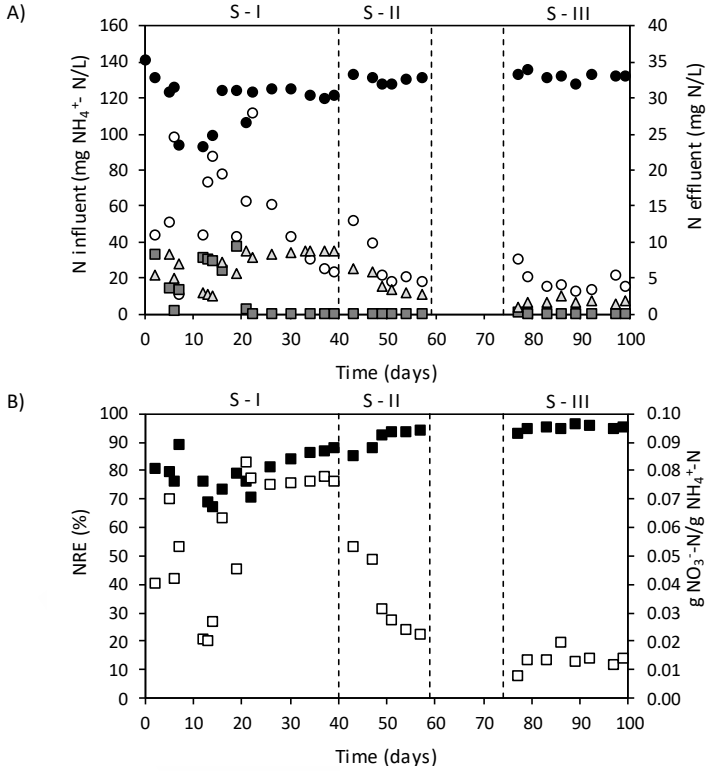

Figure 3.2. Time profiles in the SBR treating anaerobically digested blackwater for: A) ammonium (●) in the influent, and effluent nitrogen forms as ammonium (○), nitrite (■) and nitrate (▲) in mg N/L and B) nitrogen removal efficiency (NRE) in % (■) and the ratio as g/g of nitrate produced to ammonium consumed (□) observed.

Finally, during Stage III, the reactivation after a long starvation period of 15 days (simulating holiday time) was studied. Once the SBR was restarted, the NRE was rapidly recovered and maintained at 95 ± 1 % and the TN concentration in the effluent was 6.5 ± 1.3 mg TN/L, showing the robustness of the system. Thus, the long period of starvation did not negatively affect the PN/AMX processes performance since the high NRE was maintained (if only the last days of Stage II are considered) or slightly increased comparing the complete Stages II and III ($p=0.02$) (Figure 3.2).

From the beginning, the control of the aeration flow rate was revealed as the key parameter influencing the PN/AMX processes performance. During the start-up period, difficulties on adjusting the aeration flow rate caused highly fluctuating process performance, leading to peaks in the effluent nitrite concentrations up to 10 mg NO_2^--N/L (Figure 3.2). However, as airflow rate was properly controlled and maintained at approximately 1 L/min, resulting in a DO concentration of 0.1 - 0.3 mg O_2/L, process stability was achieved and maintained despite the temperature fluctuations which ranged from 14 to 21 °C (18 ± 3 °C on average) (Table 3.3). This difficulty to control the aeration is avoided in large-scale reactors since air-flow rates (i.e., DO concentrations) can be adjusted by using advanced control systems. The DO concentration has been widely reported as a critical parameter to be controlled in the one-stage PN/AMX systems treating municipal wastewater (Agrawal et al. 2018). However, scarce information is available about the PN/AMX processes performance under regular starvation and reactivation periods treating blackwater and how it can be controlled. In a previous study, operating two SBRs, treating synthetic feeding mimicking the anaerobically digested blackwater, one in continuous mode (SBR-C) and other under the same starvation and reactivation regime that the one applied in this study (SBR-D), it was also observed that the DO concentration was the most critical parameter being more challenging to control in SBR-D (Tocco et al. 2019). Furthermore, the blackwater used in the present study was more diluted than the one prepared by Tocco et al. (2019) and therefore, the aeration control was even more challenging. Similar inlet COD/N ratio was used by these authors compared to the one found in the present study, being 0.7 and 0.8 g COD/g TN, respectively. However, they achieved a nitrate production to ammonium consumption ratio in the SBR-D of 0.02 ± 0.01 g NO_3^--N/g NH_4^+-N (Tocco et al. 2019), lower than the 0.08 g NO_3^--N/g NH_4^+-N obtained in the present work with the same cycle configuration (Cycle 1). Moreover, Tocco et al. (2019) did not found significant differences in the denitrification process contribution in the PN/AMX processes comparing SBR-C and SBR-D due to the regular starvation and reactivation periods. In the present study, when the cycle configuration was changed in Stage-II (from Cycle 1 to Cycle 2) the heterotrophic denitrification process contribution was promoted confirming that the starvation periods did not limit the heterotrophic denitrification process, but the feeding composition and/or operation mode. Therefore, it was the low COD/N ratio the limitation for the heterotrophic denitrifying bacteria proliferation, and thus, the anammox pathway was maintained as the main pathway on nitrogen removal.

3.4.2. Biomass and involved activities

The ELAN® inoculum consisted of a mixture of granular and suspended biomass (Figure 3.3.A). During the SBR operation, both solid concentration and average granule size (i.e., diameter) remained almost constant at 2 g VSS/L and 1 mm (Table 3.5), respectively. As the COD in the feeding was low (< 100 mg COD/L) (Table 3.2), the development of heterotrophic bacteria, that grows preferable in the suspended fraction, was limited. Actually, a reduction in the flocculent biomass fraction (in mass fraction) was observed at the beginning of the operation (i.e., from 60 %, day 0, to 45 %, day 30) and then it was maintained at an average value of 42 ± 5 % (Figure 3.3.B).

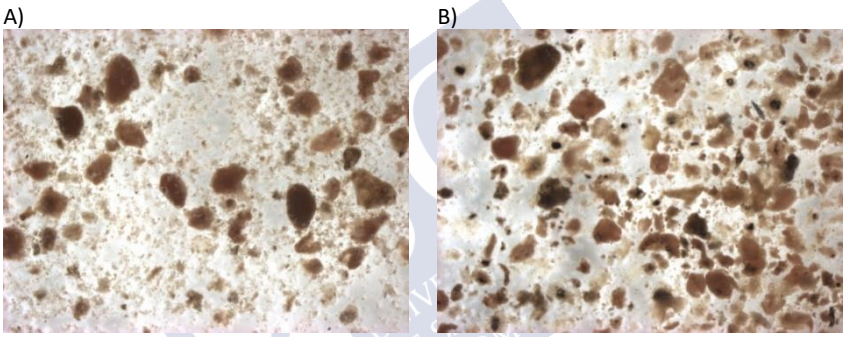

Figure 3.3. Image of biomass samples taken from the PN/AMX reactor on A) Day 0 and B) day 100. Images were taken with a zoon of 0.65 X.

Despite some biomass floatation was observed immediately after the repeated starvation periods, biomass retention was successfully achieved and biomass concentration inside the reactor remained stable. In fact, after only one cycle of operation, the biomass settled properly and the VSS concentration in the effluent was of 7 - 16 mg VSS/L. The sludge sedimentation capacity was maintained or slightly improved throughout the operation with a reduction of the SVI_{30} from 70 (inoculum) to 57 mL/g TSS (Stage III). This enhancement was also corroborated by the biomass density (Table 3.5).

On the contrary, when a similar system was operated fed with synthetic media, a considerable increase of the biomass concentration, suspended sludge fraction and progressive deterioration of the biomass setting properties were observed (Tocco et al. 2019). The improved biomass physical properties observed in the present study,

treating anaerobically digested blackwater, may be attributed to the lower readily biodegradable organic matter content as only 100 mg COD/L were present in the anaerobically digested blackwater whereas 200 mg COD/L (as acetate) were present in the feeding used by Tocco et al. (2019). No adverse effect on the biomass properties neither on the PN/AMX overall processes performance was already observed by Tocco et al. (2019) comparing the reactor with and without regular starvation and reactivation periods (SBR-D and SBR-C).

Table 3.5. Bacterial specific activities (SA), determined at 20 °C, and biomass properties throughout the operational time of SBR.

	Inoculum	**Stage-I**	**Stage-II**	**Stage-III**
Biomass concentration (g VSS/L)	1.91 ± 0.26	1.96 ± 0.15	2.05 ± 0.28	2.09 ± 0.19
Diameter (mm)	1.10 ± 0.10	1.12 ± 0.15	1.10 ± 0.20	1.15 ± 0.14
SVI_{30} (mL/g TSS)	70	61	60	57
SVI_{10} (mL/g TSS)	83	65	63	59
Density (g VSS/Lgranule)	171 ± 2	169 ± 5	175 ± 8	176 ± 3
SA_{AMX} (mg N/(g VSS·d))	210 ± 5	222 ± 8	232 ± 10	230 ± 7
SA_{AOB} (mg N/(g VSS·d))	60 ± 4	71 ± 5	75 ± 6	75 ± 7
SA_{NOB} (mg N/(g VSS·d))	n.d.	n.d.	n.d.	n.d.
SA_{aerHET} (mg COD/(g VSS·d))	60 ± 5	55 ± 6	66 ± 4	60 ± 6
SA_{HDN} (mg N/(g VSS·d))	80 ± 8	72 ± 3	81 ± 5	79 ± 8

*n.d.: no detected.

Regarding the bacterial specific activities (SA), no significant changes were observed in their respective values throughout the operational stages of the SBR (Table 3.5) ($p > 0.45$). During the whole experimental period, no SA_{NOB} was detected and nitrate concentration measured in the effluent was lower than the stoichiometrically expected one for the PN/AMX processes, confirming this data (Figure 3.2). Thus, neither the blackwater composition nor the repeated starvation-reactivation periods imposed do exert a negative effect on the PN/AMX processes performance. This is in good agreement with the work performed by Tocco et al. (2019) who did not found significant differences between the SA from biomass present in a starved and a non-starved SBRs, except for SA_{aerHET} that was lower in the case of the SBR exposed to repeated starvation and reactivation periods. SA_{AMX} determined at the reference temperature of 30 °C also remained almost stable with values of 320 ± 10 mg N/(g VSS·d) on the inoculum and 355 ± 15 mg N/(g VSS·d). The

difference between these values was attributed to the flocculent sludge washout improving the anammox sludge enrichment degree.

Despite the presence of COD (COD/N ratio of 0.8 g COD/g TN), the highest SA measured was the SA_{AMX}, showing the predominant role of this population. As the anammox activity values determined in batch tests (210 - 230 mg N/(g VSS·d)) were higher than the ones observed inside the reactor (36 mg N/(g VSS·d)), the NRR might be limited either by the applied NLR or due to the discontinuous operation with stops. Therefore, the SBR biomass had the potential to treat NLR higher than the one applied. Moreover, specific bacterial activities before and after a weekend stop were measured to assess whether the starvation periods affect the bacterial activities or not, and no significant differences were found (p=0.82; Figure 3.4).

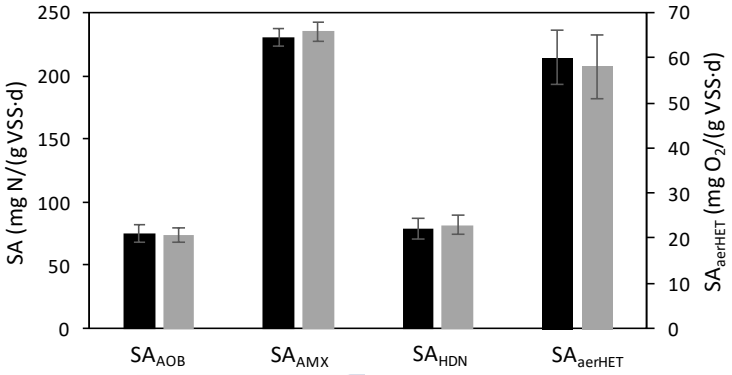

Figure 3.4. Results of ex-situ maximum specific activity tests before (■) and after (■) a weekend stop: ammonium oxidising bacteria (SA_{AOB}), anammox (SA_{AMX}), heterotrophic denitrifying bacteria (SA_{HDN}) in mg N/(g VSS·d) and aerobic heterotrophic bacteria (SA_{aerHET}) in mg O_2/(g VSS·d). All the activities were determined in triplicates at 20 °C.

Finally, the specific microbial activities were also determined on the granular and flocculent biomass fractions monthly. The obtained values at the end of the operational period are shown in Figure 3.5. As expected, bacterial segregation was observed being AOB and heterotrophic (both aerobic and anoxic) bacteria more active in the flocculent biomass, whereas granules were mainly enriched with anammox bacteria. SA_{AMX} was also detected in suspended biomass, probably due to the presence of highly active small anammox granules (< 200 µm) that were difficult to be separated from the suspended biomass.

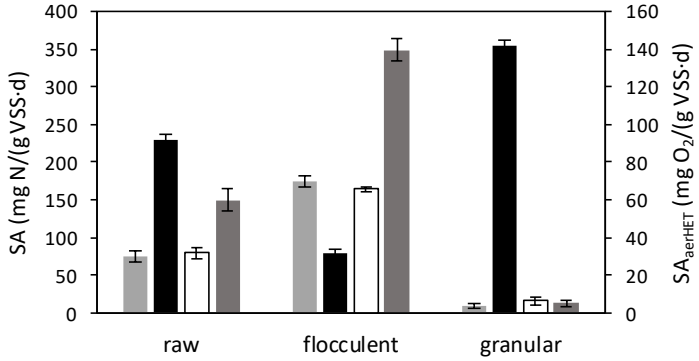

Figure 3.5. Results of ex-situ maximum specific activity tests performed on raw biomass and separated granular and flocculent biomass fractions: ammonium oxidizing bacteria (SA_{AOB}) (■), anammox (SA_{AMX}) (■), heterotrophic denitrifying bacteria (SA_{HDN}) (□) in mg N/(g VSS·d) and aerobic heterotrophic bacteria (SA_{aerHET}) (■) in mg O_2/(g VSS·d). All the activities were determined in triplicates at 20 °C.

3.4.3. Treatment of blackwater via PN/AMX processes

The results of the present study demonstrate the feasibility of a PN/AMX system to treat blackwater at room temperature (14 - 21 °C) under regular starvation and reactivation periods.

Scarce information can be found in the literature about the treatment of blackwater in PN/AMX systems performed in one-stage, where PN and AMX processes take place in the same unit (Vlaeminck et al. 2009) or two-stage , where both processes take place in different units (de Graaff et al. 2011) configurations. Furthermore, these studies were performed in systems operated in continuous mode (without stops), which would be infrequent in a decentralised modular treatment system. Furthermore, the blackwater used in the present study (from an office building with regular flushing toilets) was considerably less concentrated than the one treated in previous studies with anammox based processes (de Graaff et al. 2011, Vlaeminck et al. 2009) as they used blackwater from a demonstration site with vacuum toilets (De Graaff et al. 2010).

Among them, Vlaeminck et al. (2009) treated concentrated blackwater (1 g N/L) in a PN/AMX system achieving average NRE of 76 %, but at temperatures of 25 °C, higher than in the present study. Moreover, these authors experienced difficulties in

managing nitrite oxidising bacteria (NOB) suppression, and $NaHCO_3$ supply was required to raise the pH value and achieve NOB inhibition by free ammonia (FA) (Vlaeminck et al. 2009). In the present study, the NOB activity suppression was obtained, as confirmed by the negligible SA_{NOB} (Table 3.5). During the whole operational period, the pH fluctuated between 6.5 and 7.4, although both FA and free nitrous acid (FNA) concentrations were below the NOB inhibition thresholds (Blackburne et al. 2007). Therefore, the low DO concentration during the operational cycles combined with the starvation periods could be the responsible factors for the NOB activity suppression. Actually, Ye et al. (2019) found that NOB are much more sensitive to starvation conditions than AOB favouring its suppression.

In the study of de Graaff et al. (2011), a two-stage PN/AMX process was applied to promote the residual organic matter (approximately 400 mg COD/L) oxidation in the partial nitritation unit, avoiding the possible negative effects over anammox bacteria in the following unit. They reached NRE up to 89 % in the anammox reactor at 35 °C. However, in the present study it was demonstrated that the residual organic matter in the blackwater can be removed in the single PN/AMX unit without compromising anammox activity, despite the lower temperature and the repeated starvation/reactivation regime.

With respect to the effluent quality in the present study the produced effluent contained low COD (≤ 30 mg COD/L), low nitrogen concentration (≤ 10 mg N/L) and low solids concentration (≤ 20 mg VSS/L) accomplishing the discharge limits set on the Urban Wastewater Treatment Directive (91/271/EEC) and the minimum quality requirements for water reuse defined in the European Commission Regulation (TA(2019)0071).

3.4.4. PN/AMX system robustness under repeated starvation/reactivation periods

The feasibility of operating a PN/AMX system under regular starvation and reactivation periods at room temperature (14 - 21 °C) was proved. To the knowledge of the authors, no previous study has previously investigated all these factors together, as previous literature was focused only on the anammox activity reactivation after storage and/or at higher temperatures. In the present study, both nitritation and anammox activities were re-established immediately after substrate

supply was restored as it was also previously reported for anammox enriched biomass (Ye et al. 2018) and for long-term starvation periods in a PN/AMX system at 28 °C (Reeve et al. 2016). Only, a previous work performed by Tocco et al. (2019) investigated the influence of the repetitive anoxic starvation periods (lasting from 0.5 to 2.5 days) over the PN/AMX processes performance at room temperature. This previous work, performed with synthetic feeding to simulate the anaerobically digested blackwater, concluded that no remarkable effects on the overall process performance due to the starvation periods were observed. Moreover, Ye et al. (2018) investigated the effect of repeated short-term starvation and reactivation cycles over the anammox bacteria activity. These authors stated that repeated starvation periods (1 - 4 days) could increase the recovery rate of anammox activity, providing a pathway to enhance the resilience of the starved anammox sludge. They also found that the SA_{AMX} and tolerance of the anammox sludge was enhanced when the same starvation pattern was repeated (Ye et al. 2018). Such results are in good agreement with the findings of the present study, as with repetitive anoxic starvation periods (lasting from 0.5 to 2.5 days) the maximum specific bacterial activities (including SA_{AMX}) remained constant (Table 3.5) and no detrimental effect over the process performance was observed during the whole reactor operation (Figure 3.2). These results suggest that anammox biomass might quickly adapt to regular repeated anoxic starvation periods. On the contrary to the statement of Ye et al. (2018), in the present study, inhibition due to the starvation was not aggravated by prolonging the starvation time as no negative effect was observed after 15 days of stop. Indeed, Reeve et al. (2016) also observed that nitritation and anammox activities were re-established within one day recovery operation, at 28 °C, after 15, 33 and 62 days starvation periods.

A common approach to address the application of the PN/AMX processes to low temperature and/or nitrogen concentration was to perform a progressive adaptation to the new conditions (Agrawal et al. 2018, Cao et al. 2017). However, the benefits of progressive adaptation are uncertain (Morales et al. 2016). In the present study, the SBR was started-up directly at room temperature treating blackwater and under the repeated starvation/reactivation periods. Despite this fact, high NRE values were achieved and low heterotrophic growth was observed showing that regular stops had no negative effect on the process performance even when the biomass was not previously adapted to the operational low temperature

and blackwater composition. In fact, Tocco et al. (2019) started-up the reactor without starvation periods and once its overall process performance was stable, they implemented the starvation periods. Differently to the present work, they initially develop higher heterotrophic bacterial activities and when the load was decreased (due the stops that reduced the number of cycles per day from 8 to 4) the suspended sludge growth was limited. Therefore, results from present work indicated that a previous acclimation was not required to implement this technology in a decentralized wastewater treatment system.

3.5. Conclusions

Overall, this study demonstrated the feasibility of the application of the one-stage PN/AMX process to treat anaerobically digested blackwater originated in a decentralized system and operated at room temperatures.

The proof of concept of treating real blackwater (120 mg TN/L and 100 mg COD/L) at low temperature (17 ± 2 °C) in a PN/AMX reactor was successfully performed, with short (night-time and weekend) and long (15 days, holidays) starvation periods, achieving high nitrogen removal efficiencies up to 95 % and total nitrogen concentration in the effluent lower than 10 mg TN/L.

Furthermore, measurements of the specific activities (anammox, AOB, NOB and heterotrophic aerobic and anoxic) demonstrated that the operation mode with repeated stops did not affect them and thus, higher nitrogen loads might be treated in these systems without compromising the overall removal efficiencies and without the necessity to have a previous acclimation period.

3.6. References

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Chapter 4

Is the hydraulic load a parameter useful to uncouple AOB and NOB activities?

SUMMARY

The nitrite-shunt applied for the biological removal of nitrogen in wastewater treatment plants reduces the consumption of energy for aeration during nitrification and of organic matter for denitrification. For this purpose, the suppression of the nitrite oxidising bacteria (NOB) activity is a requisite that was successfully achieved for the treatment of nitrogen reach streams (> 200 mg NH_4^+-N/L) and at high temperature (> 25 ° C). One of the most common strategies is based on the control of the solid retention time and profits from the different growth rates of ammonium oxidising bacteria (AOB) and NOB. In this way, NOB are washed out from the system. However, when low nitrogen concentrations and/or low temperatures are applied NOB outcompete the AOB. In the present study, the nitritation process was established in a continuous nitrifying reactor (7.15 L) operated at 16 ± 1 °C and fed with 50 mg NH_4^+-N/L by shortening the operational hydraulic retention time (HRT), in the range of 7.0 to 1.5 days, to select the AOB over the NOB. Experimental data revealed that nitrite started to accumulate at HRT of 4.6 ± 0.2 days. Then, the ratio between AOB and NOB rates increased to 1.54 ± 0.22 g NH_4^+-N/g NO_2^--N when the HRT was further decreased to 1.5 ± 0.1 days. Although AOB and NOB rates were decoupled, the ammonium oxidized to nitrite was below 20 % and nitrate was the most abundant product of the nitrification process. Thus, the application of high hydraulic stress might give a competitive advantage to AOB over NOB, but NOB is not efficiently washed out. For this reason, the application of the hydraulic stress needs to be combined to another strategy to successfully achieve and maintain the nitritation process.

OUTLINE

4.1. Introduction

In the conventional activated sludge systems (CAS), long sludge retention times (SRTs) are applied to biologically remove the nitrogen with typical design SRT values between 4 and 7 days at 20 °C and from 10 to 20 days at 10 °C (Burton et al. 2014). Besides the aeration and the organic matter required in the nitrification-denitrification processes, these long applied SRT lead to lower sludge biomethane productions worsening the wastewater treatment plants (WWTPs) energy efficiency (Burton et al. 2014). Thus, the successful implementation of new processes for nitrogen removal, less energy-consuming, like the nitritation process is of high interest (Agrawal et al. 2018, Ma et al. 2016). The nitritation process has been developed for the treatment of the supernatant of sludge anaerobic digesters operated at mesophilic conditions (30 - 35 °C) by establishing a strategy based on the control of the system SRT (Lackner et al. 2014). For instance, the SHARON (single reactor for high activity ammonia removal over nitrite) process is operated at relatively high temperature (> 25 °C) and high ammonium concentration (> 200 mg NH_4^+-N/L), is based on the selective nitrite oxidising bacteria (NOB) washout due to their slower growth rate compared to that of the ammonium oxidising bacteria (AOB) (Hellinga et al. 1998, Mosquera-Corral et al. 2005, Van Hulle et al. 2007). Thus, in complete mixed SHARON reactors AOB are selectively enriched by applying short SRT values equals to hydraulic retention time (HRT) of 1 to 2 days.

Since the success of this strategy relies on the growth rate of the involved microbial populations, and kinetics are highly influenced by temperature and nitrogen concentrations (Agrawal et al. 2018, Val del Rio et al. 2019), longer SRT values are required when more complex wastewaters are treated, or the temperature is low. For example, Spagni et al. (2014) operated the nitritation process treating landfill leachate but applying an SRT of 4 to 5 days and Durán et al. (2014) applied an SRT of 3 days to treat aerobically pre-treated pig slurry at room temperature (20 - 22 °C). However, limited information is available about the optimal SRT to establish the nitritation process for the treatment of the municipal wastewater at low temperature and low nitrogen concentrations (i.e. mainstream conditions).

At mainstream conditions, the suppression of the NOB becomes difficult by applying short SRT (Agrawal et al. 2018) even at relatively high temperature (25 °C)

and SRT of 6.5 ± 4.5 days (Regmi et al. 2014). These difficulties were mainly ascribed to the niche differentiation of NOB genera that are mostly related to the low substrate concentrations (Regmi et al. 2014). The genus *Nitrobacter* is r-strategist and therefore they have lower substrate affinity and faster growth rate, whereas the genus *Nitrospira* is k-strategist and have lower growth rate but higher substrate affinity. Therefore, *Nitrospira* is the most abundant NOB genera when the liquid media substrate concentration is low such as it is the case of the mainstream in the WWTPs (Nogueira and Melo 2006, Regmi et al. 2014, Winkler et al. 2017). The higher *Nitrospira* spp. abundance explains the fact that strategies such as the dissolved oxygen (DO) concentration control at low values (< 1.5 mg O_2/L) fail to establish the nitritation process (Ma et al. 2016, Regmi et al. 2014). However, the influence of this niche differentiation is not the sole cause of the failure of the different strategies at mainstream conditions, but also the higher activation energy for AOB than for NOB. Thus, AOB activity and growth rate are more affected by the decrease of temperature in comparison with NOB (Burton et al. 2014, Soliman and Eldyasti 2018).

Moreover, at low temperature, most of the approaches to establish the nitritation process were firstly tested in one-stage systems where partial nitritation and anammox (PN/AMX) processes co-occur (Agrawal et al. 2018, Cao et al. 2017). Thus, the SRT strategy was applied combined with other such as DO concentration control or intermittent aeration, to promote the anoxic conditions for the anammox process to take place (Gilbert et al. 2014, Hu et al. 2013). Indeed, recently, hybrid PN/AMX systems were assayed where anammox biomass preferentially grows as biofilm whereas the flocculent sludge is mainly enriched on AOB and, in lesser extent, NOB (Laureni et al. 2019, Malovanyy et al. 2015). It was stated that by controlling the SRT of the flocculent biomass, NOB can be washed-out from the system (Han et al. 2016, Veuillet et al. 2014). Nevertheless, the low values of DO concentration applied in these systems can reduce the effectiveness of the SRT strategy. However, there are not studies focused only on the performance of the nitritation process in complete mixed reactors that tested the SRT as the main control parameter to select the AOB over the NOB at low temperature and low nitrogen concentration conditions. This information would be useful for both nitritation and simultaneous PN/AMX hybrid reactors operation.

4.2. Objectives

The main goal of the present study is to assess the effect of the HRT, from 7 to 1.5 days, on the competition between AOB and NOB in a continuous stirred nitrifying reactor operated at low nitrogen concentration (50 mg NH_4^+-N/L) and low temperature (16 ± 1 °C).

4.3. Materials and Methods

4.3.1. Reactor setup and operational conditions

A continuous stirred tank reactor (CSTR) with a working volume of 7.15 L was operated to perform the nitrifying process (Figure 4.1). The reactor temperature was maintained at 16 ± 1 °C using a thermostatic bath (JP Selecta Frigiterm) that pumped cooled water through the reactor external jacket. The complete mixture was achieved with a mechanical stirrer (IKA RW20) at a rotating speed of 105 - 150 rpm.

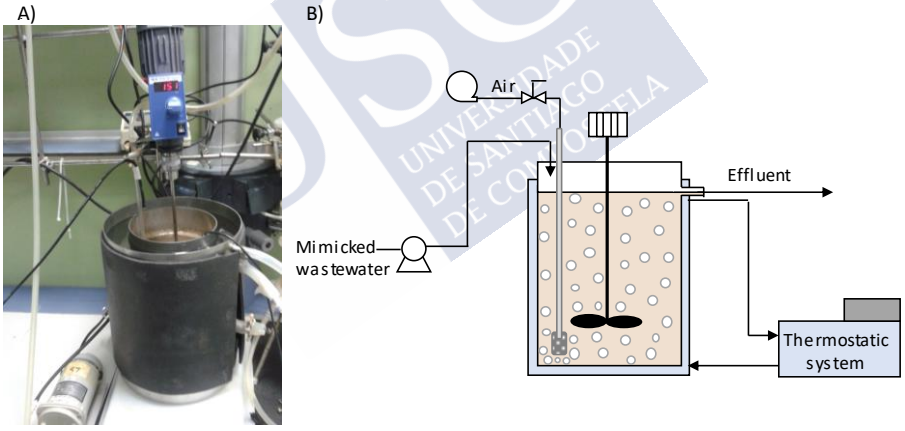

Figure 4.1. A) Nitrifying CSTR image and B) Scheme of the nitrifying reactor set-up.

Aeration was continuously supplied from the bottom of the reactor using a small air bubble diffuser and it was manually adjusted (from day 0 to 110) by means of an airflow meter (P model, Aalborg) to maintain the DO concentration in the range from 1.5 to 2.0 mg O_2/L. From day 111 onwards, a DO controller (Hanna HI 8410) was used with a set-point of 0.8 mg O_2/L and hysteresis value of 0.5 mg O_2/L. The pH value was not controlled but monitored and it ranged from 5.04 to 8.25. The feeding solution supplied using a peristaltic pump (flow 0.7 to 4.8 mL/min) whereas the

effluent was continuously discharged through an overflow pipe. HRT and SRT were intended to remain almost the same, although biofilm attachment on the walls of the reactor was observed. To minimise the HRT and SRT differences, the reactor walls were periodically washed to remove this biofilm.

The reactor was fed with a synthetic mineral medium containing an average ammonium concentration of 50 mg NH_4^+-N/L mimicking municipal wastewater after the organic matter removal step (Table 4.1). From day 20 onwards, the $NaHCO_3$ concentration was increased from 400 to 520 mg/L in order to avoid alkalinity limitations. The reactor was inoculated with biomass collected from a CAS reactor operated at a municipal WWTP where nitrification and denitrification processes occurred.

Table 4.1. Synthetic feeding composition.

Compound	mg/L
NH_4Cl	191
K_2HPO_4	40
H_2KPO_4	15
$NaHCO_3$	400 - 520
$MgSO_4$	40
Trace solution*	4.2 mL/L

*Vishniac and Santer (1957)

The CSTR was operated for 158 days divided into six different stages (Table 4.2) according to the applied HRT that was step-wise decreased from approximately 7 to 1.5 days to evaluate the possibility to out-select the NOB by limiting the corresponding SRT. Considering that the dilution rate is equal to the biomass growth rate (μ) in a CSTR, the growth rate was modified by varying the inflow rate and thus the dilution rate.

Table 4.2. Summary of the operational conditions during the different stages.

Stages	Days	HRT (d)	Dissolved oxygen(mg O_2/L)	Stirring speed (rpm)
I	0 - 13	6.7 ± 0.1	2.67 ± 2.10	150
II	14- 32	5.6 ± 0.2	2.66 ± 1.28	120
III	33 - 46	4.5 ± 0.2	1.97 ± 1.36	105
IV	47 - 56	3.3 ± 0.1	1.13 ± 0.71	105
V	57 - 137	2.1 ± 0.1	1.06 ± 0.68	105
VI	138 - 158	1.5 ± 0.1	0.88 ± 0.33	105

4.3.2. Ex-situ specific activity tests in batch mode

The maximum specific activity (SA) of ammonium and nitrite oxidising bacteria (SA_{AOB} and SA_{NOB}, respectively) were determined by collecting the biomass from the reactor in different operational days and following the methodology described by Lopez-Fiuza et al. (2002). Respirometric bath assays were performed using a biological oxygen monitor (BOM, Ysi Inc. model 5300) equipped with oxygen selective probes (YSI 5331). The batch tests were performed in triplicate and at the reactor operational temperature (15 °C). Detailed determination method is described in Chapter 2 section 2.2.6.3.

4.3.3. Analytical methods

Influent and effluent streams were sampled to follow the process performance three times per week. All samples were filtered using a 0.45 µm pore size filters prior to analysis. Spectrophotometric methods were applied to determine the ammonium (Bower and Holm-Hansen 1980), nitrite and nitrate (APHA-AWWA-WEF, 2012) concentrations. Dissolved inorganic carbon (IC) concentration was measured with a Shimadzu analyser (TOC-L-CSN). The DO concentration and temperature in the bulk liquid were on-line measured using a luminescent DO probe (LDO, Hach Lange). The pH was measured using an electrode connected to Crison 506 measurer. The concentration of the total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Methods (APHA-AWWA-WEF, 2012) with a periodicity of once per month to determine the solid concentration inside the reactor and at least twice per week in the effluent. Further information about the analytical methods is provided in Chapter 2.

4.3.4. Calculations

Free ammonia (FA) and free nitrous acid (FNA) concentrations were calculated using the equations proposed by Anthonisen et al. (1976) shown in section 2.4.1 in Chapter 2 “Materials and Methods”. All the calculations are explained in Chapter 2 too in the section corresponding to nitritation reactors (2.4.3) including the most relevant parameters: ammonium oxidation ratio (AOR), nitrite accumulation ratio (NAR) and the AOB (rAOB) and NOB (rNOB) rates.

Moreover, the SRT_{min} for AOB, defined as the minimum residence time at which the AOB are washed out from the system faster than they grow, was calculated according to Equation 4.1 (Burton et al. 2014):

$$SRT_{min} = \mu_{max,AOB} - b_{AOB} \quad \text{Eq. 4.1}$$

Being $\mu_{max,AOB}$ the maximum specific growth rate of AOB and b_{AOB} the specific endogenous decay rate of AOB at the operational temperature. The temperature dependence of these parameters was estimated following Equations 4.2 and 4.3 and considering a $\mu_{max,AOB}$ of 0.48 d^{-1} and b_{AOB} of 0.024 d^{-1}, at 20 °C (reference temperature).

$$\mu_{max,AOB,T} = \mu_{max,AOB,T}\ (1.072^{T-20}) \quad \text{Eq. 4.2}$$

$$b_{AOB,T} = b_{AOB,T}\ (1.029^{T-20}) \quad \text{Eq. 4.3}$$

being 1.072 and 1.029 the temperature dependency coefficient for $\mu_{max,AOB}$ and b_{AOB}, respectively and T the operational temperature (15 °C).

4.4. Results and discussion

4.4.1. Hydraulic load effect on AOB and NOB competition

The hydraulic load applied to the reactor was progressively increased by reducing the operational HRT. The reactor was started-up with an HRT of 6.7 ± 0.1 days (Stage I). Besides nitrification, and despite the relatively high average DO concentration (Table 4.2), denitrification also occurred since total nitrogen (TN) in the effluent (31 ± 13 mg TN/L) was lower than in the influent (56 ± 1 mg TN/L). During the first days more than 90 % of the incoming nitrogen was oxidised to nitrate and this was partially denitrified (Figure 4.2).

The AOR progressively decreased, increasing the ammonium concentration in the effluent, together with the biomass concentration inside the reactor that lowered from 2.36 ± 0.17 to 1.05 ± 0.02 g VSS/L in only 6 days (Figure 4.2.C). At this HRT, as it was expected, nitrite accumulation was not detected (Figure 4.2.A). The main cause was the applied HRT like those used in the CAS systems (between 4 and 7 days at 20 °C) where the complete nitrification process takes place (Burton et al. 2014). Consequently, the pH value decreased to values as low as 5.0 and the IC concentration to less than 5 mg IC/L (Figure 4.3).

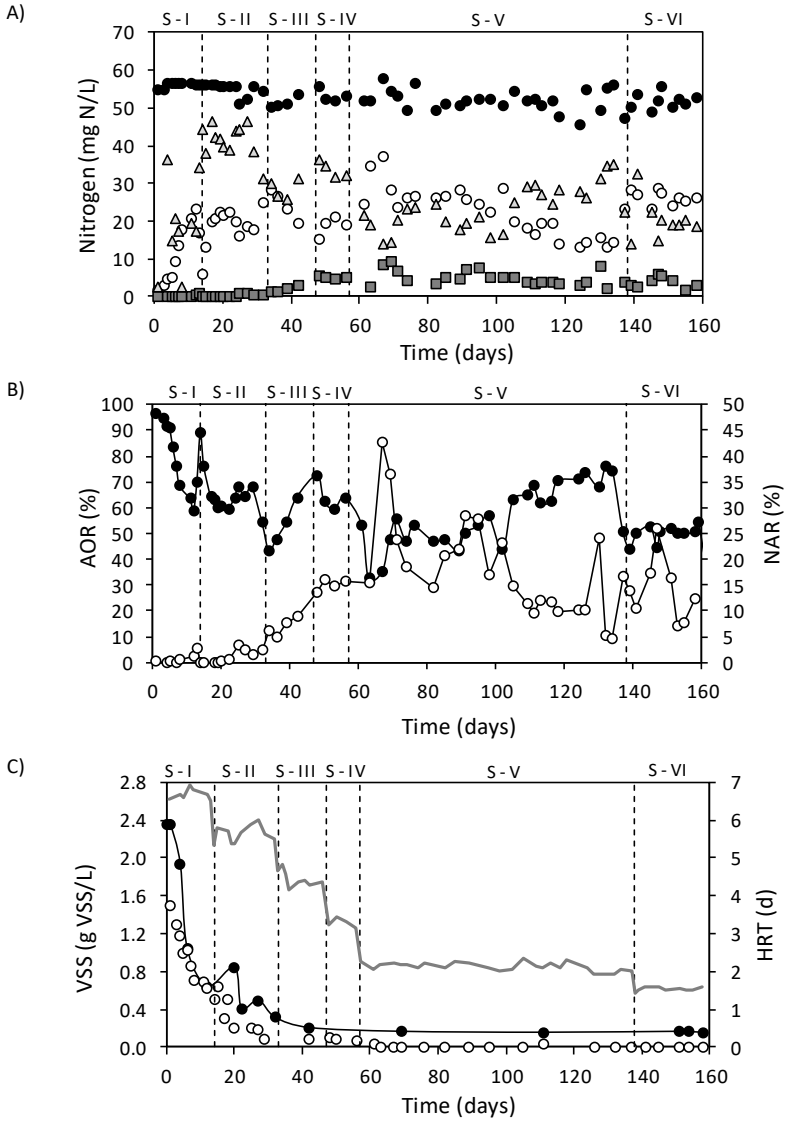

Figure 4.2. Evolution of A) nitrogen compounds: concentration of NH_4^+ in the influent (●) and NH_4^+ (○), NO_2^- (■) and NO_3^- (▲) in the effluent in mg N/L; B) ammonium oxidation ratio (AOR, (●) and nitrite accumulation ratio (NAR, ○) in %; C) biomass concentration inside the reactor (●) and in the effluent (○) in g VSS/L and hydraulic retention time (HRT) in days (grey line).

To counteract the pH decrease inside the reactor (average effluent values of 5.7 ± 0.5, Figure 4.3), caused by the alkalinity depletion during the nitrification process, on day 20 of Stage II (HRT of 5.6 ± 0.2 days) the $NaHCO_3$ added in the feeding was increased. Thus, the incoming nitrogen to inorganic carbon ratio decreased from 0.98 ± 0.05 to 0.74 ± 0.05 g NH_4^+-N/g IC. Consequently, the pH value increased to 6.8 ± 0.8 diminishing the possible inhibitory effect on the AOB activity. It was widely reported that the optimal pH for the nitritation process ranges from 7.5 to 8.0, whereas the AOB rates decrease at pH values lower than 7.0 (Burton et al. 2014, Soliman and Eldyasti 2018). For pH values near 6.0 the FA concentration, that is the actual substrate for AOB, was almost negligible and the expected rAOB would be approximately 10 - 20 % of the rate at neutral pH (Burton et al. 2014). Indeed, during Stage II, the AOR continued to decline, reaching 54 % at the end of this stage, compared to that at the beginning (Figure 4.2.B). This effect might be explained by the VSS concentration decrease (Figure 4.2.C), the alkalinity limitation and/or the achieved low pH values (Figure 4.3).

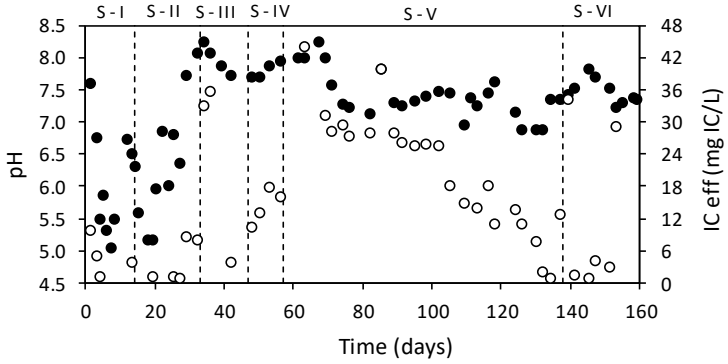

Figure 4.3. Evolution of reactor pH value (●) and inorganic carbon (IC) concentration (○) in mg IC/L.

Later, the HRT was fixed at 4.5 ± 0.2 days (Stage III). The nitrite started to accumulate (approximately 3 mg NO_2^--N/L) (Figure 4.2.A). The AOR increased from 43 % to 64 % (Figure 4.2.B) and the pH value was maintained at average values of 8.0 ± 0.2 (Figure 4.3). Despite the accumulation of nitrite was scarce, leading to a maximum NAR of 9% (Figure 4.2), it was relevant as NOB are traditionally known to be able to consume nitrite much faster than AOB produce it and thus, in complete nitrification systems, nitrite concentrations are lower than 0.10 mg NO_2^--N/L (Burton

et al. 2014). Moreover, both rAOB and rNOB increased during this operational stage (Figure 4.4) indicating that the low biomass concentration was not responsible for the nitrifying capacity limitation, but presumably the alkalinity limitation.

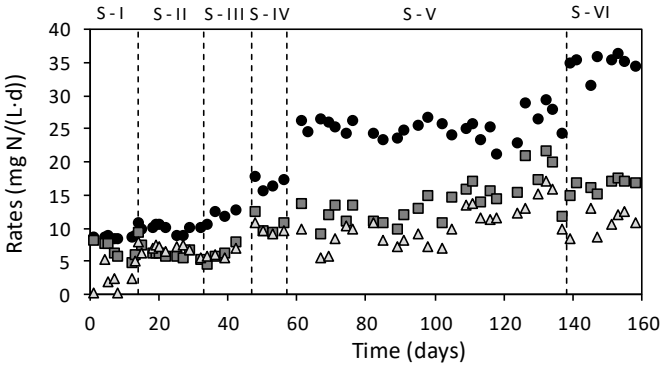

Figure 4.4. Evolution of the nitrogen loading rate (NLR, ●), ammonium oxidising bacteria rate (rAOB) (■) and nitrite oxidising bacteria rate (rNOB, ▲) in mg N/(L·d).

With the progressive decrease of HRT first to 3.3 ± 0.1 (Stage IV) and then to 2.1 ± 0.1 days (Stage V), the slight nitrite accumulation remained at an average AOR of 56 ± 12 % (Figure 4.2). The applied nitrogen loading rate (NLR) was 25 ± 2 mg TN/(L·d) and the achieved rAOB and rNOB were 14 ± 3 mg NH_4^+-N/(L·d) and 10 ± 3 mg NO_2^--N/(L·d), respectively (Figure 4.4). During this stage, both rAOB and rNOB increased with the consequent effluent IC concentration decrease but pH was maintained above 7 (Figure 4.3). The rAOB inside the system was greater than the rNOB, and the NAR was stabilized at average values of 17 ± 9 % (Figure 4.2).

The DO concentration inside the reactor fluctuated significantly until day 110 of operation due to the difficulty to manually adjust the low airflow required to aerate the small volume of the reactor, especially tough at low temperature. In fact, only the mechanical stirring was able to provide DO concentrations between 0.5 and 1.0 mg O_2/L. These fluctuations were successfully mitigated with the DO control implementation on day 111 (Figure 4.5.A and Table 4.2) and despite the achieved NAR percentage still experienced high variations, no correlation was found between both parameters (Figure 4.5.B).

Finally, the HRT was further shortened to 1.5 days (Stage VI), but the NAR was not significantly improved. At the end of the operation, the ratio between rAOB and

rNOB (rAOB/rNOB) was 1.54 ± 0.21, but effective NOB activity suppression was never achieved (Figure 4.2 and Figure 4.4). The decrease of the HRT to 1.5 days in this stage did not promote the nitrite accumulation and therefore it was not further reduced. Nonetheless, it was shown that it is possible to decouple AOB and NOB activities by applying a high hydraulic load in a CSTR. In this sense, no other previous study had reported the HRT values that lead to this difference in rates at low temperature and low nitrogen concentrations.

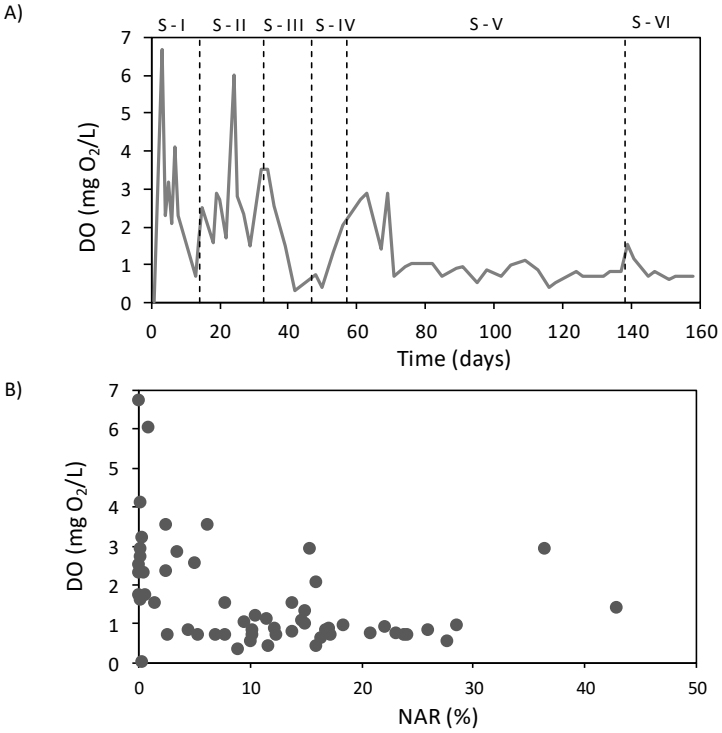

Figure 4.5. A) Time profile of the dissolved oxygen (DO) concentration, in mg O_2/L, in the bulk liquid of the CSTR. B) Correlation between DO concentration and nitrite accumulation ratio (NAR) in percentage.

During the experimental period, the applied food to microorganisms (F/M) ratio varied from 0.003 to 0.206 g N/(g VSS·d). The latter is a value higher than those applied to CAS systems (Burton et al. 2014). The increase of the F/M caused the decrease of the AOR (from 96 % in the start-up to 56 % Stage VI) (Figure 4.2.B) but also the high increase of the SA_{AOB} that varied from 10 (day 0) to 130 (day 109) and

finally to 1,140 mg NH_4^+-N/(g VSS·d) on day 155. The SA_{AOB} increase might indicate the progressive selection of nitrifying populations in the reactor. Regarding the SA_{NOB}, it was under the detection limit in the case of the inoculum (but NOB were present as nitrification took place in the reactor of origin) reaching maximum values of 290 mg NO_2^--N/(g VSS·d) during the CSTR operation.

4.4.2. Effectiveness of SRT to achieve the nitritation process

The lowest HRT tested in the CSTR was of 1.5 days. This value is like that used in the SHARON reactors which operate at higher temperatures (< 30 °C). Considering that the bacterial growth rate diminishes with the temperature decrease, in the present study would not be reasonable to operate at lower HRT values (Hellinga et al. 1998, Mosquera-Corral et al. 2005). Indeed, considering the $\mu_{max,AOB}$ of 0.34 d^{-1} and b_{AOB} of 0.028 d^{-1} at 15 °C, the resulting SRT_{min} would be 3 days (calculated according to Equation 4.5). In this sense, Durán et al. (2014) established and maintained the nitritation process in a CSTR operated with HRT = SRT of 3 days at room temperature (20 - 22 °C) but treating pig slurry pre-treated in an aerobic granular plant characterised by relatively high ammonia content (close to 400 mg NH_4^+-N/L).

However, in the present study HRT lower than the estimated SRT_{min} were applied without causing the total biomass washout. This fact is explained by the differences existing between the biomass concentration inside the reactor and the one determined in the effluent (Figure 4.2.C). For this reason, in practice, SRT and HRT were not the same and biomass retention occurred due to the biomass attachment onto the reactor walls. To minimise this effect, biomass detachment was periodically performed (twice per week) by cleaning the reactor walls. In spite of this action, the VSS concentration in the reactor remained at 150 - 200 mg VSS/L whereas the effluent concentration was lower of 100 mg VSS/L from day 29 onwards. Thus, the actual performance of the reactor was not as an ideal CSTR and the SRT was higher than the one expected by the applied HRT.

Summing up the increase of the hydraulic load provokes nitrite accumulation at HRT lower than 4.5 days. But this single action was not enough to maintain the desired nitrite accumulation. This result is in good agreement with previous studies, which stated that SRT control would be not enough to completely suppress the NOB

but its combination with other strategies such as intermittent aeration might be useful to establish the nitritation process at low temperature (Agrawal et al. 2018, Regmi et al. 2014).

4.4.3. FA and FNA concentrations contribution to decouple AOB and NOB rates

Besides SRT, FA and FNA inhibitory action over AOB and NOB were also used to promote the NOB suppression (Durán et al. 2014, Vadivelu et al. 2007) as they are more sensitive to these nitrogenous species. The FA and FNA concentrations depend on the pH in the bulk liquid. Reported NOB inhibitory concentrations are of 22 µg HNO_2-N/L and approximately 0.1 - 10 mg NH_3-N/L, respectively (Vadivelu et al. 2007). However, as the average pH value from day 33 onwards (when nitrite started to be accumulated) was 7.5 ± 0.4, maximum FNA concentration achieved was 3 µg HNO_2-N/L not enough to cause the NOB inhibition (Figure 4.6).

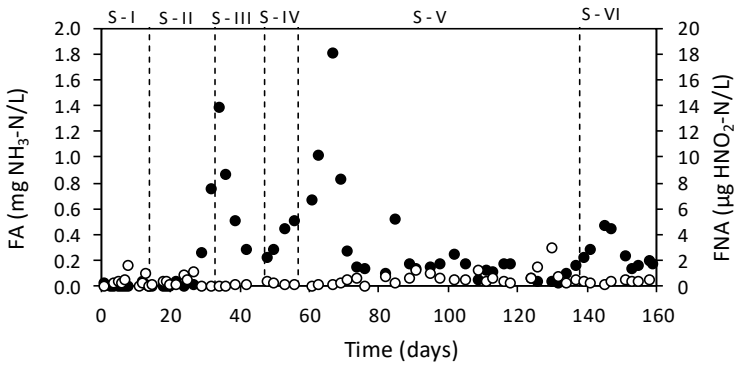

Figure 4.6. Evolution of the free ammonia (FA) in mg NH_3-N/L (●) and free nitrous acid (FNA) in µg HNO_2-N/L (○) during the reactor operation.

Several authors observed the inhibition of NOB by FA accumulation treating wastewaters with high nitrogen concentrations in systems operated at moderate to high temperatures (Durán et al. 2014, Pichel et al. 2019, Zhang et al. 2018). For instance, Wang et al. (2017) and Duan et al. (2019) achieved the nitritation process at mainstream conditions by exposing the sludge to inhibitory FA concentrations but in an external unit that operated at high nitrogen concentration (200 mg NH_3-N/L) and moderate temperature (22 °C). In the present study, despite FA concentrations higher than 0.1 mg NH_3-N/L were achieved from the end of Stage II, they were not

high enough to suppress the NOB activity and nitrate production was still observed (Figure 4.6).

4.4.4. Evolution of the AOB and NOB rates and its practical implications

Despite, in the present study, the complete and stable nitritation process was not successfully achieved, it was shown that the application of high hydraulic stress might give a competitive advantage to AOB over NOB. The ratio between rAOB and rNOB (rAOB/rNOB) progressively increase with the HRT decreased reaching maximum values of 1.54 ± 0.22 in Stage VI (Figure 4.7). This fact indicated a potential capacity for nitrite accumulation, but the NAR was limited to values lower than 20 % (Figure 4.7). The value of rAOB/rNOB ratio in Stage I was not included in Figure 4.7 since nitrogen loss was observed during the reactor start-up.

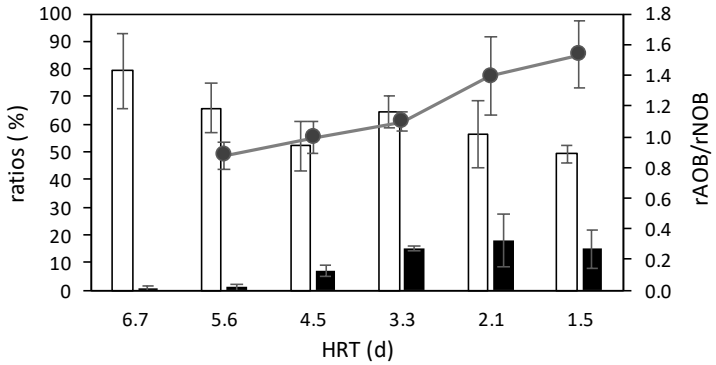

Figure 4.7. Evolution of the ammonium oxidation ratio (AOR) (□) and the nitrite accumulation ratio (NAR, ■) in percentage and the ratio between the rate of ammonium oxidising bacteria (rAOB) and nitrite oxidising bacteria rate (rNOB, ●).

Thus, results demonstrated that it is possible to decouple the AOB and NOB activities at low temperature and low nitrogen concentrations by applying low HRTs in a continuous system. One of the main limitations of the system might be the fact that the actual SRT was not the same as the HRT due to the biomass growth attached to the reactor walls. This knowledge could be interesting for the operation of biofilm-based systems since NOB tend to grow in flocculent sludge (hybrid systems) or in small granules (Morales et al. 2016, Veuillet et al. 2014). With this information, it seems possible that if the reactor is completely mixed and the applied HRT is low, AOB could be promoted over NOB.

4.5. Conclusions

Large hydraulic loads (HRT < 4.5d in a CSTR) promote the AOB rate over the NOB one allowing to activate the nitrite accumulation at mainstream conditions (16 ± 1 °C and 50 mg N/L). However, the nitritation process was not fully developed since only 20 % of the ammonium oxidised was converted to nitrite whereas the remaining 80% ended up as nitrate.

Low applied HRT need to be combined with other NOB suppressing factors to enable the development and maintenance of the nitritation process.

The biomass growth attached to the reactor walls hinders to operate the system as CSTR (SRT = HRT) at this scale and therefore to determine a limit SRT to foster the AOB growth over the NOB one. If this phenomenon could be avoided, the nitrite accumulation ratio is expected to be increased.

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Chapter 5

Nitrite oxidising bacteria suppression based on *in situ* free nitrous acid production

SUMMARY

The free nitrous acid (FNA) is a known nitrite oxidising bacteria (NOB) inhibitor at concentrations over 0.02 mg HNO_2-N/L. It is accumulated inside the reactor when large nitrite concentrations and low pH are present. In these occasions, the nitratation process is suppressed. At mainstream conditions, this simple alternative was never explored before. In the present study, stable nitritation was achieved operating a 2-L sequencing batch reactor (SBR) at 16 ± 1 °C, and fed with 50 mg NH_4^+-N/L. The FNA concentrations reached values inhibitory for NOB (up to 0.06 mg HNO_2-N/L) and they were generated profiting from the consequent pH decrease associated with the alkalinity consumption and the nitrite produced by the nitritation process itself. The stimulation of nitritation process was evaluated using two inhibitors: sodium azide and nitrite. The microbiological analysis of the biomass revealed that, throughout the operational period with inhibitory FNA levels, the NOB populations (dominated by *Nitrospira spp.*) were effectively washed out from the reactor. The success of this strategy relies on the wastewater composition in terms of nitrogen to inorganic carbon (IC) ratios. Different ratios (0.5 - 1.0 g N/g IC) were evaluated. Obtained results indicated that ratios below 0.6 g N/g IC, the *in situ* accumulated FNA concentrations were not high enough to suppress the NOB activity. In this situation, it takes approximately 40 days to develop noticeable NOB activity confirming the robustness of this strategy. The observed delay on the NOB development is expected to enable the establishment of corrective actions to avoid the nitritation process destabilisation. In the present research study, an analysis of the influence of ammonium and alkalinity concentrations was also performed to define those scenarios which allow to produce inhibitory FNA concentrations.

OUTLINE

5.1. Introduction

The stable operation of nitritation process has been revealed as one of the main challenges at mainstream conditions. The difficulties of avoiding the nitrite oxidising bacteria (NOB) activity to occur hinder the application of the combined partial nitritation-anammox (PN/AMX) processes at low temperature and low nitrogen concentrations (Agrawal et al. 2018).

Different strategies have been proposed to induce the suppression of the NOB activity at temperatures over 15 °C (Ge et al. 2015) mostly based on the control of the sludge retention time (SRT) or on the different affinities for oxygen of ammonium oxidising bacteria (AOB) and NOB (Agrawal et al. 2018, Val del Rio et al. 2019). The later relies either on the control of the dissolved oxygen (DO) concentration, aeration phase length or the exploitation of the lag-phase exhibited by NOB after transient anoxic and aerobic conditions by applying intermittent aeration strategies (Ge et al. 2015, Jiang et al. 2019, Regmi et al. 2014, Yang et al. 2017). Contrary to these observations, results from other studies indicate that NOB activity cannot be wholly suppressed, due to the NOB higher oxygen affinity and growth rate than AOB at low temperature (under 20 - 25 °C) (Agrawal et al. 2018, Regmi et al. 2014). Additionally, most of these previous research works were performed in one-stage PN/AMX systems, where the operational conditions need of optimisation to suppress NOB activity and to promote anammox activity. In these one-stage systems, low nitrogen removal efficiencies (NRE) are achieved due to nitrate production, associated to the anammox process, and limited DO concentration, imposed to avoid the NOB development, that limits the ammonium oxidation rates (Laureni et al. 2016, Lotti et al. 2015). Thus, to operate the PN and the AMX processes in two different reactors is expected to favour their separated optimisation facilitating the achievement of the global process stability (Pérez et al. 2015).

Nowadays, scarce information is available about research studies focused on the NOB suppression in single nitritation units operated at mainstream conditions. Some studies have maintained the nitritation process at these conditions by controlling the DO to total ammonia nitrogen (TAN) concentration ratio (DO/TAN) (Liu et al. 2018, Reino et al. 2016); or by using strategies based on the fact that inhibitory thresholds to free ammonia (FA) (Wang et al. 2017, Zhang et al. 2018) and free nitrous acid (FNA) are lower for NOB than AOB.

As an example, the feasibility to achieve and maintain the NOB suppression at mainstream conditions (40 - 57 mg NH_4^+-N/L and 22 °C) by exposing a fraction of the nitrifying sludge to the FNA produced in an additional unit placed in the side-stream line (high nitrogen concentration and mesophilic conditions) was demonstrated (Duan et al. 2018, Wang et al. 2016, Wang et al. 2014).

The FNA concentration required to inhibit 50% of AOB activity was found to be in the range 0.42 - 1.72 mg HNO_2-N/L (Zhou et al. 2011), much higher than the 0.02 mg HNO_2-N/L needed for the complete inhibition of NOB activity (Blackburne et al. 2007). Therefore, the use of FNA appears as a suitable alternative to inhibit the NOB at mainstream conditions, which need to be further explored to establish those conditions that guarantee its production inside the nitritation unit. The NOB suppression becomes more challenging when the inoculated sludge has already a significant NOB activity, which makes the initial accumulation of required nitrite concentrations difficult. In this case, the use of an inhibitory compound for NOB, such as sulphide, hydrazine, hydroxylamine or azide, can be adequate (Ge et al. 2015).

When sodium azide is chosen, the different inhibition thresholds (IC_{50} values of 40 mg NaN_3/L and 0.025 mg NaN_3/L, over AOB and NOB respectively (Lopez-Fiuza et al. 2002)) can be exploited to inhibit selectively the NOB activity with absent or minimal effect on AOB. Actually, this compound is frequently used to perform AOB batch activity tests (Guisasola et al. 2005). However, the long-term effect of sodium azide on NOB activity, to research its inhibitory effect or the possible acclimation of the biomass to this compound, has not been studied yet. Another possibility is to directly supply nitrite inside the reactor (Cui et al. 2019), but its possible consumption by NOB hinders the nitritation process establishment.

5.2. Objectives

The main objective of this study was to evaluate the performance of a nitritation unit, with flocculent biomass, operated at 16 °C and fed with 50 mg NH_4^+-N/L, where NOB activity was suppressed by the *in situ* accumulation of inhibitory FNA concentrations.

Furthermore, the addition of a chemical inhibitor (sodium azide) and nitrite were evaluated as potential strategies to initiate the nitrite accumulation necessary to achieve the desired FNA concentration levels.

5.3. Materials and Methods

5.3.1. Reactor setup and operation

A glass laboratory sequencing batch reactor (SBR), with a working volume of 2 L and useful height to diameter (H/D) ratio of 0.38, was used to perform the nitritation process (Figure 5.1). It was provided with a set of two peristaltic pumps to introduce the feeding solution, through the top, and to discharge the effluent, at a medium height in the column reactor. The volume exchange ratio (VER) was of approximately 50%. Compressed air (KNF Labport pump) was supplied through an air diffuser at the bottom of the reactor to guarantee the complete mixture and to supply the oxygen required for the biological reactions. Variable airflow rates, ranging from 0.3 - 1.5 L/min, were supplied to maintain the DO concentration in the range from 0.2 to 8.0 mg O_2/L. The pH values were not controlled and varied from 6.3 to 8.0. The reactor temperature was maintained at 16 ± 1 °C using a thermostatic bath (JP Selecta Frigiterm).

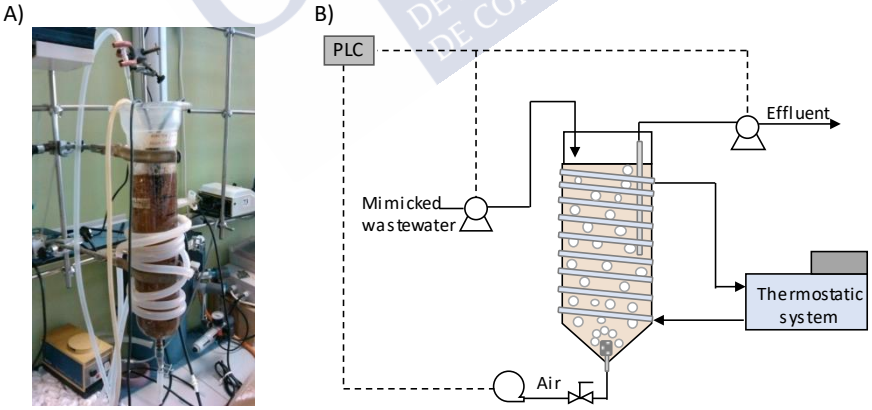

Figure 5.1. A) Image of the nitritation SBR B) Scheme of the SBR set-up.

The SBR operated in 3-hour cycles (Figure 5.2) comprising: 158 min of continuous feeding and aeration, 20 min of settling and 2 min of effluent withdrawal.

The operation of the different periods of the SBR cycle was controlled by a programmable logic controller (PLC, Siemens S7-224CPU).

Feeding			
Aeration			
Settling			
Discharge			
Time (min)	**158**	**20**	**2**

Figure 5.2. SBR cycle configuration

The inoculum consisted of 9.1 g VSS/L of sludge taken from a one-stage PN/AMX laboratory reactor operated at 16 °C and fed with 50 mg NH_4^+-N/L (Morales et al. 2016). At the moment of the collection, the PN/AMX process performance in the source reactor was not stable due to the existence of significant NOB activity.

During the first 110 days of operation, the SBR was fed with the same kind of wastewater as the reactor origin of the inoculum that was supernatant collected from an anaerobic sludge digester placed in a municipal wastewater treatment plant (WWTP). This supernatant was diluted with tap water to achieve the desired concentration of 50 mg NH_4^+-N/L and supplemented with sodium bicarbonate (0.57 g $NaHCO_3$/L) to avoid alkalinity limitations maintaining an inorganic carbon (IC) concentration of 128 mg IC/L. Later, to have a better control of the feeding media composition, a synthetic media containing 50 ± 7 mg NH_4^+-N/L supplemented with 0.4 - 0.8 $NaHCO_3$, 0.040 K_2HPO_4, 0.015 KH_2PO_4, 0.040 $MgSO_4$ in g/L and 0.2 mL/L of a trace solution (Vishniac and Santer, 1957) was used as feeding to the SBR. The feeding was prepared with tap water containing average concentrations of 2.5 ± 0.1 mg IC/L and 1.8 ± 0.6 mg NO_3^--N/L. The hydraulic retention time (HRT) was fixed at 0.25 days and the nitrogen loading rate (NLR) at 200 mg N/(L·d).

The reactor operated during 690 days, in total, divided into seven operational stages (I-VII) depending on the alkalinity of the feeding (IC concentration) and the addition or not of an external NOB inhibitor (sodium azide or nitrite) (Table 5.1). The Stage I (0 - 64 days) corresponded to the establishment of the nitrification process. In Stage II (65 - 110 days), the feeding was supplemented with 5 mg NaN_3/L to inhibit NOB activity. In stages I and II, the IC concentration was of 123 ± 11 mg IC/L. Then in Stage III (111 - 210 days), this concentration was decreased to 57 ± 4 mg IC/L to limit the alkalinity for the nitritation process establishment. This condition remained during Stage IV (211 - 384 days) when the NaN_3 addition was stopped. In Stage V

(385 - 440 days), the IC concentration was increased to 99 ± 6 mg IC/L, to restore the conditions for complete nitrification occurrence. Then, in Stage VI (441 - 489 days), the IC supply was diminished again to 52 ± 3 mg IC/L, to analyse the influence of the alkalinity limitation. Finally, in Stage VII (490 - 690 days) nitrite as $NaNO_2$ was supplemented to the feeding at a concentration of 78 ± 5 mg NO_2^--N/L to test another compound to activate the *in situ* FNA accumulation. The supplied nitrite concentration was added in excess to assure that NOB activity in the system (351 mg NO_2^--N/(g VSS·d)) was not enough to fully oxidise it to nitrate and that the remaining nitrite allowed for the accumulation of FNA concentrations inhibitory for NOB.

Table 5.1. Operational conditions and influent characteristics of the different operational stages.

Stage	Days	NH_4^+-N/IC (g N/ g IC)	Inhibitor addition	Objective
I	0 - 64	0.39 ± 0.07	No	Total nitrification
II	65 - 110	0.38 ± 0.05	NaN_3 (5 mg/L)	Nitrite accumulation
III	111 - 210	0.88 ± 0.09	NaN_3 (5 mg/L)	Nitritation
IV	211 - 384	0.90 ± 0.07	No	FNA inhibitory levels
V	385 - 440	0.52 ± 0.04	No	Alkalinity increase
VI	441 - 489	1.01 ± 0.06	No	Alkalinity limitation
VII	490 - 690	0.93 ± 0.04	$NaNO_2$ (80 mg N/L)	FNA inhibitory levels restoration

5.3.2. Batch specific activity tests

Sludge samples were collected periodically from the reactor to determine the maximum specific activities (SA) of AOB and NOB (SA_{AOB} and SA_{NOB}, respectively). Both activities were determined by respirometric assays using a Bench model Oxygen Meter (YSI 5300) with oxygen selective probes (YSI 5331) according to the methodology described by Lopez-Fiuza et al. (2002). Full details of the used protocol are provided in Chapter 2, section 2.2.6.3.

5.3.3. Analytical methods

The DO concentration and temperature in the bulk liquid were measured on-line using a luminescent DO probe (LDO, Hach Lange). The pH value was measured using an electrode connected to a measurer (Crison 506). Liquid samples, collected from the reactor (influent and effluent) three times a week, were filtered through 0.45 µm pore size filters before analysis. Spectrophotometric methods were applied

to determine the ammonium (Bower and Holm-Hansen 1980), nitrite and nitrate (APHA-AWWA-WEF, 2012) concentrations. Dissolved total organic and inorganic carbon concentrations (TOC and IC, respectively) were measured with a Shimadzu analyser (TOC-L-SCN). Samples from the reactor and the effluent were collected to determine the biomass concentration as total suspended solids (TSS) and volatile suspended solids (VSS), and the sludge volume index (SVI), according to standard methods (APHA-AWWA-WEF, 2012). All the analytical methods are described in detail in Chapter 2 "Materials and methods".

Main bacterial populations present in the biomass samples were identified by the Fluorescence *in situ* Hybridization (FISH) technique and 16S rRNA gene-based amplicon analysis (Base Space, Illumina®) according to the procedure described in Chapter 2, section 2.3. The FISH probes used were: EUB338mix for all *Bacteria*, Ntspa712 for *Nitrospira sp.* and Nit3 for *Nitrobacter spp*.

5.3.4. Calculations

The FNA concentration was estimated according to Anthonisen et al. (1976) using Equation 2.11 (Chapter 2). The SRT was calculated as the VSS concentration in the reactor times the HRT and divided by the VSS concentration in the effluent. Other parameters such as the ammonium oxidation ratio (AOR) and nitrite accumulation ratio (NAR) were calculated according to section 2.4.3 in Chapter 2.

A sensitivity analysis was carried out, using Matlab® software, to determine the impact of the inlet ammonium concentration (from 10 to 100 mg NH_4^+-N/L) and the inlet ammonium to inorganic carbon concentration (NH_4^+-N/IC) ratio (from 0.5 to 1.5 g N/g IC) on the FNA accumulation. These ranges were set considering the ammonium and IC concentrations typically found in municipal wastewater produced in areas with low-moderate alkaline freshwater. It was considered that 2 mol of IC are consumed per 1 mol of ammonium oxidised to nitrite. If the IC is supplied in excess (NH_4^+-N/IC ratio < 0.6 g N/g IC) a full conversion of ammonium to nitrite is achieved and some IC is left. If the IC is not sufficient (NH_4^+-N/IC ratio > 0.6 g N/g IC) a fraction of ammonium is not oxidised. In both cases, the pH is estimated from the remaining concentrations of ammonium and IC, and the respective CO_2-carbonate-bicarbonate and ammonium-ammonia equilibria. With the obtained pH values and the nitrite concentrations, the corresponding FNA concentration was estimated.

5.4. Results

5.4.1. Nitritation reactor operation

The SBR was operated for 690 days (Figure 5.3). During the first 64 days of operation (Stage I), the nitrification process was established. Ammonium concentration in the influent ranged from 35 to 60 mg NH_4^+-N/L (Figure 5.3.A). After the first 20 days, ammonium was oxidised entirely to nitrate while nitrite was not detected as an intermediate compound (Figure 5.3.A). The reached AOR was 97 ± 5 % (Figure 5.3.B). Measured SA_{AOB} and SA_{NOB} in the inoculated biomass were 50 ± 6 mg NH_4^+-N/(g VSS·d), and 72 ± 12 mg NO_2^--N/(g VSS·d), respectively (Table 5.2). The DO concentration inside the reactor was not controlled and significantly oscillated from 0.3 to 3.0 mg O_2/L (Figure 5.4).

In Stage II, immediately after the addition of 5 mg/L of sodium azide, an inhibitory effect on the previously developed NOB activity was observed and the accumulation of nitrite experienced a progressive increase up to approximately 35 mg NO_2^--N/L, while nitrate concentration proportionally decreased (Figure 5.3.A). Approximately 45 days of sodium azide supply were required to eliminate most of the NOB activity inside the system and to achieve a NAR close to 100 % (Figure 5.3.B). The inlet NH_4^+-N/IC ratio was approximate of 0.38 g N/g IC in Stages I and II, which is low enough to accomplish the complete nitrification resulting in surplus of IC in the effluent with average concentrations of 57 ± 12 mg IC/L (Figure 5.5).

In Stage III, the supplied bicarbonate concentration decreased (0.88 g N/g IC) limiting the AOR to average values of 61 ± 11% (Figure 5.3.B). Besides the NaN_3 acted as inhibitor of the NOB activity, in this stage, the decrease of pH value (due to alkalinity limitation) promoted the accumulation of FNA up to 0.01 mg HNO_2-N/L (Figure 5.3.C). The IC concentration in the effluent decreased from 66 ± 8 to 7 ± 2 mg IC/L (Figure 5.5). The effluent was composed, as average, by 18.4 ± 5.7 mg NH_4^+-N/L, 25.9 ± 5.6 mg NO_2^--N/L and 2.6 ± 1.0 mg NO_3^--N/L. Most fraction of the nitrate concentration measured was already present in the tap water used for the feeding media preparation, and therefore its production by NOB was considered negligible and the NAR remained at average values of 99 ± 1 % (Figure 5.3.B). This observation was confirmed by the undetectable SA_{NOB} measured in the performed respirometric assays and the SA_{AOB} of 68 ± 28 mg NH_4^+-N/(g VSS·d) (Table 5.2).

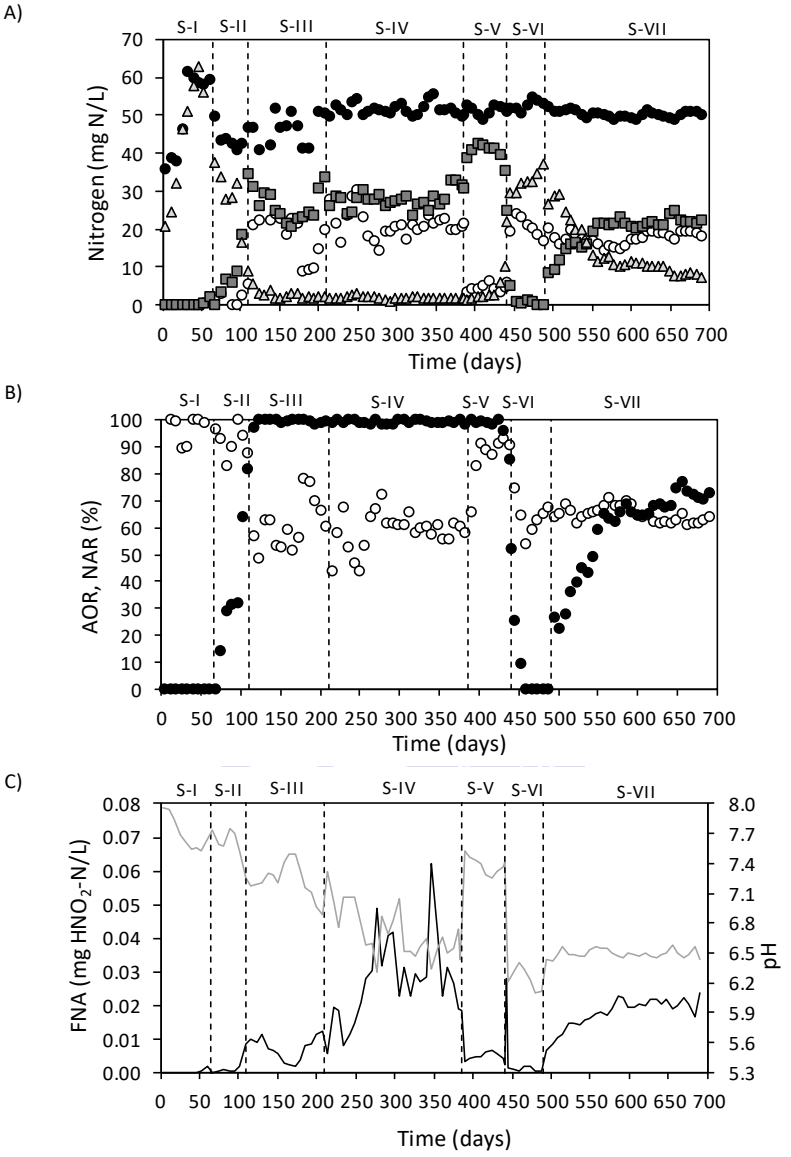

Figure 5.3. A) Evolution of nitrogen compounds in the SBR: concentrations of NH_4^+ in the influent (●) and NH_4^+ (○), NO_3^- (▲) in the effluent and NO_2^- (■) accumulated, in mg N/L; : B) Ammonium oxidation ratio (AOR, ○) and nitrite accumulation ratio (NAR, ●) in percentage; C) Evolution of FNA concentration (–) in mg HNO_2-N/L and pH value (–) at the end of the operational cycle.

In Stage IV, the supply of sodium azide was stopped and the AOR was maintained at average values of 59 ± 13%. The NOB activity repression due to the single action of the *in situ* FNA accumulation was studied at concentrations from 0.01 (first 40 days of the stage) to approximately 0.06 mg HNO_2-N/L (days 250 - 384) (Figure 5.3.C). The interruption of the sodium azide addition in the feeding provoked the pH decrease, with the consequent increase of FNA concentrations. The pH drop is owing to the release of hydroxyl ions when azide is in aqueous solution (pKa = 4.6) (Khataee and Pakdehi 2014). During the whole stage, the NOB activity in the reactor was negligible (again the nitrate present in the effluent came from the tap water) and it was not detected from the respirometric experiments either, while the SA_{AOB} increased up to 220 mg NH_4^+-N/(g VSS·d) (Table 5.2). The composition of the effluent in stage IV was 21.3 ± 3.6 mg NH_4^+-N/L, 27.9 ± 2.4 mg NO_2^--N/L (average NO_2^-/NH_4^+ molar ratio of 1.3), 1.9 ± 0.4 mg NO_3^--N/L and 5.8 ± 3.6 mg IC/L. The length of this stage (174 days) together with the applied HRT (0.25 days) provided a long enough experimental period to discard any residual effect of NaN_3 on the observed NOB inhibition.

Table 5.2. Ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) specific activities (SA_{AOB} and SA_{NOB}, respectively) determined by respirometric assays at 16 °C.

Sample collection		**SA_{AOB}**	**SA_{NOB}**
Stage	Day	mg NH_4^+-N/(g VSS·d)	mg NO_2^--N/(g VSS·d)
I	0	50 ± 6	72 ± 12
III	203	68 ± 28	N.D.
IV	238	103 ± 39	N.D.
	288	220 ± 11	N.D.
V	400	195 ± 52	N.D.
	412	178 ± 2	149 ± 4
VI	457	416 ± 65	291 ± 13
	483	393 ± 36	351 ± 22
VII	512	398 ± 26	186 ± 8
	598	382 ± 14	93 ± 6
	640	388 ± 21	52 ± 12

N.D.: Not detected

Therefore, the results obtained throughout Stage IV indicated that, at the conditions of low ammonium concentration and temperature, to achieve a stable

nitritation without the presence of NOB activity is possible thanks to the inhibitory effect of the FNA formed *in situ* in the bulk liquid. This inhibitory FNA concentration was achieved due to the synergist effect of the nitrite concentration accumulated in the system and the low pH value. It is worth to pointed out that the DO concentration during this stage shows high fluctuations reaching values up to 6.5 mg O_2/L and with average values of 2.2 ± 1.5 mg O_2/L (Figure 5.4).

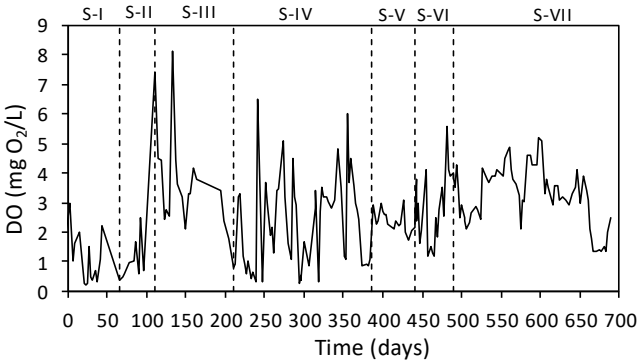

Figure 5.4. Evolution of the dissolved oxygen (DO) concentration, in mg O_2/L, measured inside the reactor.

To confirm this finding, in Stage V (385 - 440 days), the IC concentration in the feeding was doubled being high enough to achieve the complete nitrification (Figure 5.5). During this stage, the AOR increased up to 91 ± 4 %. At the beginning of this stage, the NAR remained close to 100 %, but as the pH value rose, the FNA concentration in the bulk liquid decreased (Figure 5.3.C) to values under those identified as inhibitory for NOB of 0.02 mg HNO_2-N/L (Blackburne et al. 2007). However, the development of significant NOB activity inside the reactor took approximately 40 days and nitrate production was observed only after day 424 of operation (Figure 5.3.A) decreasing the NAR to 52 % at the end of the stage (Figure 5.3.B). Throughout this period, the average effluent composition was 4.8 ± 1.1 mg NH_4^+-N/L, 42.0 ± 1.3 mg NO_2^--N/L, 2.1 ± 0.5 mg NO_3^--N/L and 35.9 ± 4.0 mg IC/L. The absence of NOB activity during the first days of Stage V was also confirmed by respirometric assays, being on day 400 (16 days after IC change) the value of SA_{NOB} still negligible (Table 5.2). However, a few days later (on day 412) the measured SA_{NOB} was of 149 ± 4 mg NO_2^--N/(g VSS·d) (Table 5.2), despite it became significant inside

the reactor only after day 424 (Figure 5.3.B), being of approximately 60 mg NO_2^--N/(g VSS·d). Once nitrate was initially produced, it increased exponentially.

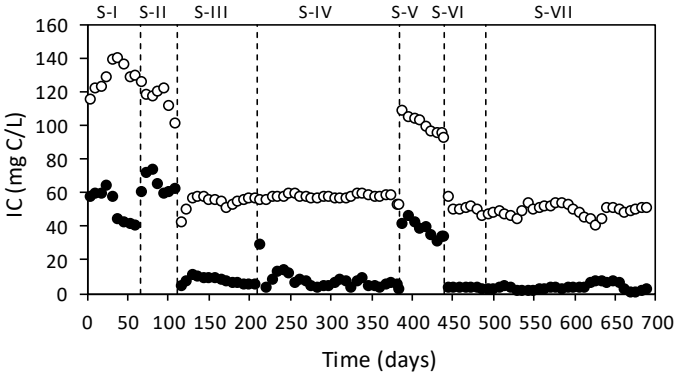

Figure 5.5. Evolution of the inorganic carbon (IC) concentration, in mg IC/L, in the SBR influent (○) and effluent (●).

This observation is very appealing since a control strategy based on the FNA concentration will allow anticipating the NOB appearance with enough time to avoid problems of destabilisation of the nitritation process.

Later, in Stage VI (441 - 489), the IC in the influent was decreased again (Figure 5.5) to see if the alkalinity limitation by itself could lead to the nitritation establishment. With this change in the feeding, the AOR progressively decreased to average values of 64 ± 8 % (Figure 5.3.B). Despite obtaining the lowest pH values (6.23 ± 0.27), FNA inhibitory levels were not achieved as nitrite in the effluent was scarce (1.3 ± 1.1 mg NO_2^--N/L). Thus, NAR values were negligible (Figure 5.3). During this stage, the average effluent composition was 19.9 ± 2.9 mg NH_4^+-N/L, 32.9 ± 5.3 mg NO_3^--N/L and 2.0 ± 1.5 mg IC/L. Therefore, only with alkalinity limitation, the NOB suppression was not possible. During this stage, both SA_{AOB} and SA_{NOB} were similar and significantly increased up to 393 mg NH_4^+-N/(g VSS·d) and 351 mg NO_2^--N/(g VSS·d), respectively (Table 5.2).

Finally, during Stage VII (490 - 690) sodium nitrite was supplied in the SBR feeding (78 ± 5 mg NO_2^--N/L) to enhance FNA accumulation. Consequently, nitrate production immediately decreased, and nitrite started to accumulate inside the reactor from 0.1 to 8 mg NO_2^--N/L in 3 days (Figure 5.3.A), as result of ammonium oxidation (average AOR of 65 ± 5 %) and after subtracting the nitrite present in the

feeding. Despite the nearly immediate accumulation of the FNA concentration in the system, from day 560 onwards the nitrite concentration in the effluent was approximately 20 mg NO_2^--N/L while nitrate depletion was not complete. The pH value remained constant during the whole stage; thus FNA progressively increased following the same pattern as the nitrite accumulation and it was stabilised at 0.02 ± 0.002 mg HNO_2-N/L. The average effluent composition in this reached steady-state was: 18.9 ± 4.3 mg NH_4^+-N/L, 20.3 ± 7.3 mg NO_2^--N/L, 15.8 ± 9.1 mg NO_3^--N/L and 4.1 ± 3.0 mg IC/L. During this stage, the NAR was limited to values of approximately 70%. Different reasons should be considered to explain this fact, as the long SRT of the biomass (higher than 100 days). However, the VSS concentrations in the effluent of Stage VII are comparable to those from the previous stage (Figure 5.6) challenging the complete and efficient NOB washout. The most likely reason for this failure on the NOB suppression can be the relatively low FNA concentrations achieved. The maximum FNA concentration during this Stage VII was limited to 0.024 mg HNO_2-N/L, whereas during stage IV, the FNA reached values up to 0.06 mg HNO_2-N/L (Figure 5.3.C). The nitrite concentration added in the feeding was partially consumed by NOB and thus more nitrite should be added to obtain higher FNA levels. Presumably, higher FNA concentration would speed up the nitrate production depletion by inhibiting the NOB activity, which could be achieved by the addition of higher nitrite concentrations in the influent.

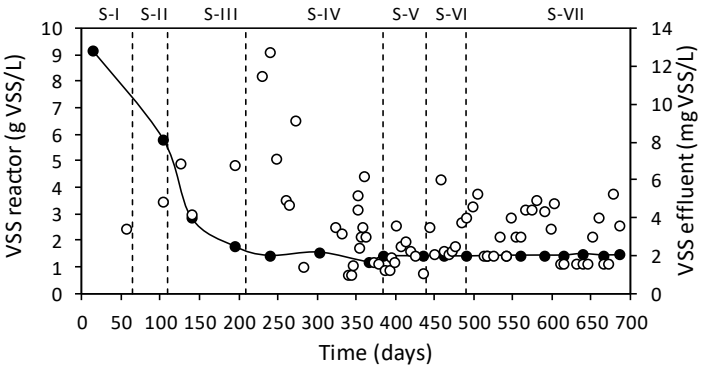

Figure 5.6. Evolution of the biomass concentration inside the reactor (●), in g VSS/L, and in the effluent (○), in mg VSS/L.

5.4.2. Long-term effect of sodium azide on AOB

Sodium azide is known to inhibit both, AOB and NOB activities, with IC_{50} values of 40 and 0.025 mg NaN_3/L, respectively (Lopez-Fiuza et al. 2002). Furthermore, this compound is reported to inhibit the NOB anabolism, meaning that when it is present these bacteria cannot survive (Vadivelu et al. 2007). Due to its so different inhibitory effect over both populations, NaN_3 at low concentrations (1.56 mg NaN_3/L) is commonly used to remove the SA_{NOB} from a nitrifying sludge when the maximum SA_{AOB} is determined by batch tests (Guisasola et al. 2005).

The effect of the sodium azide concentration used in this study (5 mg NaN_3/L), over the SA_{AOB} was previously tested by batch respirometric assays and no detrimental effect was observed with differences lower than 5% in the measured SA_{AOB} values. Additionally, its long-term effect on the SA_{AOB} was verified directly in the conditions of the experiment performed in the present study. In this sense, the SA_{AOB} of the biomass sample collected from the reactor at the end of Stage III (during the addition of 5 mg NaN_3/L) did not diminish compared to that of the inoculum and was of 68 mg NH_4^+-N/(g VSS·d) (Table 5.2).

Therefore, the variations in the measured AOR (Figure 5.3.B) were probably caused by the imposed operational conditions, mainly due to the different alkalinity values present in the medium, and cannot be attributed to any adverse effect of NaN_3 presence.

5.4.3. Biomass characteristics and NOB populations

The SBR was inoculated with 9.1 g VSS/L of flocculent biomass. From the reactor start-up until day 140 of Stage III, a fast decrease of the inoculated biomass concentration, to values of 2.8 g VSS/L, occurred. Then, the rate of biomass loss decreased, and the concentration of solids at the end of Stage III was approximately 2.0 g VSS/L. Throughout the rest of the reactor operation, the biomass concentration inside the reactor was almost constant and approximately 1.4 ± 0.2 g VSS/L (Figure 5.6).

Biomass morphological changes were not observed being small floc agglomerates (Figure 5.7). The average solid concentration in the effluent was of 5 ± 2 mg VSS/L. The SVI ranged between 20 and 30 mL/g TSS, showing the excellent

settleability properties of the flocculent sludge. The estimated average SRT was higher than 100 days.

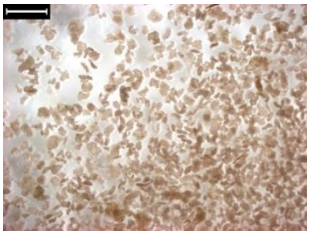

Figure 5.7. Image of nitritation flocculent biomass on day 310 of operation. The size bar represents 2 mm.

The initial sharp decrease of biomass concentration (Figure 5.6) was probably due to the washout of microbial populations present in the inoculum but not retained inside the reactor due to the imposed operational conditions. In Stage I, probably the anammox bacteria (present in the inoculum) were mainly removed and in Stages II and III the NOB populations.

To study the evolution of NOB populations inside the reactor, the 16S rRNA gene-based amplicon analysis was applied to biomass samples collected in different operational days (Figure 5.8). The most abundant NOB genus in the present study was *Nitrospira* and the obtained abundance percentage values were: 2.0 % (day 0), 27.9 % (day 44), 3.5 % (day 248), 3.5 % (day 303), 0.4 % (day 336), 0.01 % (day 372) and 0.01 % (day 414). These results indicated that the NOB were developed during Stage I (sample from day 44) being an abundant population when the full nitrification process took place. Then, with the addition of the sodium azide in Stages II and III, the NOB activity was significantly reduced together with their abundance, proved by the absence of positive results from FISH analysis. However, it was in Stage IV (samples 248, 303, 336 and 372) when their abundance was drastically reduced and maintained at very low values at the beginning of the Stage V (0.01 %, day 414). These results indicate that the strategy followed in Stage IV, maintaining FNA concentrations up to 0.02 mg HNO_2-N/L inside the reactor, was successful in reducing the NOB abundance.

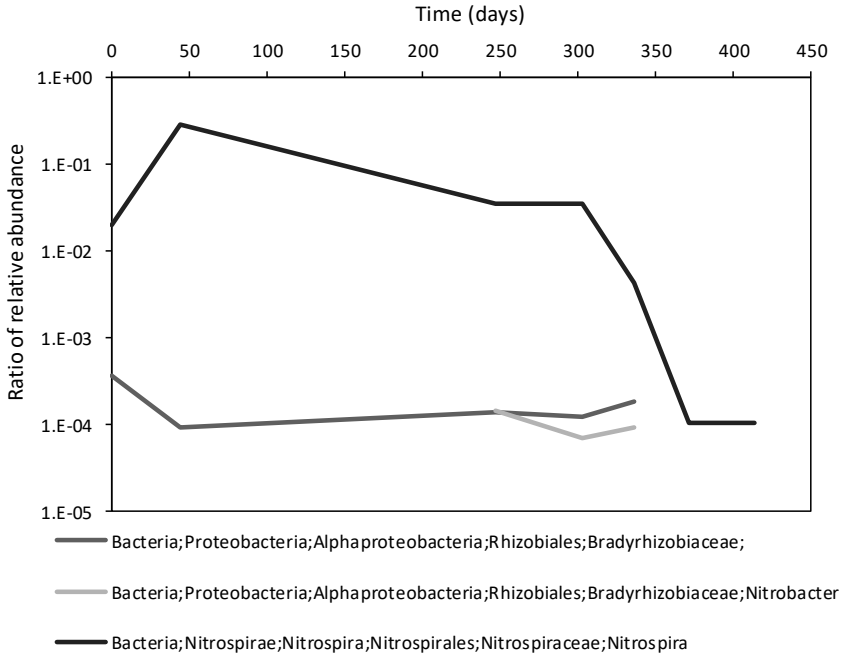

Figure 5.8. Ratio of relative abundance for the bacterial populations (at genus level) present in sludge samples collected at different operational days of the SBR.

By the FISH technique *Nitrospira sp.* population was analysed in samples of Stage I (day 0), Stage IV (day 248), Stage V (day 414), Stage VI (days 458 and 469) and Stage VII (days 518, 602, 668 and 690). The microscopy visualisation showed a positive result in the inoculum sample (Figure 5.9) and no positive signal on day 248, indicating that *Nitrospira* sp. were active in Stage I and inactive in Stage IV. The samples visualised from Stage V to Stage VII, when the NOB activity was again observed inside the reactor, showed the presence of Nitrospira sp. again. Regarding *Nitrobacter spp.,* identified by 16S rRNA gene-based amplicon analysis, the abundance obtained in the analysed days was lower than 0.015 %, while after applying the FISH probe specific for *Nitrobacter spp.*, a negative signal was obtained for the samples analyzed throughout the entire operational period. These observations indicate that the developed NOB belong mainly to *Nitrospira* outcompeting *Nitrobacter* genus.

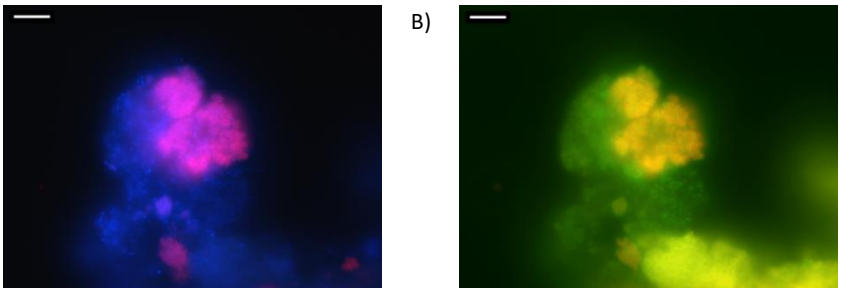

Figure 5.9. FISH images of the biomass used as inoculum with the applied probes: (a) *Nitrospira* sp. (NTSPA712, pink) and DAPI (blue); (b) *Nitrospira sp.* (NTSPA712, orange) and Bacteria domain (EUB338mix, green). Size bar represents 10 μm.

5.5. Discussion

5.5.1. FNA accumulation as a strategy to obtain stable nitritation

Among the bacteria involved in the nitrification process, AOB are significantly less sensitive to FNA than NOB (Vadivelu et al. 2007). In the present study, FNA concentrations high enough to inhibit NOB activity (> 0.02 mg HNO_2-N/L) but not enough to inhibit AOB activity (< 0.40 mg HNO_2-N/L) were reached (Figure 5.3.C), during the operation of the SBR, due to the synergist effect between the nitrite accumulated and the remained alkalinity.

The concentration of FNA depends on the values of temperature, nitrite concentration and pH, according to Equation 2.11 in Chapter 2 (Anthonisen et al. 1976). The pH is the variable with the exponential effect, while the temperature is the parameter that affects FNA concentration to a lesser extent (Figure 5.10). Although, the lower the temperature, the higher the FNA concentration achieved, contrary to what occurs with the free ammonia (FA) concentration (Equation 2.10 in Chapter 2, (Anthonisen et al. 1976)). In this way, the low temperature of the mainstream (between 5 - 20 °C) plays in favour of the FNA instead of the FA accumulation.

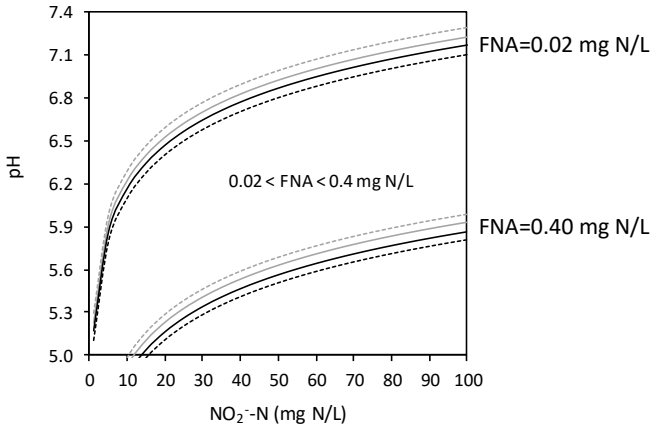

Figure 5.10. Graphical representation of the FNA concentration profile obtained using the equation proposed by Anthonisen et al. (1976) applied at different temperatures: 5 °C (--), 10 °C (—), 15 °C (—) and 20 °C (--). Top lines represent the NOB inhibition threshold (FNA = 0.02 mg HNO_2-N/L) whereas the lines in the bottom correspond to the AOB inhibition threshold (FNA = 0.4 mg HNO_2-N/L). The area comprised between both groups of lines (for each temperature) represents the operational conditions for inhibiting NOB without significantly affect the AOB activity.

The FNA concentration is linearly dependent on the nitrite concentration. Thus to maximize the nitrite concentration inside the nitritation unit can be of interest. For this reason, a fraction between the 50 and 100 % of the influent flow to the partial nitritation (PN) reactor containing ammonium requires to be nitritified. The fraction of this stream, after been treated in the nitritation reactor (containing nitrite or a variable mixture of nitrite and ammonium), will be mixed with the untreated influent (containing only ammonium) to achieve the adequate NO_2^-/NH_4^+ ratio (1.3 g N/g N) to feed the subsequent anammox process.

Afterwards, once the nitrite is accumulated inside the nitritation unit, the remaining alkalinity, which determines the pH value, is the crucial parameter to maintain an appropriate FNA concentration. Similarly to the case of the temperature, and according to the equations proposed by Anthonisen et al. (1976), the lower the pH, the higher the FNA concentration (Figure 5.10), contrary to the response corresponding to FA concentration. As the nitritation process consumes alkalinity, the natural trend of the process is to decrease the pH respect to the

feeding. Therefore, to maintain the nitritation process based on the FNA concentration instead of on the FA one can be an advantage.

However, the possible acclimation of NOB to increasing FNA and FA concentrations and the reversibility of the inhibitory effect of both compounds is a drawback to take into account (Ge et al. 2015). In the present study, once the nitritation was obtained by FNA accumulation, its long-term influence was assessed and no acclimation of NOB was detected. These results are in good agreement with Wang et al. (2016) who maintained the nitritation process at the conditions of 22 °C and 57 mg NH_4^+-N/L with the inhibition of NOB by FNA for 280 days. Their strategy was based on the generation of FNA inhibitory concentrations in an external unit. As these authors stated, this alternative implies optimisation of: 1) the sludge fraction from the nitritation exposed to FNA; 2) the contact time with FNA in the external unit; and 3) the frequency of the sludge treatment. Applying a similar strategy, Duan et al. (2019) reported the adaptation of the NOB to both FNA and FA inhibitory concentrations after the long-term nitritation process operation. To avoid such a situation, these authors proposed to alternate the FNA and FA treatment.

There is no agreement about the synergist effect of IC limitation and inhibition caused by FNA on AOB activity. As an example, Torà et al. (2010) found, by respirometric assays performed at 30 °C, that inorganic carbon limitation enhanced the inhibitory effect of FNA on an AOB enriched culture whereas Vadivelu et al. (2006a) stated the opposite. In the present study, no inhibitory effect on AOB activity was observed. The FNA values obtained (Figure 5.3.B) were sufficiently high to affect NOB but too low to provoke AOB inhibition, as the maximum FNA concentration reached was of 0.06 mg HNO_2-N/L. Opposite to this, Wang et al. (2016) applied a FNA based strategy (concentrations up to 1.82 mg HNO_2-N/L) to maintain the nitritation process and they observed inhibition of 32 % of the AOB activity. Producing the FNA in the mainstream reactor, it is challenging to achieve concentrations as high as those inhibitory for AOB that would limit the nitritation reactor capacity.

The novelty of the strategy proposed in the present research work is the *in situ* production of the inhibitory FNA concentration taking advantage of the natural pH decrease and the production of nitrite due to the ammonium oxidation process, as well as the low temperature at mainstream conditions. In this way, two units of the

system could be avoided compared with the strategy of the external FNA production proposed by Wang et al. (2016).

5.5.2. Influence of the inlet ammonium to inorganic carbon ratio

Most of the alternatives suggested to maintain the nitritation process at mainstream conditions up to date rely on pH control at basic values (mainly close to 8) (Isanta et al. 2015, Jiang et al. 2019, Reino et al. 2016, Wang et al. 2016). On the contrary, the strategy proposed in the present study to suppress the NOB activity based on the FNA concentration does not always require a direct pH control. It depends as well on the inlet NH_4^+-N/IC ratio. Wang et al. (2016) already pointed out the importance of the wastewater alkalinity on the effectiveness of the FNA to inhibit the NOB.

Durán et al. (2014) also observed that the FNA caused the suppression of NOB from a reactor where the nitritation took place at 20 °C and 400 mg NH_4^+-N/L. They based the operation of this system on the control of the NH_4^+-N/IC ratio, which was maintained at values of approximately 0.82 g N/g IC, close to the values used in the present study (Table 5.1). However, in their case, both temperature and ammonium concentration were larger than those found in the mainstream.

With this in mind, four possible scenarios can be described as a function of the inlet NH_4^+-N/IC ratio (Table 5.3), considering that the stoichiometric values needed to oxidise the 100 % and 55 % (required for the anammox process) of the total incoming ammonium concentration to nitrite are 0.6 and 1.0 g N/g IC, respectively.

If the NH_4^+-N/IC ratio is lower than 0.6 g N/g IC (case A), for example, Stages I, II and V, there is an excess of alkalinity for the nitritation process and 100 % of the inlet ammonium content could be oxidised to nitrite. Once the ammonium is consumed, the excess of alkalinity and aeration would cause an increase of pH (by CO_2 stripping) making the FNA concentration negligible. Thus, the application of the *in situ* FNA accumulation strategy in those regions characterised by hard waters would not be suitable or would require the pH control inside the reactor with the associated high costs. If this strategy is still applied, only half of the total wastewater flow would be treated in the nitritation unit, decreasing the chemicals supply, and all the ammonium would be oxidised favouring the alkalinity consumption and

maximising of the nitrite accumulation. The untreated 50% of the flow is bypassed directly to the subsequent anammox reactor.

Table 5.3. Treatment strategies in the nitritation unit depending on the inlet NH_4^+-N/IC ratio.

Case	NH_4^+-N/IC ratio (g N/g IC)	Stream to PN unit (%)	Ammonium oxidised to nitrite (%)	Action required
A	<0.6	50	100	Bypass and pH control
B	0.6 - 0.8	50 - 100	50 - 100	Bypass to anammox unit
C	0.8 - 1.0	100	50	None
D	> 1.0	100	50	Alkalinity supply

Isanta et al. (2015) and Reino et al. (2016) achieved and maintained a stable partial nitritation, in a granular biomass system, treating 70 mg NH_4^+-N/L and with a ratio of 0.62 g N/g IC for more than 400 days at 12.5 °C and for more 250 days at 10 °C, respectively. In both studies, the pH was artificially maintained at a value of 8 and they applied the same strategy based on the control of DO/TAN concentrations ratio resulting in ammonium in excess in the bulk liquid. This strategy appears as more appropriated to treat effluents with large alkalinity contents (case A) as pH is high enough to favour the FA accumulation suppression the NOB.

In the case of NH_4^+-N/IC ratios between 0.6 and 0.8 g N/g IC (case B), the inlet ammonium oxidised to nitrite can vary between the 100 % and 50 %, respectively. The percentage of ammonium oxidised will determine the fraction of the flow stream treated in the nitritation unit and the fraction bypassed to the anammox reactor. Wang et al. (2016) applied a FNA-based strategy using synthetic wastewater characterised by an NH_4^+-N/IC ratio of 0.62 g N/g IC, and they observed the oxidation of the total incoming ammonium to nitrite. Later, these authors limited the ammonium oxidation to 50% by reducing the DO concentration and controlling the aeration duration as an alternative to bypass part of the inflow stream.

If the NH_4^+-N/IC ratio is between 0.8 and 1.0 g N/g IC (case C), for example Stages III, IV, VI and VII, the alkalinity value is adequate to partially oxidise the ammonium to nitrite and to obtain an effluent with an NH_4^+/NO_2^- molar ratio of 1.3, suitable to be treated in the subsequent anammox unit. In both cases, 100 % of the streamflow enters the nitritation unit, and 50 % of the ammonium content is oxidised to nitrite.

Finally, when the NH_4^+-N/IC ratio is higher than 1.0 g N/g IC (case D), there is not enough alkalinity for the nitritation process, and external alkalinity supply is required to reach a ratio around 1 g N/g IC.

Additionally, the sensitivity analysis (Figure 5.11) indicated that at NH_4^+-N/IC ratio of 0.6 g N/g IC the maximum FNA concentration is achieved. At values lower than this ratio, the remaining excess of alkalinity causes the increase of pH (up to 8.4) due to the CO_2 stripping in aerated conditions, making the FNA concentration negligible. At values higher than 0.6 g N/g IC, the alkalinity is limited and not all the ammonium concentration can be oxidised, but the estimated pH value is low (approximately 5.6), which allowed the obtaining of inhibitory FNA concentrations over 0.02 mg HNO_2-N/L (Figure 5.11). The lower NH_4^+-N/IC ratio (limit of 0.6 g N/g IC) and the higher ammonium concentrations, the higher FNA concentrations reached. These FNA concentrations can reach values higher than 0.42 mg/L, that might inhibit the AOB activity (Vadivelu et al. 2007), when the ratio is next to 0.6 g N/g IC and the ammonium concentration is higher than 90 mg NH_4^+-N/L.

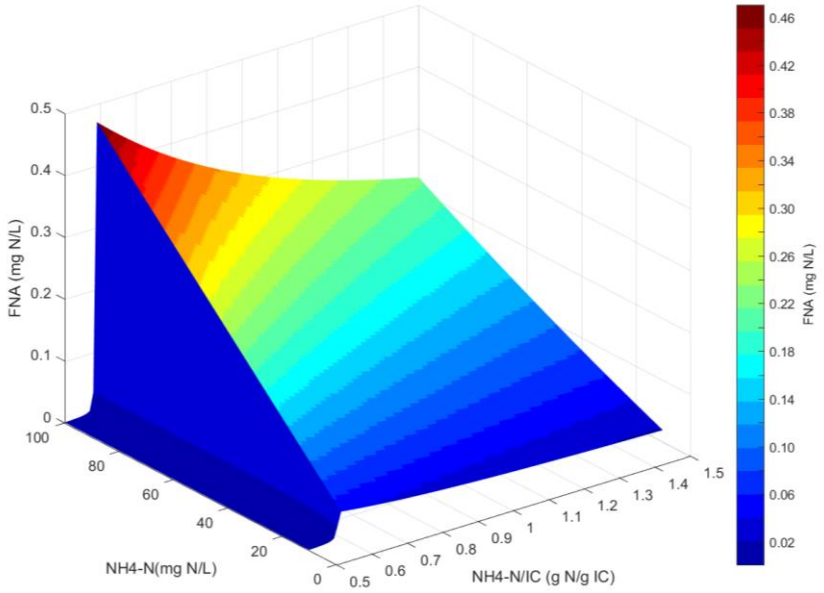

Figure 5.11. Sensitivity analysis of the impact of the change of the ammonium to inorganic carbon ratio (NH_4^+-N/IC in g N/g IC) and ammonium concentration (in mg N/L) on the free nitrous acid (FNA) accumulation.

5.5.3. Nitrite accumulation activation

To define the appropriate procedure required to start up a nitritation system is essential for a feasible application of the proposed FNA strategy.

If the sludge used as inoculum for the nitritation unit contains low or negligible SA_{NOB}, during the start-up, it is possible to accumulate nitrite inside the reactor enough to reach inhibitory FNA concentrations and to maintain the process during the rest of the operation. The direct use of sludge from a nitritation unit operated in sidestream conditions or the use of an AOB enriched sludge, without or with low NOB presence, as inoculum would lead to the faster nitrite accumulation. Then, if the NH_4^+-N/IC ratio is appropriated, the FNA concentrations inhibitory for NOB can be reached and maintained.

However, to have available an AOB enriched sludge is not always possible, and commonly activated sludge is used as inoculum. In this case, the use of an "activator" of the nitrite accumulation can be a solution, like a specific NOB inhibitor (Ge et al. 2015). Wang et al. (2015) restored a nitrate build-up in a one-stage PN/AMX reactor by adding hydroxylamine as a NOB specific chemical inhibitor. Nevertheless, they observed that this effect was reversible when the addition of the inhibitor was stopped.

In the present study, performed at a laboratory scale, a NOB inhibitor (sodium azide) acted as "activator" allowing the establishment of the inhibitory FNA concentrations for NOB activity suppression. The sodium azide effects on the anammox activity have been previously assessed in batch experiments providing different results depending on the state of aggregation of the biomass (Pedrouso et al. 2017). The batch tests indicated that a concentration of 5 mg NaN_3/L (used in the present study) caused a 50 % inhibition on the flocculent anammox biomass activity and only a 15 % on the granular one. Moreover, this inhibition was reversible in both cases (Pedrouso et al. 2017). Although, the application of sodium azide at laboratory scale allowed enhancing the FNA inhibition mechanism, another strategy must be applied to promote the nitritation process at full-scale, to avoid the use of sodium azide (due to its toxicity). For example, during Stage VII, the nitrite was successfully tested as an activator for FNA accumulation with an immediate effect over the NOB activity. However, as nitrite was supplied under aerobic conditions, it was partially consumed and it was not possible to completely inhibit the NOB indicating that

probably a higher nitrite concentration supply was required. Cui et al. (2019) obtained a stable nitritation process (for 66 days at 17 - 27 °C) by treating the nitrifying sludge with intermittent nitrite dosing (5 - 30 mg NO_2^--N/L) under anoxic conditions for 32 days. Therefore, the nitrite dosage seems to be more effective under anoxic conditions since in aerobic regime, the nitrite added might be partially utilized by NOB promoting their activity and decreasing the expected FNA accumulation.

Another strategy can be the exposure of the inoculum for a known time to FNA inhibitory concentrations. For example, Jiang et al. (2018) evaluated the exposure of the inoculum to FNA concentrations, of 1.2 mg HNO_2-N/L for 18 h, and reported a decrease of 57 % of the AOB activity while NOB activity was undetectable. Thus, this sludge treatment step might be carried out in the nitritation reactor before the start-up promoting the *in situ* FNA production. Jiang et al. (2019) also achieved the NOB activity suppression by *in situ* producing FNA inhibitory concentrations but treating high nitrogen concentrations (200 - 650 mg NH_4^+-N/L) at high temperature (32 °C). In this case, the FNA was sporadically generated, when the NOB population percentage amounted to more than 10 % of the total biomass, by stopping for 12 h the $NaHCO_3$ dossing, which provoked the corresponding pH decrease from 7.8 ± 0.05 to 6.1 - 6.3. However, this strategy seems challenging to implement as molecular techniques to determine the percentage of a bacterial population (like quantitative polymerase chain reaction, qPCR) are not commonly implemented in the WWTPs.

Another strategy relies on starting-up the nitritation process at temperatures over 20 °C where AOB activity and growth rate are favoured over those of NOB, and then decrease the temperature. This strategy was used by Isanta et al. (2015) who achieved the PN after 35 days of operation treating 70 mg NH_4^+-N/L at temperatures higher than 20 °C and then they moved to mainstream conditions decreasing the temperature. Ye et al. (2019) used the starvation stress to promote the nitrite accumulation (at 26 °C and treating 100 mg NH_4^+-N/L) based on the ability of the AOB to better cope with the starvation conditions and their shorter recovery time compared to the NOB.

Therefore, neither a precise selection of the inoculum would be required nor a continuous toxic compound addition, but the pre-treatment of the sludge or the application of high loads that promote the initial nitrite accumulation. Once FNA

accumulation begins and inhibitory concentrations are achieved, the NOB suppression is maintained.

5.5.4. Effective NOB washout

The microbiological analysis showed that throughout Stages II to IV the NOB (dominated by *Nitrospira* genus) practically disappeared, which indicates an effective wash out of these bacteria and not only their inhibition by the presence of the FNA. Indeed, it has been reported that concentrations of 0.02 mg HNO_2-N/L completely inhibited the biosynthesis of *Nitrobacter spp.* (Vadivelu et al. 2006b) and *Nitrospira spp.* are known to be more sensitive to FNA than NOB-like *Nitrobacter* (Blackburne et al. 2007). This selective removal of the NOB presents some advantages: (1) feasibility to operate the process at a relatively high DO concentration, which is advantageous to achieve a high nitritation rate; and (2) robustness of the nitritation process stability, even operating at conditions favorable for the NOB activity development, because these bacteria are not present inside the reactor (or they are in a minimal concentration). If the operational conditions become adequate for the NOB activity development, for example, a seasonal increase of the wastewater alkalinity, several days will be required to reach a level of NOB activity high enough to destabilise the nitritation process.

Meanwhile, corrective action can be implemented to avoid this negative evolution. As an example, in the present study, such a destabilisation needed approximately 40 days in Stage V when the FNA concentration was decreased. This is also an advantage if an incident occurs during the reactor operation, e.g., an increase of the DO concentration. In the present study, despite the DO concentration reached values as high as 6 - 7 mg O_2/L in some operational days of Stage IV (Figure 5.4), the nitrite oxidation rate to nitrate was maintained negligible. Isanta et al. (2015) found, operating a nitritation reactor at mainstream conditions (12.5 °C and 70 mg NH_4^+-N/L), that an event of high DO concentration (8 mg O_2/L) maintained during few hours, was immediately reflected in the appearance of NOB activity. This was probably because these bacteria were present in enough number and sufficiently active in the reactor to take quick advantage of the favourable operational conditions.

Furthermore, Isanta et al. (2015) used an inoculum with *Nitrobacter* and hypothesized that their strategy is not adequate to suppress *Nitrospira* genus. Although *Nitrobacter* are considered the key NOB in nitrifying wastewater units at high nitrogen concentrations (González-Martínez et al. 2014), the predominant NOB in full-scale plants is *Nitrospira spp.* (Nogueira and Melo 2006, Vázquez-Padín et al. 2009). Nitrospira like-NOB was the population suppressed in the present study by FNA. These findings agree with Wang et al. (2016), who claimed that FNA inhibition was the main factor responsible for the elimination of *Nitrospira* while DO limitation led to the *Nitrobacter* washout. This fact may be explained as *Nitrobacter spp.* are r-strategists and they are well adapted to high nitrite concentrations and have lower oxygen affinity compared to *Nitrospira* spp. (k-strategists) (Vázquez-Padín et al. 2009). *Nitrobacter spp.* were present in low abundance from the start-up of the reactor, but they were outcompeted by *Nitrospira spp.* (Figure 5.8).

5.6. Conclusions

In the present study, long term nitritation at mainstream conditions, low temperature and low nitrogen concentration (16 °C and 50 mg NH_4^+-N/L), was established and maintained, in a suspended biomass SBR without dissolved oxygen concentration control.

The promotion of FNA concentrations higher than 0.02 mg HNO_2-N/L inside the reactor allowed maintaining the nitritation process stable and washing out effectively the NOB (*Nitrospira spp.*). Furthermore, it was proved that if the operational conditions favor the NOB activity (low FNA concentration) more than 40 days are required to detect significant nitrate concentrations. In this case, as it will last several weeks to have significant NOB activity inside the reactor, appropriate control actions can take in time.

The addition of a specific NOB inhibitor (sodium azide at 5 mg/L) or nitrite enables the start-up of the nitrite accumulation to produce FNA *in situ*, without affecting the AOB activity, in case that the inoculum already contains NOB activity. However, the use of sodium azide at full scale is not feasible.

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Chapter 6

Performance of nitritation and organic matter oxidation processes with municipal wastewater

SUMMARY

The nitrite oxidising bacteria (NOB) activity can be suppressed by the action of *in situ* produced inhibitory free nitrous acid (FNA) concentrations (> 0.02 mg HNO_2-N/L). In the present study, a 2-L sequencing batch reactor (SBR) was started up with a sludge enriched on ammonium oxidising bacteria (AOB) and the nitritation process was established and maintained by the *in situ* FNA accumulation without the need of chemical addition for its initiation. Then, the feasibility of the *in situ* FNA accumulation strategy when treating primary settled municipal wastewater, containing 18 - 55 mg NH_4^+-N/L and total organic carbon (TOC) of 20 - 50 mg TOC/L, was tested at 15 ± 1 °C. Nitrite accumulation ratios of practically 100 % were observed and the nitritation process stability was maintained at long-term (354 days from which 191 days treating municipal wastewater) achieving FNA concentrations ranging from 0.02 to 0.20 mg HNO_2-N/L. Minimum pH values of 5.6 were reached without exerting adverse effects neither on the nitritation process nor on the specific AOB activity that was maintained at approximately 200 mg NH_4^+-N/(g VSS·d). The presence of organic matter in the feeding, pointed out as a drawback for the implementation of the PN/AMX processes, did not affect the nitritation process stability and it was removed (with an efficiency of 80 %) in the nitritation unit. This fact gives to this strategy a great opportunity since the previous units do not need to have strict control of their removal efficiencies and for this reason, their operation is more flexible. To cope with the changes of nitrogen and organic matter concentrations in the municipal wastewater, the optimisation of the SBR cycle length is required being recommended a variable cycle length.

OUTLINE

6.1. Introduction

Nitrogen removal via the nitrite pathway, like the partial nitritation and anammox (PN/AMX) processes, has gained great interest since it helps to maximise the overall energy efficiency in the municipal wastewater treatment plants (WWTPs). In this way, the aeration and the organic matter requirements for the biological nutrient removal process decrease (Morales et al. 2015). When these processes are applied for the treatment of municipal wastewater, the COD, which is commonly present (7 - 12 g COD/g N after primary settling), represents a shortcoming. Due to its presence, heterotrophic organic oxidising bacteria develop and compete with the nitrifying bacteria for dissolved oxygen (DO) hindering the establishment of the process in steady-state conditions. Indeed, at these cases, the presence of organic matter (that is not removed in the previous stage dedicated to that purpose) has been pointed out as one of the main drawbacks to implementing the PN/AMX processes (Agrawal et al. 2018, Pedrouso et al. 2018). Scarce information is available about the role of the organic matter on the successful long-term performance of the nitritation process treating municipal wastewater (< 100 mg TN/L) at low temperature (< 25 °C) since most of the studies available were performed using synthetic wastewater (Agrawal et al. 2018).

One of the most widely applied strategies to develop the nitritation process relies on controlling the duration of the aerobic reaction phase based on the ammonia valley. In sequencing batch reactors (SBRs), as soon as ammonium oxidation was complete, an inflexion point (ammonia valley) in the pH profile was observed since the oxidation of the nitrite to nitrate consumes less alkalinity (Guo et al. 2009). The change from negative to positive in the value of dpH/dt was used to terminate the aeration. This strategy was successfully applied at low temperature achieving a nitrite accumulation ratio (NAR) higher than 95 % (Guo et al. 2010, Jin et al. 2019). However, if the alkalinity is limited to oxidise all the ammonium (i.e., nitrogen to inorganic carbon (N/IC) ratios higher than 0.6 g N/g IC) the pH will only decrease, and no ammonia valley takes place. In this case, another strategy must be chosen.

Among other strategies to implement the nitritation process at mainstream conditions, the feasibility of those based on the higher sensitivity to free nitrous acid

(FNA) concentrations of nitrite oxidising bacteria (NOB) than ammonium oxidising bacteria (AOB) (Zhou et al. 2011). This approach was researched by exposing the sludge from the nitritation reactor to inhibitory FNA concentrations in an external unit (Duan et al. 2018, Wang et al. 2016) or producing it *in situ* inside the nitritation reactor (Chapter 5). As an advantage, the *in situ* production of the FNA does not require an additional external unit and the consequent pumping of the sludge. However, as it was explained in Chapter 5, in this approach the nitritation process is limited by the N/IC ratio of the incoming wastewater that must be higher than 0.6 g N/g IC. Thus, this strategy is limited by the wastewater hardness.

The wastewater alkalinity depends mostly on the local source of freshwater and the minerals leaching from the soil (Burton et al. 2014). Water streams flowing over volcanic rocks will be soft while water drilled into porous rock, such as limestone, which are readily soluble in the groundwater, are normally hard. Areas with complex geology can produce varying degrees of hardness of water over short distances. Figure 6.1 shows the water hardness distribution for freshwater (in southern-central Europe) as no information about the wastewater alkalinity distribution is available. The reported standard increment in the alkalinity content in the wastewater due to the water usage is 112 - 220 mg $CaCO_3$/L (i.e., 27 - 53 mg IC/L) (Burton et al. 2014) regardless the region. Thus, the wastewater hardness distribution is expected to show a similar pattern to the freshwater as it is the main influential pattern.

Although, in general, the *in situ* production of the FNA strategy would be not adequate for hard water, more than 50 % of the water in Europe is classified as soft to slightly hard. In these regions this strategy could be directly applied without the addition of chemicals. When the untreated wastewater is hard (g N/g IC > 0.6 g N/ g IC) the alkalinity depletion during the nitritation process could not be enough to consume the buffering capacity and the pH would not decrease to produce the FNA inhibitory concentrations required inside the reactor. Thus, in these conditions, the feasibility of the nitritation process based on the *in situ* FNA production would be questionable. Therefore, upstream processes that produce a change in the ratio of N/IC concentration such as the chemical enhanced pre-treatment (CEPT) should be considered as an alternative to removing the organic matter content prior to the implementation of the PN/AMX processes (Taboada-Santos et al. 2019).

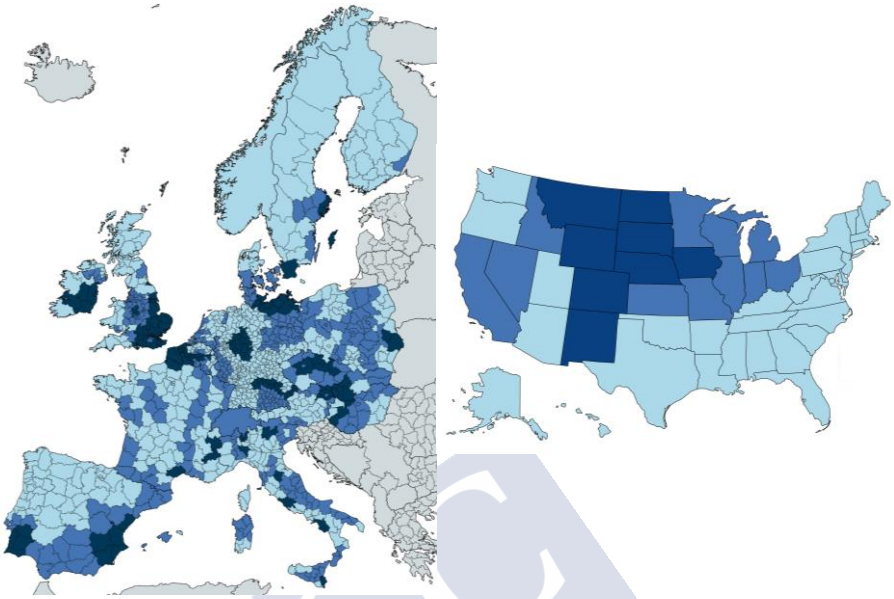

Figure 6.1. Water hardness distribution in Europe (left) and United States of America (right): soft (■, < 100 mg $CaCO_3$/L or 24 mg IC/L), slightly and moderate hard (■, 100 - 200 mg $CaCO_3$/L or 24 - 48 mg IC/L), hard to extremely hard (■, > 200 mg $CaCO_3$/L or 48 mg IC/L) water. Grey colour (■) means no data.

Different start-up strategies were proposed to promote the nitrite accumulation depending on whether the NOB were active in the inoculum (requiring the addition of a NOB specific inhibitor, for example, Chapter 5 or not (Liu et al. 2017). But no information is available about the long-term suppression of NOB activity by *in situ* produced FNA inhibitory concentrations when the used inoculum presents negligible NOB activity, to operate in the same reactor the nitritation and organic matter removal processes at mainstream conditions

6.2. Objectives

The present study aims at demonstrating the feasibility of achieving and maintaining in the same unit the long-term operation of the nitritation and organic matter oxidation processes in stable conditions, when treating municipal wastewater at low temperature (15 °C).

First, the effectiveness of the NOB suppression by the *in situ* FNA production was assessed by inoculating the reactor using a sludge without NOB activity. The absence of NOB activity was monitored during the treatment of mimicked municipal wastewater.

Then, the feasibility of treating primary-settled municipal wastewater was studied promoting the COD removal together with the NOB activity suppression. Additionally, the effect of the starvation and reactivation periods over the system was assayed.

6.3. Materials and Methods

6.3.1. Reactor setup and operational conditions

A 2-L nitritation reactor was operated as an SBR with a volume exchange ratio fixed at 50 %. The temperature was maintained at 15 ± 1 °C by pumping cooled water through the reactor external jacket by means of a thermostatic bath (Frigiterm, P Selecta). Air was supplied to the SBR through an air pump (KNF Labport) coupled to a coarse bubble diffuser to guarantee the complete mixture and oxygen supply for the biological reactions (Figure 6.2). Neither DO concentration nor pH were controlled and ranged from 0.5 – 7.5 mg O_2/L (airflow was manually regulated) and 5.4 – 7.5, respectively.

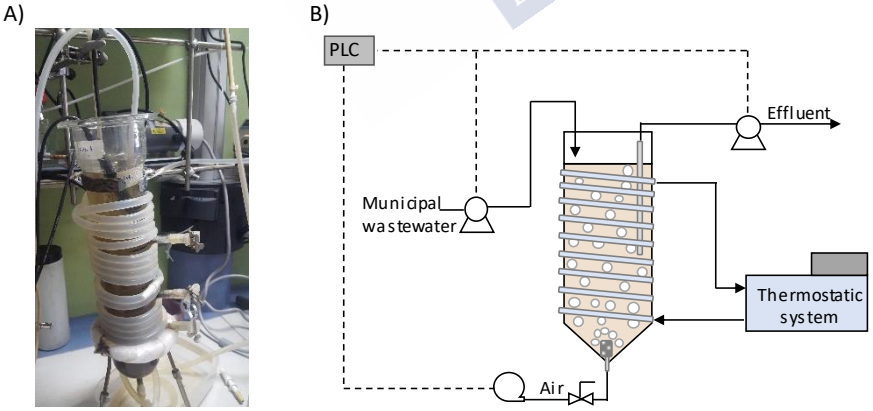

Figure 6.2. A) Nitritation SBR image and B) Scheme of the reactor set-up.

The SBR was seeded with 2.2 g VSS/L of nitrifying suspended sludge from a reactor that treated high saline wastewater (approximately 18 g NaCl/L) with total nitrogen (TN) concentrations of 150 - 200 mg TN/L and operated at 30 °C (Giustinianovich et al. 2018). The biomass was inoculated after 21 days of starvation at room temperature (20 °C). The measured maximum specific activity (SA) of AOB (SA_{AOB}), at 15 °C, was 11 ± 2 mg NH_4^+-N/(g VSS·d) whereas NOB activity (SA_{NOB}) was not detected.

The operational period lasted 354 days divided into four stages. In Stage I, the same synthetic medium used in Chapter 5 was fed to the reactor for the start-up. This mineral medium contained 50 mg NH_4^+-N/L (supplied as NH_4Cl), 0.4 g $NaHCO_3$/L, 0.040 g K_2HPO_4/L, 0.015 g KH_2PO_4/L, 0.040 g $MgSO_4$/L and 0.2 mL/L of a trace solution (Vishniac and Santer 1957). Afterwards, primary settled municipal wastewater was fed during Stages II - IV (Table 6.1). At the end of Stage II, the sludge from the SBR with nitritation capacity was stored anoxically in the reactor medium at 4 °C for 25 days and it was used later in Stage III to re-start the nitritation unit. Finally, in Stage IV the applied organic matter and nitrogen loads decreased compared to previous stages due to the decrease of those component concentrations in the used municipal wastewater.

Table 6.1. Feeding characteristics during the different operational stages.

Stage	Days	TN (mg N/L)	pH	IC (g IC/L)	NH_4^+-N/IC (g N/g IC)	TOC (mg/L)
S-I	0 – 137	50 ± 3	7.70 ± 0.10	55 ± 6	0.89 ± 0.02	-
S-II	138 – 182	33 ± 2	6.92 ± 0.09	41 ± 7	0.80 ± 0.05	40 ± 4
S-III	207 - 310	45 ± 10	7.20 ± 0.25	67 ± 8	0.68 ± 0.08	45 ± 9
S- IV	311- 354	20 ± 1	7.01 ± 0.09	26 ± 2	0.61 ± 0.02	22 ± 3

IC: inorganic carbon; TN: total nitrogen; TOC: total organic carbon.

The SBR was operated using three different cycle distributions (Figure 6.3) controlled by a programmable logic controller (PLC, Siemens S7-224CPU). From day 0 to 142 the 3-hour cycle was distributed as: 158 min of simultaneous feeding and aeration, 20 min of settling and 2 min of effluent withdrawal (Figure 6.3.A). On day 143 the cycle configuration was modified, and the aerated feeding lasted for 60 min, while 98 min were used (Figure 6.3.B) just for aeration to decouple organic matter oxidation from the nitritation process. Finally, on day 334 the total cycle length was

reduced from 180 min to 144 min (Figure 6.3.C) shortening the hydraulic retention time (HRT) from 6.0 h to 4.8 h.

A)

Feeding				
Aeration				
Settling				
Discharge				
Time (min)	**158**		**20**	**2**

B)

Feeding				
Aeration				
Settling				
Discharge				
Time (min)	**60**	**98**	**20**	**2**

C)

Feeding				
Aeration				
Settling				
Discharge				
Time (min)	**46**	**76**	**20**	**2**

Figure 6.3. SBR-cycle configurations: A) days 0 - 142, B) days 143 - 333 (stopped from 182 to 207) and C) days 334 - 354.

6.3.2. Biomass batch specific activity tests

Respirometric batch tests, of biomass samples collected from the SBR, were periodically carried out to assess the maximum SA_{AOB} and SA_{NOB} as well as the aerobic heterotrophic activity (SA_{aerHET}). The oxygen uptake rate (at 15 °C) was measured according to the methodology described by Lopez-Fiuza et al. (2002), using a biological oxygen monitor (BOM, YSI Inc. model 5300) device equipped with oxygen selective probes (YSI 5331). All the activity tests were performed in triplicate as described in section 2.2.6.3 from Chapter 2.

6.3.3. Analytical methods

Influent and effluent samples were periodically taken from the SBR. The pH values were measured with an electrode (pH1) connected to a Hach Sension$^+$ meter with temperature compensation. Liquid samples were filtered through 0.45 µm pore size filters prior to analysis. Spectrophotometric methods were used to determine the ammonium (Bower and Holm-Hansen 1980), nitrite and nitrate (APHA-AWWA-WEF, 2012) concentrations. Total organic and inorganic carbon (TOC and IC)

concentrations were measured with a Shimadzu analyser (TOC-L CSN). TN concentration was measured also in the TOC-L CSN L analyser coupled with a TNM-L Unit. The chemical oxygen demand (COD) was measured according to the Standard Methods (APHA-AWWA-WEF, 2012) to characterise the municipal wastewater. DO concentration and temperature in the bulk liquid were continuously measured (LDO HQ40d, Hach Lange). The concentration of volatile and total suspended solids (VSS and TSS) in samples collected from the reactor and its effluent were determined according to the Standard Methods (APHA-AWWA-WEF, 2012). Single operational cycles were also monitored in specific operational days to evaluate the evolution of the concentrations of the different compounds inside the reactor. Full description of the analytical methods is provided in Chapter 2.

6.3.4. Calculations

The FNA concentration, the NAR and the ammonium oxidation ratio (AOR) were calculated according to equations described in Chapter 2.

6.4. Results

6.4.1. Nitritation establishment by natural *in situ* FNA accumulation

The SBR was operated for 354 days fed with low ammonium concentrations (< 50 mg NH_4^+-N/L) and at 15 °C (mainstream conditions) (Figure 6.4).

During the start-up (Stage I), despite the initial low SA_{AOB} of the inoculated biomass (Figure 6.5), the nitritation process was established fast. The AOR was already 20 % on day 3 and reached 60 % after 5 days of operation (Figure 6.4.A). Then, the nitritation process was successfully maintained. Average effluent concentrations of 20 ± 3 mg NH_4^+-N/L and 26 ± 3 mg NO_2^--N/L were measured, while nitrate production was negligible leading to NAR of 100 % (Figure 6.4). The average IC concentration in the effluent was 10 ± 2 mg IC/L, and despite it was not totally depleted (Figure 6.6.A) it was close to the alkalinity affinity constant value of 6 mg IC/L, commonly considered in the activated sludge modelling (ASM2) (Henze et al. 2006). Therefore, the AOR could be either partially limited by the alkalinity concentration, by the DO concentration (Figure 6.6.B) and/or the cycle duration.

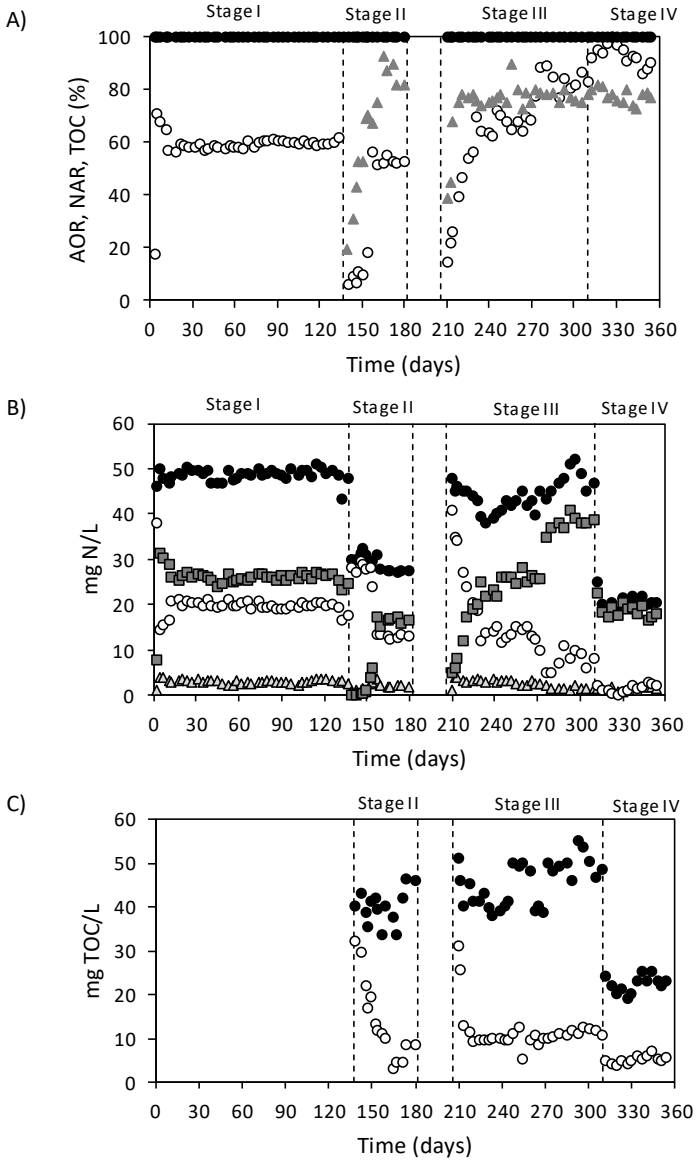

Figure 6.4. Evolution of: A) Nitrite accumulation ratio (NAR, ●), ammonium oxidation ratio (AOR, ○) and total organic carbon (TOC) oxidation ratio (▲), expressed as percentages; B) Concentrations of NH_4^+ in the influent (●) and NH_4^+ (○), NO_2^- (■) and NO_3^- (▲) in the effluent, expressed as mg N/L; C) TOC concentration in the influent (●) and effluent (○), expressed as mg TOC/L.

Furthermore, the progressive increase of the AOB activity during Stage I was also confirmed by batch activity tests where SA_{AOB} values up to 207 ± 9 mg NH_4^+-N/(g VSS·d)) were measured, while SA_{NOB} was never detected (Figure 6.5). From these results, and considering the biomass concentration inside the reactor close to 2 g VSS/L (Figure 6.5), the reactor treatment capacity was approximately 400 mg NH_4^+-N/(L·d)) much higher than the applied nitrogen loading rate (NLR) of 200 mg NH_4^+-N/(L·d)) and therefore this could be further increased.

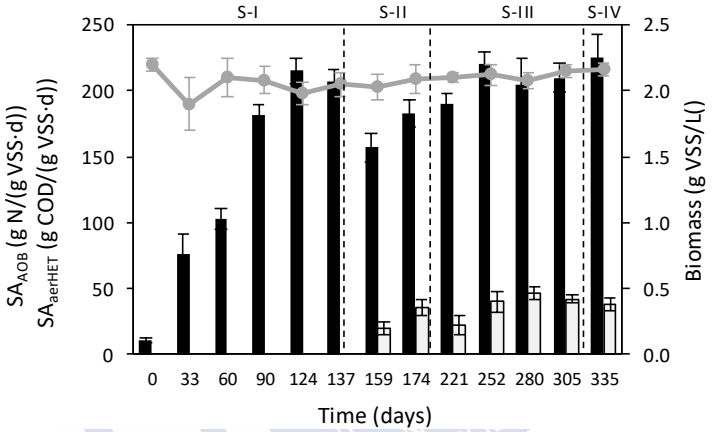

Figure 6.5. Evolution of the maximum specific ammonium oxidising bacteria activity (SA_{AOB}, ■) and specific aerobic heterotrophic activity (SA_{aerHET}, □) in batch tests, and biomass concentration inside the nitritation reactor (●) during the different Stages (S). Note that SA_{NOB} was also determined but it was under the detection level of the measurement method during the whole operational period. The bacterial activity tests were performed at 15 °C in triplicate.

The fact that the average concentration of VSS in the effluent was 6 ± 2 mg VSS/L and that the biomass concentration inside the reactor, throughout the whole stage, remained almost constant (Figure 6.5) indicated that the biomass washout (48 ± 16 mg VSS/d) was compensated by the growth of the microorganisms. Indeed, the AOB growth rate (as the main active bacterial population in the SBR) was estimated according to the stoichiometric reaction (Burton et al. 2014) being 35 ± 2 mg VSS_{AOB}/d. Having this in mind, the estimated SRT was longer than 80 days, showing the good biomass retention capacity of the system.

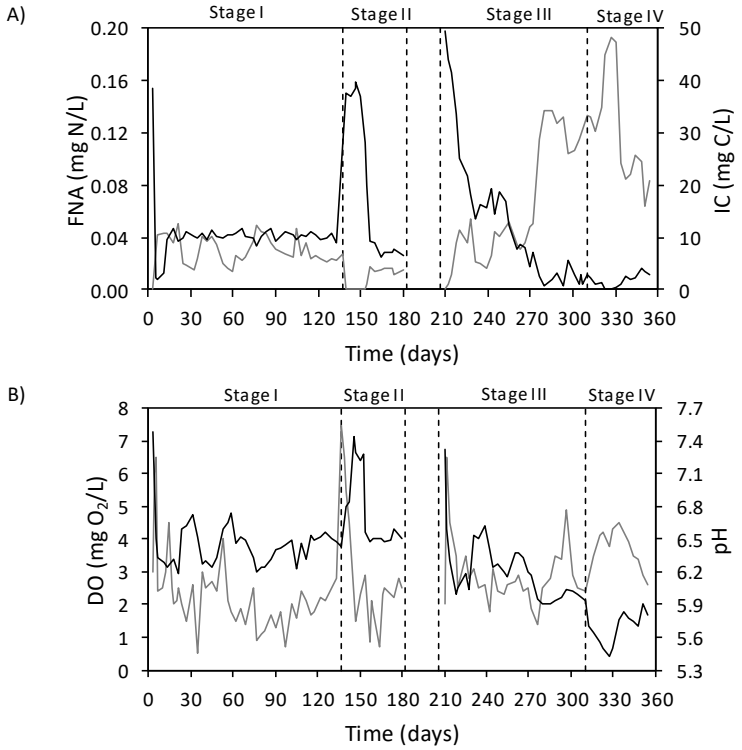

Figure 6.6. Evolution of A) Free nitrous acid (FNA) concentration (—), in mg HNO_2-N/L, and inorganic carbon (IC) concentration (—), expressed as mg C/L, in the SBR effluent; B) Dissolved oxygen (DO) concentration (—) expressed as mg O_2/L, and pH values (—) measured inside the reactor.

Although the inhibition caused on the SA_{NOB} by salt concentrations in the feeding above 4 - 8 g NaCl/L or the existence of starvation periods has been reported to be reversible (Ge et al. 2015, Liu et al. 2017, Val del Rio et al. 2018), this activity remained suppressed in the present nitritation reactor due to the *in situ* FNA accumulation at concentrations higher than 0.08 mg HNO_2-N/L (Figure 6.6.A). As stated previously in Chapter 5, when SA_{AOB} is higher than SA_{NOB} it is possible to maintain the nitritation process without the addition of an inhibitor. In these conditions, nitrite accumulates in the system, which leads to FNA inhibitory concentrations (if the ratio of N/IC ranges from 0.6 to 1.0).

6.4.2. Performance of the nitritation and organic matter oxidation

Primary settled municipal wastewater was fed to the SBR in Stage II. It was characterised by N/IC ratio of 0.80 g NH_4^+-N/g IC and an organic matter concentration of 40 mg TOC/L (with a COD/TOC ratio of 2.6 ± 0.1 g COD/g TOC) (Table 6.1). This change in the feeding provoked that initially no ammonium oxidation occurred, with the consequent increase of the DO concentration (from average values of 2.5 mg O_2/L to 6.0 mg O_2/L) as AOB did not consume it (Figure 6.6.B) neither it is consumed for organic matter oxidation due to the limited heterotrophic bacteria presence. For this reason, on day 143, the SBR operational cycle configuration was changed decreasing the period length for feeding and maintaining a period with only aeration (Figure 6.3.B), to separate the organic matter oxidation from the ammonium oxidation. After 15 days of this change, more than 70 % of the incoming organic matter was removed and the AOR increased again to approximately 50 % while NOB activity was negligible (Figure 6.4.A). The lower IC consumption leads to higher pH values in the effluent compared to Stage I (Figure 6.6).Therefore, the FNA concentration decreased close to the reported NOB inhibition concentrations of 0.02 mg HNO_2-N/L (Figure 6.6.A). However, the NAR remained at 100 % (Figure 6.4.A).

Batch tests performed, with the biomass from the SBR, confirmed that the nitritation activity took place throughout the whole operational time (Figure 6.5) despite the SBR operate for 44 days at FNA concentrations under the inhibitory values as SA_{NOB} was not detected. At the beginning of Stage II, when the organic matter started to be fed, the SA_{AOB} measured in batch tests decreased from 207 ± 9 to 157 ± 11 mg NH_4^+-N/(g VSS·d), presumably due to the development of the fast-growing aerobic heterotrophic bacteria. However, in these conditions, the SA_{AOB}, which partially recovered at the end of the stage to values of 183 ± 10 mg NH_4^+-N/(g VSS·d), was enough to maintain the nitritation process performance. In the meanwhile, the SA_{aerHET} reached values of 35 ± 6 mg COD/(g VSS·d) (Figure 6.5). Moreover, the biomass concentration inside the SBR remained at values of approximately 2 g VSS/L (Figure 6.5), while it increased slightly in the effluent but with values under 15 mg VSS/L. Despite the presence of organic matter and the heterotrophic bacteria development, the stablished long SRT (approximately 20 - 30 days) enabled the maintenance of the nitritation process stability.

6.4.3. Oxidation processes restoration after anoxic starvation

Between Stages II and III, there was a period of 25 days when the reactor was stopped, and the biomass was stored at 4 °C. Then, in Stage III, the SBR was re-started. It took approximately 12 days to recover the oxidation of 70 % of the TOC (Figure 6.4.C). This fact might be explained by the biomass starvation during the anoxic storage period. Once organic matter removal was re-established, the AOR rose exponentially and it stabilised at 60 % after 6 days (day 225) without nitrate production (Figure 6.4.A and Figure 6.4.B). Nan et al. (2019) observed, from batch experiments, that starvation periods (of 5 days before or 12 hours after the FNA treatment) enhance the resistance of the NOB to FNA. However, the starvation period before Stage III did not seem to affect the NOB suppression in the present study.

As the incoming N/IC ratio decreased from Stage II (0.80 ± 0.05 g N/g IC) to Stage III (0.68 ± 0.08 g N/ g IC) (Table 6.1), more ammonium was oxidized consuming more IC and decreasing the pH to average values of 6.1 ± 0.2 (reaching minimum values of 5.8) (Figure 6.6). This fact combined with the higher nitrogen concentration fed, that lead to higher nitrite concentrations in the effluent (Figure 6.4.B), caused the increase of the FNA concentration to average values of 0.06 ± 0.04 mg HNO_2-N/L reaching maximum concentrations of 0.13 mg HNO_2-N/L (Figure 6.6.B). These values were much higher than those reported as inhibitory for NOB (0.022 mg HNO_2-N/L) (Zhou et al. 2011). AOB activity remained unaffected by the exposure to these FNA concentrations. Indeed an increase in the SA_{AOB} was determined by respirometric tests getting average values of 210 mg NH_4^+-N/(g VSS·d) (Figure 6.5).

At the end of Stage III, the AOR further increased from average values of 67 ± 7 % (days 225 - 276) to 85 ± 3 % (Figure 6.4.B). In days 225 – 276 the AOR could be limited by the reaction time established by the fixed duration of the operational cycle and/or by the operational conditions imposed such as the aeration flow rate. The airflow rate was manually regulated by a valve, which was more opened after day 276, increasing the DO availability, although it was not reflected with a DO concentration increase inside the reactor (Figure 6.6.B), which means that more oxygen was consumed. Whereas at the end of the stage the average N/IC ratio of 0.68 g N/g IC (Table 6.1) was only enough to oxidise approximately the 85 % of the ammonium, therefore the AOR was limited by the IC concentration (Figure 6.6.A).

To deeply evaluate the nitrogen conversion in the reactor, on day 266 one SBR operational cycle was monitored (Figure 6.7.A and Figure 6.7.B). Once the feeding phase ended, pH, ammonium and IC concentrations dropped following a similar pattern. Consequently, nitrite concentration rose from 14 to 30 mg NO_2^--N/L leading to the *in situ* FNA accumulation. The FNA concentrations were higher than 0.02 mg HNO_2-N/L only in the last 40 min of the cycle (Figure 6.7.B). Even in these conditions, nitrate production was not detected and therefore its concentration was almost zero during the whole cycle (being the same that was already present in the feeding). The IC was almost completely consumed and at the end of the cycle, its concentration was 2 mg IC/L (Figure 6.7.A). Therefore, the AOR was limited by the almost fully depleted alkalinity. Henze et al. (2006) already predicted in the activated sludge model, ASM2, the limitation of the nitrification process rates at low inorganic carbon concentrations under 6 mg IC/L (based on the Monod kinetics 0.5 mmol HCO_3^-/L).

6.4.4. Optimisation of the operational cycle to treat very low nitrogen concentrations

In Stage IV, diluted wastewater was fed to the system as it was collected during raining periods (winter time). Consequently, considering that the supplied airflow was not changed along with the individual cycles, the average DO concentration sharply increased at the end of the cycle (up to 6 mg O_2/L) due to the decrease of its consumption to transform the substrate present. The N/IC ratio was 0.61 ± 0.02 g N/g IC (Table 6.1), close to the stoichiometric ratio of 0.59 g N/g IC required for the complete oxidation of ammonium to nitrite (Burton et al. 2014), and thus, both ammonium and IC were almost fully consumed (Figure 6.4.B and Figure 6.6.A). Due to the lack of enough alkalinity to buffer the system pH values as low as 5.3 were obtained in the effluent and in turn, the FNA concentration rose up to 0.20 mg HNO_2-N/L (Figure 6.6.A).

The cycle monitored on day 331 (Figure 6.7.C and Figure 6.7.D) proved the fact that ammonium and inorganic carbon were consumed before the end of the reaction period. IC concentration decreased from 20 to close to 0 mg IC/L and therefore the pH values sharply dropped. Thus, the FNA concentration augmented up to concentrations higher than 0.30 mg HNO_2-N/L that might partially inhibit the AOB activity. It has been reported that 0.4 mg HNO_2-N/L resulted in a 50 % suppression of the AOB activity according to Zhou et al. (2011) whereas Jiang et al. (2018)

observed the 57 % of the AOB activity loss by exposing the sludge to FNA concentrations as high as 1.2 mg HNO_2-N/L. Although, as far as alkalinity is available, full ammonium abatement is not recommended and concentrations of 2 - 5 mg NH_4^+-N/L should remain to avoid limiting the AOB growth rate (Burton et al. 2014, Laureni et al. 2019, Soliman and Eldyasti 2018). However, in SBR systems this effect should not be relevant since from the major part of the cycle higher ammonium concentrations were present (Figure 6.7.C). Furthermore, nitrate production was not observed probably due to the high FNA concentration leading to a NAR of 100 % during the whole operational period.

In order to optimise the performance of the cycle and to avoid part of the cycle that is inactive since all the ammonium was already oxidised, on day 334, the cycle length was shortened to 144 minutes (Figure 6.3.C). By doing this, the average pH values slightly increased (from 5.56 ± 0.10 to 5.79 ± 0.06) (Figure 6.6.B) and the AOR decreased obtaining ammonium concentration in the effluent of 2 - 3 mg NH_4^+-N/L, which indicated that the reaction phase adjusted to the duration of the cycle (Figure 6.4 and Figure 6.6). This fact was confirmed by the cycle characterisation performed on day 345, obtaining a similar pattern to that observed in Stage III (Figure 6.7.E and Figure 6.7.F). The ammonium and inorganic carbon concentrations were linearly consumed along time with the consequent decrease on the pH value. Nitrite production was observed during the whole operational cycle increasing the FNA concentration up to 0.11 mg HNO_2-N/L. The fact that ammonium oxidation was observed immediately after the feeding stop showed that this phase length was long enough to oxidise the organic matter fed to the system.

With respect to the biomass characteristics, its average concentration inside the reactor was 2 g VSS/L. Solid concentrations in the effluent were under 20 mg VSS/L. The SA_{AOB} was over 200 mg NH_4^+-N/(g VSS·d) which indicated that, despite the presence of organic matter, the ammonium oxidation capacity inside the reactor was 400 mg N/(L·d) enough to oxidise the supplied ammonium (Figure 6.5). The measured SA_{aerHET} was of 40 mg COD/(g VSS·d). Considering these results, the HRT might be further reduced increasing the applied nitrogen load up to average values of 150 ± 42 mg NH_4^+-N/(L·d).

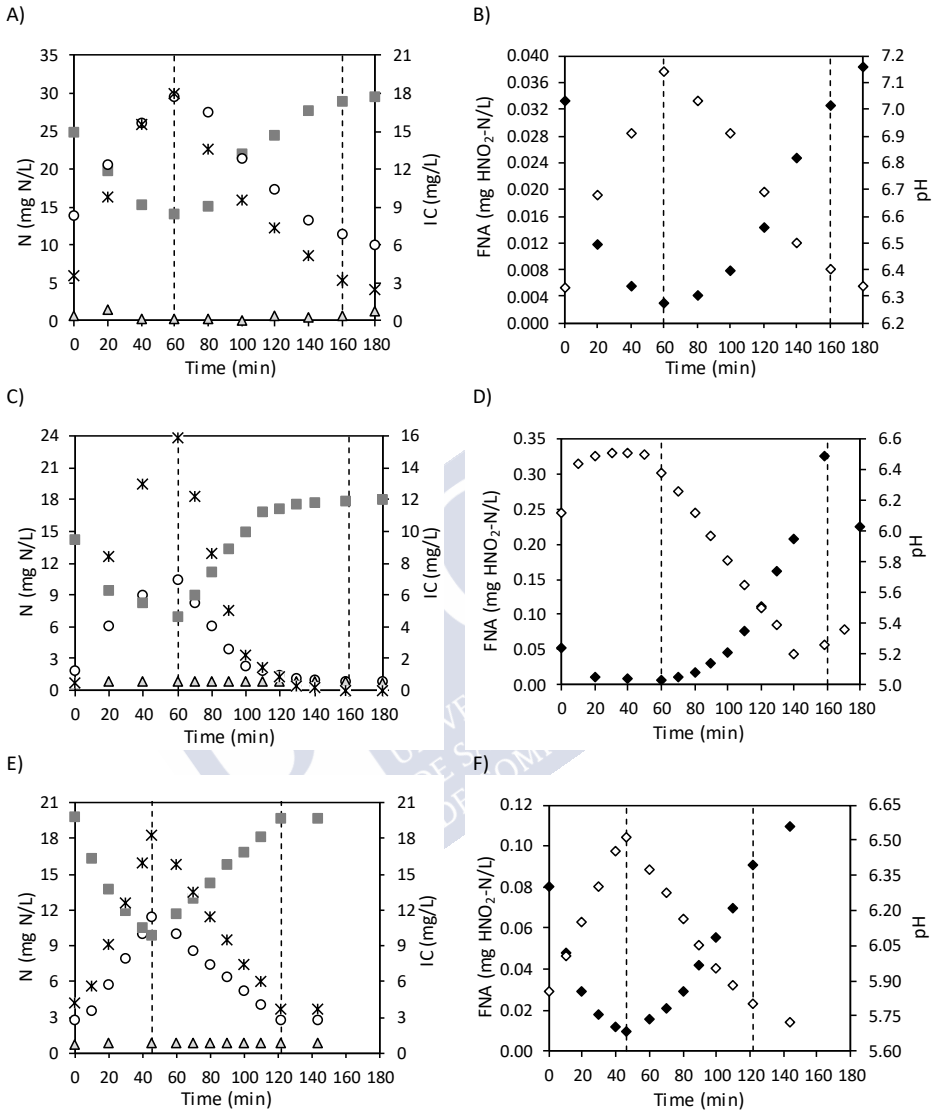

Figure 6.7. SBR cycle characterization on day 266 (Stage III) (A, B) and on day 331 (C, D) and day 345 (E, F) (Stage IV). A, C, E) Evolution of ammonium (○), nitrite (■) and nitrate (Δ) concentrations expressed as mg N/L and inorganic carbon (IC) concentration as mg IC/L (*). B, D, F) Evolution of the pH value (◊) and FNA concentration (♦) expressed as mg HNO_2 -N/L.

6.5. Discussion

6.5.1. Nitritation process treating municipal wastewater in the presence of organic matter

The implementation of the nitritation process at mainstream conditions requires to suppress the activity of NOB and overcome the competition of the organic matter oxidisers for the dissolved oxygen. Thus, the strategy to perform this process must be selected depending on the wastewater composition.

When dealing with wastewater streams with low alkalinity, the approach where FNA is accumulated is feasible. However, the studies based on the NOB activity suppression by FNA were mainly carried out using synthetic wastewater with no presence of organic matter. Jiang et al. (2018) treated municipal wastewater, containing 365 ± 40 mg COD/L, at decreasing temperatures from 25 to 15 °C and DO concentration controlled at 1 mg O_2/L (Table 6.2). These authors achieved NAR higher than 95 % and organic matter removal efficiencies of 30 % by treating the inoculated sludge with FNA. Although NOB activity was not detected in their study, it needs to be considered that the operational period at low temperature (< 20 °C) was too short to guarantee the long-term process performance. Ma et al. (2017) also achieved the stable nitritation process by treating part of the sludge with FNA in an external unit but the long-term stability was an issue lasting only 32 days. Better results were achieved in the present study, which shows for the first time that the organic oxidation and nitritation processes can take place in the same unit operated at 15 °C, achieving values of NAR close to 100 % and COD removal efficiencies of 80 % for 191 days.

In the present study, where the nitritation process was maintained by FNA *in situ* production, no adverse effects associated with the presence of the organic matter (besides the organic matter mineralisation) were observed. This fact is in good agreement with other reported research works (Table 6.2) where the only effect related to the organic matter presence was the higher DO consumption to oxidise it. In this study, the higher DO consumption was not reflected on the DO concentration in the bulk liquid since the aeration flow was periodically manually adjusted mainly focused on guaranteeing the reactor mixture. Thus, the defined prerequisites of organic matter content in the fed wastewater to apply the partial

nitritation combined to the anammox process (TOC/N < 0.5 - 0.8 g TOC/g N, Cao et al. (2017)) should be revised by performing an environmental-economic study taking into account the energy balance of the WWTP and the associated costs of removing the organic matter in a separate unit to achieve the required removal efficiency. For instance, for very diluted wastewater streams to implement a specific unit for organic matter removal could not be necessary (Pedrouso et al. 2018). The pre-concentration is a powerful strategy to reduce the organic load in the waterline, while the energy recovery is enhanced as more organic matter is transformed into biogas in anaerobic digesters. However, difficulties to achieve high removal efficiencies with low organic matter concentrations, and thus the required low organic matter to nitrogen ratio, at low temperature are well-known (Agrawal et al. 2018, Cao et al. 2017, Hoekstra et al. 2019). In the present study average organic matter removal of 80% was achieved in aerobic conditions limiting the possible growth of heterotrophic denitrifying bacteria that would compete for nitrite with the anammox bacteria in the following anoxic unit.

6.5.2. Robustness and feasibility of the *in situ* FNA inhibitory concentration strategy

In the present research work, the long-term nitritation process (354 days, 191 treating municipal wastewater) was achieved by maintaining *in situ* FNA inhibitory concentrations, from 0.02 to 0.20 mg HNO_2-N/L, treating primary settled wastewater at low temperature (15 ± 1 °C). Despite the wastewater composition variability, this strategy proved to be very robust even when in Stage II the FNA concentration was lower than 0.02 mg HNO_2-N/L. Furthermore, when FNA concentrations rose up to 0.3 mg HNO_2-N/L (Stage IV), AOB activity was not suppressed.

These results confirm the robustness of the *in situ* FNA strategy and are in good agreement with those obtained in Chapter 5, using the same strategy but treating synthetic wastewater. Then, more than 40 days were required to detect NOB activity when FNA concentration went down the inhibitory values. On the contrary, Duan et al. (2019) applying an *ex situ* FNA treatment to the nitritifying sludge observed that NOB activity recovered as soon as the FNA treatment was stopped. Afterwards, despite the sludge treatment was restart, the NAR recovery was limited to 30 %. Therefore, it seems that the strategy of NOB activity suppression by *in situ* FNA inhibitory concentration is more robust than *ex situ* treatment.

Table 6.2. Literature review of published studies on the nitritation process treating municipal wastewater at low temperature (< 25 °C).

Municipal wastewater		**Operational conditions and process performance**					
mg NH_4^+-N/L	mg COD/L	Reactor V - Type	T (°C)	NAR (%)	Days of stability	Strategy to maintain the nitritation process	**Reference**
18.2 - 52.0	55 - 146	2 L - SBR	15	100	191	*In situ* FNA accumulation	This study
44.5 - 72.6	159 - 241	3 L-SBR	17 - 27	98	66	Nitrite stress in starvation conditions	[1]
32.1 -79.3	62 -262	10 L-SBR	23 - 25	98	260	Real time control ammonium valley	[2]
48.3 ± 12.7	235 ± 143	9 L-SBR	Room T	80	78	External sludge ultrasonic treatment and DO control 0.6 - 0.8 mg O_2/L	[3]
50.3 ± 4.9	365 ± 40	10 L-SBR	15 - 25	95	150	FNA seeding sludge treatment and DO control 1 mg O_2/L	[4]
39.9 - 78.7	91 - 232	10 L-SBR	12 - 17	97	150	Anoxic/Oxic mode and short SRT	[5]
43.2 -74.3	131 - 241	10 L-SBR	18 -23	95	80	DO 5 - 7 mg O_2/L Anaerobic/aerobic	[6]
41.2-78.3	160 - 300	7 m^3-SBR	17 - 26	95	180	Aeration control by real time control based on blower frequency and pH	[7]
58.1 ± 12.8	215 ± 56	10 L-SBR	12 - 25	90	166	Aeration length controlled by ammonia valley	[8]
58.1 ± 12.8	215 ± 56	10 L-SBR	12 -25	90	240	Aeration length controlled by ammonia valley	[9]
60.05	142.3	54 m^3-SBR	12 - 25	95	180	Aeration controlled by ammonia valley and DO breakpoint	[10]

DO: dissolved oxygen; FNA: free nitrous acid; NAR: nitrite accumulation ratio; T: temperature; V: Volume.

References: [1] Cui et al. (2019); [2] Jin et al. (2019); [3] Zheng et al. (2019); [4] Jiang et al. (2018); [5] Zhang et al. (2018); [6] Zhang et al. (2017); [7] Gu et al. (2012); [8] Guo et al. (2010); [9] Guo et al. (2009); [10] Yang et al. (2007).

Moreover, with the *in situ* FNA production strategy no long-term adaptation of NOB to the suppression factor has been observed, despite this has been widely reported as a key challenge (Duan et al. 2019). Indeed, Ma et al. (2017) noticed a dominant NOB population shift from *Nitrospira* to *Nitrotoga* genus after the establishment of the nitritation process by treating the sludge in a FNA treatment unit using 0.75 mg HNO_2-N/L. These authors observed that when the incoming nitrogen concentration decreased by 28 %, the excess of aeration caused the decrease of NAR from 75 to 10 %. After the increase of ammonium concentration in the influent, NAR was not reestablished, and the increase of the FNA concentration (from 0.25 to 1.00 mg HNO_2-N/L) and the treatment of a sludge fraction (from 10 to 30 %) caused an AOB activity reduction whereas nitrate was the main oxidation product (Ma et al. 2017). These authors suggested a NOB acclimation to the FNA treatment, and they found an increase in the FNA threshold needed to suppress the NOB activity. In this line, Duan et al. (2019) reported the adaptation of the NOB to both FNA and FA inhibitory concentrations, when applied in an external unit, after long-term operation of the nitritation process. To avoid this behaviour, these authors proposed to alternate the FNA and FA treatment. On the contrary, the development of NOB activity was not observed in the present study with the strategy based on the FNA *in situ* produced treating neither synthetic wastewater (Chapter 5) nor municipal wastewater (the present Chapter).

Additionally, the establishment and maintenance of the partial nitritation by the *in situ* FNA strategy are independent of the DO concentration, and for this reason, it was not controlled in the present study (Figure 6.6). Thus, the DO concentration only affected the oxidation rate, which was maximised by increasing the DO addition. Indeed, these results also reinforce previous reports that have pointed out the drawback in relying exclusively on oxygen kinetics to achieve NOB suppression (Agrawal et al. 2018, Val del Rio et al. 2019). In this way, when the ammonium concentration decreases or a failure in the feeding pump occurred, NOB activity was not immediately observed as it happened when other strategies were applied (Jin et al. 2019, Ma et al. 2017, Reino et al. 2016). For example, Jin et al. (2019) observed the immediate nitrate production after a failure in the ammonium valley control system that leads to the increment of the DO concentration to values higher than 2 mg O_2/L. The nitritation process stability was only recovered by shortening the SRT. This strategy succeeded since the operational temperature was

higher than 20 °C, but as at lower temperatures the NOB growth rate is higher than the AOB one the recovery of the NAR would not be presumably feasible.

Moreover, most of the strategies based on the NOB inhibition by FNA require pH adjustment and sludge pumping to an external unit where a stream containing FNA is supplied. By the *in situ* production of the FNA, this extra unit is not needed and the sludge suffers from less mechanical stress. Moreover, more extreme conditions are applied when the external unit is used that might also cause AOB activity reduction (Duan et al. 2018, Jiang et al. 2019, Ma et al. 2017, Wang et al. 2017).

As a final feature of the *in situ* FNA accumulation strategy, the alkalinity of the wastewater (N/IC ratio) must be considered. It was already explained in Chapter 5 that for incoming wastewater ratios between 0.6 to 1.0 g NH_4^+-N/g IC, the limited alkalinity decreases the pH leading to high FNA concentrations. The feasibility of this strategy was demonstrated in the present study using municipal wastewater with N/IC ratios between 0.61 - 0.80 g NH_4^+-N/g IC. After the alkalinity was fully depleted the pH dropped and FNA concentrations as high as 0.20 mg HNO_2-N/L accumulated inside the reactor. Thus, the application of the *in situ* FNA inhibitory concentration strategy in those regions characterised by hard freshwater (N/IC < 0.6 g NH_4^+-N/g IC) would not be suitable or would require of pH control with the associated high operational costs. In these cases, a suitable strategy is to overload the system in order to accumulate ammonia and increase the pH value (due to CO_2 stripping) inside the system in order to inhibit NOB activity by free ammonia. Considering hard wastewater case, most alternatives proposed so far to maintain the nitritation process at mainstream conditions rely on pH control at basic pH (mainly close to 8) (Jiang et al. 2019, Reino et al. 2016). Apart from the wastewater hardness, the incoming nitrogen and inorganic carbon concentrations are also influenced by the previous treatment stages in the wastewater treatment plant. The most common approaches to remove the organic matter content from the wastewater are: the high rate activated sludge (where alkalinity is not modified), the wastewater anaerobic digestion (alkalinity is produced) and/or chemical enhanced pre-treatment (CEPT) (consumes alkalinity). Thus, the treatment train must be considered globally as for example, if hard wastewater is treated by CEPT, the resulting nitrogen to inorganic carbon concentration ratio entering the nitritation system could be adequate for the NOB suppression based on *in situ* FNA production. In this way not only the

wastewater hardness. but the previous processes it is submitted, need to be considered carefully to define the correct operational conditions of the subsequent nitritation unit.

6.6. Conclusions

In the present study, the nitritation process was successfully started-up using an inoculum with negligible SA_{NOB} since it was previously exposed to high NaCl concentrations (approximately 18 g NaCl/L) and to a starvation period. These inhibition factors are reversible, but the NOB suppression was maintained even if they were not being applied in the system. Thus, the feasibility of establishing the nitritation process by the *in situ* FNA accumulation-based strategy directly using an AOB enriched seeding sludge (without the addition of chemical inhibitory compounds) was proven for the first time.

The nitritation process was established and maintained in an SBR by the *in situ* FNA accumulation-based strategy at concentrations up to 0.2 mg HNO_2/L, treating municipal wastewater containing organic matter (up to 1.2 g TOC/N) and operating the system at low temperature (15 ± 1 °C).

To cope with the changes of nitrogen and organic matter concentrations in the municipal wastewater, the optimisation of the length and distribution of the operational cycle in the SBR is required.

The obtained results showed that a nitrite accumulation ratio close to 100% and an organic matter removal of 80 % can be achieved in the same unit. Moreover, the long-term stability (354 days from which 191 days were treating municipal wastewater) and robustness of the nitritation process were proven despite the influent wastewater composition fluctuations with N/IC ratios varying from 0.61 to 0.89 g NH_4^+-N/g IC.

Organic matter had no adverse effect over the nitritation process stability and, thus, a previous unit for organic matter removal might be not required depending on the potential valorisation of the organic matter content. This fact gives to this strategy a great opportunity since allows to be more flexible with the required removal efficiencies in the previous units.

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Chapter 7

Assessment of the anammox process performance

SUMMARY

The anammox process revealed itself as a promising alternative to biologically remove the nitrogen increasing the energy efficiency of the wastewater treatment plants. Limited studies are available on the operation of reactors where only the anammox process takes place for the treatment of municipal wastewater. In this chapter, a 5-L anammox granular reactor was operated at 15 ± 1°C treating low nitrogen loaded wastewater. The reactor was inoculated with sludge from a one-stage partial nitritation and anammox reactor treating high strength wastewater at 30 °C. Then, it was started-up treating synthetic feeding (25 mg NH_4^+-N/L and 25 mg NO_2^--N/L) directly at 15 °C, showing that an acclimation period to progressive reach the mainstream conditions is not needed and, consequently, shortening the start-up periods. The long-term anammox process stability was proved treating both, synthetic wastewater with decreasing alkalinities and nitritified primary settled municipal wastewater. The low influent pH values (6.2 ± 0.1) reached when municipal wastewater was fed did not affect the process stability. On the other hand, residual organic matter concentrations augmented the nitrogen removal efficiency from 80 % (with the synthetic medium) to 92 % achieving total nitrogen (TN) effluent concentrations below 10 mg TN/L. Specific anammox activity (SA_{AMX}) increased from 53 ± 11 to 78 ± 8 mg N/(g VSS·d) during the operation with synthetic media while it was worsened (58 ± 6 mg N/(g VSS·d) when municipal wastewater was fed due to the development of heterotrophic denitrifying bacteria. Finally, the effect of pH (6 - 8), temperature (15 - 30 ° C) and organic matter concentration (0 - 75 mg TOC/L) over the SA_{AMX} was evaluated. Results indicated that pH and temperature and their interactions exerted significant influence on the SA_{AMX} value while the TOC concentrations itself did not significantly change the SA_{AMX} at short-term.

OUTLINE

7.1. Introduction

The contribution of the partial nitritation and anammox (PN/AMX) processes, to the achievement of energy autarky in wastewater treatment plants (WWTPs), when implemented in the mainstream is nowadays undeniable (Kartal et al. 2010). In special considering that 80 % of the nitrogen processed in the plant goes through the mainstream line. However, up to now, an anammox based process has been never implemented at full scale in these conditions (Agrawal et al. 2018, Hoekstra et al. 2019, Winkler and Straka 2019). When this combined process was first evaluated, the anammox step was pointed out as the sensitive one (Jin et al. 2012). The main identified limiting factors that challenge its application are the slow growth rate of the anammox bacteria, exacerbated at low temperature, and the low biomass yield associated to the low nitrogen concentrations in mainstream (Agrawal et al. 2018, Morales et al. 2015b). Thus, to tackle these issues the maximisation of the biomass retention inside the reactor is fundamental.

Biofilm systems are used to accumulate large biomass concentrations and operate at long solid retention times (SRTs). Due to their slow-growth rates, anammox bacteria tend to form microbial aggregates, and therefore, easily form granules without the need for carrier material addition. Moreover, substrate gradients establish inside the granules (Morales et al. 2015a), which are even more relevant when dealing with low concentrations. In case PN/AMX processes take place in a single unit this fact allows for the establishment of an internal granular anoxic core where anammox activity takes place while ammonium oxidising bacteria (AOB) operate in the external layers (Morales et al. 2015a, Morales et al. 2016).

In addition, operation at temperatures below the optimal range (varying from 30 to 40 °C, Jin et al. (2012)) is expected to exert a detrimental effects on the anammox population activity similar to those happening with AOB, as they have similar activation energy values of 68 and 70 kJ/mol, respectively (Burton et al. 2014). However, recent studies indicated that the anammox energy activation (Ea) cannot be considered constant for the temperature range from 15 to 30 °C, as it is generally accepted for AOB in the activated sludge models (ASM) (Henze et al. 2006). For this reason, AOB and anammox activities are unbalanced, limiting the specific treatment capacity of the system (Lotti et al. 2015).

To implement the PN/AMX processes in separate units represents a beneficial solution to optimise both of them independently (Pérez et al. 2015). In such circumstances, in the anoxic reactor, anammox bacteria competition is absent or minimised with nitrite oxidising bacteria (NOB), since dissolved oxygen (DO) would not be available; and with heterotrophic denitrifying bacteria, as negligible organic matter concentrations will be present.

The feasibility of performing the anammox process at low/moderate temperature was assessed in batch tests, which indicated that the specific anammox bacteria activity decreases by about 10 times when temperature drops from 30 to 10 °C (De Cocker et al. 2018), and in long-term operation using different reactor configurations (De Cocker et al. 2018, Dosta et al. 2008, Reino et al. 2018). Most available studies, up to now, have been performed by subjecting the inoculum to long acclimation periods (several months) until conditions of low temperature and low total nitrogen (TN) concentrations were reached (De Cocker et al. 2018, Reino et al. 2018, Sánchez Guillén et al. 2016). Few studies reported on the stable operation of anammox reactors at mainstream conditions and the achieved nitrogen removal rates (NRR) were usually low ranging from 27 to 60 mg TN/(L·d) (Hendrickx et al. 2014, Sánchez Guillén et al. 2016). Whereas limited research works were performed using municipal wastewater (Laureni et al. 2015, Lotti et al. 2014b, Ma et al. 2013, Reino et al. 2018) and the achieved nitrogen concentrations in the effluent were up 40 mg TN/L, higher than 10 mg TN/L the commonly established discharge limit used, among others, in the European Union for sensitive areas.

As in most research works synthetic media were fed to the reactor, the alkalinity concentrations fixed mimicked those values generally found in high-strength wastewater (De Cocker et al. 2018, Reino and Carrera 2017). However, this situation is difficult to find in municipal wastewater, so in practice, the inorganic carbon (IC) concentration in the mainstream is low and might even limit the anammox reaction (Burton et al. 2014, Seuntjens et al. 2018). Anammox are chemolithoautotroph bacteria, which utilise IC as sole carbon source for growth (Strous et al. 1999). So, they become vulnerable to the IC limiting conditions (Wang et al. 2019, Zhang et al. 2016). With this in mind, Kimura et al. (2011) investigated the effects of the IC on the anammox bacteria and defined as optimal for the anammox process a minimal ratio of 5 mg NH_4^+-N/mg IC and they recommend to maintain it higher than 10 mg NH_4^+-N/mg IC, meaning that at lower ratios the process

efficiency diminished. Contrary, Liao et al. (2008) found that the NRR increased when influent ammonium to IC concentration decreased from 0.56 to 0.37 mg NH_4^+-N/mg IC whereas it was inhibited at 0.28 mg NH_4^+-N/mg IC. Nevertheless, these studies were performed at high temperature (≥ 30 °C). So further investigation is required considering that in mainstream conditions the alkalinity could be completely depleted in the previous nitritation process, depending on the ammonium to IC concentration ratio of the used wastewater and the applied nitritation strategy (Chapters 5 and 6).

Finally, as anammox bacteria are sensitive to environmental conditions such as pH, temperature, DO concentration, organic matter or substrate concentrations (Cao et al. 2017) the effects of each parameter might be different when mainstream conditions are imposed. Daverey et al. (2015) evaluated the simultaneous effect of temperature and pH and observed that the optimal pH increases at low temperature. Since the nitritation process consumes alkalinity, the low pH of the stream entering the anammox reactor might be an issue at low temperature, as well as the residual organic matter concentration which could favour the denitrification process development. The individual effects of these factors have been widely studied (Daverey et al. 2015, Ma et al. 2016, Tomaszewski et al. 2017) but scarce information and unclear conclusions are available on the interaction of combined inhibitory conditions, like low pH, organic matter presence and low temperature.

7.2. Objectives

The main objective of this study is to evaluate the long-term performance and stability of a granular anammox reactor operated at mainstream conditions, 15 °C and 50 mg TN/L, and inoculated with biomass which was not previously acclimated to low nitrogen neither lower temperature.

First, the effect of alkalinity supply over the long-term reactor performance was assessed using a synthetic feeding containing different ammonium to alkalinity ratios.

Then, the performance of the anammox reactor fed with the effluent from a nitritation reactor (Chapter 6) was operated to evaluate the influence of the residual organic matter concentration, in terms of nitrogen removal and biomass properties such as particle diameter, relevant for the biomass retention.

Additionally, the influence of individual and combined effects of temperature, organic matter concentration and pH on the specific anammox activities was evaluated in batch experiments.

7.3. Materials and Methods

7.3.1. Reactor description and operating conditions

A 5-L sequencing batch reactor (SBR) with a volume exchange ratio fixed at 25 % (Figure 7.1) was used to perform the anammox process. The temperature was controlled at 15 ± 1 °C, by using a thermostatic jacket where water from a water bath (Frigiterm, JP) was pumped, and pH was not controlled ranging from 6.1 to 8.9. The complete mixture inside the reactor was provided by a mechanical stirrer with a rotating speed of 60 - 80 rpm. A slow flow of Argon gas (95 % Ar and 5 % CO_2) was bubbled in the liquid media to ensure anoxic conditions. A set of two peristaltic pumps was used to introduce the feeding solution on the top of the reactor and to discharge the effluent at the desired height level. The anammox reactor was seed with granular PN/AMX sludge from a 1.2-m^3 ELAN® pilot plant (Morales et al. 2015a), which treated, at approximately 30 °C, the supernatant from an anaerobic sludge digester located in a municipal wastewater treatment plant (Guillarei, Spain).

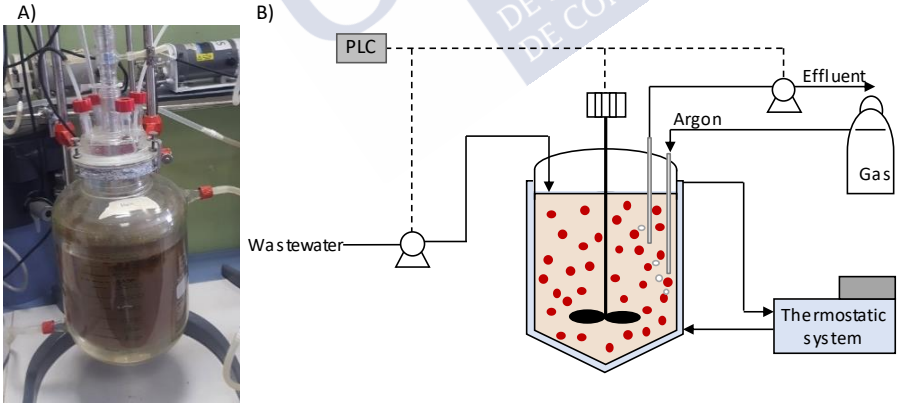

Figure 7.1. Image and scheme of the anammox SBR.

The SBR operation lasted 485 days. Two types of feeding were used: synthetic media, between days 0 and 392 (S-I to S-VI), and municipal wastewater from day 393 onwards (S-VII to S-VIII).

The synthetic feeding composition was adapted from Dapena-Mora et al. (2004) mimicking the effluent produced in a nitritation reactor containing 25 mg NH_4^+-N/L (as NH_4Cl) and 25 mg NO_2^--N/L (as $NaNO_2$). Nitrate was supplied, 0 to 25 mg NO_3^--N/L (as KNO_3), to prevent anaerobic conditions, which could lead to biomass methanization (Dapena-Mora et al. 2004). The feeding media was supplemented with, in mg/L: 96 – 1,250 of $KHCO_3$ (to provide variable alkalinities), 147 of KH_2PO_4, 300 of $CaCl_2 \cdot 2\ H_2O$, 200 of $MgSO_4 \cdot 7\ H_2O$, 11 of $FeSO_4 \cdot 7\ H_2O$, 8 of EDTA-Na·2 H_2O and 0.2 mL/L of trace solution (Vishniac and Santer 1957).

From day 393 onwards, the anammox reactor oxidised with primary settled municipal wastewater, which was partially treated in the nitritation reactor of Chapter 6. During this period, up to 85 % of the ammonium fed to this nitritation unit was oxidised to nitrite. Therefore, this effluent was diluted with raw primary settled municipal wastewater (containing only ammonium as nitrogen) to obtain a stream with a nitrite to ammonium ratio of approximately 1.2 g NO_2^--N/g NH_4^+-N (Table 7.1) slightly lower than the stoichiometric value of 1.32 g NO_2^--N/g NH_4^+-N for the anammox process (Strous et al. 1999) to maintain the nitrite as limiting substrate.

Table 7.1. Primary settled municipal wastewater composition after nitritation process and fed to the anammox reactor prepared after mixing with the primary settled wastewater.

Parameter	Primary settled wastewater		Nitritation reactor effluent		Anammox reactor influent	
	S- VII	S-VIII	S- VII	S-VIII	S- VII	S-VIII
NH_4^+ (mg N/L)	45 ± 4	24 ± 9	11 ± 3	2 ± 2	20 ± 3	12 ± 4
NO_2^- (mg N/L)	0	0	31 ± 6	21 ± 7	24 ± 3	14 ± 5
NO_3^- (mg N/L)	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 1
TOC (mg TOC/L)	48 ± 5	26 ± 9	10 ± 2	6 ± 2	19 ± 4	14 ± 4
IC (mg IC/L)	65 ± 6	30 ± 2	5 ± 4	2 ± 1	5 ± 4	2 ± 1
pH	7.1 ± 0.1	7.1 ± 0.2	6.1 ± 0.1	5.7 ± 0.2	6.2 ± 0.1	5.9 ± 0.2

IC: inorganic carbon; TOC: total organic carbon; S: Stage, see Table 7.2 for details.

The reactor operational period was divided into 8 different Stages (Table 7.2), fed with synthetic media (S-I to S-VI) and with primary settled municipal wastewater (S-VII to S-VIII). In Stage I nitrate was supplied to the feeding and its concentration was gradually decreased in Stages II and III. Then, during Stages IV to VI, the alkalinity concentration fed was also step-wise decreased to have values for this parameter close to mainstream conditions. Finally, during Stage VII and VIII, a nitritified

municipal wastewater was treated in the SBR and in Stage VIII the cycle duration was shortened in order to treat higher load.

Table 7.2. Summary of the operating conditions and feeding composition throughout the operational stages.

Feeding	Stages	Days	Nitrate mg NO_3^--N/L	Alkalinity mg IC/L	NH_4^+/IC ratio g N/g IC
Synthetic media	I	0 – 118	25	130	0.19
	II	119 - 154	10	130	0.19
	III	155 - 197	0	130	0.19
	IV	198 - 248	0	65	0.38
	V	249 - 338	0	30	0.83
	VI	339 - 392	0	10	2.50
Municipal wastewater	VII	393 -432	1	6	3.67
	VIII*	433 - 485	1	2	7.50

* The cycle length was shortened from 6 to 4 hours.

The SBR cycle lasted 6 hours (S-I to S-VII) distributed according to Dapena-Mora et al. (2004): 300 min of mixed feeding and reaction, 30 min of mixing, 15 min of settling and 15 min of effluent withdrawal (Figure 7.2.A). The reactor was operated in a semi-continuous mode since the feeding lasted for more than 80 % of the total cycle length. In this way, the existence of peaks of high substrate concentrations was avoided. The different cycle phases were controlled by a programmable logic controller (PLC, Siemens, S7-224 CPU). Finally, in Stage VIII the cycle length was shortened to 4 hours (Figure 7.2.B) and the hydraulic retention time (HRT) shortened from 24 to 16 hours.

A)

Feeding				
Mixing				
Settling				
Withdrawal				
Time (min)	**300**	**30**	**15**	**15**

B)

Feeding				
Mixing				
Settling				
Withdrawal				
Time (min)	**180**	**30**	**15**	**15**

Figure 7.2. Distribution of the operational cycle: A) Cycle duration of 6 hours applied from day 0 to 432 and B) Cycle duration of 4 hours applied from day 433 onwards.

7.3.2. Microbial activity batch tests

Maximum specific anammox activity (SA_{AMX}) was determined according to Dapena-Mora et al. (2007) as detailed in Chapter 2. Activity tests were performed at 30 °C (as reference temperature) and 15 °C once per month with biomass samples collected from the SBR. Tests at 20 and 25 °C were performed as well, but with less frequency, to assess the effect of the temperature changes over the SA_{AMX}. Based on the SA_{AMX} method a modification was applied, by adding nitrate or nitrite (50 mg N/L) and acetate (100 mg COD/L) as substrate, to determine the activity of heterotrophic denitrifying (SA_{HDN}) bacteria at 30 °C, in the periods when the reactor was fed with municipal wastewater. All the activity tests were performed in triplicate. Detailed procedure is described in Chapter 2, section 2.2.6.

7.3.3. Response surface methodology

Additionally, batch activity tests were performed with the biomass from the reactor taken from days 410 - 420 to assess the effect of temperature, pH and organic matter content over the SA_{AMX} using a response surface methodology (RSM).

Statistical methods are powerful tools to find the optimal values of process parameters along with their interactive effects on the response. A three-level-three-factor Box-Behnken design (BBD) (Ferreira et al. 2007) was used for the assessment of the influence of temperature, pH and total organic carbon (TOC) concentrations over the SA_{AMX}. Temperature was evaluated in the range of the optimal value for the test (30 °C) and the operational temperature of the reactor (15 °C), organic matter concentration between 0 and 75 mg TOC/L as they are typical values found in mainstream effluents and pH in the range of 6 to 8. The natural and coded values of the selected independent variables are shown in Table 7.3.

Table 7.3. Variables and levels of each parameter assayed according to the Box-Behnken experimental design.

Parameter	Units	Code	Low (-1)	Medium (0)	High (1)
Temperature	°C	x_1	15.0	22.5	30.0
pH	-	x_2	6.0	7.0	8.0
TOC	mg TOC/L	x_3	0	37.5	75.0

A total of 15 experiments (in triplicate), including the three replicates in the central point (E7, E8 and E9), were conducted (Ferreira et al. 2007). The full experimental design is shown in (Table 7.4). To assess the reproducibility of the results, the experiments were repeated and consequently, the final number of experiments was 30. The substrate concentrations (ammonium and nitrite) were maintained at 70 mg N/L each, according to Dapena-Mora et al. (2007). The organic matter (as sodium acetate) was added with the substrates and pH value was adjusted to the target value by adding NaOH or HCl in the washing step. Biomass concentration in the vials was similar with average values of 2.6 ± 0.1 g VSS/L.

Table 7.4. Defined Box-Behnken factorial experimental design.

Test	Temperature (°C)	TOC (mg/L)	pH
E1	15.0	0	7
E2	15.0	37.5	6
E3	15.0	37.5	8
E4	15.0	75.0	7
E5	22.5	0	6
E6	22.5	0	8
E7	22.5	37.5	7
E8	22.5	37.5	7
E9	22.5	37.5	7
E10	22.5	75.0	6
E11	22.5	75.0	8
E12	30.0	0	7
E13	30.0	37.5	6
E14	30.0	37.5	8
E15	30.0	75.0	7

The relationships between the response (SA_{AMX}) and the independent variables tested were analysed by linear regression and fitted to a second-order polynomial model using Equation 7.1.

$$Y = b_{0j} + \sum_{i=1}^{3} b_{ij} x_i + \sum_{i=1}^{3} \sum_{k=1}^{3} b_{ikj} x_i x_k \qquad \text{Eq. 7.1}$$

Where Y represents the predicted response (SA_{AMX}), b_{0j}, b_{ij}, and b_{ikj} are the regression coefficients calculated from the experimental results by the least-squares method, and x_i and x_k ($k \geq i$) are the independent variables in coded values, with variation

ranges from -1 to 1. The statistical analysis was performed using the analysis of variance (ANOVA), including the F-test value, which established the global model significance, the lack of fit and the determination coefficients (R^2) and the adjusted R^2 ($R^2_{adjusted}$). The significant factor affecting each dependent variable was selected according to the Student t-test establishing a 95 % confidence level. The statistical software IBM SPSS 24 was used to generate the regression analysis and analysis of factor contribution. Excel tool was used to plot the response surface and contour plots.

7.3.4. Analytical methods

Liquid samples from influent and effluent of the SBR were periodically taken to follow the process performance. All samples were filtered using 0.45 µm pore-size filters before analysis. Spectrophotometric methods were applied to determine the ammonium (Bower and Holm-Hansen 1980), nitrite and nitrate (APHA-AWWA-WEF, 2012) concentrations. Dissolved total organic and inorganic carbon concentrations (TOC and IC, respectively) were measured with a Shimadzu analyser (TOC-L-CSN). Total nitrogen (TN) concentration was determined in the same Shimadzu analyser with a TNM-L Unit. The pH was measured using an electrode connected to Crison 506 measurer. Concentration of the total suspended solids (TSS), volatile suspended solids (VSS) and sludge volume index at 30 min (SVI_{30}) were determined according to Standard Methods (APHA-AWWA-WEF, 2012). The average diameter of the granules and size distribution were periodically measured by means of a stereomicroscope (Stemi 2000-C, Zeiss) incorporating a digital camera (Coolsnap, Roper Scientific Photometrics) for image acquisition and then these images were processed using the Image ProPlus® software. Details of all the analytical methods are provided in Chapter 2.

7.3.5. Calculations

Nitrogen loading rate (NLR) (without considering the nitrate supplied in the influent) is calculated according to Equation 7.2 and NRR and nitrogen removal efficiency (NRE) were calculated from the concentration of the nitrogen forms in the SBR influent and effluent according to the section 2.4.4 in Chapter 2. The specific nitrogen loading and removal rates (sNLR and sNRR) were estimated as the NLR and NRR, respectively, divided by the average VSS concentration inside the reactor.

$$NLR = \frac{(NH_4^+ - N + NO_2^- - N)_{inf}}{HRT}$$ Eq. 7.2

7.4. Results and discussion

7.4.1. Anammox process establishment and maintenance

7.4.1.1. Anammox reactor start-up

The stable performance of the anammox process was quickly achieved fed with the synthetic media containing a nitrite to ammonium ratio of approximately 1 g NO_2^--N/NH_4^+-N and with a NLR of 50 mg TN/(L·d) (Figure 7.3.A). As the inoculum came from a one-stage PN/AMX system (Morales et al. 2015a), it was already enriched in anammox bacteria but also AOB. At the beginning of the operation, nitrate was consumed together with the decrease of the biomass concentration from 1.6 to 1.2 g VSS/L, which took place in 20 days. This might be explained by the lysis of the aerobic bacteria (like AOB) happening during the first operational days as no oxygen was available for them. Then, the organic matter coming from the biomass death was used to denitrify the fed nitrate. Once no organic matter was available, heterotrophic denitrifying activity decayed and significant nitrate consumption was no longer observed from day 10 onwards. The VSS concentration in the reactor remained almost constant from day 50 onwards.

As nitrite was fixed as the limiting substrate, even if it was fully depleted, ammonium was always left at concentrations of approximately 5 mg NH_4^+-N/L. In these conditions, the total nitrogen (TN) concentration in the effluent was 11 ± 2 mg N/L (Figure 7.3.B). During this stage (except for the first 10 days), the obtained average values of nitrite to ammonium consumed ratio and nitrate produced to ammonium consumed ratio were 1.26 ± 0.12 g NO_2^--N/g NH_4^+-N and 0.34 ± 0.09 g NO_3^--N/g NH_4^+-N, respectively (Figure 7.4). In particular, the first ratio fits well with the anammox stoichiometry (Lotti et al. 2014a, Strous et al. 1999). The produced nitrate to consumed ammonium ratio (Figure 7.4) is too high indicating the presence of NOB, which might profit from small concentrations of dissolved oxygen entering the non-hermetically closed reactor.

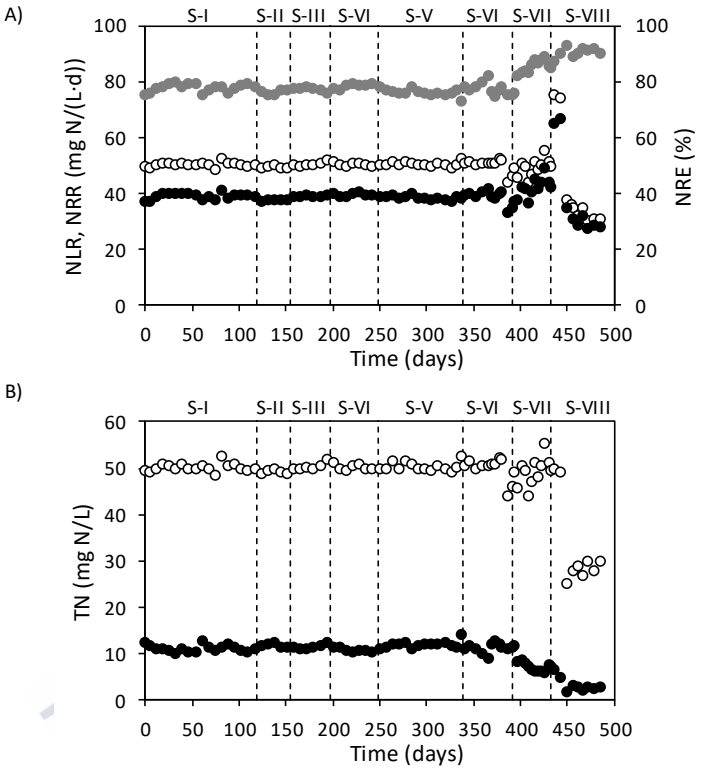

Figure 7.3. Evolution throughout the operational time of A) applied nitrogen loading rate (NLR, ○) and achieved nitrogen removal rate (NRR, ●), in mg TN/(L·d) and nitrogen removal efficiency (NRE, ●) in percentage; B) total nitrogen (TN) concentration in the influent (○) and effluent (●) in mg TN/L.

The NRE ranged from 74 to 79 % leading to a NRR of 38 ± 4 mg TN/(L·d) (Figure 7.3.A). The maximum achievable NRE according to the anammox stoichiometry would be between 89 % (Strous et al. 1999) and 92 % (Lotti et al. 2014a). It is limited by the nitrate produced simultaneously to the dinitrogen gas. In the present study, obtained maximum NRE was limited to 78 % (Strous et al. 1999) and 86 % (Lotti et al. 2014a), due to the ammonium added in excess in the feeding.

During Stages II and III, the nitrate addition to the feeding was first reduced (from 25 to 10 mg N/L) and then stopped (Table 7.2) since the anammox process was properly established and the produced nitrate by the anammox process was enough to maintain the anoxic environment. The average NRE slightly decreased from Stage

I (77.7 ± 1.5 %) to Stage II (76.1 ± 0.8), with a p-value of 0.04, but it recovered in Stage III (77.2 ± 0.7 %). As expected, no significant statistical differences regarding the NRE were detected when Stage III is compared with previous stages (Figure 7.3 and Figure 7.4) with p-values of 0.7 for Stage II and 0.3 in the case of the comparison with Stage III. Thus, it can be stated that the reduction of nitrate fed to the systems has no effect over the system performance.

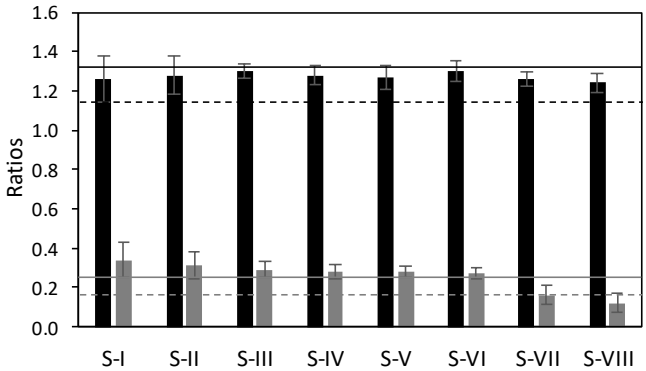

Figure 7.4. Evolution of the nitrite to ammonium consumed ratio (■) and nitrate produced to ammonium consumed ratio (■) in the different operational stages. Horizontal lines represent the stoichiometric values: solid lines according to Strous et al. (1999) and dashed lines according to Lotti et al. (2014a).

Moreover, the applied sNLR was always below the SA_{AMX} proving that the system was never overloaded (Figure 7.5). No remarkable differences on the SA_{AMX} were observed from Stage I to Stage III with values of 53 ± 11 and 65 ± 9 mg N/(g VSS·d) on day 0 and day 183, respectively, with p values for the SA_{AMX} higher than 0.10. Therefore, the NRR was limited by the applied NLR as it was also confirmed by the negligible nitrite concentration in the effluent (< 0.01 mg NO_2^-/L during the whole operational period).

Dosta et al. (2008) operated an anammox reactor at decreasing temperatures (from 30 to 15 °C) fed with a NLR of 270 mg N/(L·d). These authors observed that nitrite accumulated in the reactor at 15 °C causing the total loss of the system efficiency. The complete efficiency of the system was only restored when the NLR was diminished to 50 mg N/(L·d). Similar NLR was applied in the present study (Figure 7.3.A) but the SA_{AMX} indicated that higher NRR might be achieved (Figure 7.5). The

average SA_{AMX} values obtained in the present study at 15 °C doubled the ones obtained by Dosta et al. (2008) of 20 mg/(g VSS·d) at the same operational temperature, but after acclimation, which indicates that in fact it is not needed.

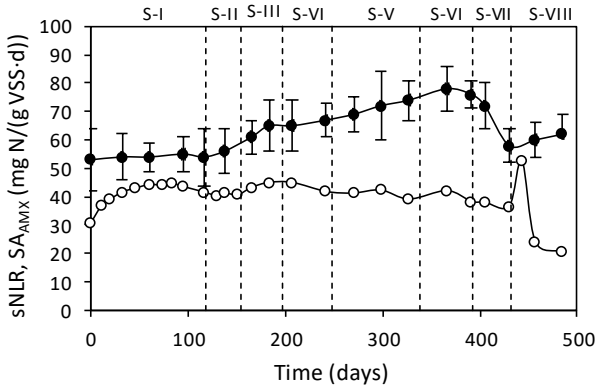

Figure 7.5. Evolution of the applied specific nitrogen loading rate (sNLR, ○) to the reactor and the maximum specific anammox activity (SA_{AMX}, ●) obtained in batch tests at 15 °C.

7.4.1.2. Anammox process performance at decreasing alkalinity concentrations

Most part of the operational time with the synthetic media it contained high alkalinity concentrations (Table 7.2). However, due to the nitritation process, this alkalinity would have been consumed. Indeed, the strategy to achieve successful nitritation that has been proven in Chapters 5 and 6 is based on the alkalinity limitation. For this reason, to evaluate the effect of low alkalinity on the anammox process performance is of interest. Different research works studied the short-term N/IC optimal ratio finding that the anammox activity decreased when the ratio is higher than 10 g NH_4^+-N/g IC (Kimura et al. 2011) or lower than 0.28 g NH_4^+-N/g IC (Liao et al. 2008). Nevertheless, Strous et al. (1999) and Lotti et al. (2014a) reported stoichiometric NH_4^+-N/IC consumption ratios of 17.7 and 16.4 g NH_4^+-N/g IC, respectively, during the anammox process.

At the present study, no significant effect was observed over the NRR ($p = 0.38$) (Figure 7.3) due to the different IC concentrations (Table 7.2) in the feeding, which ranged between 0.19 and 2.50 g NH_4^+-N/g IC. Kimura et al. (2011) reported a decrease on the NRR in an anammox reactor fed with 160 mg NH_4^+-N/L and 200 mg NO_2^--N/L when the IC concentration decreased to 8 mg IC/L (10 mg N/mg IC) and

found an apparent inorganic carbon affinity constant of 1.2 mg IC/L. In the present work, the highest N/IC ratio tested was 2.50 g N/g IC and the IC concentration was higher than 5 mg IC/L, therefore, no limiting effect was expected and found.

The decrease of the influent IC concentrations, in Stages IV to VIII, caused a decrease on the buffering capacity and a slight decrease of the influent pH (from 7.8 ± 0.1 to 7.3 ± 0.2). However, the effect of variable IC concentrations on the pH in the reactor bulk liquid was hard to assess, since the Argon gas bubbling (used to prevent the entry of oxygen) might cause alkalinity depletion (due to CO_2 stripping) increasing the pH value (up to 9), or the entrance of oxygen to the system would contribute to the pH descent (close to 6.5) because of the AOB activity. Nonetheless, the pH in the effluent did not seem to be directly affected by the alkalinity depletion in the feeding as it remained at average values of 7.3 ± 0.3.

The NRE was 82 ± 3% (Figure 7.3.A) and a ratio of the nitrite to ammonium consumed of 1.28 ± 0.05 g NO_2^--N/g NH_4^+-N and a nitrate produced to ammonium consumed ratio of 0.28 ± 0.03 g NO_3^--N/g NH_4^+-N (Figure 7.4) were obtained in Stages IV-VI, close to the stoichiometric values (Lotti et al. 2014a, Strous et al. 1999). As nitrite accumulation was not observed, again the NRR was limited by the applied NLR (Figure 7.3.A) and the NRE was limited by the equimolar ammonium and nitrite concentrations in the feeding causing an excess of ammonium compared with the needed according to the anammox stoichiometry. The TN concentration in the effluent was not affected by the alkalinity depletion and it remained at values of 11 ± 3 mg TN/L (Figure 7.3.B, p=0.81).

Compared with the inoculum, the SA_{AMX} augmented ($p= 3\ 10^{-7}$) up to 78 ± 8 mg N/(g VSS·d) at the end of Stage VI (Figure 7.5). Whether this SA_{AMX} improvement was due to the alkalinity depletion in the feeding or due to the increase of the anammox enrichment level is uncertain. However, it is worth to point out that, contrary to other studies, the SA_{AMX} did not decrease despite the long-term operation of the reactor at low temperature (15 °C) and low nitrogen concentrations (Agrawal et al. 2018, Cao et al. 2017). A comparison between the sNLR and the SA_{AMX} values revealed that, at the end of the Stage VI, the system was able to treat almost double of the applied sNLR (40 mg TN/(L·d)) (Figure 7.5).

7.4.1.3. Dependence of the anammox activity with temperature

The SA_{AMX} is known to be highly affected by temperature (Agrawal et al. 2018), experiencing a decrease when the temperature of operation diminishes. In the present study, this behaviour was proven when the SA_{AMX} of biomass samples collected from the reactor was measured at different temperatures (Figure 7.6). Throughout the operational period, all SA_{AMX} measured at 15 °C resulted in the lowest values but they significantly increased from the start-up to Stage VI, with p-value of $3\ 10^{-7}$, whereas the SA_{AMX} values at 30 °C decreased from 270 ± 11 to 200 ± 10 mg N/(g VSS·d) ($p = 2\ 10^{-15}$). Nevertheless, no significant differences were found for the measured SA_{AMX} values at 20 °C (p = 0.07) and 25 °C (p = 0.17).

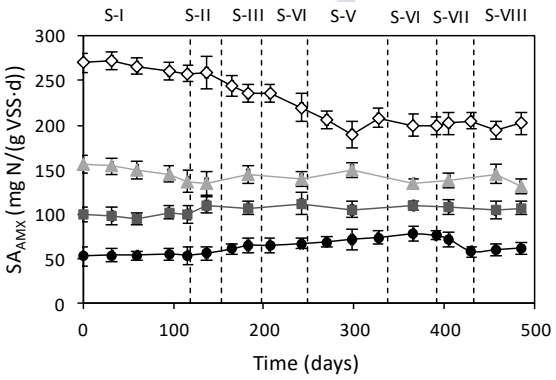

Figure 7.6. Evolution of the maximum specific anammox activities (SA_{AMX}) throughout the reactor operation measured at different temperatures: 15 °C (●), 20 °C (■), 25 °C (▲) and 30 °C (◊).

To better understand the effect of the temperature, as an example, Figure 7.7.A shows the SA_{AMX} values of the inoculum (considered acclimated to 30 °C) obtained at different temperatures and those determined for a biomass sample collected on day 370 from the SBR (considered acclimated to 15 °C). The values of the SA_{AMX} for biomass adapted to 15 °C are higher at low temperature (15 and 20 °C), and their diminishing tendency is smoother than in the case of the inoculum. Furthermore, in the case of the inoculum the SA_{AMX} at the reference temperature (30 °C) is more than 5 times higher than at 15 °C, whereas this ratio decreased to 2.6 for sample on day 370 (Figure 7.7.A). In the present study, the highest SA_{AMX} was always obtained at 30

°C, whilst Hu et al. (2013) reported that the optimal temperature of the anammox biomass after long-term operation (300 days) at 12 °C changed from 35 °C to 25 °C.

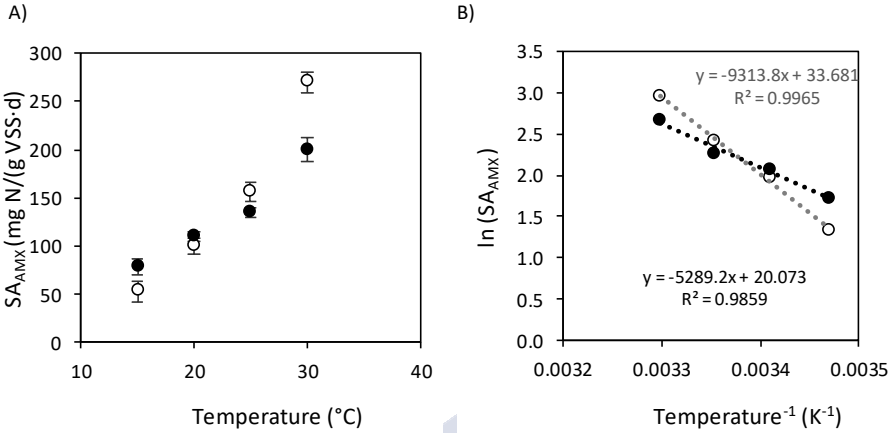

Figure 7.7. A) Maximum specific anammox activity (SA_{AMX}) temperature dependency from samples collected on day 0 (○) and day 370 (●); B) Arrhenius linearization of the SA_{AMX} at different temperatures determined for the samples collected on day 0 (○) and day 370 (●).

The fact that SA_{AMX} values measured at different temperatures fit with the Arrhenius equation was widely reported (Ma et al. 2019, Reino et al. 2018). In this way, the SA_{AMX} expected at low temperature might be predicted from the SA_{AMX} determined at optimal temperatures. However, Lotti et al. (2015) found that models, like the Arrhenius equation, cannot be applied for the complete range of temperature values since the effect of this parameter is not satisfactorily described if the activation energy is considered constant. These authors reported a descent of the activation energy throughout the time of exposition to low temperature. However, in the range of the temperatures tested in the present study (15 - 30 °C) a good correlation was found after applying the Arrhenius model to the data of SA_{AMX} obtaining R^2 values higher than 0.98 (Figure 7.7.B). In this range, apparent activation energy (Ea) of 77 KJ/mol was calculated for the inoculum, close to the 70 KJ/mol reported by Strous et al. (1999). However, its value was of 44 KJ/mol on day 370 which reflected the variations of SA_{AMX} due to temperature changes (Figure 7.8) which justified the obtained SA_{amx} values. This might be correlated with an increase in the enrichment level or due to adaptation to the low temperature applied in the SBR.

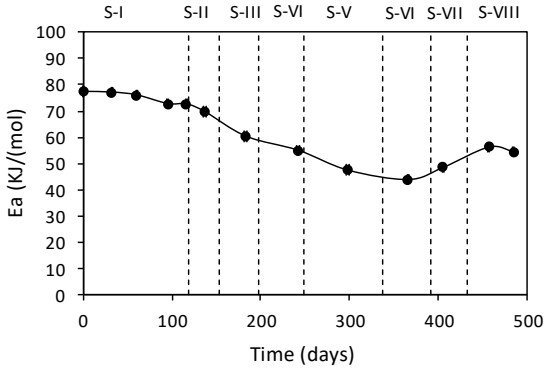

Figure 7.8. Evolution of the estimated activation energy (Ea) of the anammox biomass throughout the reactor operation.

Adaptation of the anammox biomass to operate at low temperature was also found by other authors (Dosta et al. 2008, Lotti et al. 2015) since this is a natural phenomenon among bacteria. Some authors recommend to slowly adapt the sludge to low temperatures (De Cocker et al. 2018, Reino et al. 2018). However, the decrease of the maximum specific anammox activities was similar to that observed in studies where the biomass was step-wise acclimated or directly exposed to mainstream conditions (Morales et al. 2016). Results demonstrated that the progressive anammox adaptation to low temperature might be not required and in this way the start-up periods could be shortened.

After the long-term operation, at the end of Stage VI, the SA_{AMX} measured at 15 °C augmented (Figure 7.5), amounting to 39 % of that one measured at 30 °C to the same sample (Figure 7.6). Similarly, De Cocker et al. (2018) reported that the anammox activity measured at 15 °C was the 22.4 % of the value obtained at 30 °C operating an anammox SBR at temperatures decreasing from 30 to 10 °C in a period of 257 days. They also reported the partial recovery of the anammox activity which reached values up to 92 mg N/(g VSS·d) at 10 °C similar to those of 99 mg N/(g VSS·d) corresponding to 30 °C. This fact might be explained by the progressive enrichment of the biomass inside the reactor leading to an anammox populations abundance increase from 80.5 to 87.2 % and the biomass concentration diminishing from 0.76 to 0.24 g VSS/L.

7.4.2. Anammox process performance treating nitritified municipal wastewater

Finally, in Stages VII-VIII the anammox reactor was fed with a mixture of the effluent from the nitritation reactor, treating municipal wastewater (Chapter 6), with the primary settled wastewater to achieve an influent nitrite to ammonium ratio of 1.2 g NO_2^--N/g NH_4^+-N. During Stage VII, the NRE was slightly increased to average values of 88 ± 5 % due to the presence of low concentrations of organic matter in the feeding (Table 7.1), mainly from the fraction of the fed stream which was not treated in the nitritation reactor. The applied NLR was maintained at average values of 58 ± 9 mg TN/(L·d) (Figure 7.3.A).

It is worth to note that the pH in the influent, and therefore in the effluent, of the reactor decreased to average values of 6.2 ± 0.1. These values were lower than those indicated for the anammox process (Cao et al. 2017). In fact, Tomaszewski et al. (2017) recommend operating the anammox reactors in a range of pH from 7.0 to 7.5 to maintain the process stability. In these conditions, the decrease of the SA_{AMX} of the biomass was measured after switching the feeding to municipal wastewater (Stage VII). It was of 72 ± 8 mg N/(g VSS·d) in the first 10 days of this stage and it decreased to 58 ± 6 mg N/(g VSS·d) after 40 days of operation in these conditions. Both values were obtained at 15 °C (Figure 7.5). Similarly, Laureni et al. (2015) observed a decrease on the anammox activity to 40 mg N/(g TSS·d) after feeding the reactor with municipal wastewater containing up to 20 mg N/L and 47 mg sCOD/L and operated at 12 and 29 °C. These authors attributed this change to the development of other bacterial populations such as the heterotrophic denitrifying bacteria due to the presence of organic matter in the wastewater. This might be the case in the present study; however the existence of an inhibition associated to the low operational pH should be also considered.

The heterotrophic denitrification process contribution to the total nitrogen removal can be estimated by the decrease of the nitrate concentration in the effluent of the anammox reactor with respect to the concentration produced by the anammox process. In the present case, the ratio of nitrate produced to ammonium consumed was of 0.12 - 0.16 g NO_3^--N/g NH_4^+-N) under the stoichiometric one of 0.26 g NO_3^--N/g NH_4^+-N (Strous et al. 1999) for the anammox process but close to the one proposed by Lotti et al. (2014a) of 0.16 g NO_3^--N/g NH_4^+-N (Figure 7.4). Since the major fraction of the anammox feeding corresponded to the effluent of the

nitritation reactor (> 70% in volume), the concentration of the organic matter was low (< 20 mg TOC/L, Table 7.1) and limited the nitrate removal. Therefore, the growth of the heterotrophic denitrifying bacteria is not relevant enough to hinder the anammox bacteria activity. The total nitrogen concentration in the effluent during this stage decreased to 6 mg TN/L (Figure 7.3.B). Thus, it accomplished the EU discharge limits for nitrogen. As a result, the denitrifying process developed due to the presence of low concentrations of organic matter in the feeding media acted as a polishing process for the effluent of the reactor by slightly decreasing the nitrogen concentration. This low contribution of the heterotrophic denitrification corresponded to very low maximum activities which were under the detection limit of the batch experiments even when the test was repeated at 30 °C (using both nitrate and nitrite as substrates). This observation corroborated the fact that the anammox process was the main pathway of nitrogen removal.

Finally, during on day 433, the cycle length was shortened (Figure 7.2) to reduce the HRT and increase the applied load from 58 ± 9 to 75 ± 1 mg TN/(L·d). Again, all the nitrite supplied was depleted indicating that the system could cope with even higher loads. Nevertheless, this NLR was maintained only for 12 days due to the decrease in the municipal wastewater load (as happened in Chapter 6, Stage IV). The average total nitrogen concentrations ranged between 20 and 25 mg TN/L (Figure 7.3) leading to an average applied NLR of 33 ± 3 mg TN/(L·d) (Figure 7.3.A). In this Stage VIII, the NRE was 91 ± 2 %. At this point, it was not possible to further decrease the HRT (16 h) due to the limited availability of the effluent from the nitritation reactor that treated 10 L/d. Low HRT values of 0.28 h were applied by Ma et al. (2013) treating the secondary clarifier effluent amended with nitrite and ammonium achieving a NRR of 2.28 g TN/(L·d) with nitrite and ammonium removal efficiencies of 92 and 78%, respectively. In the present study, higher HRT were applied. However, the objective was to assess the feasibility of the anammox process at mainstream conditions and future research efforts would need to prove the HRT limit meeting the required discharge limits.

Results demonstrated that it was possible to perform the nitritation process, based on the in-situ FNA production strategy, followed by the anammox one to remove nitrogen from municipal wastewater at low temperature, even at the low pH values and alkalinity of operation. The N/IC ratio of the influent to the anammox reactor was between 3.67 and 7.50 g NH_4^+-N/g IC (Table 7.1), enough to perform the

anammox process. The effluent produced from the anammox reactor fulfilled the discharge standards in the EU (Figure 3.B).

The NRR achieved of 40 ± 10 mg TN/(L·d) was much lower than that reported by Reino et al. (2018) and Lotti et al. (2014b) of 1,200 ± 500 mg TN/(L·d) and 510 mg N/(L·d), respectively. These differences might be associated to the low biomass concentration achieved in the present study (1.4 ± 13 g VSS/L) in comparison with their systems (16.8 ± 0.5 g VSS/L and 6.7 g VSS/L, respectively). Reino et al. (2018) reported a decrease of more than 50 % of the SA_{AMX} (at 11 °C) after replacing the synthetic feeding by municipal wastewater. It was approximately of 60 mg N/(g VSS·d), still higher than the one obtained in the present study. This fact might be explained by the substrate limiting conditions applied in the latter study. In addition, the TN concentration of the effluent produced in Lotti et al. (2014b) and Reino et al. (2018) was higher than 10 mg TN/L.

Limited information is available about the long-term performance of anammox reactors treating municipal wastewater and fulfilling the nitrogen discharge limits. As an example, Jin et al. (2019) using a two-stage system managed to produce an effluent containing 5 - 12 mg TN/L, but they controlled the temperature of the anammox reactor at 29 - 30 °C, far from the common values at the mainstream. In the present study, treating primary settled wastewater, TN and TOC concentrations were decreased from 38 ±8 to 5 ± 2 mg TN/L and 40 ± 8 to 4 ± 3 mg TOC/L achieving a high-quality effluent.

7.4.3. Biomass retention

The biomass concentration initially decreased from 1.6 g VSS/L (day 0) to 1.2 g VSS/L (day 32). Later, the biomass concentration remained constant, until Stage VI, indicating that the solids wash-out (5 -10 mg VSS/L in the effluent) was compensated by the anammox growth (Figure 7.9). Once municipal wastewater was used as feeding the concentration increased again to the initial values (Stages VII and VIII. In spite of the occurrence of unstable periods, mainly due to problems with the Argon flow rate that provoked the changes of pH, the system maintained its good biomass retention capacity and flotation events like those reported by Dapena-Mora et al. (2004) were not observed. This might be explained by the almost complete depletion of the nitrite supplied in the feeding (Campos et al. 2017).

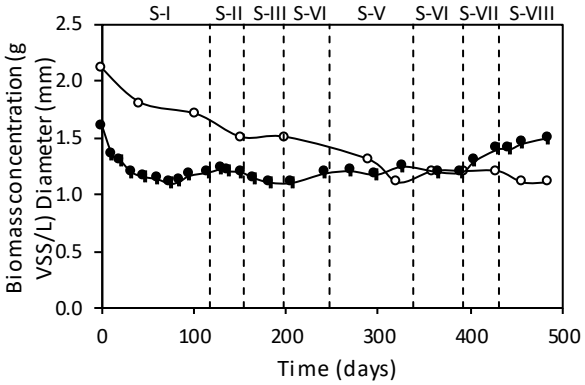

Figure 7.9. Evolution of the biomass concentration inside the reactor (●), in g VSS/L, and average diameter of the granules (○), in mm.

Concerning the biomass physical characteristics, the diameter progressively decreased from 2.1 mm in the inoculum to 1.1 mm at the end of the operation (Figure 7.9). Despite this fact, the sludge volume index decreased to average values of 60 ± 5 mL/ g TSS. The concentration of VSS in the effluent was lower than 10 mg VSS/L during the operational period with the synthetic feeding, showing the capacity of the system to retain biomass. During the last two stages, with municipal wastewater, the biomass concentration in the effluent slightly increased (from 10 mg VSS/L to 20 mg VSS/L), due to the heterotrophic flocculent biomass developed due to the presence of residual organic matter in the feeding.

Concerning the influence of the reactor type on the biomass retention, SBR systems based on granular biomass are widely applied at lab and full-scale. However, in the present study the accumulation of biomass was limited whereas higher biomass concentrations were reached in up-flow anaerobic sludge bed anammox reactors (Lotti et al. 2014a, Reino et al. 2018). For example, Reino et al. (2018) achieved a biomass accumulation from 10 to 17 g VSS/L for 100 days treating nitrite amended pre-treated municipal wastewater containing 70 mg /L. Nevertheless, the different NLR applied might contribute more than the reactor type since in the present study the SBR was fed under substrate limiting conditions which hindered the biomass growth. The low sNLR applied compared with the biomass capacity could be one of the reasons that lead to the deterioration of the granules integrity and to the subsequent increase of the VSS in the effluent, according to (Sánchez

Guillén et al. 2016). The measured average granule size decreased indicating the increase of number of small granules (**Figure 7.9** and Figure 7.10).

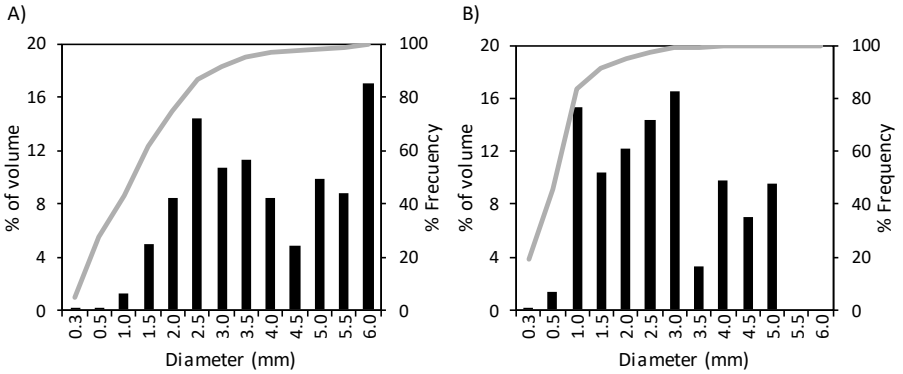

Figure 7.10. Granules size distribution in percentage of volume (bars) and in percentage of frequency (line) for A) Inoculum and B) end of the operation.

Moreover, during the operation with municipal wastewater, a flocculent biomass fraction developed corresponding to between 10 and 15% of the total biomass concentration. Moreover, the ratio of VSS/TSS remained at 0.78 ± 0.06 during the whole reactor operation were not significantly affected using municipal wastewater as feeding. This absence of effect was statistically corroborated as p = 0.3. This fact indicates that inorganic solids did not accumulate inside the reactor.

7.4.4. Effect of pH, COD and temperature over the specific maximum anammox activity

The SA_{AMX} of the biomass from the reactor increased in the period with synthetic media and decreased when municipal wastewater was fed (Figure 7.5). This drop might be attributed either to the pH decrease, that was 6.2 ± 0.1, or to the presence of organic matter responsible for the development of denitrifiers, which could outcompete the anammox bacteria (Cao et al. 2017). Up to now, there is no agreement on the pH value which is optimum for the anammox process operated at low temperature and on the influence of this parameter over the tolerance to COD concentrations of this population.

The SA_{AMX} obtained from the experiments defined according to the Box-Behnken design (Table 7.4) revealed that this parameter was severely affected by

the low pH, being barely detected in the experiments carried out at pH 6 even at high temperature (30 °C) (Figure 7.11), in good agreement with the results found by Tomaszewski et al. (2017).

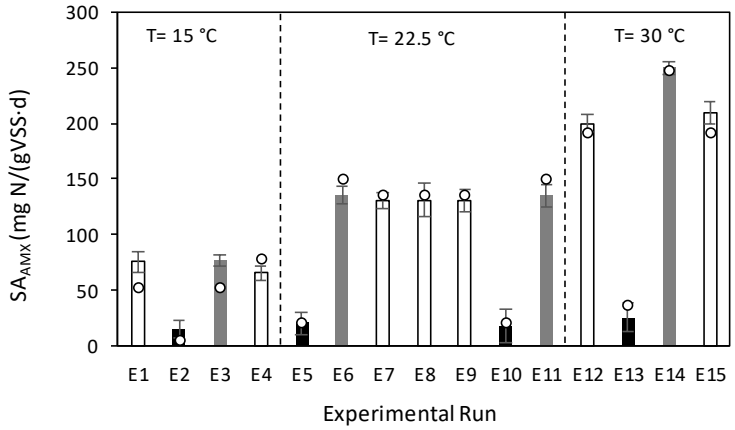

Figure 7.11. Maximum specific anammox activity (SA_{AMX}) results obtained from the Box-Behnken design experiments. Colours indicate different pH values: 6.0 (■), 7.0 (□) and 8.0 (■). Refer to Table 7.4 to see the used total organic carbon (TOC) in each experimental run.

By applying the multiple regression analysis to the experimental data, a second-order polynomial equation was defined, which allow predicting the SA_{AMX} value at a particular temperature, TOC concentration and pH (in the tested interval). Those terms that were not significant at a 95 % of confidence (i.e., significance higher than 0.05) were excluded from the model one by one starting from the lowest significant one (backward method) (Table 7.5). The obtained polynomial equation, using the coded variables, is shown in Equation 7.3.

$$SA_{AMX} = 134.4 + 56.7\ T + 60.8\ pH + 41.0\ T{\cdot}pH - 50.2\ pH^2 \qquad \text{Eq. 7.3}$$

The value of the coefficient of determination (R^2) of 0.975 indicated that the quadratic polynomial model proposed for SA_{AMX} represents accurately the actual relationship between the responses. The ANOVA analysis from the model showed an adjusted determination coefficient (R^2_{adj}) of 0.966 and a high Fisher's F value (equal to 99) suggesting a high degree of correlation between the experimental and predicted values (Figure 7.12). The points cluster close to the diagonal line (45°) indicating a good fit of the model.

Table 7.5. Model estimation by multiple regression and ANOVA analysis.

Model number	Excluded variable	Significance of excluded variable	Fisher's F- value	R^2	$R^2_{adjusted}$	Estimation error
1	none	-	32.743	0.983	0.953	16.06
2	TOC	0.967	44.186	0.983	0.961	14.67
3	TOC_pH	0.948	58.868	0.983	0.967	13.58
4	TOC^2	0.620	75.536	0.983	0.970	12.95
5	T_TOC	0.462	94.774	0.981	0.971	12.65
6	T^2	0.125	99.238	0.975	0.966	13.78

The results of the Box-Behnken were further subjected to Student's test (t) (Table 7.68). Low values of t and P indicate a high significance of the corresponding model term. The organic matter concentration (TOC, x_2) and its interactions with the other variables were excluded from the model as they were not significant ($p < 0.05$). The same occurred in the case of the quadratic interaction for temperature while pH interaction with temperature and its square term were found to be significant (Figure 7.12 and Table 7.6) indicating that SA_{AMX} was very sensitive to these factors.

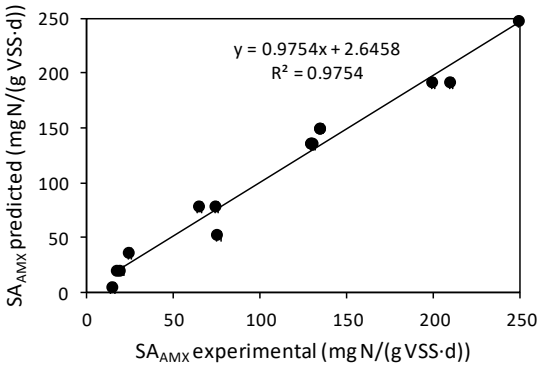

Figure 7.12. Experimental versus predicted values for the maximum specific anammox activity (SA_{AMX}) obtained according to Box-Behnken experimental design.

Daverey et al. (2015) studied the interaction of pH (5.38 - 9.62) and temperature (21.9 - 43.1 °C) over the SA_{AMX}. These authors also found that the pH value was the most influential factor. Although the temperature was not significant in their model its effect cannot be neglected. Indeed, when they validated the obtained model at low temperature, the SA_{AMX} at 15 °C and pH value of 6.5 was zero

whereas at 25 °C was 9 mg N/(g VSS·d). Tomaszewski et al. (2017) stated that despite a statistical correlation between temperature and pH over the SA_{AMX} was not found, the optimal pH range was narrower at low temperatures. Contrary to those findings, the interaction between temperature and pH in the model obtained in the present study was significant (Table 7.6).

Table 7.6. ANOVA results for the quadratic model and estimated regression coefficients for SA_{AMX} obtained in the Box-Behnken experimental design

	Coefficient	**S.D**	**t**
constant	134.429	5.210	25.801
Temperature	56.750	4.874	11.644
TOC	NS	NS	-0.049
pH	64.750	4.874	13.286
Temperature_TOC	NS	NS	0.497
Temperature_pH	41.000	6.892	5.948
$Temperature^2$	NS	NS	0.491
TOC_pH	NS	NS	0.229
TOC^2	NS	NS	-0.199
pH^2	-50.179	7.134	-7.033

NS: non-significant coefficient for a 95 % confidence level; S.D: standard deviation; t: T-Student.

The analysis of the response surface (Figure 7.13) obtained for the dependent variables helps to visualise the relationships between the SA_{AMX} and the experimental levels of tested variables. The surface plots are graphical diagrams of the regression equations showing the effects of two factors, while one factor is maintained at a fixed level. In this case, as organic matter concentration did not significatively affect the SA_{AMX}, only pH and temperature were used in response surface methodology. SA_{AMX} increased with temperature and the maximum SA_{AMX} was measured at 30 °C and pH 8. Thus, pH highly influenced the SA_{AMX} which rises with the increase of the pH value. The contour plot further revealed that the interaction between temperature and pH is also significant.

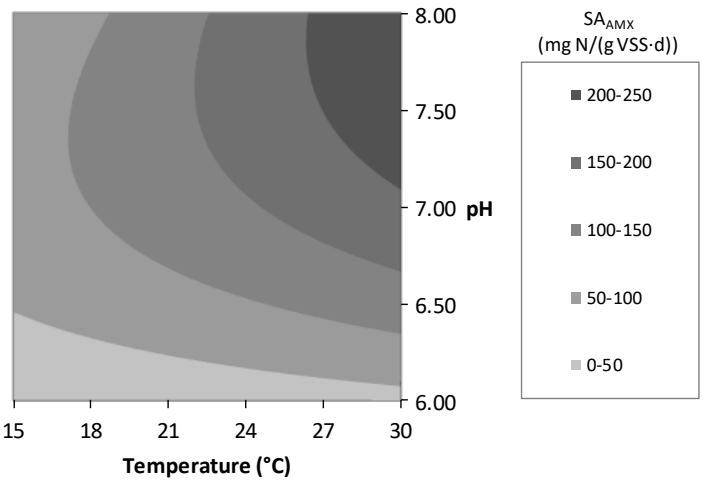

Figure 7.13. Surface and contour plots showing the interactive effects of temperature and pH on the specific anammox activity (SA_{AMX}).

As the SA_{AMX} at pH values of 6.0 was almost negligible but the anammox reactor was able to treat large NRE at pH values of 6.2 ± 0.1, experiments were carried out at these conditions (and at pH 6.5), in triplicate, to validate the adequacy of the model. Data from the SA_{AMX} at different temperatures in the standard buffer solution (pH 7.8) were also used to validate the model. The predicted and the experimental values are compared in Table 7.7. Organic matter was not added as it was found to be irrelevant in the SA_{AMX} values. The experimental values obtained were close to the predicted ones, confirming the validity of the model except for the low pH values for which the error was 26 % and 62 % at 30 °C and 15 °C, respectively. This fact might be explained by the low SA_{AMX} observed that challenge to measure the SA_{AMX} with precision.

Table 7.7. Experimental and predicted values of the experiments used to validate the model.

pH	T (°C)	SA_{AMX} (mg N/(g VSS·d))		Error (%)
		experimental	predicted	
6.0	15	10	3.75	62.5
6.0	30	28	35.25	26.0
6.2	15	30	26.56	11.5
6.2	30	73	74.46	2.4
6.5	15	56	53.26	4.9
6.5	30	130	125.76	3.2
7.8	15	60	64.56	7.6
7.8	20	128	124.26	2.9
7.8	25	190	183.96	3.2
7.8	30	253	243.66	3.7

7.5. Conclusions

Results demonstrate that for the establishment of the anammox process at low temperature and nitrogen concentration an acclimation period might not be needed shortening the start-up periods.

The possibility of stable operation of anammox reactors at relatively low temperatures (15 °C) has been proved treating both synthetic wastewater and nitritified primary settled wastewater reaching a total nitrogen effluent concentration lower than 10 mg TN/L. The presence of low organic matter concentrations contributed to an increase of the nitrogen removal efficiency polishing the effluent. The composition of the produced effluent will meet discharge requirements only after a precise adjustment of the influent composition in terms of nitrite and ammonium to avoid any of them limit the removal of the other.

Although the temperature of operation affects the maximum specific anammox activity once the biomass is adapted to low temperatures its effect becomes less relevant. This means that anammox biomass adapted to operate at low temperature exhibits higher SA_{AMX} than non-acclimated biomass.

Anammox biomass was successfully retained in the system and biomass washout was compensated by the anammox biomass growth. Anammox activity

measured to the biomass fed was higher when the reactor was with synthetic media than with the municipal wastewater. When municipal wastewater was fed to the system, the SA_{AMX} slightly decreased since heterotrophic denitrifying bacteria were developed due to the presence of residual organic matter.

The anammox activity is highly influenced by pH and its effect is also affected by temperature. The higher the temperature and pH, the higher the SA_{AMX} measured in batch tests.

7.6. References

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Chapter 8

Performance of one-stage partial nitritation-anammox processes in an IFAS system

SUMMARY

The partial nitritation-anammox (PN/AMX) processes implementation in the mainstream of wastewater treatment plants require further research to overcome the nitrite oxidising bacteria (NOB) development, the low rates and effluent quality achieved and to improve the anammox biomass retention. With this purpose, the integrated fixed-biofilm activated sludge (IFAS) reactor was tested as a promising configuration to develop the one-stage PN/AMX processes. Efficient nitrogen removal (72 ± 11 %) was achieved, at pilot scale, for anaerobically pre-treated municipal wastewater at low temperature (from 21 to 15 °C), with a nitrogen removal rate of 37 ± 3 mg TN/(L·d) at 15 °C. Microbial population segregation was observed as the ammonium oxidizing bacteria were more abundant in the activated sludge, while anammox bacteria were primarily located in the biofilm. Thus, PN and AMX processes occur primarily in different biomass fractions. NOB activity was similar between both fractions and its specific activity decreased more than that of other populations when the operating temperature was reduced. Furthermore, the IFAS operational strategy (aerobic/anoxic periods) allowed for an efficient NOB activity suppression inside the reactor which amounted to 10 - 20 % of the maximum potential activity. Thus, despite the relatively high COD/N ratio of 2.5 ± 0.3 g COD/g N, the anammox process was the predominant nitrogen removal pathway. In regards to the effluent quality, nitrogen concentrations of 12 ± 5 mg TN/L were obtained showing that further optimisation is required to meet the standard discharge limits in the European Union.

OUTLINE

8.1. Introduction

The main identified bottlenecks in implementing the partial nitritation-anammox (PN/AMX) processes operating at mainstream conditions, are the achievement of the stringent discharge limits, the active suppression of nitrite oxidising bacteria (NOB) growth, the overcoming of the competition with heterotrophic denitrifiers when organic matter is present and the sufficient anammox bacteria retention inside the reactor (Cao et al. 2017, Hoekstra et al. 2018). The feasibility to operate the PN/AMX processes at mainstream conditions, considering most of the previously mentioned limitations, has been researched in laboratory-scale units fed with synthetic or nitrogen supplemented wastewater (Agrawal et al. 2018, Cao et al. 2017).

However, there are very few documented examples explaining the PN/AMX processes performance treating municipal wastewater at low temperature (Agrawal et al. 2018, Hoekstra et al. 2018, Pedrouso et al. 2018), where the presence of residual organic matter (as chemical oxygen demand, COD) concentrations involves COD/N ratios higher than those used in the studies with synthetic media (usually prepared without COD). Consequently, in these cases the heterotrophic denitrifying bacteria might outcompete anammox bacteria.

Furthermore, biofilm-based technologies are used to provide a long solid retention time (SRT) to counteract the slow growth rate of anammox bacteria at low temperature, allowing for the retention of high biomass concentrations inside the system (Agrawal et al. 2018). Moreover, the NOB appearance in single-stage PN/AMX systems was suggested to be associated to the development of flocs or small granules which are also produced in biofilm systems (Morales et al. 2016, Winkler et al. 2012).

Recently, hybrid systems like integrated fixed-biofilm activated sludge (IFAS) reactors, where sludge is a mixture of suspended and biofilm biomass, have been explored to perform the PN/AMX processes simultaneously (Veuillet et al. 2014). The IFAS reactors have been found to be more flexible, efficient and resistant against both concentration and hydraulic shock loads than other activated sludge processes (Regmi et al. 2011). The ability of these systems to decouple the SRT of different populations arose as a promising approach to improve the process stability joining the advantages of both one-stage and two-stage PN/AMX configurations (Han et al.

2016, Veuillet et al. 2014). Due to mass transfer limitations, anammox bacteria preferentially grow in the biofilm while the ammonium oxidising bacteria (AOB) and NOB are mainly developed as suspended biomass (Veuillet et al. 2014). Thus, IFAS reactor provides the opportunity to experience a more versatile operation and potentially obtain a selectively NOB washout, while anammox bacteria remain in the system. This segregated washout is possible by the different SRT corresponding to the suspended and biofilm biomass fractions, respectively (Han et al. 2016, Veuillet et al. 2014). Up to now, only a few studies are available on the application of different operational strategies to treat municipal wastewater in IFAS reactors operated at low temperature (Table 8.1), being the control of the aeration conditions crucial.

Table 8.1. Comparison of PN/AMX reactors taking place in an IFAS system to treat municipal wastewater at mainstream conditions.

Operational conditions					System Performance			
T (°C)	Volume (L)	TN (mg N/L)	sCOD/N (g/g)	HRT (h)	NRE (%)	ANRE (%)	NO_3^- $_{produced}$ to NH_4^+ $_{consumed}$ ratio	**Reference**
25	200	45	1.8	15	70	89	0.25	[1]
17	200	47	1.5	39	44	86	0.49	[2]
15	12	21	2.0	14	63	89	0.27	[3]
25	160	44	1.3	7 - 10	82	90	0.04	[4]
21 - 15	200	43	2.5	19	72	81	0.11	This study

ANRE: ammonium nitrogen removal efficiency; HRT: hydraulic retention time; NRE: nitrogen removal efficiency;.sCOD/N: soluble chemical oxygen demand to nitrogen ratio; T: temperature; TN: total nitrogen.

References: [1] Malovanyy et al. (2015a); [2] Trojanowicz et al. (2016); [3] Laureni et al. (2016); [4] Yang et al. (2017).

Among these studies, Yang et al. (2017) operated a plug-flow IFAS system, at 24 - 26 °C, fed with aerobically pre-treated wastewater at an applied total nitrogen loading rate (NLR) of 125 mg TN/(L·d). These authors manage to generate an effluent containing total nitrogen (TN) concentrations lower than 10 mg TN/L (commonly established discharge limit) by applying a strategy based on the residual ammonium concentration (3 - 5 mg NH_4^+-N/L). In addition, the long-term process stability required the strict control of the airflow rate to maintain the microaerobic conditions (0.15 to 0.36 mg O_2/L) (Yang et al. 2017). For this purpose, intermittent aeration was

also widely used. In this sense, Wang et al. (2018) operated an IFAS reactor where simultaneous PN/AMX and denitrification processes took place. Air was intermittently supplied for 1 min followed by 10 min of non-aerated reaction. In these conditions, these authors achieved a nitrogen removal efficiency (NRE) of 72 % in an IFAS system fed with synthetic wastewater and operated at 25 °C. Malovanyy et al. (2015a) optimised the intermittent aeration strategy (15 min ON/45 min OFF) obtaining a NRE up to 70 % for anaerobically pre-treated wastewater in a continuous IFAS system at 25 °C. Trojanowicz et al. (2016) run a similar system at 17 °C but the obtained NRE was limited to 44 %, mainly due to the nitrate produced by the NOB activity, probably caused by the low operational temperature. Laureni et al. (2016) treated low loaded wastewater at 15 °C achieving an NRE of 63 %. However, they applied microaerobic conditions to suppress the NOB activity, which limited the ammonium oxidation rate and therefore the nitrogen removal rate (NRR) was approximately of 23 mg TN/(L·d). Although several studies are available, some questions still need to be addressed like the improvement of the effluent quality to meet discharge limits or the long-term stability maintenance of the PN/AMX processes to develop the process to a further stage.

8.2. Objectives

The main objective of this study is to evaluate the performance and stability of a one-stage PN/AMX processes taking place in a pilot-scale IFAS system operated at mainstream conditions. As a novelty, the reactor was operated at low temperatures (from 21 to 15 °C) with the added challenge of treating anaerobically pre-treated wastewater with a sCOD/N of 2.5 ± 0.3 g/g while meeting the effluent discharge requirements (< 10 mg TN/L).

Additionally, a detailed study of the microbial population segregation between suspended and biofilm fractions is presented to understand the reasons for the NOB activity limitation inside the reactor.

8.3. Materials and Methods

8.3.1. IFAS pilot plant setup

An IFAS pilot-scale reactor with a working volume of 200 L and filled, 40% of the volume, with AnoxKaldnes K1 biofilm carriers characterised by an effective

surface area of 500 $m^2/m^3_{carrier}$ was used (Figure 7.1). The suspended sludge, leaving the unit, settled in a sedimentation tank (100 L) from which the concentrated sludge was recirculated back to the reactor at a recirculation ratio of 1 (100 % of inflow rate). The IFAS reactor was operated as a continuous stirred-tank reactor with intermittent aeration (alternation between aerobic and anoxic periods).

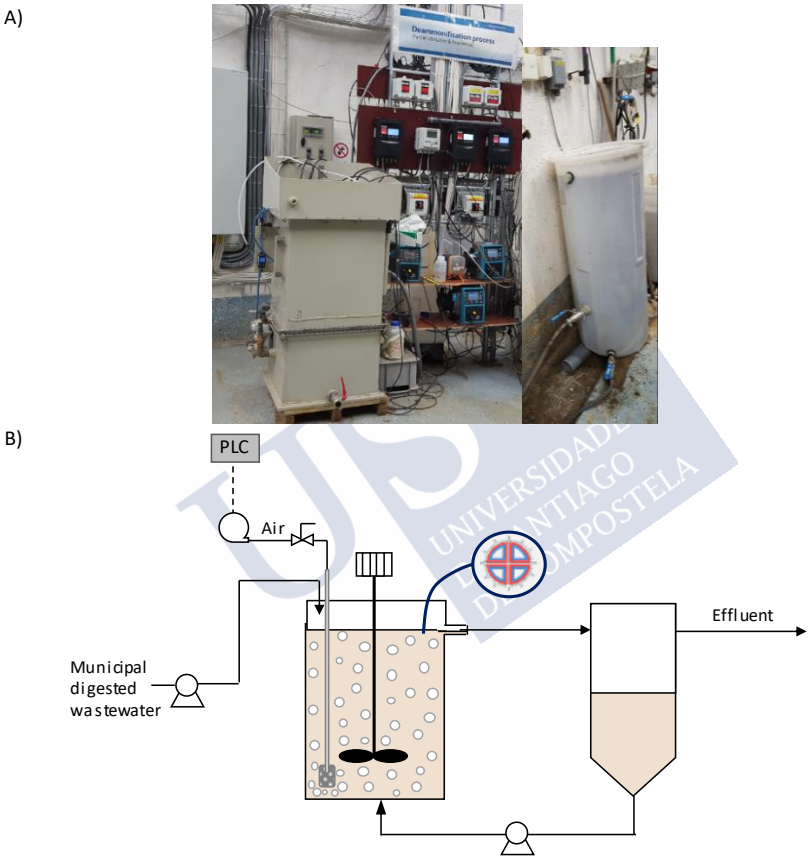

Figure 8.1. A) Image of the reactor and the settling tank and C) scheme of the pilot-scale IFAS reactor.

The dissolved oxygen (DO) concentration inside the reactor, during the aerated periods, was fixed at a set point of 1.5 mg O_2/L, which resulted in low average daily DO concentrations. This set point was regulated by a PID (proportional-integral-derivative) controller (Cerlic AB, Sweden), which adjusted the airflow rate. The pH was not controlled and was 6.95 ± 0.20. The PN/AMX processes performance was

monitored by online measurements of temperature, DO concentration (O2X, Cerlic, Sweden), pH (pHX, Cerlic, Sweden), suspended solids (SS) concentration (ITX, Cerlic, Sweden), oxidation-reduction potential (ORP) (ReX, Cerlic, Sweden), ammonium (AmmonLyt®plus 700 IQ, WTW, Germany) and nitrate (NitraLyt®plus 700 IQ, WTW, Germany) concentrations inside the reactor. Additionally, the reactor was also equipped with conductivity sensors of both influent and mixed liquor. All data from the on-line sensors were recorded and collected in a data acquisition system.

8.3.2. IFAS operational conditions

The IFAS reactor was operated for years, before beginning the present research study. During those periods, it treated low strength nitrogen streams at temperatures of 21 - 25 °C (Malovanyy et al. 2015a, Malovanyy et al. 2015b). In the present study, the reactor was operated for 100 days, treating similar streams but at lower temperature. The operation was divided into three stages as temperature was step-wise decreased from 21 °C (Stage I, days 0 - 33) to 18 °C (Stage II, days 34 - 79) and finally to 15 °C (Stage III, days 80 - 100). The temperature was continuously monitored and regulated by both immerse cooler and heater connected to a thermostat (Julabo AB, Sweden). Moreover, the intermittent aeration pattern was changed from 15 min aerobic/45 min anoxic periods to 20 min ON/40 min OFF from day 38 onwards.

The IFAS reactor was located at the Hammarby Sjöstadsverk research facility, which is managed by the Royal Institute of Technology (KTH) in Stockholm (Sweden). This facility is located directly on top of the Henriksdal WWTP and provided with a direct discharge point for municipal wastewater, which was used as feeding. The municipal wastewater was pre-treated in a primary settler and a subsequent up-flow anaerobic sludge blanket (UASB) reactor (6.3 m^3) operated at 20 °C to remove the organic matter (Malovanyy et al. 2015a, Malovanyy et al. 2015b). Then, a fraction of the UASB effluent was filtrated through a 20 µm pore size filter and stored in an equalisation tank (2 m^3) to reduce the incoming solids concentration and to mitigate the wastewater composition fluctuations. Afterwards, it was fed to the IFAS unit with the following composition: 38 - 48 mg NH_4^+-N/L, 110 - 138 mg sCOD/L, 235 – 283 mg $CaCO_3$/L, 5 – 22 mg VSS/L and average pH values of 7.5 ± 0.1.

The hydraulic retention time (HRT) in the IFAS reactor was maintained constant at 19 h, and therefore 260 L of water were treated daily resulting in an average NLR of 56 ± 3 mg TN/(L·d), varying only due to the fluctuations of the inlet nitrogen concentration. The SRT of the system was not controlled as biomass was only removed during sampling collection. However, the sludge age could be adjusted by periodically wasting the sludge.

8.3.3. Analytical methods

To monitor the pilot plant performance and to check and calibrate the online sensors, both influent and effluent were periodically sampled. Prior to the analysis, samples were filtered using 0.45 µm pore size filters. Then, the concentrations of chemical oxygen demand (COD), total nitrogen (TN), ammonium, nitrite, nitrate and alkalinity were measured spectrophotometrically with Dr Lange test kits (Hach Lange, Germany) in a Photolab® 6600 UV-Vis. Potassium and chloride ion concentrations were also spectrophotometrically determined (with WTW Spectroquant in a Dr Lange ION 500) to calibrate the ammonium and nitrate online sensors, respectively. Concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the Standards Methods (APHA-AWWA-WEF, 2012) in the influent, effluent and the mixed liquor from the bioreactor. The biomass concentration, TSS and VSS, corresponding to the biofilm was determined by the mechanical detachment of the biomass (Figure 8.2) from a randomly selected known number of carriers. A detailed description of all the analytical methods is provided in Chapter 2.

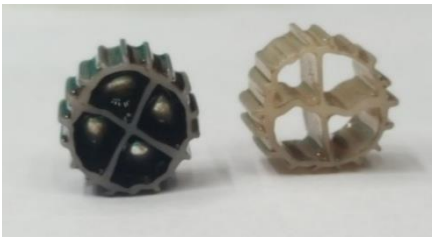

Figure 8.2. Image of the used AnoxKaldnes K1 biofilm carriers colonised with biomass (left) and without biomass (right).

8.3.4. Microbial activity batch tests

To follow up the microbial processes performance inside the reactor, activity tests were carried out on both biofilm and suspended biomass samples taken out from the IFAS reactor. The specific anammox activity (SA_{AMX}) was determined according to the methodology described by Dapena-Mora et al. (2007) using an online pressure transducer (GHM 5155 Greisinger, Germany). A modification of the SA_{AMX} method was also used to determine the activity of heterotrophic denitrifying (SA_{HDN}) bacteria by adding nitrate (50 mg N/L) and acetate (100 mg COD/L) as substrate. Activities of aerobic heterotrophs (SA_{aerHET}), AOB (SA_{AOB}) and NOB (SA_{NOB}) were determined by an oxygen uptake rate (OUR) based method according to Surmacz-Gorska et al. (1996). All these batch assays were performed at the corresponding IFAS reactor operational temperature at the moment of the biomass collection. Moreover, SA_{AMX} was also determined at 30 °C, considered as reference temperature for anammox bacteria activity measurement. Detailed protocols are provided in Chapter 2.

8.3.5. Identification of microbial populations

The main active bacterial populations present in the suspended sludge and biofilm biomass were identified in samples collected from the IFAS reactor on day 75 (Stage II). For this purpose, the fluorescence *in situ* hybridisation (FISH) molecular technique was applied following the protocol described by Amann et al. (1990). Biomass was manually detached from the surface of K1 carriers to avoid bacterial deactivation. The specific oligonucleotide probes used (Table 8.2) were 5'-labelled with the fluorochromes FITC (fluorescei-5-isocyanate) or Cy3 (Carbocyanine 3). Details of the oligonucleotide probes are available at probeBase (Greuter et al. 2016). DAPI (4,6-diamindino-2-phenylindole) was used as universal dye for all DNA. Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Scientific Photometrics) coupled to an Axioskop 2 Plus epifluorescence microscope (Zeiss, Germany). The relative abundances of AOB, NOB and anammox bacteria were estimated by semi-quantitative counting of the ratio between their specific microbial populations biovolume and the total bacterial biovolume using the DAIME software (Daims et al. 2006).

Table 8.2. List of 16S rRNA-targeted oligonucleotide probes used for biomass FISH analysis.

Probe name	Target organism
EUB338 I	Most Bacteria
EUB338 II	Planctomycetales
EUB338 III	Verrucomicrobiales
Nso190	Betaproteobacterial ammonia-oxidizing bacteria
Nso1225	Betaproteobacterial ammonia-oxidizing bacteria
NIT3*	Nitrobacter spp.
Ntspa712*	Most members of the phylum Nitrospirae
NTG840	Nitrotoga arctica
Amx368	All anammox bacteria
Amx820	Candidatus Brocadia anammoxidans and Candidatus. Kuenenia stuttgartiens
BAN162	Candidatus Brocadia anammoxidans

* For these probes, the specific competitor was applied together with the probe.

8.3.6. Calculations

Statistical differences between the results obtained in the three operational stages at different temperatures were tested by one-factor analysis of variance (ANOVA) using the statistical software R version 3.5.2 (The R Foundation Statistical Computing). Mass balances were performed using the Equations described in Chapter 2 section 2.4.6. Full description of the calculations is provided in Chapter 2.

8.4. Results

8.4.1. Nitrogen removal in the IFAS reactor at decreasing temperatures

The operational strategy imposed to the IFAS pilot-plant, continuous feeding and intermittent aeration cycles, was defined to promote the performance of anoxic processes and exploit the lag phase of NOB bacteria activity after oxygen starvation. The NOB suppression was crucial for the correct performance of both processes at the low temperatures to be tested of 21, 18 and 15 °C.

Initially (Stage I), the daily average DO concentration was of 0.45 mg O_2/L (Table 8.3) and the PN/AMX processes performed stable from day 18 onwards. The concentration of TN in the effluent at the end of this stage was lower than 10 mg TN/L corresponding to 4.0 ± 1.5 mg NH_4^+-N/L, 3.0 ± 2.2 mg NO_3^--N/L and less than 0.2 mg NO_2^--N/L (Figure 8.3.A). Ammonium nitrogen removal efficiency (ANRE) and

NRE reached values of 80.0 ± 13.4 % and 72.5 ± 12.7 %, respectively (Figure 8.3.B and Table 8.3). The observed nitrate production to ammonium consumption ratio was only 0.094 ± 0.057 g NO_3^--N/g NH_4^+-N, which indicated a negligible NOB activity inside the reactor (Table 8.3). Furthermore, the NRE increased reaching average values of 85 % in the last 10 days of this period.

Table 8.3. Average values for the different parameters monitored in the IFAS reactor during the three operational stages performed at decreasing temperatures.

	S-I (21 °C)	**S-II (18 °C)**	**S-III (15 °C)**
Phase length (days)	0 - 33	34 - 79	80 -100
Daily DO (mg O_2/L)	0.45 ± 0.05	0.58 ± 0.19	0.64 ± 0.07
pH	7.05 ± 0.04	6.83 ± 0.13	7.09 ± 0.23
NRR (mg TN/(L·d))	40.04 ± 5.05	42.47 ± 7.80	37.35 ± 3.32
NRE (%)	72.5 ± 12.7	74.1 ± 11.4	65.8 ± 4.9
ANRE (%)	80.0 ± 13.4	84.4 ± 11.4	77.5 ± 10.5
$(NO_3^-)_{produced}/(NH_4^+)_{consumed}$	0.094 ± 0.057	0.113 ± 0.091	0.125 ± 0.091
$NO_2^-N_{eff}$ (mg N/L)	0.10 ± 0.07	0.29 ± 0.13	0.54 ± 0.23
TN_{eff} (mg N/L)	10.9 ± 5.6	10.8 ± 5.3	15.0 ± 2.3
COD_{eff} (mg/L)	32.8 ± 0.7	44.5 ± 3.4	46.0 ± 4.9
VSS_r (g/L)*	1.03 ± 0.12	1.05 ± 0.15	1.03 ± 0.09

ANRE: ammonium nitrogen removal efficiency; COD: chemical oxygen demand; NRE: nitrogen removal efficiency; NRR: nitrogen removal rate; TN: total nitrogen; VSS: volatile suspended solids. Sub-indexes "eff" and "r" refer to concentrations measured in the effluent and inside the reactor, respectively.

* Biomass concentration corresponding only to the suspended fraction.

On day 34 the temperature was decreased to 18 °C (Stage II) resulting in an immediate decrease of the NRE with the consequent increase of ammonium concentration up to 13 mg NH_4^+-N/L, whereas nitrate concentration remained similar to the values measured at the end of Stage I (< 1 mg NO_3^--N/L) (Figure 8.3.A). The relatively short oxygen supply periods and the low DO concentration applied (1.5 mg O_2/L), resulted in daily average DO concentrations of 0.49 mg O_2/L, which might be limiting the ammonium oxidation. Thus, on day 39 the intermittent aeration length was increased from 15 min to 20 min and the anoxic phase reduced from 45 to 40 min, to diminish AOB activity limitations. Then, the average daily DO

concentration increased to 0.58 mg O_2/L (Table 8.3). Consequently, the ANRE increased and the NRE stabilised at average values of 74 %. In addition, the differences between the NRE and ANRE values corresponding to Stages I (21 °C) and II (18 °C) were statistically insignificant according to the results from ANOVA ($p = 0.925$) and Kruskal-Wallis ($p = 0.485$) analysis performed at a level of confidence of 95 % ($p < 0.05$ for significance). Thus, no effect can be attributed to the temperature reduction, from 21 to 18 °C, over the performance of the PN/AMX processes

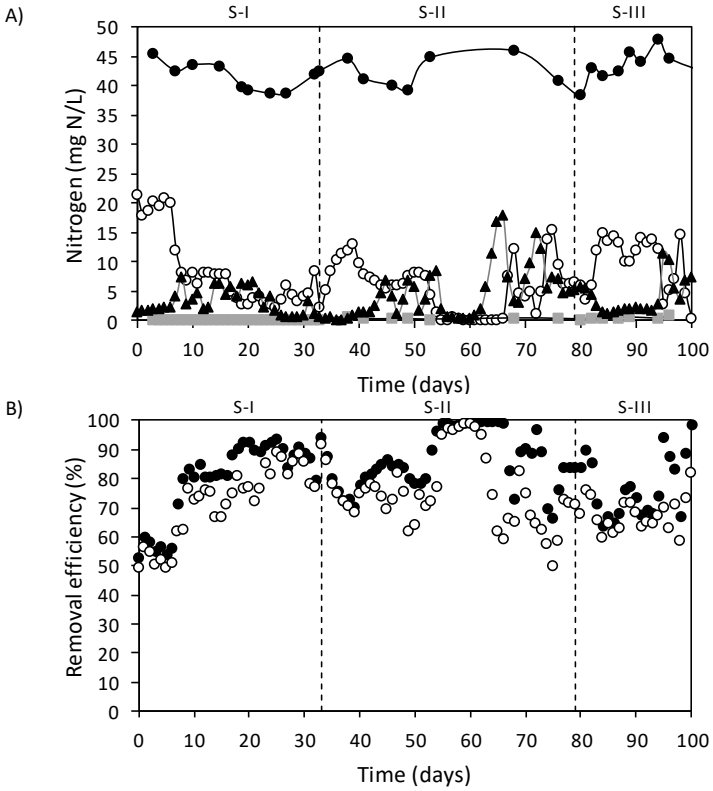

Figure 8.3. IFAS reactor performance: A) Evolution of the concentrations of ammonium in the influent (●) and ammonium (○), nitrite (■) and nitrate (▲) in the effluent, in mg N/L. B) Evolution of ammonium removal efficiency (ANRE, ●) and nitrogen removal efficiency (NRE, ○) in %.

Finally, the temperature was further diminished to 15 °C (Stage III). NRR of 43 mg TN/(L·d) was reached, with the maximum NRE of 75.6 % and average values of 65.8 ± 4.9 % (Figure 8.3.B and Table 8.3). At 15 °C, both ANRE and NRE decreased

compared to previous Stages (Kruskal-Wallis p=0.044 and p = 0.0006, compared to Stages I and II respectively). NRE obtained in this last Stage III were significantly different from those measured in the previous ones with 99.92 % of confidence. Whereas the post-hoc Wilcoxon test pointed out that the ANRE from Stages II and III were significantly different (p = 0.040), while no significant differences were found when Stage I and III were compared (p = 0.074).

The nitrate production to ammonium consumption ratio slightly increased from 0.094 g NO_3^--N/g NH_4^+-N in Stage I to 0.125 g NO_3^--N/g NH_4^+-N in Stage III (Kruskal-Wallis, p = 0.036). Furthermore, significant differences in this ratio corresponding to the operation at 18 °C and 15 °C were found (Wilcoxon, p = 0.027). Nitrate concentrations in the effluent fluctuated, reaching sporadic nitrate production to ammonium consumption ratios as high as 0.35 g NO_3^--N/g NH_4^+-N. These results indicated the existence of NOB activity in the biomass from the IFAS reactor, as well as the fact that its activity was suppressed inside the reactor due to the imposed operational strategy, which leads to minimum values of 0.05 g NO_3^--N/g NH_4^+-N.

During the whole operational period, effluent nitrite concentration was almost negligible with a maximum value of 0.89 mg NO_2^--N/L (Figure 8.3.A and Table 8.3), whereas ammonium was still present, indicating that the nitritation process was limited. Although the nitrite accumulation was scarce, a particular upward trend of the nitrite concentration was observed with the temperature decrease. Thus, at low temperature, AOB activity was presumably favoured over the nitrite consuming bacteria activities (either anammox bacteria or NOB). Although the nitrate production to ammonium consumption ratio was, in Stages I and II, close to the stoichiometric value of 0.11 g NO_3^-N/g NH_4^+-N, according to the PN/AMX reactions, in Stage III, at 15 °C, it was frequently higher, diminishing the NRE (Table 8.3).

Besides nitrogen, an average COD removal rate of 82 ± 22 mg sCOD/(L·d) was achieved via both aerobic and anoxic routes. Thus, the COD concentration in the effluent remained on average as 43 ± 6 mg sCOD/L, for the whole operational period. This value is lower than the discharge limit in the European Union of 125 mg COD/L. The influent sCOD/N ratio in the influent to the IFAS reactor was relatively high (2.5 ± 0.3 g sCOD/N) and close to the limit values recommended to guarantee the correct performance of the autotrophic processes (Agrawal et al. 2018, Cao et al. 2017), and avoid the development of the heterotrophic denitrifying bacteria which might

outcompete the anammox bacteria. However, the ratio corresponding to the COD and N removed obtained was of 2.1 ± 0.5 g sCOD/g N. considerably lower than that generally needed for heterotrophic denitrification of 5 g sCOD/N (Burton et al. 2014). Consequently, the PN/AMX processes were the main route for the obtained nitrogen removal. This is another advantage of the hybrid reactors that usually tolerate higher COD concentration than other one-stage PN/AMX systems. In spite of this, the further optimisation of the operation of the UASB reactor, with current limited COD removal efficiency of 50%, would help to improve the PN/AMX system performance.

8.4.2. Intermittent aerobic/anoxic cycle characterisation

Although the reactor was fed in continuous mode, the alternating imposed aerobic/anoxic conditions produced the evolution of the different operational parameters monitored throughout each operational cycle. To evaluate the performance of the IFAS reactor in the three periods operated at 21, 18 and 15 °C, three consecutive operational cycles were monitored on days 20, 74 and 94, respectively (Figure 8.4). Ammonium and nitrate concentrations varied depending on the DO concentration inside the reactor. The biofilm biomass present inside the reactor was expected to be active in both, aerobic and anoxic, periods since the gradient of DO concentration created anoxic environments in the inner zones of the biofilm. The maximum penetration depth of the DO depended on the DO concentration in the media and of course, was zero during the anoxic periods. In these conditions, ammonium was consumed in the aerobic (via AOB and anammox activity) and anoxic (via anammox activity) periods. However, due to the continuous feeding, the ammonium concentration decreased during the aeration periods and increased in the anoxic ones. Ammonium consumption by AOB and anammox bacteria was counterbalanced by the ammonium continuously fed to the system softening the concentration variations. Nitrate concentration increased in the aerobic periods and decreased in the anoxic ones. This concentration decrease was due to both the presence of heterotrophic denitrification activity and a dilution effect caused by the continuous feeding (as influent did not contain nitrate), which counterbalanced its production by the anammox process. The pH values also varied with the aeration pattern decreasing during the aerated phases due to the occurrence of the nitritation process and increasing in the anoxic phases due to the denitrification activity and the effect of new influent coming to the system.

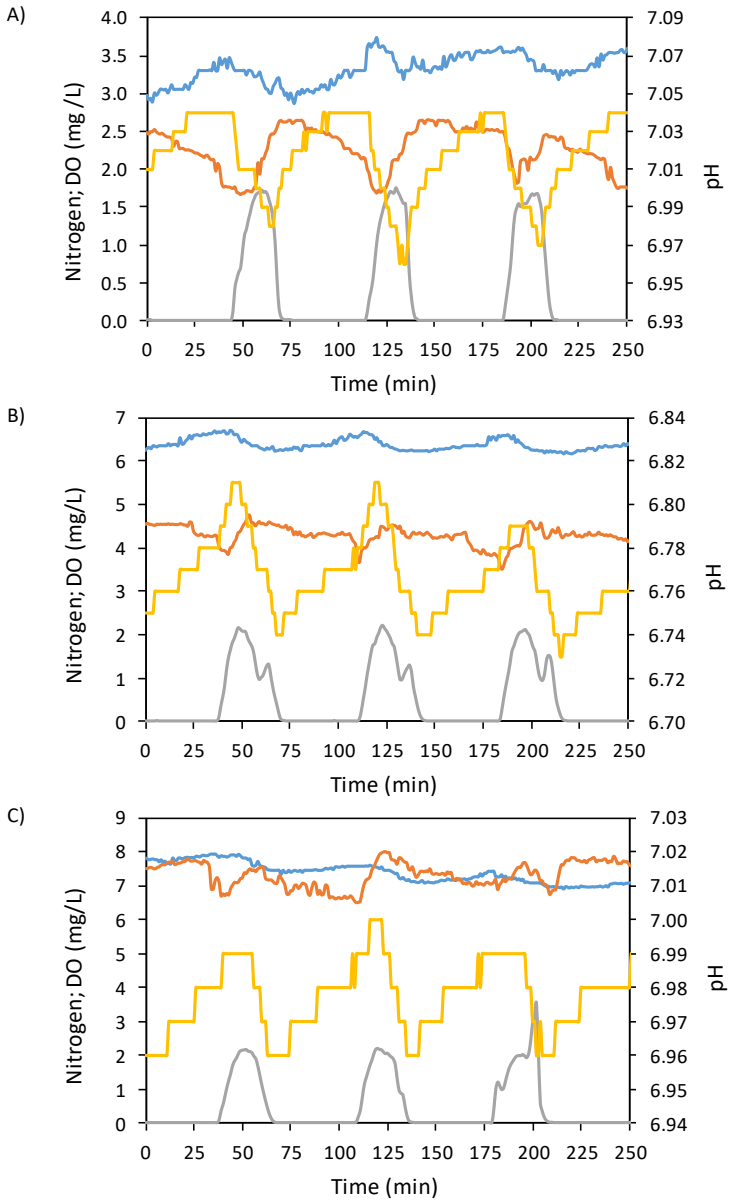

Figure 8.4. Evolution of pH (——), and concentrations of DO (——), ammonium (——) and nitrate (——) monitored in 3 representative aeration/anoxic cycles with 15 min ON and 45 min OFF in A) Stage I (Day 20); and with 20 min ON and 40 min OFF in B) Stage II (Day 74) and C) Stage III (Day 94).

The concentration profiles obtained during the measured cycles in the three stages (Figure 8.4) together with the known amount of nitrogen fed, allowed estimating the consumption and production rates for ammonium and nitrate in the aerated and anoxic phases (Table 8.4).

Table 8.4. Maximum observed conversion rates during the operational cycles performed at different temperatures.

	21 °C	**18 °C**	**15 °C**
	day 20	**day 74**	**day 94**
NH_4^+-N decrease in aerated phase (mg N/L·d))	59	60	70
NH_4^+-N decrease in anoxic phase (mg N/(L·d))	33	32	44
NO_3^--N increase in aerated phase (mg N/(L·d))	26	25	30
NO_3^--N decrease in anoxic phase (mg N/(L·d))	13	15	8
NLR (mg TN/(L·d))	51	53	58
NRR (mg TN/(L·d))	44	39	40

NLR: nitrogen loading rate; NRR: nitrogen removal rate.

Despite the higher DO solubility at 18 °C compared to that at 21 °C, the nitrate production rate in the aerated phase (associated to the NOB activity) remained unaltered and the temperature did not negatively affect the rest of the observed nitrogen conversion rates (Table 8.4). The achieved NOB activity repression correlated with the appearance of a lag phase in the nitrate production after the increase of the DO concentration (Figure 8.4). Moreover, it is worth to note that the aerated phase lasted only the 25 % (Stage I) and 33 % (Stage II and III) of the total intermittent aeration cycle of 1 hour (Table 8.3). Therefore, the actual transformation in the aerobic phase accounted for less than the one in the anoxic phase. For example in Stage I, the nitrate concentration increase during the aerobic phase would be 6.6 instead of 26 mg TN/(L·d) in the anoxic one, while the ratio of ammonium transformation in the anoxic compared with the aerated phase would be 1.7 g $NH_4^+{}_{anoxic}$/g $NH_4^+{}_{aerobic}$ instead the 0.6 g $NH_4^+{}_{anoxic}$/g $NH_4^+{}_{aerobic}$ considering the ratio between rates. These results are in good agreement with the scarce nitrite accumulation observed during the reactor operation (Figure 8.3).

At the lowest temperature tested of 15 °C (Table 8.4), ammonium consumption and nitrate production rates significantly increased, probably due to the higher DO availability. At 15 °C, the oxygen solubility increased and complicated the control of

the DO concentration leading to DO concentrations higher than 1.5 mg O_2/L, the fixed set point, for more than 10 min. Even son, it reached values up to 3 mg O_2/L. Then it remained close to zero for only 30 of the 40 min of the anoxic phase length, resulting in average daily concentrations of 0.64 ± 0.07 mg O_2/L, higher than in previous stages (Table 8.3). In these conditions, the control of the DO concentration at the desired set point became difficult. Together with the increase of aerobic ammonium oxidation activity, when temperature decreased from 21 to 15 °C, the ammonium removal in the anoxic phase also increased by 25 % (Table 8.4). This increase in the ammonium removal was associated with the more substantial amount of nitrite available for the anammox bacteria. Furthermore, at the beginning of the anoxic phase, some aerobic transformation might occur since DO concentration decrease more slowly until reaching the required anoxic conditions. Considering that the ammonium conversion increased at decreasing temperatures, it can be stated that the nitrate production was the limiting step to improve the NRE in the system.

8.4.3. Maximum specific microbial activities

In the IFAS reactor, as it is a hybrid system, suspended and biofilm biomass coexisted. Both suspended solids concentration (approximately 1 g VSS/L, Table 8.3) and biofilm biomass concentrations (18 g VSS/m^2) remained practically constant during the reactor operation. Furthermore, the major part of the biomass (78 % of total biomass measured as VSS) grew attached to the carrier surface.

The contribution of biofilm and suspended biomass fractions to the different specific microbial activities was evaluated by batch activity tests. As expected, as the temperature decreased, the specific activities of the different microbial groups decreased (Figure 8.5), therefore the potential nitrogen removal capacity in the reactor diminished. Furthermore, to better understand the importance of the different bacterial population distribution and competition, several ratios among the measured specific activities were calculated (Figure 8.6).

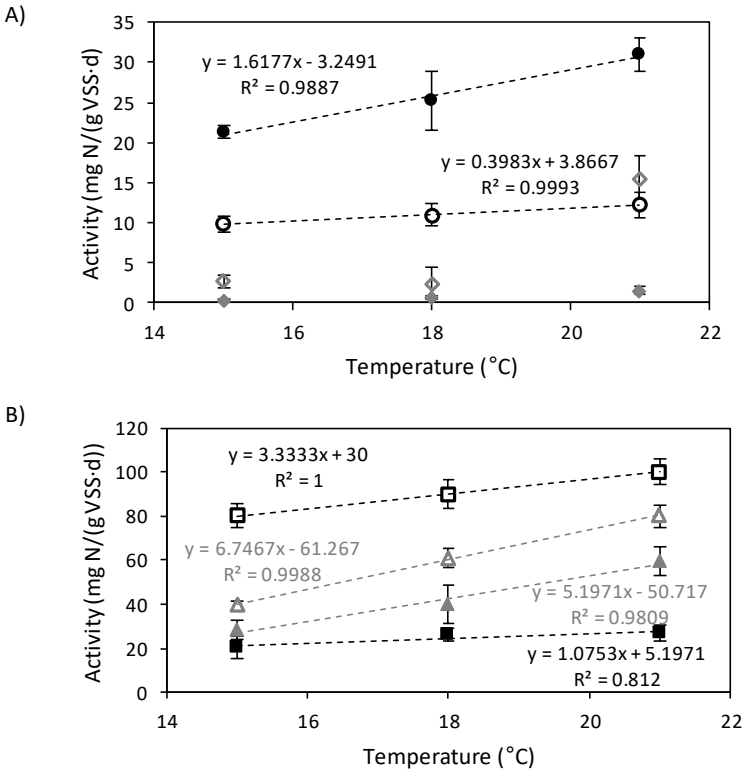

Figure 8.5. Evolution of the specific activities (SA) of the biomass from the IFAS reactor operated at decreasing temperatures. A) Specific anammox activity of the biofilm (●) and suspended biomass (○), specific denitrifying activity of the biofilm (◆) and suspended biomass (◊). B) Nitrifying activities: AOB activity of the biofilm (■) and suspended biomass (□), NOB activity of the biofilm (▲) and suspended biomass (△). All the activities are expressed as mg N/(g VSS·d) and are average values of experiments in triplicate.

8.4.3.1. Anammox activity

From the results obtained from the anammox activity tests, it can be stated that the biofilm biomass was mostly enriched in anammox bacteria. The anammox $SA_{biofilm}/SA_{floc}$ ratio was considerably higher than those corresponding to the other bacterial populations (Figure 8.6.A). The maximum SA_{AMX} in the biofilm was at least twice as high as the one measured in the suspended sludge for the three tested temperatures (Figure 8.6.A). This difference slightly diminished with the decrease of

temperature, indicating that the suspended biomass was less affected by this change as the slope of the regression line fitting the measured SA was four times lesser than that corresponding to the biofilm biomass (Figure 8.5.A). Although the SA_{amx} in the biofilm fraction was different depending on the temperature, the value measured in the biofilm at 30 °C, considered as the reference temperature, was of 83 ± 3 mg N_2-N/(g VSS·d) in Stage I, and of 61 ± 8 mg N_2-N/(g VSS·d) in Stages II and III. This almost constant reference activity in the three stages indicates that the differences in the SA_{AMX} values should be attributed to the temperature changes inside the reactor.

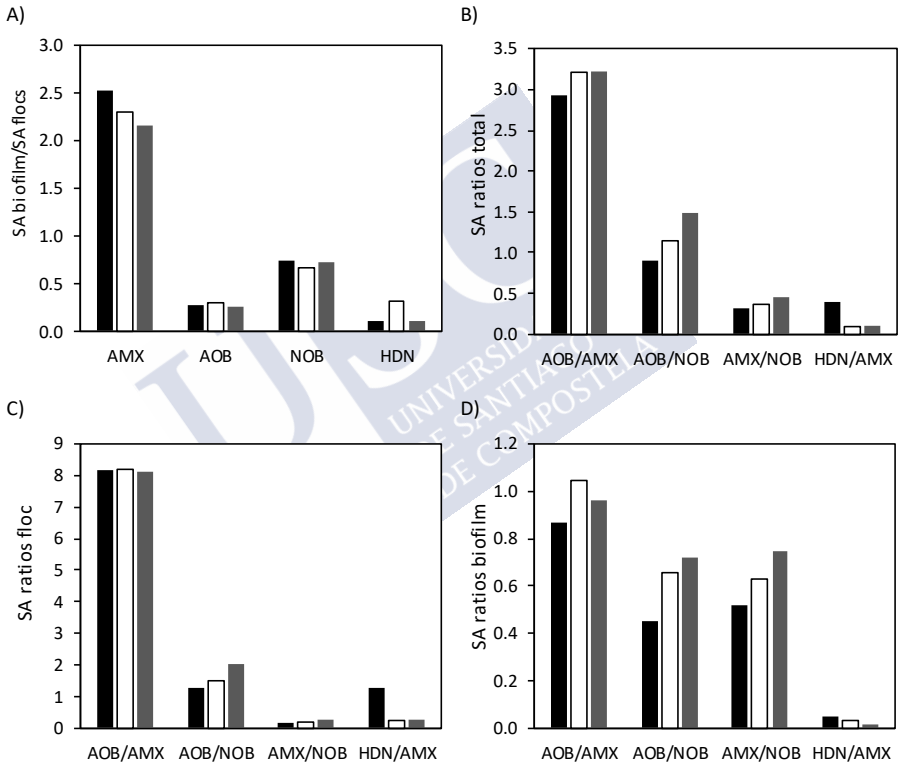

Figure 8.6. Evolution of the specific activities (SA) of the main bacterial populations present in the biomass from the IFAs reactor operated at decreasing temperatures: 21 °C (■), 18 °C (□) and 15 °C (■): anammox bacteria (AMX), ammonium oxidising bacteria (AOB), nitrite oxidising bacteria (NOB) and heterotrophic denitrifying bacteria (HDN). A) Ratio between the SA of the biofilm and the flocculent sludge (floc), B) SA ratios considering both fractions, C) SA ratios of the flocculent sludge and D) SA ratios of the biofilm.

8.4.3.2. Heterotrophic activities

Although the aerobic heterotrophic activity is not depicted in Figure 8.5, the obtained results showed that it was present throughout the operational period at average values of 100 ± 8 mg O_2/(g VSS·d) and 20 ± 3 mg O_2/(g VSS·d) for the suspended and biofilm biomass, respectively. Thus, the SA_{HDN} occurred preferentially in the suspended biomass fraction (Figure 8.6.A). $SA_{biofilm}/SA_{floc}$ maximum values were of 0.3 at 18 °C. However, the SA_{HDN} was less than 10 % of the SA_{AMX} (Figure 8.6.B), except for the period at 21 °C (Figure 8.5.A). Thus, it can be stated that nitrogen removal in the IFAS reactor was mostly attributable to the anammox bacteria activity.

8.4.3.3. Nitrifying activities

Both the SA_{AOB} and SA_{NOB} in suspended biomass were higher than in the biofilm with ratios of $SA_{biofilm}/SA_{floc}$ below 1 (Figure 8.6.A). In fact, in the suspended sludge the SA_{AOB} activity was higher than the SA_{NOB} (Figure 8.6.C) whereas in the biofilm the behaviour was quite the opposite (Figure 8.6.D). In addition, AOB activities in suspended sludge and biofilm biomass were more similar than NOB ones. In both biomass fractions the SA_{NOB} activity was the most affected one by the temperature decrease as observed by the increase of the SA_{AOB}/SA_{NOB} ratio (Figure 8.6.B). The average rate of descent of the SA_{NOB} was estimated as 6 mg N/(g VSS·d·°C), which doubled that of the AOB value, constituting a potential advantage to maintain the process stability (Figure 8.5.B). This observation contradicts the results from other studies reporting that NOB suppression is even more challenging at low temperature. The NOB are supposed to be less temperature-sensitive than AOB and anammox (due to their lower activation energy) (Trojanowicz et al. 2016). However, in the present study, the decrease in the SA_{NOB} cannot be attributed only to the temperature descent, since the biomass used for the assays was collected at different operational days and the microbial population distribution might evolve due to the different imposed SRT and lower biomass growth rates due to the diminishing of temperature.

For the anammox activity, the relatively high SA_{NOB} lead to SA_{AMX}/SA_{NOB} ratios below 1. This fact indicates that NOB could compete with the anammox bacteria for nitrite. However, as SA_{NOB} was the activity most affected by the temperature, meaning that the ratio SA_{AMX}/SA_{NOB} increased by 30 % throughout the reactor

operation (from 0.31 to 0.46). In this way, lowering the temperature might benefit the performance of the PN/AMX processes.

Moreover, the ratio SA_{AOB}/SA_{AMX} was above 1 (Figure 8.6.B) indicating that despite nitrite production was limited during the reactor operation, the biomass had the potential capacity to produce more to provide it as a substrate for the anammox bacteria. This ratio remained almost unaltered with the temperature decrease. Such high values, up to 8, in the flocculent sludge (Figure 8.6.C) shown that this biomass fraction is highly enriched in AOB whereas the SA_{AOB}/SA_{AMX} ratio in the biofilm biomass was approximately 1.

8.4.3.4. Maximum conversion capacity achievable inside the reactor

Considering the separated concentrations of the two biomass fractions (suspended and biofilm) inside the IFAS reactor and the corresponding measured specific activities, the maximum conversion capacities (as mg TN/(L·d)) of each microbial population were calculated (Figure 8.7). As the fraction of biomass attached to the carrier surface is much higher than the suspended sludge one, the biofilm is the main contributor to the TN removal capacity. Considering the whole biomass inside the system, the total AOB capacity was 1.8 -2.0 times the anammox capacity, which is the desirable situation to reach high NRE (Figure 8.8).

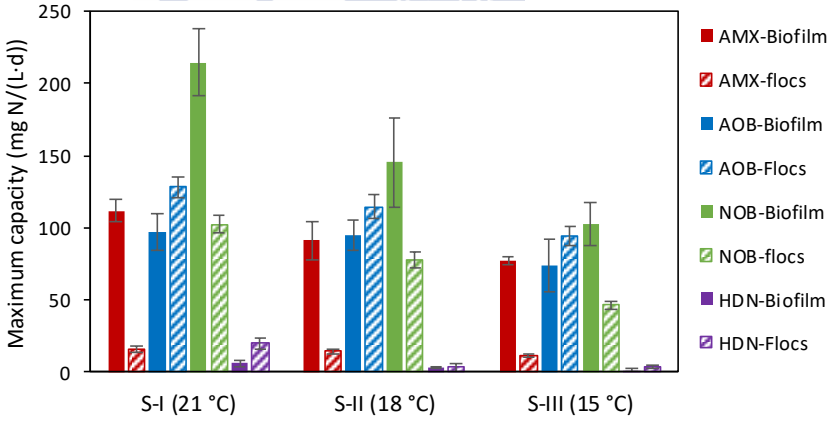

Figure 8.7. Maximum conversion capacity for the different bacterial populations present in suspended sludge (flocs) and biofilm biomass: anammox (AMX), ammonium oxidising bacteria (AOB), nitrite oxidising bacteria (NOB) and heterotrophic denitrifying (HDN).

Additionally, NOB activity decreased the most with the descent of temperature and consequently, its nitrogen transformation capacity (Figure 8.7). Therefore, the ratio of AOB/NOB considering both biomass fractions progressively increased from 0.7 in S-I (21 °C) to 1.1 in S-III indicating the promotion of the AOB while the NOB capacity decreased a 53 % (Figure 8.7 and Figure 8.8). Indeed, at 15 °C the AOB was the population with the highest total capacity with average values of 167 mg TN/(L·d) while the NOB one was of 145 mg TN/(L·d). The limitation of NOB activity was also detected during the reactor operation since the measured NOB activity amounted to 10 - 20 % of the maximum potential capacity obtained from the batch tests. The much lower heterotrophic denitrification capacity, compared with the anammox one (ratios lower than 0.05 in Stages II and III, Figure 8.8), corroborated that the main route of nitrogen removal in the IFAS system was the autotrophic pathway. Moreover, much lower contribution of the heterotrophic denitrification capacity in NRE would be expected as the reactor is not completely anoxic and COD supplied is limited (Table 8.4).

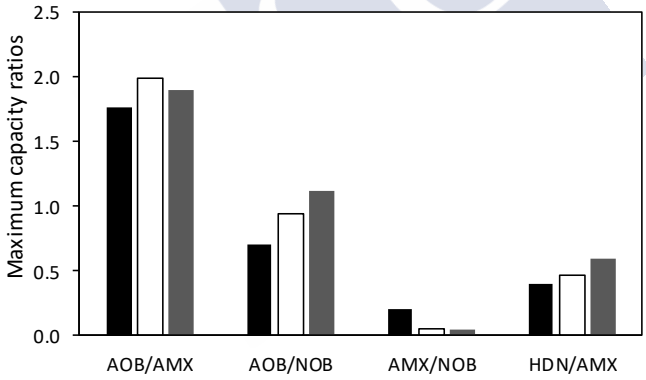

Figure 8.8. Evolution of the maximum conversion capacity ratios of the different bacterial populations: anammox (AMX), ammonium oxidising bacteria (AOB), nitrite oxidising bacteria (NOB) and heterotrophic denitrifying (HDN) at decreasing temperatures: 21 °C (■), 18 °C (□) and 15 °C (■).

8.4.4. Microbiological characterisation

The microbial population segregation suggested by the different specific microbial activities (Figure 8.6) detected in each biomass fraction was also confirmed by the microbial characterisation analysis of a sample collected in Stage II (Figure 8.9 and Table 8.5).

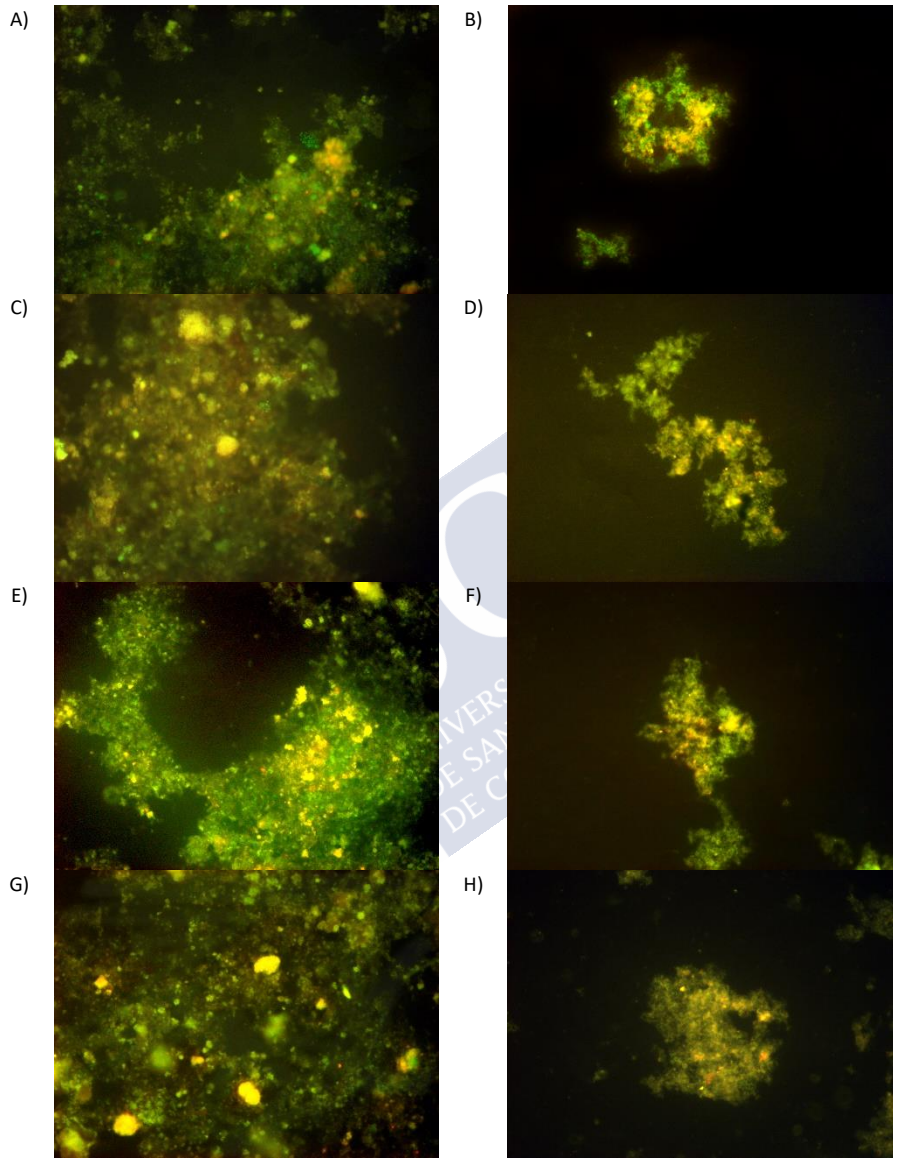

Figure 8.9. FISH images of the biomass sample collected from the IFAS reactor illustrating the distribution of AOB (Nso190: Cy3 dye, orange) in A) biofilm and B) suspended sludge; anammox (AMX820: Cy3 dye, orange) in C) biofilm and D) suspended sludge; *Nistrospira sp.* (Ntspa712: Cy3 dye, orange) in E) biofilm and F) suspended sludge and *Nitrobacter spp.* (Nit3: Cy3 dye, orange) in G) biofilm and H) suspended sludge. In all the images Bacteria domain (EUB338 mix) was labelled with FITC dye (green).

Anammox bacteria represented approximately 33 % of the active bacterial community in the biofilm whereas they only amounted to 12 % of the suspended sludge fraction. This result confirms the fact that the SA_{AMX} measured in the biofilm was twice the activity in flocculent biomass (Figure 8.6). *Candidatus* "Brocadia Anammoxidans" was the predominant anammox specie detected (representing 90 % of the total active anammox).

Table 8.5. Relative abundances of the anammox, ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) populations identified by FISH in biofilm biomass and the suspended sludge (flocs) fractions collected in Stage II from the IFAS reactor.

Group (%)		Biofilm	Suspended Sludge
	Anammox	33.3 ± 6.5	12.1 ± 3.9
	AOB	16.9 ± 4.0	42.9 ± 6.1
	Nitrospira sp.	21.3 ± 4.3	11.4 ± 3.2
NOB	*Nitrotoga arctica sp.*	0.9 ± 1.1	2.13 ± 1.9
	Nitrobacter spp.	20.9 ± 1.8	3.6 ± 1.8

In the case of the suspended sludge fraction, AOB relative abundance was significantly higher (42.9 ± 6.1 %) than in the biofilm biomass (16.9 ± 4.0 %). NOB were detected in both biofilm and suspended biomass. However, differences among the dominant species were observed. *Nitrospira* was the predominant NOB genus in the suspended sludge at a relative abundance of 11.4 % whereas in the case of the biofilm both *Nitrospira sp.* and *Nitrobacter spp.* presented similar abundances (Table 8.5). *Nitrospira* is the most commonly found specie in reactor systems operated at mainstream conditions as these bacteria present high affinity for substrates being able to survive in nitrite limited environments (Cao et al. 2017). *Nitrotoga arctica sp.* was also detected in both fractions but at low percentages.

8.5. Discussion

8.5.1. Nitrogen removal rate and produced effluent quality

In the present study, stable nitrogen removal (72 ± 11 % in average) took place while average NRR of 39 ± 6 mg TN/(L·d) were achieved in the PN/AMX IFAS pilot-plant treating anaerobically pre-treated municipal wastewater at low temperature

(21 - 15 °C) (Figure 8.3). Reached NRR were in the range of those reported for other systems and/or operated at higher temperatures. As an example, Laureni et al. (2016) operated an SBR lab-scale IFAS system under microaerobic conditions (Table 8.1), and manage to reach NRR of 23 mg TN/(L·d). They hypothesised that the SBR cycle should be optimised by decreasing the HRT to increase achieved NRR. At pilot scale, Pedrouso et al. (2018) also obtained comparable NRR (33 mg TN/(L·d)) treating primary settled municipal wastewater in a granular-biomass SBR at uncontrolled temperature ranging from 12 to 18 °C. Han et al. (2016) obtained higher NRR of 60 mg TN/(L·d), but the operational temperature was also considerably higher (19 - 31 °C). Similarly, Malovanyy et al. (2015a) operated the same IFAS system used in the present research work but at higher temperature (25 °C) achieving a NRR of 52 mg TN/(L·d).

Precisely at 15 °C, a relatively high NRE was achieved in the present study (65.8 ± 4.9 %), but further optimization of the reactor operation is required to meet the discharge limits as the total nitrogen concentration in the effluent was 12 ± 5 mg TN/L (> 10 mg TN/L). In this stage, either ammonium or nitrate contributed to the TN concentration in the effluent. The low effluent quality was already pointed out as one of the drawbacks of the continuously fed PN/AMX systems by Hoekstra et al. (2018) who operated a 4-m^3 complete mixed reactor where NRR as high as 97 mg TN/(L·d) at 13.4 ± 1.1 °C was reached. In this sense, the plug flow reactors (Lotti et al. 2015, Yang et al. 2017) or SBRs (Laureni et al. 2016, Pedrouso et al. 2018) are presumably more suitable for effluent quality optimisation than the continuous complete mixed ones. It was demonstrated that using a residual ammonium concentration (2 - 5 mg NH_4^+/L) as set point either to terminate the reaction (Laureni et al. 2016) or to adjust the airflow rate (Yang et al. 2017) or the HRT (Malovanyy et al. 2015a), higher NRE are achieved. Indeed, Yang et al. (2017) obtained NRE of 82 % at temperatures of 24 - 26 °C (Table 8.1), in a system where also the denitrification process took place since the nitrate concentration in the effluent was lower than that expected considering the PN/AMX processes. In this way, the effluent quality was improved.

8.5.2. Overcoming AOB activity limitation

Although the obtained AOB/AMX ratios in terms of both activities and corresponding maximum conversion capacity were higher than 2 (Figure 8.6 and Figure 8.8), indicating that enough nitrite could be supplied, the ammonium oxidation was identified as one of the main limiting steps during the reactor operation.

A possible explanation is the fact that the SA_{AOB} in the biofilm might be restricted by DO transfer limitation and even if it is high in the flocculent sludge, the contribution of this fraction to the global capacity could be insufficient to oxidise enough ammonium to nitrite for the anammox process. Moreover, the length of the aeration phase of 15 or 20 min might also be too short to reach this required ammonium oxidation. In addition, increasing the length of the aerated phase might also promote the NOB activity, which is an undesired effect. Only during Stage III the observed accumulation of low nitrite concentrations (Table 8.3) indicates that AOB was not the limiting capacity anymore. This fact is explained by both the AOB growth and the NOB suppression, being more nitrite is available for the anammox bacteria.

Another possible action would be the optimisation of the previous UASB reactor performance to decrease the COD concentration of the influent to promote the AOB activity inside the IFAS reactor. By lowering the COD concentration of the incoming flow, the aerobic heterotrophic activity would decrease and therefore more DO would be available for the nitritation process.

Additionally, in the case of continuously fed IFAS systems, as they are hybrid systems, it has to be considered that the slowest growing organisms present in the sludge grow on the carrier material while the rest of the bacterial populations grow as suspended biomass. This means that, in the present case, AOB, NOB and heterotrophic bacteria grow mainly in the suspended sludge fraction and anammox bacteria are enriched on the carriers. Obtained results support this statement as the suspended biomass fraction presents a specific AOB activity 4 times higher than that of the biofilm (Figure 8.6). Therefore, if the suspended biomass concentration augments the ammonium oxidation capacity of the system would increase too, improving the nitrogen removal of the system. However, to increase this biomass fraction was difficult, since it presented poor settling properties and tended to float in the sedimentation tank. This fact was due to the accumulated N_2 gas produced by

the heterotrophic denitrification process, which was not appropriately released. However, the system was sufficiently enriched in AOB and anammox bacteria as proven by the estimated AOB/NOB capacity ratio of approximately 1 (Stages II and III) and the AOB/AMX capacity ratio higher than 1.7 (Figure 8.8) to justify the nitrogen was mainly removed via PN/AMX processes. Therefore, IFAS provides alternative control strategies to improve the ammonium removal and overcome one of the main challenges in mainstream conditions.

8.5.3. NOB activity suppression

To improve the process stability and effluent quality to suppress the NOB activity is a requisite. In previous studies, Malovanyy et al. (2015a) operated an IFAS reactor and observed that 60 % of the aerobic activity took place in the suspended sludge, while almost the total anammox activity was found in the biofilm. With this in mind, to limit the nitritation activity would be possible, without affecting the anammox bacteria present in the biofilm, by controlling the concentration of flocculent activated sludge. However, the control of the suspended biomass concentration must be applied carefully to avoid damaging the AOB activity.

In the present study, the complete suppression of NOB activity based on the selective wash out of the flocculent biomass fraction (Han et al. 2016, Veuillet et al. 2014) was difficult, since NOB were also well integrated into the biofilm (Table 8.5, Figure 8.9 and Figure 8.6). As NOB are aerobic, they should be located in the outer layers of the biofilm. Therefore, they might be washed-out by increasing the shear stress imposed on the system to improve the detachment of the loose parts of the biofilm surface. Then, NOB would be washed-out by adjusting the SRT of the flocculent biomass as it happens in a well-segregated IFAS reactor. It was already shown in Chapter 4 that AOB activity might be promoted over NOB one in a continuous stirred tank reactor operated at low temperature (15 °C) by applying high hydraulic loads. Moreover, as an effect of the temperature decrease the NOB conversion capacity diminished in approximately 50 % for both suspended sludge and biofilm from Stage I to Stage III (Figure 8.7) indicating that despite NOB are present, the operation conditions were adequate to suppress them.

As an additional consideration, it was generally accepted that AOB have oxygen affinities higher than NOB and thus PN/AMX reactors should be operated at low DO

concentration to decrease the NOB growth rate and improve the NRE (Lackner et al. 2014). However, at mainstream conditions, the use of a strategy based on just the DO control was not suitable for NOB suppression since at low temperatures NOB have oxygen affinities higher than AOB (Regmi et al. 2014, Val del Rio et al. 2019). In these conditions the NOB dominant genus was *Nitrospira* and not *Nitrobacter* (Cao et al. 2017).

Although intermittent aeration was widely applied to create transient anoxic conditions, which help the NOB out-selection (Malovanyy et al. 2015a, Regmi et al. 2014) the induced lag phase, occurring at the beginning of the aeration phase, failed to completely suppress the NOB activity at low temperature (Agrawal et al. 2018, Cao et al. 2017, Malovanyy et al. 2015a, Regmi et al. 2014). In the present study, with the imposed aeration strategy the NOB activity inside the reactor was limited to 20 % of the SA_{NOB}. So, NOB were not completely wash-out from the system. In this sense, Malovanyy et al. (2015a) evaluated the effect of different ratios between the lengths of aerated and non-aerated phases to suppress the NOB activity. The best PN/AMX processes performance achieved in their system (at 25 °C) was with an intermittent aeration pattern of 15 min ON and 45 min OFF, a DO setpoint of 1 mg O_2/L and a sCOD/N ratio equal to 1.8 g/g, achieving a NRR of 55 mg TN/(L·d) with a NRE of 70 %. However, the effectiveness of the intermittent aeration strategy also depends on the reactor type and operational conditions. For example, in the present study it was demonstrated that at lower temperatures (18 and 15 °C) the aeration pattern of 20 min ON and 40 min OFF was more appropriate.

Other strategies for NOB suppression have been proposed (Agrawal et al. 2018). Among them, the operation of the system at high free ammonium (FA) concentrations lead to a good NOB suppression since AOB have no substrate limitation and NOB are inhibited at lower FA concentrations than AOB (Blackburne et al. 2007, Vadivelu et al. 2007). Other authors proposed the use of free nitrous acid (FNA) to inhibit NOB as it is substantially more harmful to NOB than for AOB (Vadivelu et al. 2007). However, with the pH value of the reactor media (ranging from 6.64 to 7.52) neither FA (0.1 mg NH_3-N/L) nor FNA (0.02 mg HNO_2-N/L) inhibitory concentrations were achieved. Average values were of 0.03 ± 0.02 mg NH_3-N/L and 0.10 ± 0.08 µg HNO_2-N/L, respectively. Inhibitory FA concentrations would be only possible if large ammonium concentrations were present inside the reactor, but in this way, the NRE would not be high enough to accomplish the stringent disposal

requirements. To obtain inhibitory FNA concentrations in a one-stage system would be difficult as anammox bacteria consume nitrite and they are more sensitive to this compound than NOB. This problem might be avoided if the reactor was an SBR or a plug flow reactor where gradient concentrations are easily achieved.

8.5.4. Online monitoring

Concerning the biological treatment of wastewater, the ultimate goal when establishing a control strategy is to reach stable operational conditions, high removal efficiency, and minimal energy consumption for aeration. The control strategy relies on either one or a combination of different control parameters that are continuously measured throughout the system operation. The most commonly used control parameters for PN/AMX processes are pH and/or DO concentration (Lackner et al. 2014). In the present study, DO control was implemented; however, to control the nitrogen concentration in the effluent is usually preferred. For this purpose, ammonium nitrogen and nitrate nitrogen sensors required constant monitoring, proper maintenance (at least once a week) and calibration. From the obtained results, a good correlation between online sensors and laboratory analysis was found (Figure 8.10). However, it would be interesting to find correlations of those parameters with others such as pH or conductivity, which are more reliable and cheaper measurements. Malovanyy et al. (2015a) and Pedrouso et al. (2018) already used a pH set point to control the HRT of one-stage PN/AMX system due to its good correlation with the ammonium concentration, as it was observed in this study throughout the monitoring of the aerobic/anoxic phases (Figure 8.4).

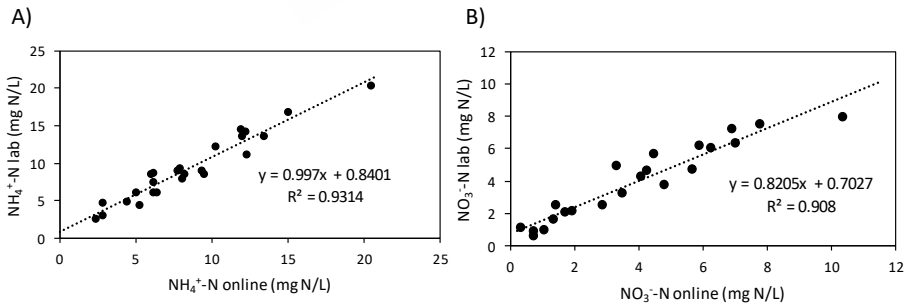

Figure 8.10. Correlations between the concentrations determined in laboratory analyses and those measured by the online sensors A) in mg NH_4^+-N/L and B) in mg NO_3^--N/L.

Conductivity is also a common parameter used in the control strategy when wastewater streams with high nitrogen concentrations are treated (Lackner et al. 2014). However, in the mainstream wastewater treatment, the changes in this parameter occur in a narrow range and therefore it is not suitable for controlling the PN/AMX processes, at least in a continuous system (Figure 8.11).

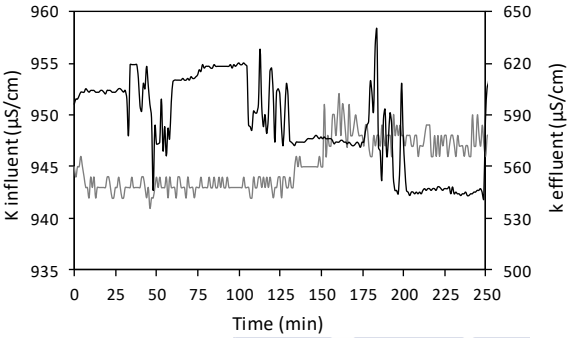

Figure 8.11. Evolution of the influent (—) and effluent (—) conductivity (k) values, in µS/cm, collected on day 94 (Stage III).

Yang et al. (2016) used a redox probe to monitor the PN/AMX processes at sidestream conditions since it is a cheap sensor and it is more sensitive to the changes in the system than other process control parameters such as DO. However, in the present study, no good correlation between the process performance and the ORP online values was found probably due to, as it was previously reported, it is not an adequate parameter when the process is not in steady-state conditions. Yang et al. (2017) found that the nitrogen removal rate showed a stronger linear correlation with the airflow rate than the one found with the DO concentration and obtained nitrogen removal efficiencies up to 82 % (Table 8.1). These authors also found that nitrate production was significantly affected by residual ammonium observing low nitrate production (4 %) when the ammonium concentration was higher than 1 mg NH_4^+-N/L.

8.6. Conclusions

The feasibility of operating one-stage mainstream PN/AMX processes in an IFAS reactor configuration was proved at pilot scale. Anaerobically pre-treated municipal wastewater was treated at decreasing temperatures (from 21 to 15 °C) at a

maximum NLR of 56 ± 3 mg TN/(L·d) and achieving an average NRE of 72 ± 11 %. At 15 °C, the process stability was maintained reaching average NRR of 37 ± 3 mg TN/(L·d), comparable with that observed in conventional nutrient removal systems operated in mainstream conditions.

The quality of the effluent generated, to fit the appropriated quality for discharge, depending on the nitrate produced due to NOB activity and to a lesser extent on the ammonium and nitrite removal efficiencies. The AOB conversion capacity inside the reactor exceeds that of the anammox biomass indicating that if an action is taken to increase its occurrence the treated nitrogen load in the system could augment. For this purpose, the recycling ratio of the biomass from the sedimentation tank could be increased to augment the concentration of AOB in the system.

Microbial population segregation was observed being the AOB and NOB more abundant in the flocculent fraction while the biofilm was mostly composed by anammox bacteria. During the IFAS operation, NOB were present, but their activity was successfully limited observing less than the 20 % and 10 % of the maximum potential NOB activity inside the reactor at 21 and 15 °C, respectively. The successful suppression of most of the NOB activity was achieved by the applied intermittent aeration strategy.

Nitrite oxidising capacity decreased during the IFAS operation, but further optimisation to control the nitrate production is required to improve the NRE.

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Chapter 9

Testing a two-stage partial nitritation and anammox pilot-scale plant implemented in a WWTP

SUMMARY

The results achieved at laboratory scale in the two-stage partial nitritation (PN) and anammox (AMX) configuration led to test this configuration at pilot-scale. The PN/AMX system (two reactors of 600 L each) was installed in a municipal WWTP provided with a full-scale high rate activated sludge (HRAS) system for the removal of the wastewater organic matter content. The PN/AMX system was operated without temperature control (ranging from 11 to 28 °C) and facing the same wastewater characteristic fluctuations that faced the municipal WWTP. The wastewater ammonium content (varying from 22 to 63 mg N/L) was partially oxidised to nitrite in the PN reactor. NOB were successfully suppressed due to the *in situ* free nitrous acid (FNA) accumulation (0.015 - 0.2 mg HNO_2-N/L). A control aeration strategy, based on the pH value, was implemented to obtain the desired nitrite to ammonium ratio in the effluent (1.2 g NO_2^--N/g NH_4^+-N) to feed the subsequent anammox reactor. Nitrite accumulation remained stable without evidence of NOB activity for the whole operational period (118 days), except in the start-up. The effluent from the PN unit was fed to the anammox reactor achieving a global nitrogen removal efficiency of 80 % with a nitrogen removal rate of 47 mg TN/(L·d) limited mainly by the HRT required for the successful biomass retention in the PN unit. Regarding the effluent quality, 12 ± 3 mg TN/L were obtained in the effluent but already 5 mg NO_3^--N/L came in the wastewater from the HRAS. Finally, a study of the potential savings comparing the conventional activated sludge (CAS) system and the combination of the HRAS and PN/AMX processes were performed. Results demonstrated that the implementation of the autotrophic nitrogen removal processes enables to double the energy recovery as more organic matter is driven for the anaerobic sludge digester to produce biogas.

OUTLINE

9.1. Introduction

In the last years, autotrophic partial nitritation and anammox (PN/AMX) based systems have provided promising results when operated for the removal of the nitrogen present in the mainstream of the wastewater treatment plants (WWTPs) (Agrawal et al. 2018, Cao et al. 2017). Besides the low temperature and low nitrogen concentration (commonly studied parameters), the mainstream presents other characteristics such as high variability, in terms of fluctuating temperatures, nitrogen and organic matter loads and flow rates, due to daily and seasonal variations and rainfall periods (Agrawal et al. 2018, Han et al. 2016). Up to now, scarce information is available about the operation of PN/AMX processes treating municipal wastewater at pilot-scale facing the mentioned fluctuations and in general they are performed in a single-unit system (Hoekstra et al. 2019, Pedrouso et al. 2018). Indeed, most of these pilot-scale studies were carried out with temperature control.

Another issue to consider during the operation of these systems is the presence of organic matter in excess that drives the heterotrophic denitrifying bacteria development, which competes with the ammonia oxidising bacteria (AOB) for dissolved oxygen (DO) and with anammox bacteria for nitrite. For this reason, a prior stage for organic matter removal, known as A-stage, is required to favour the subsequent autotrophic nitrogen removal pathway by PN/AMX processes (known as B-stage), and to improve the energy efficiency of the WWTPs by maximising the organic matter use for biogas production (Agrawal et al. 2018, Guven et al. 2019).

Moreover, in the B-stage, the overgrowth of the nitrite oxidising bacteria (NOB) is challenging to control in these conditions as, at low temperature, NOB have higher growth rates and lower oxygen affinities than AOB (Agrawal et al. 2018). As an example, Seuntjens et al. (2016) achieved nitrogen removal efficiencies (NRE) of 51 ± 23 % in a one-stage PN/AMX reactor treating the effluent from a high rate activated sludge (HRAS) reactor at 20 °C. These authors estimated an organic matter to nitrogen consumption ratio (COD/N) of approximately 3 g COD/g N, which limited the nitrogen removal via nitrite to 49 - 62 %. Similarly, Lotti et al. (2015) evaluated the simultaneous PN/AMX processes in a plug-flow granular system operated at 19 °C treating aerobically pre-treated wastewater (2.3 g sCOD/g N) obtaining a nitrogen removal rate (NRR) of approximately 160 mg TN/(L·d) with a NRE of 39 ± 8 %. These authors observed difficulties in maintaining the appropriated balance of microbial

populations and detected the development of NOB whereas heterotrophic denitrifying bacteria did not outcompete the anammox bacteria (Lotti et al. 2015).

Without temperature control, Han et al. (2016) operated a PN/AMX reactor treating the effluent from an HRAS reactor, in operation after a chemical enhanced primary treatment (CEPT), and characterised by a COD/N ratio between 1 and 3 g COD/g TN. These authors managed to treat NRR of approximately 100 mg TN/(L·d) with the temperature ranging from 19 to 31 °C. Also, Pedrouso et al. (2018) operated a granular PN/AMX reactor to treat primary settled wastewater, at 12 - 18 °C, with influent nitrogen concentrations ranging from 6 to 25 mg TN/L and COD/N ratios between 1.5 and 4.4 g COD/N. These authors achieved NRE of 50 % using a strategy based on pH control, fixing the set point at 6.0 without any chemical supply, and concluded that the large fluctuating wastewater composition should be considered as a possible reason hindering the fulfilment of the effluent discharge limit (10 mg TN/L in the European Union (EU) for sensitive areas and big WWTPs). Hoekstra et al. (2019) also pointed out the influence of the temperature changes, and achieved a NRR of 223 ± 29 mg TN/(L·d) in summertime (23 ± 1 °C) whereas it was limited to 95 ± 16 mg TN/(L·d) in wintertime (13 ± 1 °C). The decrease in the NRR was attributed either to an increase of NOB activity or a decrease on the AOB one, presumably due to the exerted control at low values of the DO concentration to promote the anammox activity.

Recently, the potential of the two-stage PN/AMX configuration has been proven at laboratory scale treating both synthetic (Reino and Carrera 2017, Zhang et al. 2019) and municipal wastewater (Chapter 6, Jin et al. 2019, Reino et al. 2018, Zhao et al. 2018). Nevertheless, limited information is available about the performance of the PN/AMX two-stage configuration at pilot-scale treating municipal wastewater. Yang et al. (2007) operated a nitritation unit (54 m^3) by controlling the aeration length based on the ammonia valley concept and DO concentration. In this way, they reached an effective NOB suppression during 180 days at temperatures ranging from 12 to 25 °C. Gu et al. (2012) also maintained stable nitritation as well (at 17 - 26 °C) for 180 days by regulating the aeration supplied to the reactor using a real-time control based on the blower frequency modification and pH value. In both studies, wastewater streams without previous organic matter concentration/removal were treated. Information about neither the PN nor the anammox process performance treating A-stage effluents in separate

units at pilot scale is available. Only, Zeng et al. (2016) treated nitrified sewage in an anammox pilot-scale (160 L) anaerobic biofilter at ambient temperature (18.0 - 21.5 °C) achieving a NRR of 99 mg TN/(L·d). Besides all these research works indicate that the long-term stability of the PN/AMX processes fed with municipal wastewater at pilot and full scale is still uncertain and several challenges need to be addressed before the PN/AMX processes are applied at mainstream conditions.

9.2. Objectives

This study aims at exploring the feasibility of a pilot-scale two-stage PN/AMX system to treat municipal wastewater at not-controlled ambient temperature (11 to 28 °C). The robustness of the system to cope with the fluctuations of the characteristics of the treated wastewater was evaluated as it was implemented *in situ* in the WWTP. The effluent from the HRAS system, characterised by 22 to 63 mg NH_4^+-N/L (average 52 ± 2 mg NH_4^+-N/L) and 25 - 135 mg COD/L (average 75 ± 8 mg COD/L), was used to feed the PN/AMX system.

The performance of each PN and AMX unit was evaluated and optimised separately. Special attention was paid to the accomplishment of the discharge limit (< 10 mg TN/L) of the effluent produced in the system and aspects related to the full-scale application (e.g., energy savings, costs) were discussed.

9.3. Materials and Methods

9.3.1. Valdebebas WWTP

The Valdebebas municipal WWTP was selected to test the feasibility of the pilot-scale two-stage PN/AMX system. It is located in Madrid (Spain), managed by the public entity Canal de Isabel II and operated by the company FCC Aqualia. This WWTP has a treatment capacity of approximately 260,000 population equivalents and an average wastewater flowrate of 52,000 m^3/day. This facility is adequate to evaluate the applicability of the autotrophic nitrogen removal processes since its biological process consists of an HRAS system to remove the organic matter without affecting the nitrogen concentration of the wastewater. In this HRAS system, the aeration was mechanically supplied. The hydraulic retention time (HRT) was low (7 ± 1 h) to enhance the adsorption of COD, from the wastewater, onto the biomass to maximise biogas production during the subsequent anaerobic digestion of the

separated sludge. Phosphorus is chemically removed by ferric chloride ($FeCl_3$) dosage in these aeration tanks (approximately 3,500 kg/day), just before going to the secondary settlers also improving the sludge settleability. The effluent from the HRAS unit was characterised by annual average values of 66 ± 11 mg COD/L, 62 ± 11 mg TN/L, 43 ± 10 mg NH_4^+-N/L, 3 ± 1 mg NO_3^--N/L, 0.9 ± 0.1 mg TP/L and 18 ± 4 mg SST/L. The low COD/N ratio in the HRAS effluent (approximately 1 g COD/g TN) indicated the excellent process performance and its adequacy to be fed into a PN/AMX system.

9.3.2. Pilot plant set-up and operation

Two pilot-scale rectors (600 L each), one aerobic for the partial nitritation process (PN unit) and another anoxic for the anammox process (AMX unit), were operated without temperature control in the Valdebebas WWTP (Figure 9.2). The HRAS effluent was stored in a 220 L intermediate tank from which wastewater was fed to the system.

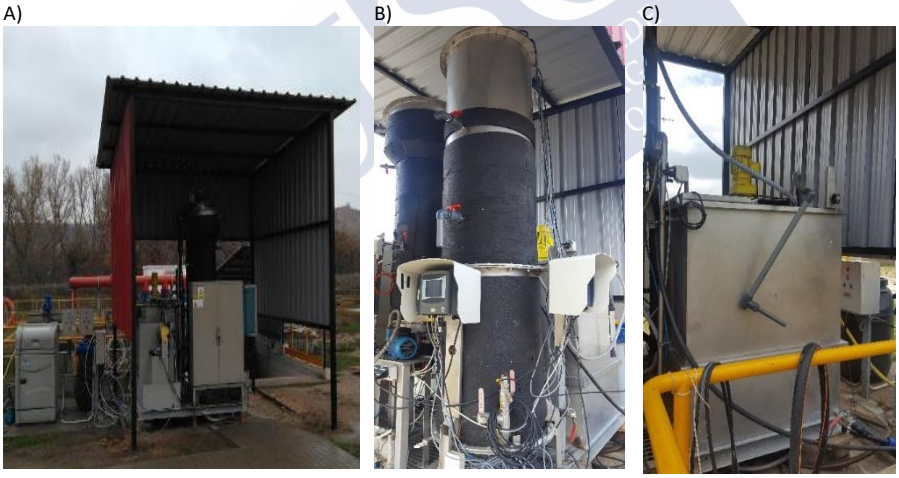


Figure 9.1. Images from: A) the PN/AMX pilot-plant, B) PN unit and C) AMX reactor.

9.3.2.1. Partial nitritation reactor

First, the PN unit (Figure 9.1.A and B) was started-up treating the full-scale HRAS effluent. This reactor was seeded with sludge from a conventional activated sludge (CAS) system located in La Gavia WWTP (Madrid, Spain) as it was the nearest

WWTP also managed by Canal Isabel II where biological nitrogen removal processes took place. After a week facing problems of sludge retention, the reactor was reinoculated by reintroducing the sludge washed-out from the system mixed with the flocculent fraction of ELAN® biomass (enriched in AOB but with presence of NOB too) coming from a one-stage PN/AMX reactor treating the supernatant of the sludge digester operated at mesophilic conditions (i.e. sidestream PN/AMX reactor), located in Guillarei WWTP (Tui, NW Spain) (Morales et al. 2018).

A set of two peristaltic pumps were used to feed (from the top) and discharge (by adjusting the final liquid level) the reactor, respectively (Figure 2). Dissolved oxygen was supplied by introducing air through diffusers placed at the bottom of the reactor. The applied airflow rate ranged between 5 and 10 L/min. The DO concentration was monitored, but not controlled, utilising an online sensor (Hach LDO). Moreover, the pilot plant was equipped with online probes to measure pH (pHD, Hach) and ion-selective electrodes to determine ammonium and nitrate concentrations (AN-ISE, Hach). All the probes were connected to a sc100™ controller (Hach) for data acquisition.

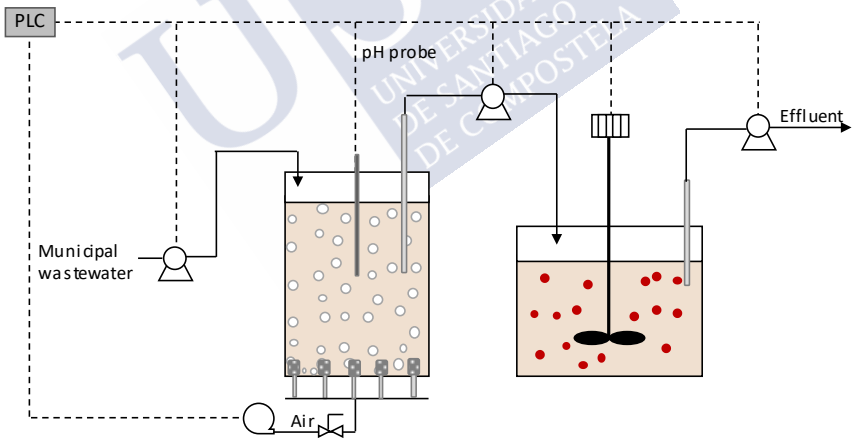


Figure 9.2. Scheme of the PN/AMX pilot-plant implemented in the Valdebebas WWTP.

The pilot plant operated as a sequencing batch reactor (SBR) and the SCADA software was used to monitor the pilot-plant process and operation. The cycle distribution (Figure 9.3) was designed to promote the nitritation process and then, due to technical limitations, the anammox cycle duration was the same. The SBR

cycles from the PN unit were divided into four phases (Figure 9.3.A) comprising: aerated feeding, aerobic reaction (with variable length), settling and discharge.

A)

Feeding				
Aeration				
Settling				
Discharge				
Time (min)	**90 and level**	**Variable**	**40***	**25 or level**

B)

Stirring				
Settling				
Discharge				
Feeding				
Time (min)	**Variable**	**20**	**20**	**25 or level**

Figure 9.3. SBR-cycle configurations: A) Partial nitritation reactor and B) anammox reactor. Details about the variable phase length are provided in Table 9.2. The discharge pump from the nitritation unit directly fed the anammox reactor.

*The settling time in the partial nitritation reactor from day 0 to 16 was 20 min instead of 40 min.

The PN unit was operated for 118 days, from March to July 2018, and it comprised three different Stages according to the aerobic phase duration (Table 9.1). Initially, in Stage I (days 0 - 16) the aerobic reaction phase only lasted 10 min resulting in a short cycle duration of 145 minutes. This cycle length was imposed to reach a high hydraulic load at a hydraulic retention time (HRT) of 4.8 hours, to promote the growth of AOB over the NOB ones (as it was shown in Chapter 4). During this Stage I, the settling time was 20 min and the volume exchange ratio (VER) was set at 50 %, in order to select the AOB while NOB are washout. Moreover, the length of the total reaction phase (including feeding) was of 100 min. Later, in Stage II (days 17 - 50) the settling time increased from 20 to 40 min to improve biomass retention. During this Stage II, the length of the aeration phase was first extended to 150 min to promote the ammonium oxidation and then stepwise reduced to 90 min on day 21 to be finally set at 60 min from day 25 onwards. The cycle length was manually adjusted in order to obtain an effluent with the nitrite to ammonium ratio close to the required value for the anammox process of 1.32 g NO_2^--N/g NH_4^+-N. The resulting HRT ranged from 10.2 to 7.2 hours (Table 9.1). Finally, during Stage III (days 51 to 118), the VER was diminished to 33 % and the SBR cycle configuration was changed to one with variable duration based on a pH set-point that terminates the aeration

phase. A maximum aeration time of 120 min was set in order to finish the cycle in case that pH-set point was not reached due to pH probes failure or process instabilities. The objective of this cycle variable duration was to adjust the nitrite to ammonium ratio in the effluent to feed it to the posterior AMX unit. As the wastewater composition and temperature fluctuated, the pH-set point was periodically readjusted according to the performed periodic single cycle characterisation. In all stages the effluent withdrawal lasted 25 minutes or till the required remaining liquid level inside the reactor was reached.

Table 9.1. Different operational stages distribution for the pilot-scale PN reactor.

Stage	Duration (days)	Cycle length (min)	Aeration[a] (min)	Settling (min)	VER (%)	HRT (h)
S - I	0 -16	145	10	20	50	4.8
S - II	17 -49	310 – 215[b]	150 - 90 (day 21)- 60 (day 25)	40	50	7.2 - 10.2
S - III	50 -118	140 – 275[c]	120 min or pH	40	33	7.5 - 13.9

[a] In all cases the previous 90 min was used for the simultaneous feeding + aeration stage.

[b] Cycle length manually adjusted.

[c] Cycle length variable because the aeration phase was of 120 min (maximum) or finalised according to the defined pH set point.

9.3.2.2. Anammox reactor

Once the partial nitritation process was stable, on day 69 of operation (Stage III), the AMX unit (Figure 9.1.C) was started-up and operated for 48 days simultaneously with the PN unit. The anammox reactor was an open SBR provided with mechanical stirring (Figure 9.2). The VER was equal to 33 %, the same as the one from the PN unit, and the discharge pump from the partial nitritation unit directly fed the anammox reactor. Thus, the SBR cycles were coupled. The feeding phase lasted 25 minutes (coinciding with the discharge from the PN unit) (Figure 9.3). Then, the reactor was mixed during at least 90 min and the reaction time terminated by stopping the mechanical stirring when the defined set-point was reached on the previous PN unit (Figure 9.3.B). Thus, the reaction time in the anammox reactor was not optimised by itself. The biomass settled for 20 min and then the effluent was discharged by till the defined liquid level. The reactor was equipped with ammonium, nitrate (AN-ISE, Hach), conductivity and redox sensors connected a sc100™

controller (Hach) for data acquisition and to the SCADA software for anammox process monitoring.

The anammox reactor was inoculated with anammox-enriched granular biomass from the full-scale ELAN® reactor located in the Guillarei WWTP (Tui, NW Spain) (Morales et al. 2018) (Figure 9.4).

Figure 9.4. Image of the inoculum used for the anammox pilot-scale reactor.

9.3.3. Analytical methods

Influent and effluent streams were periodically sampled to follow the pilot plant performance and to calibrate the online sensors. Single operational cycles were monitored to evaluate the evolution of the concentrations of the different compounds in the liquid phase in order to assess the process performance and to adjust the pH set point in the PN unit. Before analysis samples were filtered through 0.45 µm pore size. The concentration of chemical oxygen demand (COD), ammonium, total phosphorous, total nitrogen, nitrite and nitrate were determined spectrophotometrically using Dr. Lange test kits (Hach Lange, Germany). Alkalinity was determined by titration according to *Standard Methods for Water Examination* as well as the concentration of the total (TSS) and volatile suspended solids (VSS) and sludge volume index (SVI) (APHA-AWWA-WEF, 2012). In order to obtain a homogenous and representative sample to determine the solid concentrations in the effluent, approximately 2 L of this stream were collected each 5 min, throughout the length of the discharge phase. Moreover, pH, temperature, conductivity and DO concentration probes were also used to corroborate the online measurements. The maximum specific nitrifying activities (both for AOB and NOB, SA_{AOB} and SA_{NOB} respectively) were determined by respirometric tests according to Lopez-Fiuza et al. (2002). All the methodologies used are described in detail in Chapter 2.

9.3.4. Calculations

All the calculations required to obtain free nitrous acid (FNA) concentration, and the corresponding mass balances for both PN and AMX units are described in Chapter 2 in sections 2.4.1, 2.4.3 and 2.4.4, respectively.

A comparative study of the implementation of the PN/AMX processes after the HRAS or the use of a CAS for nitrogen removal was performed. A flow rate of 30,000 m^3/day, the one that is treated in the HRAS was used for the calculations. The actual removal efficiencies for the primary treatment were used in both scenarios as well as for the HRAS. Nevertheless, as data for energy consumption and sludge production was not available, theoretical values were used to estimate them considering a solid retention time (SRT) of 1.5 days and an aerator efficiency of 1 kg O_2/kWh. Biomethanization of the sludge depends on the organic fraction and it was estimated that 85 % it, is converted into methane. Electrical efficiency of fuelled electric power generators was fixed at 35 % (Garrido et al. 2013). Energy requirements of the physical stages (e.g. pumping, operation of settlers, sludge dewatering units) was fixed at 0.1074 kWh/m^3. Energy price of 0.125 €/kW·h, sludge management of 200 €/T_{sludge} and 300 €/$T_{methanol}$ were used for the cost estimations.

9.4. Results

9.4.1. Partial nitritation process performance

9.4.1.1. Nitrite accumulation promotion and maintenance

During the 118 days of operation of the PN unit no COD removal was observed, indicating that COD removal in the full-scale HRAS was optimised and the COD present in the effluent, of approximately 60 mg COD/L during the whole operational period, was not easily biodegradable. As the reactor was operated during spring to summertime without temperature control, its value varied from 11 to 28 °C (Figure 9.5). The operational period was divided into three stages according to the applied cycle distribution (Table 9.1), which corresponded with the applied average daily HRT values shown in Figure 9.5.

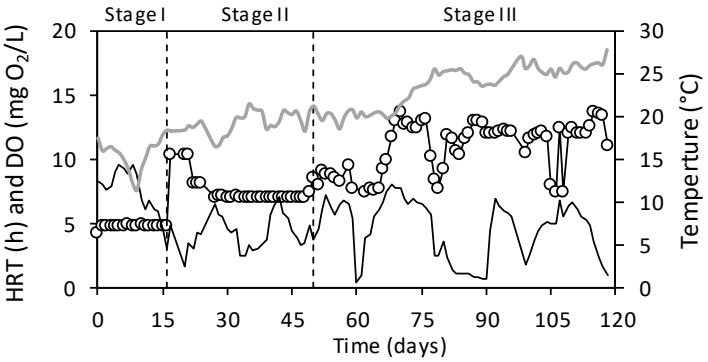


Figure 9.5. Evolution of average daily hydraulic retention time (HRT, ○), in hours, dissolved oxygen (DO) concentration (—), in mg O_2/L, and operational temperature (—), in °C.

Initially, during Stage I, the ammonium oxidation to nitrite was incipient (Figure 9.6.A). Applied NLR was as high as 248 ± 41 mg TN/(L·d) and only the first day of operation an ammonium oxidation ratio (AOR) of 72 % was achieved (Figure 9.6.B) producing 44 mg NO_3^--N/L (Figure 9.6.C). Thus, both AOB and NOB were active in the seeding sludge but did not operate properly in the reactor operational conditions. Thus, the AOR sharply decreased to values lower than 10 % (Figure 9.6.A) and therefore no significant ammonium oxidation was observed neither to nitrite nor to nitrate (Figure 9.6.C). This was presumably due to the high hydraulic load applied, with an HRT of 4.8 h (Table 9.1 and Figure 9.5) that caused the biomass washout from the system. During this Stage, a constant airflow rate of 5 L/min was supplied during the aerobic phase leading to an average DO concentration of 7.4 ± 1.8 mg O_2/L. The decrease of DO concentration occurring in the bulk liquid during this stage was mainly related to the temperature increase throughout the operational period (Figure 9.5). The nitrite accumulation ratio (NAR) progressively increased during this stage reaching values of 99 % at the end of this stage (Figure 9.6.A). Thus, the early development of the nitritation process was successfully started-up. Nevertheless, as the AOR was still limited the maximum nitrite concentration accumulated in the system was 3.9 mg NO_2-N/L (Figure 9.6.C).

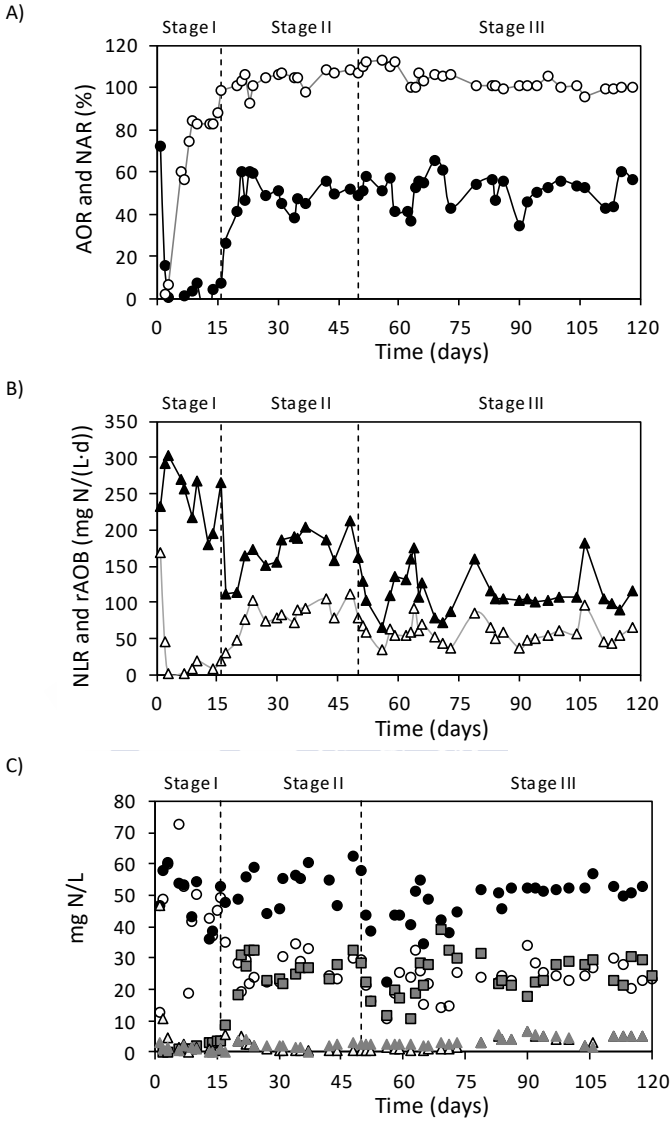


Figure 9.6. Evolution of A) ammonium oxidation ratio (AOR, ●) and nitrite accumulation ratio (NAR, ○), in %; B) nitrogen loading rate (NLR, ▲) and ammonium oxidizing bacteria rate (rAOB, Δ), in mg N/(L·d); C) nitrogenous compound concentrations throughout the partial nitritation reactor operation: ammonium in the influent (●) and effluent (○), nitrite (■) in the effluent and nitrate in the influent (▲) and effluent (△) in mg N/L.

During this Stage I, the pH in the effluent was higher than the one in the influent (Figure 9.7). This behaviour might be due to the excess of DO supplied that caused stripping of dissolved CO_2, which increased the pH in the system. The free nitrous acid (FNA) concentration in the system was negligible (Figure 9.7), therefore, the relatively high hydraulic load applied and the expected AOB abundance higher than that of NOB in the inoculum, allowed for the selection of the AOB over NOB.

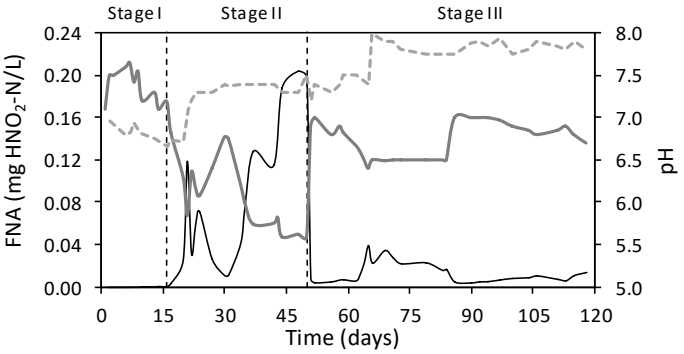


Figure 9.7. Evolution of the free nitrous acid (FNA) concentration (—) and pH values in the influent (--) and effluent (—) of the partial nitritation reactor.

Then, on day 17 the sludge that was washed-out from the system was reintroduced and a cycle characterisation (with extended aeration) was performed (from 8:00 AM to 14:00 PM) before Stage II began. The objective of this cycle characterisation was to evaluate the length of the aeration phase required to achieve high nitrite accumulation by promoting the rate of AOB (rAOB) that was limited to values below 30 mg NH_4^+-N/(L·d) (Figure 9.6.B). The feeding took approximately 40 min and during this time both ammonium and pH increased (Figure 9.8). Furthermore, the aeration in this single cycle was extended to a total duration of 360 min (phase 2, length of 270 min) and, during the whole aerated phase, the AOB rate doubled the NOB one. The pH decreased from 7.05 to 6.70, but the reached FNA concentration was still wide below 0.02 mg HNO_2-N/L, the inhibitory value for NOB (Blackburne et al. 2007) (Figure 9.8.B). Based on the results obtained with this extended aeration cycle characterisation (Figure 9.8.A, B), the total cycle duration on day 17 was extended from 145 min (Stage I) to 310 min (Stage II) in order to promote the AOB growth but limiting the NOB one.

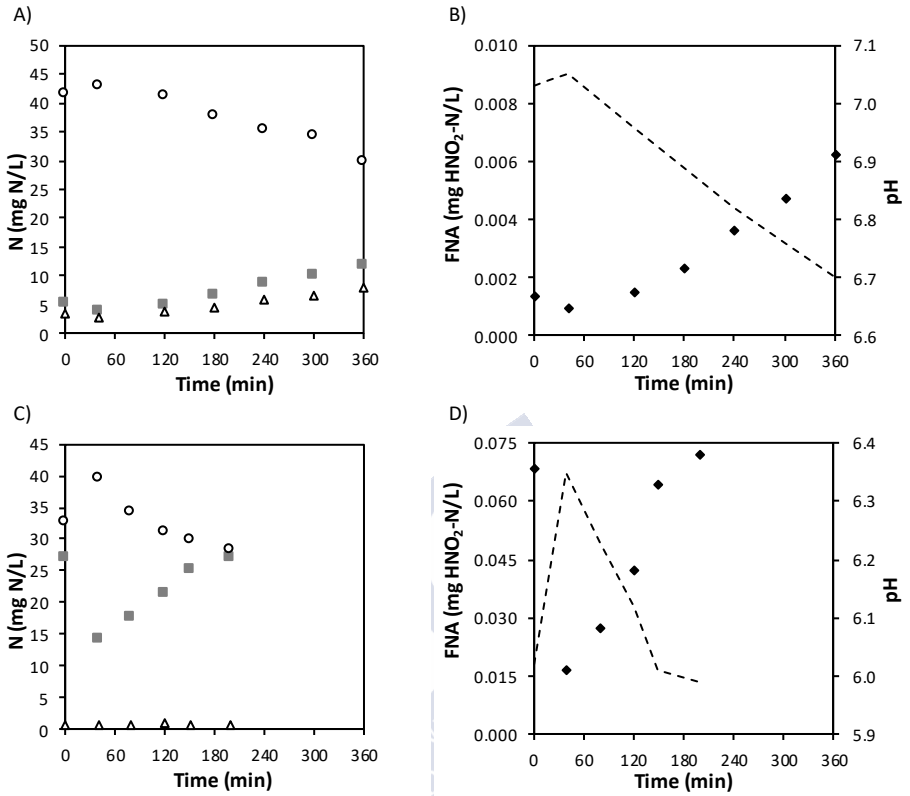


Figure 9.8. Cycle characterisations in days 17 (extended aeration) (A and B) and 35 (Stage II with shorter aeration length) (C and D). A and C) evolution of the ammonium (○), nitrite (■) and nitrate (Δ) concentrations, expressed as mg N/L. B and D) evolution of the pH value (--) and FNA concentration (♦) expressed as mg HNO_2-N/L.

During Stage II, applied NLR was diminished and NAR remained close to 100 % while nitrite concentration in the effluent progressively increased (Figure 9.6). Later, the cycle length of the cycle was manually modified by gradually reducing the length of the aeration phase (Table 9.1) resulting in a progressive HRT decrease from 10.2 to 7.2 hours (Figure 9.6). The cycle length was shortened to limit the AOR and to obtain a nitrite to ammonium ratio in the effluent close to the stoichiometric value required for the subsequent anammox process (1.15 - 1.32 g NO_2^--N/g NH_4^+-N) (Lotti et al. 2014, Strous et al. 1999). Moreover, the aeration flow rate was also increased from 5 to 7 L/min, to ensure that the AOR was not limited by the insufficient DO supplied to the bulk liquid (Figure 9.6). During this part of Stage II, successful

nitritation was achieved and maintained reaching concentrations of accumulated nitrite up to 25 ± 6 mg NO_2^--N/L (Figure 9.6.C) while suppressing the NOB activity (NAR close to 100%). Nitrate production was not observed. As an example, the cycle from day 35 is shown in Figure 9.8.C and D. In this case, FNA concentrations high enough to inhibit the NOB during the entire cycle were accumulated associated to the nitrite concentrations and pH values. Despite the low pH values of operation (in average 6.1 ± 0.1), the AOB activity does not seem to be negatively affected. Indeed, the rAOB during the aerobic phase of day 35 was 93 mg N/(L·d), whereas it was 58 mg N/(L·d) in the extended aeration cycle from day 17. The change in trends at the end of the cycle characterisation from day 35 was due to the beginning of the settling phase.

Finally, from day 50 onwards (Stage III), the cycle had variable length and the aeration phase was terminated by a pH set-point to better adjust the effluent nitrite to ammonium ratio while coping with the influent characteristic fluctuations. From the previous cycle characterisations, a good correlation between pH and ammonium concentration in the effluent was observed (Figure 9.8). Therefore, pH was selected as control parameter as the sensor used for its measurement was more reliable than those used for ammonium and nitrate determination that required to be calibrated at least once per week.

At the beginning of this Stage III, the pH in the effluent sharply increased from 5.6 to 6.6 (Figure 9.7). This change of the pH value coincided with some malfunction events in the WWTP that led to the stop of the $FeCl_3$ dosage, used for the physical-chemical phosphorus removal, which consumed alkalinity according to the following reaction.

$$FeCl_3 + 3\ (HCO_3)^- \rightarrow Fe(OH)_3 \downarrow\ + 3\ Cl^- + 3\ CO_2$$

As the alkalinity consumed during phosphorous removal diminished, its concentration in the influent to the nitritation reactor increased from 134 ± 69 to 225 mg $CaCO_3$/L. Therefore, the wastewater presented a buffering capacity higher than before and the pH in the effluent of the PN unit rose up. Due to this fact, the pH set-point established on 5.6 on day 50 was changed to 6.9 and afterwards, it was reduced progressively to 6.5. The obtained AOR during this stage was in average 51 ± 7 % (Figure 9.6.A) and the NAR remained at 100 % (Figure 9.6.A), values adequate to be fed to the anammox reactor. Indeed, NAR higher values than 100 % were

obtained in some operational days due to the difficulties on closing the mass balances due to the wastewater composition fluctuations.

The pH increase caused the decrease of FNA concentration (Figure 9.7) but no nitrate production was observed indicating that the NOB activity was successfully suppressed inside the reactor. In fact, the nitrate concentrations in the influent and effluent are almost the same (Figure 9.6.C). In this Stage III (68 days) the average FNA concentration was 0.014 ± 0.001 mg HNO_2-N/L. Thus, another factor presumably contributed to the nitritation process performance or, as it was demonstrated in Chapter 5, the strategy based in FNA concentration is robust and with good NOB suppression, in such way that when no inhibitory FNA concentrations are achieved (like in Stage III), the system is no immediately destabilised. Furthermore, during this last Stage the temperature increased reaching average values of 24 ± 2 ° C that might favour the AOB growth over the NOB one.

Moreover, the SA_{AOB} and SA_{NOB} were measured in batch tests on days 38 (Stage II) and 79 (Stage III) obtaining values for SA_{AOB} of 195 ± 23 and 360 ± 35 mg NH_4^+-N/(g VSS·d), respectively, whereas SA_{NOB} was not detected. Thus, biomass was considered to be enriched on AOB.

9.4.1.2. Biomass retention capacity

The main identified operational problem in the PN unit was associated to the biomass retention as the VSS concentration in the effluent was relatively high, with values higher than 40 mg VSS/L the most of the operational period (Figure 9.9).

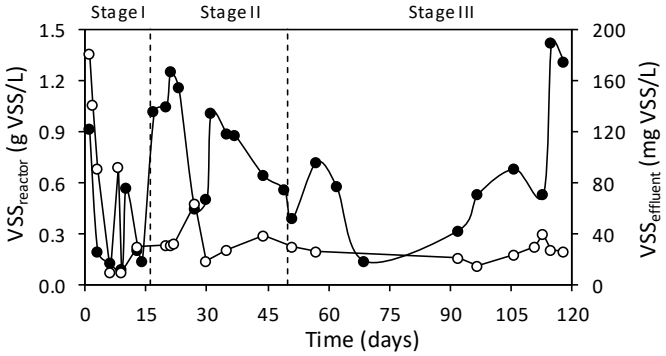


Figure 9.9. Evolution of the biomass concentration inside the reactor (●), in g VSS/L, and the effluent (○), in mg VSS/L, throughout the partial nitritation reactor operation.

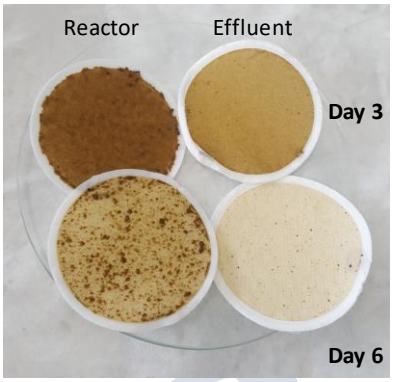
During Stage I, the low HRT applied (Figure 9.5) caused the biomass washout resulting in a VSS concentration decrease from 0.92 g VSS/L (day 0) to 0.10 g VSS/L (day 9) (Figure 9.9). Besides the biomass washout, aggregates formation was observed (Figure 9.10). The formation of the aggregates was associated to the high hydraulic stress applied that resulted in an average specific NLR of 1,423 mg N/(g VSS·d) reaching values as high as 2,900 mg N/(g VSS·d). As the biomass growth did not compensate for the biomass washout, the solids from the effluent were collected in an external tank and reintroduced back to the reactor weekly. This biomass concentration decrease trend might be attributed to the reactor configuration since the biomass washout problems were not solved even when the settling time was increased from 20 to 40 min and the cycle length extended in Stage II (Table 9.1 and Figure 9.9). In Stage III, despite the VER was reduced from 50 % to 33 %, the biomass concentration in the effluent remained almost constant with average values of 26 ± 7 mg VSS/L. On day 70 the biomass reintroduction was stopped since the anammox unit was started-up, but the nitritation process performance was kept stable (Figure 9.6) probably due to the ambient temperature by that time and longer HRT that promoted the AOB growth (Figure 9.5).

Besides the biomass washout, biomass stratification inside the reactor was also observed (Figure 9.10.B) proving that the reactor was not well mixed. This could be also an explanation of the fact that higher rAOB were not achieved. From day 52 onwards, the aeration flow rate was increased to 10 L/min to improve the reactor mixture, but the stratification phenomenon was still present. One possible reason is the fact that the air diffusers were not located at the bottom of the reactor but 20 cm over it and therefore, when the biomass settled, it was difficult to re-suspend it as air diffusers occupied a large part of the surface challenging the mixing at the bottom of the reactor.

Regarding the SVI, fluctuating values were obtained again probably due to the fact that the reactor was not properly mixed. The SVI from the seeding sludge was 150 mL/g TSS that was in the range of values commonly found in the CAS systems ranging between 100 and 200 mL/g TSS (Burton et al. 2014). During Stages I and II, the SVI remained at average values of 127 ± 14 mL/g TSS and 121 ± 21 mL/g TSS, respectively. Finally, during Stage III the SVI was slightly reduced to values of 95 ± 25

mL/g TSS. The relatively high SVI values (> 80 mL/g TSS) also indicated the moderate biomass compaction capacity explaining the observed biomass wash out.

A)



B)

Figure 9.10. Picture of: A) Evolution of the aspect of the biomass retained in the filters used for solids determination on days 3 (suspended sludge) and 6 (aggregates) of operation filtering the same volume of sample. B) Biomass samples, from left to right, taken from the top, medium-high and bottom exits from the reactor during the aerated phase on day 51.

9.4.2. Anammox process performance

Once the PN reactor performed stable, the produced effluent was directly fed to the anammox pilot-scale reactor for 48 days (Figure 9.3) resulting in an average NLR of 116 ± 22 mg TN/(L·d) (Figure 9.11.A). The AMX SBR cycle length was fixed according to the duration of the PN one (Figure 9.3.B) and, therefore, both reactors were operated with the same HRT that was in average 11.7 ± 1.6 hours (Figure 9.5). The average temperature of the anammox reactor was 24.9 ± 1.3 °C, ranging from 21 to 28 °C (Figure 9.5).

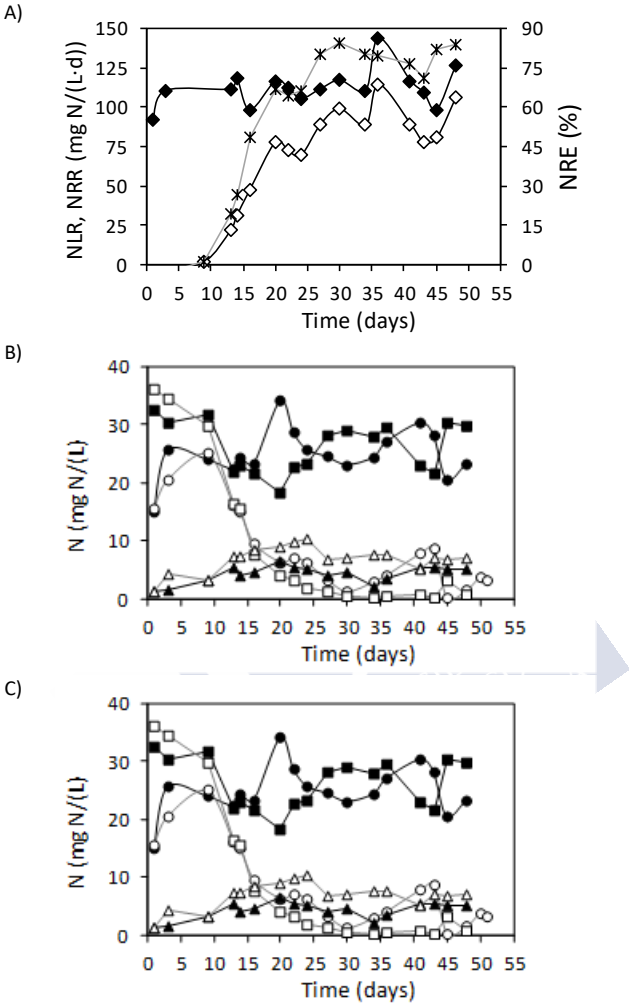


Figure 9.11. Evolution of A) nitrogen loading rate (NLR, ♦), nitrogen removal rate (NRR, ◊), in mg N/(L·d) and the nitrogen removal efficiency (NRE, *) in %. B) Nitrogenous compound concentrations throughout the anammox reactor operation: ammonium in the influent (●) and effluent (○), nitrite (■) in the influent and effluent (□) and nitrate in the influent (▲) and effluent (Δ) in mg N/L. C) Nitrite to ammonium consumption ratio (●) and nitrate production to ammonium consumption ratio (▲). Horizontal lines represent the stoichiometric values: solid lines according to Strous et al. (1999) and dashed lines according to Lotti et al. (2014).

During the first 10 days, the vigorous mechanical stirring applied caused the transfer of air through the bulk liquid surface and as a consequence provided a high DO concentration (up to 6 mg O_2/L) inside the anammox reactor. In these conditions, nitrogen removal was not observed and nitrogen removal efficiency (NRE) values were close to zero (Figure 9.11.A). Indeed, the nitrite concentration in the effluent was higher than the one in the influent, indicating that the ammonium fed to the system was partially oxidised in the system (Figure 9.11.B). However, as soon as a variable frequency drive was installed in the mechanical stirring system, the anoxic conditions were established and the anammox process occurred with simultaneous ammonium and nitrite removal (Figure 9.11). The obtained nitrogen removal rate (NRR) quickly rose to average values of 99 ± 26 mg TN/(L·d) with NRE of 80 ± 5 % (Figure 9.11.A). The nitrite concentration in the effluent was close to zero (0.8 mg NO_2-N/L) (Figure 9.11.B) indicating that the reactor might be able to treat NLR higher than those applied, but the hydraulic limitation imposed by the previous PN unit impeded the increase of the NLR. The observed specific anammox rates ranged from 110 to 214 mg N/(g VSS·d).

Once the process reached stable conditions, the nitrate produced to ammonium removed ratio was on average 0.15 ± 0.09 g NO_3^--N/g NH_4^+-N, close to the theoretical one of 0.16 - 0.26 g NO_3^--N/g NH_4^+-N (Lotti et al. 2014, Strous et al. 1999); whereas the nitrite to ammonium consumption ratio was of 1.22 ± 0.13 g NO_2^--N/g NH_4^+-N, also fitting well with the anammox stoichiometry (1.15 -1.32 g NO_2^--N/g NH_4^+-N) (Figure 9.11.C). The average total nitrogen in the effluent was 11.5 ± 2.6 mg N/L composed mainly by nitrate with average concentrations of 6.9 ± 0.8 mg NO_3^--N/L. Finally, slight COD removal occurred corresponding to average COD removal rates of 26 ± 1 mg COD/(L·d). This result indicated that the denitrification process might be taking place but it was limited by the amount of organic matter available.

Despite the low initial granular biomass concentration used to inoculate the reactor (0.4 g VSS/L), the anammox process was successfully established and relatively high NRE was achieved. The granular biomass was successfully retained inside the system with VSS concentration in the effluent lower than 10 mg VSS/L. Although the too short operational period of this reactor (48 days), together with the slow growth rate of the anammox bacteria, do not allow to categorically state that the process is able to provide long-term stable performance, the results obtained are auspicious.

9.5. Discussion

9.5.1. Effluent quality and impact of the wastewater composition

The lack of information about the performance of PN/AMX systems treating municipal wastewater under realistic conditions has been pointed out by several authors (Agrawal et al. 2018, Hoekstra et al. 2019, Pedrouso et al. 2018). This fact highlights the relevance of the results in the present study, where the feasibility of using the effluent from an HRAS to feed a pilot-scale PN/AMX system was proven. This system was able to cope with the same wastewater fluctuations as the municipal WWTP in terms of temperature and composition.

Initially, the nitrite accumulation in the PN reactor was promoted by applying a relatively high hydraulic load. This strategy was also tested in Chapter 4 where a chemostat was used to treat synthetic wastewater. Nevertheless, in the present study at pilot-scale, the biomass used as inoculum presented an expected higher abundance of AOB than NOB, which might also contribute to promoting the high NAR achieved, close to 100 %, from day 17 onwards. During the rest of the operational period, the production of nitrate was not detected in the reactor indicating that the NOB activity was successfully suppressed (Figure 9.6). Therefore, neither the continuous addition of a toxic compound (as in Chapter 5) nor a precise selection of the inoculum (as in Chapter 6) would be required, but the pre-treatment of the sludge or the application of high nitrogen loads that promote the initial nitrite accumulation. Once this starts, the FNA inhibitory levels achieved will be enough to maintain the NOB suppression. During Stage II, the cycle length was extended to promote the AOR by providing enough time for the bacteria to grow. Finally, a variable-length cycle was applied in Stage III to better cope with the wastewater fluctuations. Nevertheless, during this last operational stage, the cycle duration was almost constant too, with relatively high average HRT values of 11 ± 2 hours (Figure 9.5).

One of the main identified challenges during the operation of the PN reactor was the existing biomass wash out. Due to the reactor configuration, long settling times of 40 minutes, were required to guarantee the biomass retention. In these conditions, the applied HRT also augmented. These long settling times might also favour the NOB activity suppression since, during this time, anoxic conditions could

be present. The transient anoxic conditions are widely applied for limiting the NOB activity (Agrawal et al. 2018, Regmi et al. 2014). However, the high airflow rates applied during the aerobic phase, to improve the reactor mixture, lead to high DO concentrations and anoxic conditions were barely reached even when the blower was stopped. Moreover, higher rAOB might be achieved if the reactor mixture would be ensured for example by implementing a mechanical stirring. Agrawal et al. (2018) stated that for the successful application of the PN/AMX processes at mainstream conditions the combination of different factors might be required to restrict the NOB activity. In the present case, maintaining as the basis the application of the FNA strategy, additionally other factors might act. For example, in Stage III the combination of the relatively high temperature, moderate FNA concentration and the moderate HRT might be the responsible ones for the maintenance of the nitritation process.

Besides the frequent wastewater characteristics fluctuations, the pilot plant had also to cope with the particular events when the operation of the WWTP was compromised. As an example, on day 50 a peak of drinking water with large amounts of sand entered the full-scale plant collapsing the treatment units. To avoid process failure, the HRAS and the anaerobic sludge digester were stopped for one week, which was the time that lasted the sand removal works. However, the operation of the pilot-scale PN reactor remained stable, showing its robustness. This is a critical feature for its future implementation. Nevertheless, the operational period of the pilot-scale reactors was short, especially for the anammox unit (48 days), and further research is required in order to evaluate more extended process stability. Indeed, the operation must also be tested under colder conditions and with more diluted and fluctuating wastewater conditions (winter time).

Despite anammox bacteria were traditionally considered as very sensitive bacteria and initially pointed out as one of the main limitations to implement the autotrophic nitrogen removal processes at mainstream conditions, the anammox process was quickly established even without temperature control. Moreover, the reactor was opened to air and therefore, transient oxic-anoxic conditions were present. At the beginning of the cycle DO concentrations close to 4 mg O_2/L were measured due to the DO dissolved present in the fed stream and also to the mass transfer through the water surface in the top of the unit. Its concentration decreased to zero during the first 15 minutes of the cycle. The pH of the partial nitritation

effluent was 6.7 ± 0.3, in the lowest limit of the reported optimal pH range for the anammox bacteria of 6.5 to 8.5 (Strous et al. 1999). This stream was directly fed to the anammox reactor were a NRE of 80 % was reached. Thus, the low pH presumably did not negatively affect the process performance. The obtained NRE was higher than the one obtained by other authors treating municipal wastewater in one-stage PN/AMX pilot scale and the NRR was in the same range (Agrawal et al. 2018, Laureni et al. 2019, Lotti et al. 2015, Pedrouso et al. 2018). Han et al. (2016) obtained, at 19 -31 °C, a NRE of 70 % with a NRR of 98 mg TN/(L·d), slightly higher than the one obtained in the present study. In a continuous system, Hoekstra et al. (2019) obtained much higher NRR of 223 ± 29 mg TN/(L·d) in summertime (23 ± 1 °C) with NRE close to 75 %. There is no similar information concerning the operation of two-stage PN/AMX pilot-scale systems. In the present study, the applied NLR was limited by the partial nitritation cycle distribution and ultimately limited by the biomass retention capacity of the PN reactor. This aspect should be optimised for up-scaling purposes, as the NRR might be higher since the SA_{AOB} measured on day 79 by batch tests was 360 ± 35 mg NH_4^+-N/(g VSS·d). Furthermore, the NRR in the anammox reactor was limited by the NLR applied, which depended on the PN unit.

The wastewater composition from the last 25 days of operation of the combined PN/AMX system is presented in Table 9.2. The pH of the effluent discharged from the anammox unit was considerably lower than the one from the already existing HRAS but it was close to the neutrality. The effluent solids concentration was not increased and the COD concentration in the effluent slightly decreased, improving the effluent quality. In terms of nitrogen, the total nitrogen (as the sum of ammonium, nitrite and nitrate) concentration was 12.22 ± 3.17 mg TN/L higher than 10 mg N/L, the discharge limit established for sensitive areas in the European Union. The most abundant nitrogen form in the effluent was nitrate, from which around 62 % coming directly from the HRAS unit and only 38 % produced in the AMX unit because no nitrate production was observed in the nitritation unit but in the HRAS. This increase of the nitrate concentration in the effluent from the HRAS was associated with the increase of temperature and affected the effluent quality of the system and the maximum achievable NRE since no nitrate is removed in the PN/AMX system. The HRAS system performance must be optimised by further reducing the HRT to limit the nitrifying bacteria development. If the nitrate fed to

the PN unit is discarded to evaluate the effluent quality, the average total nitrogen concentration would be 7.74 ± 3.02 mg TN/L, below the EU discharge limits.

Table 9.2. Composition of the different streams from the two-stage PN/AMX pilot plant in the last 25 operational days.

	HRAS effluent	PN effluent	Anammox effluent
pH	7.84 ± 0.06	6.86 ± 0.09	6.89 ± 0.23
mg COD/L	75 ± 8	70 ± 11	56 ± 12
mg NH_4^+-N/L	52.48 ± 1.94	25.83 ± 3.08	3.97 ± 2.96
mg NO_2^--N/L	0	26.20 ± 3.45	0.99 ± 0.95
mg NO_3^--N/L	4.56 ± 1.44	4.50 ± 1.17	7.27 ± 1.34*
mg VSS/L	16 ± 15	26 ± 9	11 ± 4

*Note that around 62% comes from the HRAS effluent whereas less than 3 mg N/L are produced in the AMX unit.

9.5.2. Process control strategy

In the present study, all the wastewater was treated first in the PN unit and, then, fed to the AMX unit due to a limitation of available pumps to run both reactors. However, to maximise the nitrite accumulation in the nitritation reactor, favouring the NOB suppression would be of high interest.

With this objective, if the wastewater alkalinity is enough to oxidise more ammonium, it could be of interest to reach the maximum AOR achievable leading to higher nitrite concentration accumulation and alkalinity depletion, decreasing the pH and promoting the accumulation of the FNA. As it was already stated in Chapter 5, if the N/IC ratio in the incoming wastewater is higher than 0.6 g N/g IC the nitritation process can be achieved and maintained without the addition of chemicals and/or any other control action. If this ratio is lower than 0.6 g N/g IC, the aerobic HRT should be controlled, and if it is higher than 1 g N/ g IC, the AOR would be limited by the alkalinity availability.

To produce the stream with the required nitrite to ammonium ratio to feed the AMX reactor the fraction of wastewater bypassed to the PN reactor would depend on the wastewater nitrogen to alkalinity (as IC) ratio (Figure 9.12). For this purpose, the ammonium in the incoming wastewater and the ammonium and nitrite concentrations inside the nitritation reactor are measured to adjust the desired ratio close to 1.32 g NO_2^--N/g NH_4^+-N. Thus, to maximise the nitrite accumulation, the

modifiable parameters are at least one or a combination of the aerobic HRT (aerHRT) and the fraction of the wastewater bypassed (Bypass) (Figure 9.13).

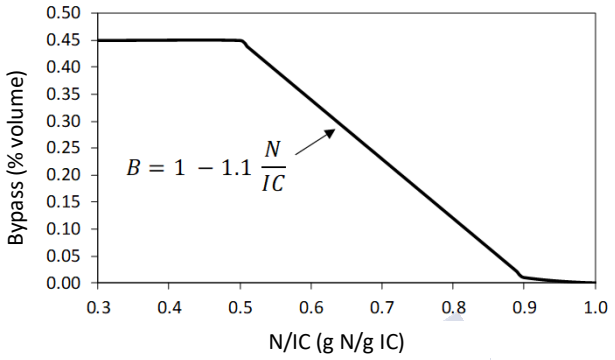


Figure 9.12. A) Scheme of the two-stage configuration system based on the nitritation maintenance by in-situ free nitrous acid (FNA) production. B) Flow of bypass stream with respect to raw wastewater driven directly to the anammox reactor, in percentage.

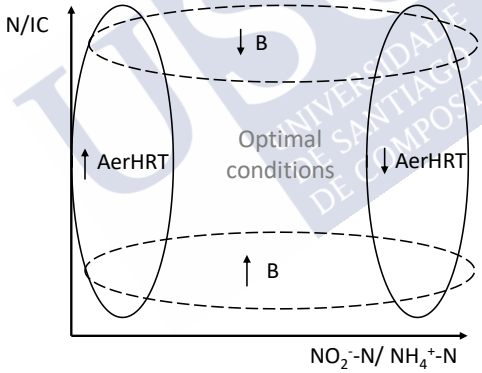


Figure 9.13. Representation of the corrective actions to return the PN unit to the optimal conditions by controlling the aerobic hydraulic retention time (aerHRT) and/or the fraction of raw wastewater bypassed to the anoxic reactor (Bypass), depending on the N/IC ratio in the feeding and the NO_2^--N/NH_4^+-N ratio in the effluent.

9.5.3. Practical implementation

The energy contained in wastewater can be recovered either by directly using anaerobic reactors in moderate climates or by indirectly applying an organic matter pre-concentration step by adsorption onto sludge, which is subsequently treated by

anaerobic digestion (Guven et al. 2019, Liu et al. 2018). In both cases, the PN/AMX systems could be applied for nitrogen removal and would allow for reducing both the energy consumption of the WWTP and the size of the reactors, when compared with CAS, since they are usually more compact units with capability to treat higher volumetric loads (Agrawal et al. 2018, Lackner et al. 2014). A study of the savings associated to the partial nitration and anammox processes combined with the already existing HRAS in the WWTP, compared to a scenario where the HRAS was converted to a CAS, where organic matter and nitrogen are removed, was performed.

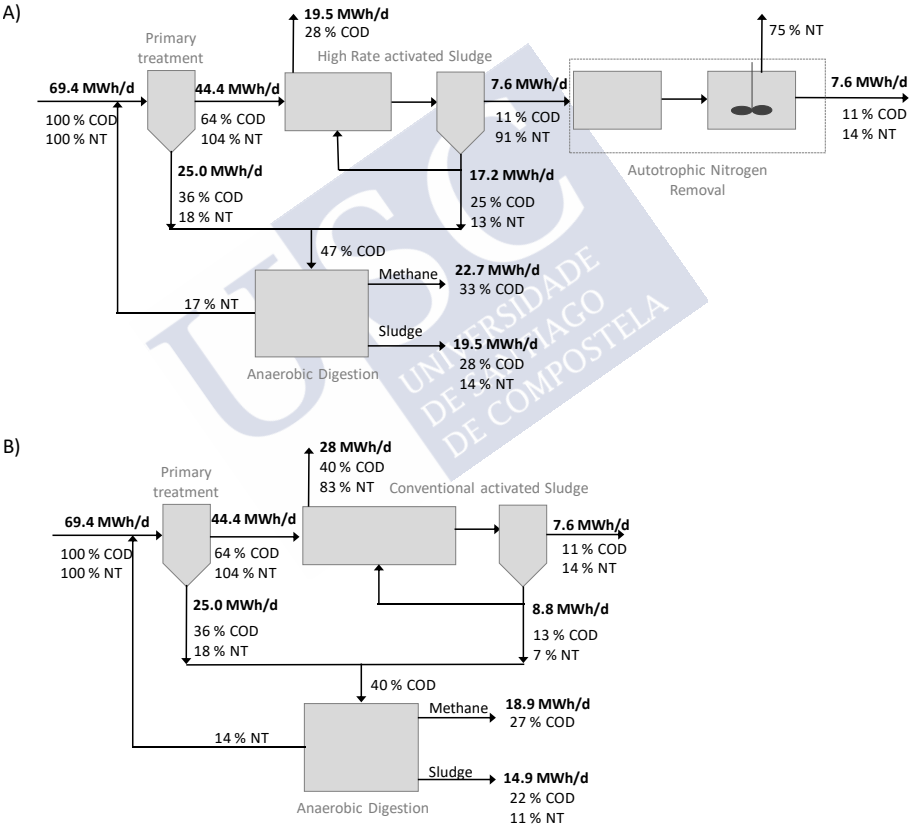


Figure 9.14. Mass (as % of COD, referred to the influent) and energy (MWh/d) balances for A) combination of HRAS and PN/AMX and B) Conventional activated sludge (CAS).

For the evaluation of the different scenarios, which results are shown in Table 9.3, the same considerations were done:

1) the already existing primary treatment in the WWTP was defined with removal efficiencies of 37 %, 55 % and 36 % of BOD_5, TSS and COD, respectively (Figure 9.14);

2) the biological treatment presented an average treatment capacity of 30,000 m^3/day and influent concentrations of 170 mg BOD_5/L, 110 mg TSS/L, 380 mg COD/L and 70 mg TN/L;

3) the annual minimum temperature of 12 °C was considered.

In the present full-scale HRAS process, approximately 82 % of the COD from the treated wastewater was removed obtaining an effluent with 11 % of slowly-biodegradable COD. The HRAS is the most advantageous scenario from the energy recovery point of view since the captured COD in the sludge is ready for the anaerobic digestion for biogas generation. Nevertheless, this scenario did not consider the nitrogen removal and approximately 90 % of TN is still present in the effluent, so it does not fulfil the discharge limits. Despite theoretical calculations shown an energy efficiency of 84 % (Table 9.3) with energy recovery of 0.6 kWh/g bCOD, the values obtained in the full-scale plant were lower with energy self-sufficiency of approximately 72 %. Indeed, these values are low compared with the values close to 4 kWh/g bCOD reported for wastewater treatment plants (Garrido et al. 2013, Shizas and Bagley 2004).

Traditionally, WWTPs were upgraded by increasing the SRT and converting the HRAS in a CAS where nitrogen is removed by nitrification-denitrification processes. In this case, the primary settled wastewater had a biodegradable COD to TN ratio of 5 g bCOD/g bTN. Thus, the organic matter present in the stream is not enough to remove all the nitrogen by heterotrophic denitrification if a consumption ratio of 7.5 g COD/g NO_3^--N (Burton et al. 2014) is considered. To achieve the desired TN limit (10 mg N/L) methanol addition was considered as external carbon source. In this scenario, the SRT was 18.5 days in order to achieve the nitrification and the HRT was increased from 2.4 to 21 h. The energy efficiency of the plant was worsened as only 21 % of the used energy was recovered, and the cost of treatment increased 3.5 times.

Table 9.3. Energy balance evaluation of implementing conventional activated sludge (CAS) or the autotrophic nitrogen removal by the combination of PN/AMX to upgrade the HRAS based WWTP to remove nitrogen.

	HRAS	**CAS**	**HRAS + (PN+ AMX)$_I$**	**HRAS + (PN + AMX)$_{II}$**
HRT (h)	2.4	26	2.4 + 11 + 11	2.4 + 5 + 14
VSS (g/L)	2	2	2 + 0.8 + 0.4	2+ 2+ 2
V reactor (m^3)	3,027	32,900	3,027+ 13,750 + 13,750	3,027 + 6,875 + 17,100
SRT (d)	1.5	18.5	-	-
Aeration requirements (KWh/m^3)	0.134	0.303	0.222	0.222
Methane (m^3 CH_4/m^3)	0.053	0.029	0.053	0.053
Energy produced (kWh/m^3)	0.204	0.111	0.206	0.206
Sludge management (kg dry TSS/m^3)	0.086	0.072	0.091	0.101
Other energy consumption[b] (kWh/m^3)	0.107	0.108	0.128	0.135
Energy efficiency (%)	84	21	59	57
Operational cost (€/m^3)	0.022	0.078 [a]	0.036	0.039

(PN+AMX)$_I$: considering the biomass concentration of the present pilot-plant study; (PN+AMX)$_{II}$: considering 2 g VSS/L in each unit.

[a] Considering the cost of methanol 300 €/kg

[b] This factor includes pumping, sludge dewatering…

The combination of the HRAS and the PN/AMX processes was proposed to fulfil the requirements for nitrogen removal. With this configuration, COD was still mainly driven to the anaerobic digestion process, but the energy was required in the autotrophic nitrogen removal units, for aeration in the PN unit and mixing in the anammox one. Initially, an average HRT of 11 hours was considered in each unit as it was the average during the pilot plant operation. In this scenario, the energy efficiency of the plant was better than with the CAS increasing from 21 to 59 % (requiring 0.145 kWh/m^3) and the operational costs diminished by 50 %.

Nevertheless, the biomass concentration in both PN and AMX units were low with values, in the present study at the pilot-scale plant, of 0.8 and 0.4 g VSS/L, respectively (scenario called $(PN+AMX)_I$). These low values were due to biomass retention problems and inoculum availability. Thus, an estimation of the volumes and HRT was carried out assuming that biomass concentrations of 2 g VSS/L was achieved in each unit (scenario called $(PN+AMX)_{II}$). For the anammox reactor, a specific anammox activity of 50 mg N/(g VSS·d) was considered in the range of the ones obtained in literature at temperatures between 10 and 15 °C (Agrawal et al. 2018). In this final scenario, 60 % of the energy self-sufficiency was achieved and the nitrogen limits would be reached. In reality, better results are expected since by further optimisation of the SBRs cycle configuration more active time will be achieved. Moreover, the energy demand for mixing the anammox reactor increased from 0.02 to 0.08 kWh/m^3 due to the large- scale reactor needed due to the low anammox activity at low temperature. Nevertheless, this might be further optimised promoting the biomass accumulation as granular sludge, because granular systems can achieve much higher biomass concentrations. Moreover, in this last scenario, the aeration in the HRAS amounts to 60 % of the aeration requirements. This unit might be also optimised by reducing the SRT in order to capture more COD in the sludge and decrease the COD that is burned to CO_2, which usually ranges from 10 to 25 % (Guven et al. 2019). Indeed, Garrido et al. (2013) in a theoretical study proposed that the combination of an HRAS plus PN/AMX systems at mainstream would lead to 111 % of energy sufficiency. However, the composition of water is different and the research work of these authors is only based on energy and mass balances calculations, without considering some aspects such as the longer SRT required due to the low temperature, like the 12 °C used in the present study, that reduced the

percentage of COD that goes to the sludge fraction and, thus, can be potentially valorized, and increased the energy consumption and CO_2 production.

Thus, further research is required in order to validate these results and to investigate the minimal HRT that might be applied during wintertime.

9.6. Conclusions

A pilot-scale two-stage PN/AMX plant was successfully operated for the first time without applying temperature control (11 – 28 °C) and fed with wastewater pre-treated in a high rate activated sludge (HRAS) system (22 – 63 mg NH_4^+- N/L).

The nitritation process was successfully achieved and maintained, despite the wastewater characteristics fluctuations, with the FNA based strategy. A variable aeration phase length (controlled by the pH value) allowed to maintain the process stability obtaining the adequate nitrite to ammonium ratio of 1.1 mg NO_2^--N/g NH_4^+-N in the produced effluent to be fed to the anammox reactor. No nitrate production was observed during the entire operation.

The anammox process was quickly established in an open pilot-scale reactor despite the low biomass concentration inoculated (0.4 g VSS/L). The quality of the effluent produced in the previous PN unit, after the imposed control strategies, did not affect the anammox reactor performance.

Thus, preliminary promising results were obtained characterised by short start-up periods and achieved NRR of 94 ± 14 mg TN/(L·d) limited mainly by the HRT required for the successful biomass retention in the PN unit.

Quality of the effluent produced in the system was of 12.22 ± 3.17 mg TN/L, limited by the nitrate produced in the HRAS system (approximately 5 mg N/L) that enters in the PN/AMX system and it is not converted on it. Without considering this nitrate, the effluent fulfilled the discharge limits established in the European Union for sensitive areas and big WWTPs (< 10 mg TN/L).

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Chapter 10

General conclusions and future challenges for the mainstream autotrophic nitrogen removal

SUMMARY

A general overview of the main outcomes of the thesis is provided here. Indeed, a perspective of the lessons learned and the main identified bottlenecks for the implementation of the partial nitritation-anammox (PN/AMX) processes at mainstream conditions are discussed. Especial attention is paid to the future research work required to spread out the anammox based processes implementation in the conditions of low temperature and low nitrogen concentrations. The conclusions extracted from each chapter are also described in this chapter.

OUTLINE

10.1. Main outcomes of the thesis

The importance of the anammox process in the field of the wastewater treatment lies on the possibility to achieve the complete autotrophic nitrogen removal to improve the economic and environmental sustainability of wastewater treatment plants (WWTPs). Despite anammox bacteria were traditionally considered as sensitive microorganisms, it was the suppression of the nitrite oxidising bacteria (NOB) one of the most challenging features to spread out the application of the partial nitritation and anammox (PN/AMX) processes to the conditions of low temperature and low nitrogen concentrations characteristics of the mainstream in WWTPs. Most of the approaches tested for the PN/AMX processes implementation at mainstream used one-stage PN/AMX configuration, whereas limited information is available about the performance of two-stage configuration. Several operational conditions were investigated in this thesis with the main goal of providing new insight for the implementation of anammox based nitrogen removal systems at mainstream conditions.

10.1.1. Is it possible to apply the anammox based processes to decentralised wastewater treatment systems?

Decentralised wastewater treatment systems are implemented in small urban agglomerations or centres like accommodation facilities, office buildings, etc. They need to be reliable, robust and cheap process solutions requiring minimum operator actions (as they are usually managed from the distance). Nitrogen removal is barely considered in small size WWTPs since they cannot take profit of the economy of scale as conventional nitrogen removal processes are energy and space-intensive processes. In these decentralised systems, source-separation sewer systems may be installed to enable for the obtainment of different wastewater fractions (mainly blackwater and greywater) that are treated separately according to their properties. In this way, treated wastewater can be reused and the resources that it contains valorised. Blackwater fraction is the more concentrated stream compared with greywater one, with high organic matter concentrations that might be valorised throughout direct anaerobic digestion (AD) (Eshetu Moges et al. 2018). From the AD, a nitrogen-rich stream is obtained that can be used for fertirrigation or submitted to nitrogen removal processes when produced reclaimed water exceeds irrigation needs or its nitrogen concentration is over the amount possibly absorbed in

agriculture due to the soil balance. Up to now, the treatment of blackwater by PN/AMX processes has not been widely assessed (de Graaff et al. 2011, Vlaeminck et al. 2009). The existing scarce studies did not consider the possibility of wastewater unavailability events that might face the decentralised systems, primarily when no treatment is needed as reclaimed water is used for irrigation and also due to users habits for example in office buildings, commercial areas, etc. In Chapter 3, it was proven that anaerobically digested blackwater can be successfully treated in a one-stage PN/AMX system capable of coping with repeated starvation and reactivation periods. The fact that this stream contains higher nitrogen concentrations and present less variable composition compared to municipal wastewater, might favour the PN/AMX processes stability and prevent for NOB activity, that did not develop inside the reactor operated at low temperature (14 -21 °C). Regardless that the operational period was short (100 days), when municipal wastewater was treated in a similar granular reactor, NOB activity development was already observed in a shorter period (60 days) (Morales et al. 2016).

10.1.2. Is it possible to suppress the NOB activity at mainstream conditions?

Due to the difficulties found on in applying the NOB control strategies to PN/AMX processes that are commonly used in sidestream to mainstream conditions (described in Chapter 1), the nitrate production is one of the significant barriers to obtain large nitrogen removal efficiencies to accomplish the nitrogen discharge standards. So far, dissolved oxygen (DO) concentration has been the most frequently controlled parameter to out select NOB (Akaboci et al. 2018, De Clippeleir et al. 2013, Laureni et al. 2016, Regmi et al. 2014). However, from previous studies, different outcomes are provided about the role of DO on the suppression of NOB. Some of them indicate that low bulk DO concentrations are suitable to control nitritation, but other studies reported that NOB could adapt to low oxygen availability and become competitive with AOB at these conditions, impeding the achievement of efficient nitrite oxidation suppression. Regarding the aeration regime, intermittent aeration was applied to exploit the lag phase exhibited by NOB after being subjected to anoxic conditions (Ma et al. 2015, Malovanyy et al. 2015). Results demonstrated that this strategy was sufficient to limit the high NOB activity inside an integrated fixed activated sludge (IFAS) reactor, but it was not efficient to wholly suppress the nitratation process (Chapter 8).

Other authors propose the control of solid retention time (SRT) to washout the NOB from the system (Laureni et al. 2019, Regmi et al. 2014). Nevertheless, at low temperature, NOB growth rates are higher than those of AOB and, thus, this strategy by itself would not be enough to establish the nitritation process (Regmi et al. 2014). With this in mind, in Chapter 4, the effect of step decrease of the SRT and the hydraulic retention time (HRT) length on the out selection of NOB was studied. The results presented in this chapter evidence that AOB is favoured over NOB activity and the nitrite accumulation takes places but in low extent, while the NOB washout was unfeasible, becoming the nitrate the main ammonium oxidation product. Thus, SRT and HRT based strategies are not able to establish the nitritation process at low temperature, but their combination with other factors such as DO control might succeed (Laureni et al. 2019). In Chapter 8, the SRT of the flocculent sludge fraction from the biomass in the IFAS system was not controlled but a strategy based on limiting its concentration is expected to provide better results. Another common approach is to expose the sludge in an external unit to free ammonia (FA) (Wang et al. 2017, Zhang et al. 2018) or free nitrous acid (FNA) (Wang et al. 2016a, Wang et al. 2014) inhibitory concentrations for NOB. Despite promising results were obtained, these strategies require to pump the sludge from the PN/AMX system in the mainstream to units in operation in the sludge line of the WWTPs, where it is mixed directly with the supernatant of the anaerobic sludge digestion to treat it with FA or with the nitritified effluent containing FNA. Several factors are under study to optimise the suspended sludge fraction washout, the exposure time or the required toxic concentrations. A more straightforward strategy based on the NOB inhibition by FNA *in situ* accumulation was proposed in Chapter 5 and then validated treating municipal wastewater (Chapter 6 and 9). Results evidenced that it is possible to achieve the nitritation process by the *in situ* created FNA levels (by the combination of the low pH values resulting from the alkalinity deletion and the produced nitrite) and maintained at long term. This finding is one of the main outcomes of the present thesis since if adequate conditions are applied no nitrate production is produced being a robust strategy even when organic matter is present (Chapter 6).

For partial nitritation in granular systems, the maintenance of residual ammonium concentrations in the bulk liquid facilitates the creation of a stratification favouring the growth of AOB. Several research works describe the successful nitrite accumulation by the control of DO to total ammonium (TAN) concentration ratio

(DO/TAN), but information is limited to the treatment of synthetic wastewater (Isanta et al. 2015, Reino et al. 2016). Finally, other authors found that the nitritation process might be achieved using real-time aeration control by terminating the aeration phase when ammonium is finished. In this sense, the most common parameter to control is the pH that decreases during the nitritation process and increases when ammonium is depleted (Yang et al. 2007). In chapter 9, the aeration phase also has a variable-length controlled by the pH value, but the objective of this control was not the NOB suppression but the adjustment of the composition of the effluent from the nitritation reactor to reach an adequate nitrite to ammonium ratio to feed the subsequent anammox process.

Considering all the different research outputs, it can be stated that it is possible to suppress the NOB at the conditions of low temperature and low nitrogen concentrations and it seems that different strategies might be adequate to obtain the stable nitritation process. However, up to now none of them has fully succeeded to maintain the nitritation process long-term stability at mainstream conditions for temperatures under 20 °C, treating real municipal wastewater.

10.1.3. Are anammox bacteria able to perform stable treating practical loads at mainstream conditions?

The low temperatures (< 25 ° C) found at mainstream conditions are known to limit the microbial activities. As the nitrifying bacteria are active in the conventional activated sludge systems, they were initially not really considered and special attention was traditionally paid to the effect of the low temperature over the anammox bacteria (Dosta et al. 2008, Lotti et al. 2015). To evaluate this effect, several studies applied progressive changes in the reactor operational conditions to move from sidestream, high nitrogen concentrations and mesophilic conditions, to mainstream conditions (Dosta et al. 2008, Reino and Carrera 2017, Sánchez Guillén et al. 2016). Nevertheless, in the present thesis, results indicated that the anammox process can operate stable without a previous adaptation of the biomass to mainstream conditions (Chapter 7). This was possible as long as enough biomass concentration accumulated inside the system, which facilitated the effective nitrogen removal by the anammox bacteria. Thus the objective of the present thesis was mainly focused on testing the performance of the PN/AMX processes operated at low temperature and nitrogen concentrations without trying to find the maximum

nitrogen removal rates (NRR) achievable. Indeed, low NRR were obtained in general limited by the applied nitrogen loading rate (NLR), as this effect can not be attributed to the presence of nitrite in the effluent or to the insufficient specific anammox activity of the biomass that was high enough to cope with the applied nitrogen loads (Chapter 3, 7 and 9). In the case of Chapter 8, the anammox activity was limited by the ammonium oxidising capacity inside the IFAS system that did not provide enough nitrite for the anammox bacteria. Reino et al. (2018) reached high NRR of 1,200 ± 500 mg N/(L·d) but these authors observed a sharp deterioration of the process performed when synthetic medium was replaced by municipal wastewater. In chapter 7, no adverse effect was observed when nitritified municipal wastewater was treated but the presence of residual organic matter helps to polish the final effluent to concentrations below 6 mg TN/L. Low NRR were achieved limited by low biomass accumulation. In chapter 9, anammox granular biomass was also used to remove the nitrogen in a pilot-scale two-unit PN/AMX system where excellent process performance was obtained in spite of the reactor being uncovered, which enabled the entrance of oxygen. The absence of negative effect might be attributed to the fact that temperature was not controlled during the period of operation, from spring to summertime, at temperatures close to 24 °C. All these results emphasise the fact that during winter/cold conditions, large anammox biomass concentrations will be necessary to sustain the required NRR.

Moreover, the determination of the specific anammox activity (SA_{AMX}) of the biomass in operation by batch tests was revealed as a powerful tool to obtain preliminary information about the influence of different operational parameters over the anammox process performance (Dapena-Mora et al. 2007). Previous research works evaluated the effects of parameters such as pH, organic matter, salts or substrates concentrations at mesophilic temperature range (30 °C) (Jin et al. 2012). However, these boundaries need to be determined at mainstream conditions to fix the corresponding maximum nitrogen removal capacities for design purposes. For example, Tomaszewski et al. (2017) found that the optimal pH changed with temperature as it was also confirmed in Chapter 7 of the present thesis. Thus, the resistance against toxic compounds might also change with temperature. In Chapter 7, it was found that pH changes (from 6 to 8) exerted a higher effect than temperature (ranging from 15 to 30 °C) and organic matter concentrations (from 0 to 75 mg/L as total organic carbon (TOC)) on SA_{AMX}.

10.1.4. How does municipal wastewater composition affect the PN/AMX processes performance?

Besides the low nitrogen concentration, the presence of organic matter has been found to influence the PN/AMX processes efficiency as it promotes the overgrowth of heterotrophic denitrifying bacteria (Xu et al. 2015). Thus, the optimisation of the previous organic matter removal step, either via wastewater anaerobic digestion (AD) or chemical oxygen demand (COD) pre-concentration to be treated in the sludge AD, is crucial to improve the WWTP energy-efficiency and the stability of the PN/AMX processes performance.

In this thesis, the residual organic matter present in the wastewater was found to improve the nitrogen removal in both PN/AMX one-stage configuration treating blackwater (Chapter 3) and the two-stage configuration (Chapter 7) by polishing the final effluent. Nevertheless, organic matter is not always beneficial as indicated by other authors who reported difficulties in suppressing the heterotrophic denitrifying bacteria that compete with anammox by consuming the nitrite produced by AOB (Seuntjens et al. 2016).

Another parameter to consider is the alkalinity of the wastewater which needs to be considered while exploring alternatives for the NOB out competition. As an example, the success of the proposed strategy based on the *in-situ* FNA accumulation relies on the decrease of the pH value due to the alkalinity consumption (Chapter 5). If the ratio of nitrogen to inorganic carbon (IC) concentrations in the wastewater is below 0.6 g N/g IC, the system has enough buffering capacity to avoid the pH decrease. In these conditions, the *in situ* FNA accumulation strategy is not feasible and for this reason, pH control is not recommended at mainstream conditions. On the contrary, the strategies based on the residual ammonium concentration usually require to control the pH value over 8.0 (Reino et al. 2016) to suppress the NOB. When the wastewater contains low alkalinity, this strategy will imply the consumption of chemicals for pH adjustment. Similarly, the strategy based on the control of the aeration length by the change of the pH trend occurring after ammonium exhaustion (Yang et al. 2007) is not feasible if the wastewater has ratios over 0.6 g N/g IC, as the complete ammonium oxidation efficiency becomes limited by alkalinity.

The alkalinity of the wastewater mainly depends on the soil characteristics and to a less extent on the previous wastewater treatment stages. For example, when chemically enhanced pretreatment (CEPT) is used for organic matter removal, coagulant addition consumes alkalinity. Thus the effect of this pretreatment must be properly assessed as it could be favourable or not depending on the selected nitritation strategy.

Moreover, it has to be considered that the municipal wastewater characteristics experience large daily and seasonal fluctuations whereas more homogeneous wastewater composition is generated in source-separation decentralised treatment systems (Chapter 3). In Chapter 6 the effect of the fluctuations of wastewater composition on the nitritation SBR performance was evaluated and the operational cycle length was pointed out as the modifiable parameter to deal with them. The results from the anammox reactor indicated that it could withstand successfully these variable nitrogen concentrations (Chapter 7). At pilot-scale, in Chapter 9, the temperature changed together with the composition fluctuation but no effect was observed either on NOB activity suppression (nitritation unit) or final effluent composition (anammox unit). In the case of the anaerobically pretreated wastewater, used to feed the IFAS system, its composition was less variable probably due to the longer HRT applied in the anaerobic wastewater digester compared to that of the high rate activated sludge (HRAS) system used in Chapter 9. In addition, in the former case, a homogenisation tank was used to mitigate the composition fluctuations.

10.1.5. Which is the most effective reactor configuration for the implementation of the PN/AMX processes to treat domestic wastewater?

The best reactor configuration for the implementation of PN/AMX processes at mainstream conditions has not been identified yet. The most widely assessed configuration is the one-stage system where both processes take place simultaneously. This configuration was shown to be more robust and to have lower investment and operational costs than the two-stage one when it was tested to treat the supernatant of anaerobic sludge digesters (Lackner et al. 2014). In the case of decentralised source-separation systems, this configuration is still feasible since despite temperature is low, the blackwater fraction contains large nitrogen concentrations and the results obtained in terms of effluent quality are adequate

(Chapter 3). Nevertheless, as it was already mentioned, the suppression of the NOB activity in the one-stage systems treating municipal wastewater at low temperature is difficult.

Hybrid systems (i.e., IFAS) combine the advantages of the one-stage ones, but PN/AMX processes take place separately in different biomass fractions, suspended and biofilm ones (Chapter 8). Obtained results indicate that the IFAS systems are suitable to perform PN/AMX processes, but the aeration control required for NOB activity suppression limits the nitrite production and consequently the nitrogen removal. Moreover, air supply needs to be carefully controlled to avoid excessive DO penetration inside the biofilm that might hinder the anammox bacteria activity. This dependency does not exist in the two-unit system as the anammox process takes place separated from the partial nitritation one.

In the two-stage configuration, the residual organic matter is oxidised in the first nitritation unit and thus the anammox process is favoured in the second one while the heterotrophic denitrification is avoided (Chapter 7 and 9). In this separated configuration the accumulation of enough anammox biomass to compensate for the SA_{AMX} decrease due to the low temperature will be presumably easier as biomass selection is not needed in this reactor.

It is essential to highlight that there is not a unique optimal treatment train system that can be applied to all circumstances. Before selecting the alternative which fits the most to each case, several issues must be carefully analysed including regulation of discharge standards, temperature, municipal wastewater characteristics, land requirements, organic matter removal efficiencies, sludge production, etc. The limits fixed by regulations for effluent discharge and reuse vary to a greater or lesser extent, depending on countries and the purpose of polished wastewater reuse.

10.2. Research gaps and future perspectives

Although the present thesis contributes to advance on the understanding of the features of the autotrophic PN/AMX nitrogen removal processes in mainstream conditions, new research questions emerged. Furthermore, the objective of this thesis was to understand how the two-stage PN/AMX configuration adequate performance can be achieved at low temperature and nitrogen concentrations

starting from laboratory scale reactors, treating synthetic wastewater, and ending-up with a pilot-scale experience, treating municipal wastewater. Nevertheless, there are still many gaps of information that need to be addressed to have a complete and detailed overview of the possible application of the mainstream autotrophic nitrogen removal processes.

First, future studies should further evaluate the maximum achievable rates, especially for the two-stage PN/AMX processes treating municipal wastewater at low temperature. These experiments should be carried out at pilot scale in reactors dealing with the regular wastewater composition and temperature variability. Particular attention should be paid to the operation during wintertime when the most challenging conditions occurred. These fluctuations are expected to have a high impact on the process performance and scarce information about them is available nowadays (Agrawal et al. 2018).

Second, the control strategies for the implementation of these processes should be optimised and *in-situ* proven, since until now their application in these conditions is still unclear. Most of the research work available was performed under controlled conditions, and the control strategies might be not feasible under dynamic conditions (Jin et al. 2019).

Third, to elucidate the contribution of each different microbial group present in the biomass inside the reactors, molecular techniques such as metagenomics and metabolomics might be applied. The role of side-populations, as well as the participation of AOB and comammox under natural conditions, would give relevant information. Up to now, most of the studies were performed treating synthetic wastewater, which limits the microbial diversity and the understanding of the role of the different microbial populations under realistic conditions (Liu et al. 2018, Persson et al. 2017, Yang et al. 2018). Metagenomic and metabolomics will give new insights not only on the population abundance but also on the expressed genes.

Fourth, the greenhouse gases (GHGs) emissions produced in the new proposed WWTP configuration should be further evaluated. Concerning the GHG emitted from the PN/AMX systems, special attention should be put on the selection of the best operating conditions to minimise N_2O production. The N_2O emissions are, in general, related to the presence of AOB and high nitrite concentrations (Campos et al. 2016, Chen et al. 2018). In PN/AMX systems its emission factor ranged from 1.2 to 4.0 % of

the ammonium converted (Castro-Barros et al. 2015, Duan et al. 2018, Jiang et al. 2019, Wang et al. 2016b). However, contradictory information is available regarding those conditions that promote N_2O emissions. Among them are the one-stage versus two-stage configuration, feeding mode (Duan et al. 2018), pH value and temperature. Moreover, most of the available data were obtained from reactors operated at mesophilic conditions with one-stage configuration (Castro-Barros et al. 2015, Jiang et al. 2019). Further research is required in this aspect to reduce the uncertainty allowing the proper evaluation of the carbon footprint of PN/AMX systems at mainstream.

Last, future investigations should also evaluate the potential effect of the pretreatment technologies pursuing the energy recovery maximisation, and the effect of different inhibitory compounds over the SA_{AMX} at different temperatures as their inhibitory thresholds could be different (Daverey et al. 2015, Tomaszewski et al. 2017).

10.3. General conclusions

The general conclusions derived from the studies carried out in the present thesis are summarised below.

Blackwater treatment (Chapter 3)

- It is possible to treat anaerobically digested blackwater (120 mg TN/L and 100 mg COD/L) in a one-stage PN/AMX reactor at room temperature (17 ± 2 °C).
- High nitrogen removal efficiencies up to 95 % and concentrations in the effluent below 10 mg TN/L were achieved despite the starvation and reactivation periods imposed simulating the lack of wastewater in a decentralised wastewater treatment located in an office building.
- Biomass segregation was observed where anammox bacteria are enriched in the granules, whereas AOB are mainly active in the flocculent fraction.

AOB and NOB activities decouplement by the applied hydraulic load (Chapter 4)

- The use of long hydraulic loads (HRT < 4.5 days) in a chemostat promoted the growth of AOB over the NOB ones, allowing for the activation of the nitrite accumulation at 16 ± 1 °C and 50 mg TN/L.
- NOB were not successfully washed out being the nitrate the main product of the ammonium oxidation, amounting to 80 % of it conversion.
- Short HRT values need to be combined with other NOB suppressing factors to allow for the establishment of the nitritation process.

NOB suppression based on *in-situ* FNA production (Chapter 5)

- The nitritation process was established and its long-term operation maintained by *in-situ* FNA production at 16 ± 1 °C and 50 mg TN/L without dissolved oxygen concentration control.
- FNA concentrations over 0.02 mg HNO_2-N/L were used to maintain the nitritation process stable and to effectively wash out the NOB.
- Strategy robustness was proven taking more than 40 days to detect significant NOB activity inside the reactor after the decrease of the FNA concentration below the inhibitory threshold.
- The ratio of N/IC determines the feasibility of applying this strategy since if it is over 0.6 g N/g IC, other actions such as the pH value control would be required making it probably inappropriate from an economic point of view.

Nitritation and organic matter oxidation processes in the same unit (Chapter 6)

- The *in situ* FNA-based strategy succeeded to establish the nitritation process treating synthetic wastewater when the seeded sludge was enriched in AOB without requiring the addition of any chemical compound.
- Despite the presence of organic matter (up to 1.2 g TOC/g N) and the low operating temperature (15 ± 1 °C) nitritation process was maintained by producing FNA concentrations up to 0.2 mg HNO_2-N/L and treating municipal wastewater.

- Organic matter had no adverse effect over the nitritation process stability and it was removed by 80 % in the same unit.
- To cope with the changes of nitrogen and organic matter concentrations in the municipal wastewater, the optimisation of the length and distribution of the operational cycle is required. For example, with the increase of the reaction phase when the organic matter concentration increases.
- The ratio of N/IC determines the feasibility of applying this strategy since if it is over 0.6 g N/g IC, actions such as the pH value control will be required, making the FNA strategy inappropriate from an economic point of view.

Assessment of the anammox process performance (Chapter 7)

- An acclimation period to the low temperature (15 ± 1 °C) and low nitrogen concentration (50 mg TN/L) might be not needed when anammox biomass from sidestream conditions is used as inoculum, which will be shortening the start-up periods.
- Anammox process was maintained stable, treating both synthetic wastewater at decreasing alkalinities and nitritified primary settled wastewater reaching a TN concentration in the effluent below 10 mg TN/L.
- The presence of residual organic matter in the influent of the anammox unit contributed to the increase of the nitrogen removal efficiency from 80 % to 90 %. Problems of heterotrophic denitrifying bacteria overgrowth were not observed because the limited organic matter/nitrogen ratio in the influent (approximately 0.5 g TOC/g TN).
- The temperature of operation (15 °C) decreased the SA_{AMX} of the inoculated biomass. However, then the anammox activity increased during the reactor operation with synthetic wastewater. When municipal wastewater was fed, SA_{AMX} slightly decreased (20 %) due to the presence of heterotrophic denitrifying bacteria.
- SA_{AMX} is highly influenced by pH and its effect is also affected by temperature.

One stage PN/AMX processes in an IFAS system (Chapter 8)

- It is feasible to treat anaerobically pre-treated municipal wastewater in a one-stage PN/AMX process in an IFAS reactor at decreasing low temperature (from 21 to 15 °C).
- Average nitrogen removal efficiencies of 72 ± 11 % were achieved and the process stability was maintained while average NRR of 37 ± 3 mg TN/(L·d) were obtained at 15 °C.
- The successful suppression of most NOB activity was achieved by applying intermittent aeration, accounting for only 10 - 20 % of its maximum capacity.
- Microbial population segregation was observed being the AOB and NOB more abundant in the flocculent biomass while the biofilm was mostly enriched on anammox bacteria.
- NOB are also integrated within the biofilm biomass and further optimisation to control the nitrate production is required.

Testing two-stage PN/AMX at pilot scale (Chapter 9)

- A pilot-scale two-stage PN/AMX plant was successfully operated for the first time without applying temperature control (11 – 28 °C) and fed with wastewater pre-treated in a high rate activated sludge (HRAS) system (22 – 63 mg NH_4^+- N/L).
- Nitritation process was successfully achieved and maintained, despite the fluctuating wastewater characteristics, by applying the *in situ* FNA based strategy.
- A variable aeration phase length (controlled by the pH value) allowed to maintain the process stability obtaining the adequate nitrite to ammonium ratio of 1.2 mg NO_2^--N/g NH_4^+-N in the produced effluent to be fed to the anammox reactor.
- Anammox process was quickly established in an open pilot-scale reactor despite the low biomass concentration inoculated (0.4 g VSS/L) achieving a NRR of 47 ± 7 mg TN/(L·d) limited mainly by the HRT required for the successful biomass retention in the nitritation unit.

- The quality of the effluent produced in the system was not enough for discharge as it contained 12 ± 3 mg TN/L. Its composition was limited by the nitrate produced in the HRAS system (approximately 5 mg NO_3^--N/L) that entered the PN/AMX system where it was not removed.

10.4. References

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