GREENHOUSE GAS EMISSIONS FROM WASTEWATER TREATMENT PROCESSES: IDENTIFYING TRIGGERING FACTORS AT LABORATORY AND FULL-SCALE SYSTEMS

Anna Ribera Guàrdia

Doctoral thesis

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Supervisor: Dr. Maite Pijuan Vilalta

Co-supervisor: Dr. Oriol Gutiérrez Garcia-Moreno

Tutor: Ignasi Rodríguez-Roda

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La Dra. Maite Pijuan Vilalta i el Dr. Oriol Gutierrez Garcia-Moreno, investigadors de l'institut Català de Recerca de l'Aigua (ICRA) i el Dr. Ignasi Rodríguez-Roda Layret, professor del Departmanet d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona (UdG),

DECLAREM:

Que el treball titulat **Greenhouse gas emissions from wastewater treatment processes:identifying triggering factors at laboratory and full-scale systems**, que presenta la llicenciada en Enginyeria Química, **Anna Ribera Guàrdia** per a l'obtenció del títol de doctora, ha estat realitzat sota la direcció de la **Dra. Maite Pijuan Vilalta** i el **Dr. Oriol Gutiérrez Garcia-Moreno**.

I, perquè així consti i tingui els efectes oportuns, signem aquest document.

Girona, 17 de juliol de 2017

Dra. Maite Pijuan Vilalta Dr. Oriol Gutiérrez Garcia-Moreno Dr. Ignasi Rodriguez-Roda Layret

Agraïments

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Table of Contents

List of publications:

Anna Ribera-Guardia, Elissavet Kassotaki, Oriol Gutierrez; Maite Pijuan. 2014. Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. Process Biochemistry **49** (12), 2228-2234.

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A. Vieira, **A. Ribera-Guardia**, R. Marques, M.T. Crespo, A. Oehmen, G. Carvalho, 2017. The link between the microbial community, gene expression and biokinetics of denitrifying polyphosphate-accumulating systems under different electron acceptor combinations. (submitted to Applied Microbiology and Biotechnology)

List of acronyms:

DAF-FM DA	4-amino-5-methylamino-2′,7-difluorofluorescein diacetate
ASP	Activated sludge process
ASR	Activated sludge reactor
ATP	Adenosine triphosphate molecule
ATU	Allylthiourea
NH_3	Ammonia
AOR	Ammonia oxidation rate
AORsp	Ammonia oxidation specific rate
NH_4^+	Ammonium
AOB	Ammonia oxidizing bacteria
BNR	Biological nutrient removal
BOD	Biological oxygen demand
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CFCs	Chlorofluorocarbons
dPAOs	Denitrifying polyphosphate accumulating organisms
dGAOs	Denitrifying glycogen accumulating organisms
N_2	Dinitrogen
N_2O_4	Dinitrogen teatroxide
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
FID	Flame ionization detector
FISH	Fluorescence *in situ* hybridisation
FA	Free ammonia
FNA	Free nitrous acid
GC	Gas chromatography
GC-ECD	GC–electron capture detector
GC-MS	GC–mass spectrometry

GHG	Global greenhouse gas
GWP	Global warming potential
GAOs	Glycogen accumulating organisms
H_2	Hydrogen
H_2S	Hydrogen sulphide
HCL	Hydrochloric acid
HRT	Hydraulic retention time
HFCs	Hydrifluorocarbons
NH_2OH	Hydroxylamine
HAO	Hydroxylamine oxidoreductase
IPCC	Intergovernmental panel on climate change
Bo	Maximum methane production capacity
CH_4	Methane
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NO_3^-	Nitrate
NaR	Nitrate reductase
NO	Nitric oxide
NoR	Nitric oxide reductase
NO_2^-	Nitrite
NOB	Nitrite oxidizing bacteria
NiR	Nitrite reductase
N	Nitrogen
NO_2	Nitrogen dioxide
NOx	Nitrogen oxide species
NOH	Nitrosyl radical
N_2O	Nitrous oxide
N2Osp	Nitrous oxide production specific rate
NoS	Nitrous oxide reductase
PO_4^{-3}-P	Orthophosphate

O_2	Oxygen
PFCs	Perfluorocarbons
PBS	Phosphate Buffer Solution
P	Phosphorus
PHA	Polyhydroxyalkanoate
PAOs	Polyphosphate accumulating organisms
PLC	Programmable logic controller
RAS	Returned activated sludge
SBR	Sequencing batch reactor
SRT	Solid retention time
SDNR	Specific denitrification rate
NaOH	Sodium hydroxide
SF_6	Sulphur hexafluoride
TKN	Total kjeldahl nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
TSS	Total suspended solids
TCA	Tricarboxylic acid cycle
VFAs	Volatile fatty acids
WWTP	Wastewater treatment plant

List of figures:

as single electron acceptors respectively (● NO_3^-, ○NO_2^-, and -N_2O).*Notice the different scale of the x-axis. It is due to the velocity of the reduction of each of the electron acceptors added depending on the batch tests.

Figure 4.3: Nitrate, nitrite and nitrous oxide specific reduction rates using acetate (a), ethanol (b) and methanol (c) respectively, with error bars showing the standard deviation associated (● NO_3^-, ○NO_2^-, and ▼N_2O).*Notice the different scale of the y-axis in the case of methanol.

Figure 4.4: Nitrate, nitrite and nitrous oxide profiles for batch tests type F using acetate (a) and ethanol (b) respectively (● NO_3^-, ○NO_2^-, and -N_2O).

Figure 4.5: Nitrate, nitrite and nitrous oxide specific reduction rates using a combination of the three carbon sources with error bars showing the standard deviation associated (● NO_3^-, ○ NO_2^-, and ▼ N_2O).

Figure 4.6: Nitrate, nitrite, nitrous oxide and COD profiles for batch type D using acetate (a) and ethanol (b) (● NO_3^-, ○NO_2^-, -N_2O and ◊ COD). Zone A refers to the time when external carbon is available and zone B refers to the time when external carbon is limited. In the case of ethanol there is a zoom for the N_2O accumulation.

Figure 5.1: Experimental acetate (■), propionate (□), phosphate (▽), nitrate (●), and nitrite (○) profiles analyzed during a typical cycle study conducted in the dPAO (a) and dGAO (b) reactors.

Figure 5.2: FISH images of the enriched dGAO biomass (left) and enriched dPAO biomass (right) used in the batch tests. In blue is shown EUBMIX (all bacteria) and in magenta is shown GAOMIX and PAOMIX.

Figure 5.3: Nitrate (●), nitrite (○), and N_2O (-) profiles for batch tests A, B and C for dPAO (a) and dGAO (b) cultures. The arrows represent the moment when NOx was added. Notice the different N_2O axis scale in Tests B compared with Tests A.

Figure 5.4: Nitrogen oxides reduction rates (● NO_3^-, ○NO_2^-, and ▼N_2O) for dPAOs (a) and dGAOs (b) cultures.

Figure 5.5: Electron consumption rates (a and b) and electron distribution (c and d) for nitrate reductase (NaR ▭), nitrite reducyase (NiR ▭) , nitric oxide reductase

(NoR ▭) and nitrous oxide reducatse (NoS ▭) for dPAO (left) and dGAO (right) cultures.

Figure 6.1: Experimental profiles of N_2O (—), NH_4^+ (●), NO_2^- (○), NO (─), DO (···) and pH (···) during a typical cycle study of the AOB reactor. Nitrate was not detected in any of the samples taken.

Figure 6.2: Experimental profiles of NO (─), N_2O (—) and NH_4^+ (●) at pH 7 and DO=1.5-2 mg O_2/L. The arrows represent the time when a pulse of ammonia was added. Nitrate was not detected in any of the samples taken.

Figure 6.3: Correlation between the specific nitric oxide production rate (a) and the specific nitrous oxide production rate (b) with the specific ammonia oxidation rate.

Figure 6.4: Biomass stained with the DAF-FM DA fluorescence probe.

Figure 6.5: Experimental profiles of N_2O (—), NH_4^+ (●), NO_2^- (○), NO (─), DO (···) and pH(···) during set 2 of tests: DO decreasing from 3 to 0.5 mgO_2/L (a) and increasing from 0.5 to 3 mgO_2/L (b). Nitrate was not detected in any of the samples taken.

Figure 6.6: Experimental profiles of N_2O (—), NO_2^- (○), NO (─), DO (···) and pH (···) of batch test 2.3: when DO was 0 mg O_2/L. Nitrate was not detected in any of the samples taken.

Figure 6.7: Experimental profiles of N_2O (—), NH_4^+ (●), NO (─), and pH (···) at DO=1.5-2 mg O_2/L in batch test 3.1: while pH is decreasing from 8 to 6.5. Nitrate was not detected in any of the samples taken.

Figure 6.8: Experimental profiles of NO (─), N_2O (—) and pH (···) at DO=1.5-2 mg O_2/L of batch tests 3.2 and 3.3: pH decreasing from 8 to 6.5 without ammonia but with biomass (a) and without biomass (b).

Figure 7.1: Plug-flow reactor configuration and zone of study. The black dots represent the plant DO sensors located in aeration zone 1 and aeration zone 4. The squares represent the online ammonia sensors at the inlet of the plug-flow reactor and in aeration zone 2. The white dots represent the place where the gas hoods were placed. The arrows represent the direction of the wastewater flow.

Figure 7.2: N_2O emissions from aerobic zones 1, 2 and 3 of the plug-flow reactor in November (left), January (center) and March (right).

Figure 7.3: CH_4 emissions from aeration zones 1, 2 and 3 from the plug-flow reactor in November (left), January (center) and March (right).

Figure 7.4: Typical ammonium (─) and N_2O patterns (┄) in the aerobic zone 2 of the plug-flow reactor found during the monitoring period of November (a) and the monitoring period of March (b).

Figure 7.5. Daily N_2O (┄), ammonium (●), nitrite (▲) and nitrate (○) concentration profiles measured in aerobic zone 2 measured in the 7th and 8th of March.

Figure 7.6: Electricity consumption (a) and direct (N_2O ▬, CH_4 ▨ and total direct emissions ▨) and indirect CO_2 emissions (▩)(b) from the plug-flow reactor of the WWTP along the monitoring period.

Figure SI.1: Correlation between the specific NO production rate and ammonia concentration (a) and the specific N_2O production rate and the ammonia concentration (b).

Figure SI.2: Correlation between ammonia oxidation rate and the different ammonia concentrations.

Figure SI.3: Experimental profiles of N_2O (┄), NO_2^- (○), NO (─) and pH (···) of batch test 3.4: with distilled water and changing the pH set point from 8 to 7. Nitrate was not detected in any of the samples taken.

Figure SI.4: Experimental profiles of N_2O (┄), NO_2^- (○), NO (─) and pH (···) of batch test 3.5: without biomass and adding base (NaOH) and HCL to see the effect on NO production. Nitrate was not detected in any of the samples taken.

Figure SI.5: Profile of the temperature of the wastewater from November till late February of aeration zone 2 (-) and influent of the reactor (-).

Figure SI.6: Daily pattern of N_2O (┄), ammonium (●), nitrite (▲) and nitrate (○) concentration profiles measured in aerobic zone 2 measured in the 8th and 9th of March (a) and aeration zone 3 measured in the 2nd and 3rd of March (b).

List of tables:

Summary:

In the last decades, global greenhouse gas (GHG) emissions have increased due to human activities. Climate change and acceleration of the global warming are the consequences of this increase. Lately it has been reported in scientific studies the relevance of GHG emissions from wastewater treatment plants (WWTP). Nitrous oxide (N_2O) and methane (CH_4) are the main GHG directly emitted from these systems. Nitric oxide (NO) can also be emitted during wastewater treatment and it is a potent ozone-depleting compound and toxic for living organisms. N_2O and CH_4 have a global warming potential of 298 and 21 times higher than CO_2. Therefore, even low levels of emissions of these gases can be important and increase the overall carbon footprint of a WWTP.

The production of N_2O and NO is still under debate. These gases can be emitted through the nitrification and denitrification process. During nitrification they can be produced when ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOBs) and during denitrification they are produced in the reduction of nitrate to dinitrogen gas. On the other hand, methane can be produced under anaerobic conditions when organic matter is degraded. GHG emissions should be determined and mitigation strategies should be implemented in WWTP in order to reduce their impact.

In this thesis, different studies were performed in order to identify some of the factors triggering N_2O and NO production during nitrification and denitrification in wastewater. The N_2O production when using different combination of electron acceptors during denitrification was studied. Firstly, three different external carbon sources (acetate, ethanol and methanol) where used in a mixed denitrifying culture. Secondly, a denitrifying polyphosphate accumulating organism (dPAO) and a denitrifying glycogen accumulating organism (dGAO) enriched cultures were used to assess the effect of using an internal carbon source (polyhydroxyalkanoates, PHA) for denitrification on the N_2O production. Results indicated that electron competition during the reduction of different nitrogen oxides is a significant factor in ordinary heterotrophic denitrification processes using external carbon sources as the electron donor, but not in PHA-driven denitrification processes conducted by dPAO or dGAO. Results also showed that generally, higher N_2O accumulation was detected in the tests conducted with dGAO

than those conducted with dPAO, especially when nitrite was used as electron acceptor. Therefore, special attention is needed on those systems were the nitrite pathway is promoted since the abundance of dGAO will not only affect the effectiveness of the P removal process of the plant but also will most likely increase its overall N_2O emissions.

Later, the factors affecting N_2O and NO production in a partial nitrification sequencing batch reactor (SBR) were studied. The effect of DO at a constant pH level and the effect of pH at a constant DO level were explored. Also, the relationship between NO production and the ammonia oxidation rate (AOR) as well as the N_2O production rate and the AOR were studied. Results showed that these relationships were linear and exponential, respectively. This investigation highlighted the importance of also monitoring NO emissions since they may lead to N_2O emissions.

The last investigation of this thesis was a long-term full-scale study in the WWTP of Girona in order to assess the N_2O and CH_4 emission dynamics of one of its plug-flow reactors. Results showed seasonal and spatial variations on N_2O emissions on the plug-flow reactor but only spatial variations on CH_4 emissions. Temperature seemed to affect the nitrification process leading to zero N_2O emissions in winter time and higher emissions during autumn and spring time. On the other hand, methane was dissolved in the wastewater and was coming mainly from the inlet wastewater and the reject wastewater stream. Once it entered the aeration tanks of the plug-flow reactor it was stripped and emitted to the atmosphere. Finally, the direct emissions quantified during the study were compared with the indirect CO_2 emissions coming from the energy consumption and the overall carbon footprint of the plug-flow reactor was assessed.

Resum:

En les últimes dècades els gasos d'efecte hivernacle (GEH) han augmentat degut a les activitats humanes. El canvi climàtic i l'acceleració de l'escalfament global són les conseqüències d'aquest increment. Últimament s'ha comunicat en els estudis científics la rellevància de les emissions dels GEH de les estacions depuradores d'aigua residual (EDAR). L'òxid nitrós (N_2O) i el metà (CH_4) són els principals GEH emesos directament per aquest tipus de sistemes. L'òxid nítric (NO) també pot ser emès durant el tractament de les aigües residuals i és un potent compost i destructor de la capa d'ozó i tòxic per als organismes vius. El N_2O i el CH_4 tenen un coeficient d'escalfament global de 298 i 21 vegades major que el diòxid de carboni (CO_2). Per tant, fins i tot a nivells baixos d'emissions aquests gasos poden ser importants i augmentar la petjada de carboni total d'una EDAR.

La producció de N_2O i NO encara està en debat. Aquests gasos es poden emetre a través dels processos de nitrificació i desnitrificació. En el procés de la nitrificació es poden produir quan l'amoni s'oxida a nitrit pels bacteris amoni oxidants (AOBs) i en el procés de la desnitrificació, durant la reducció del nitrat o nitrit a nitrogen gas. Per una altra banda, el metà es pot produir en condicions anaeròbies quan la matèria orgànica es degrada. Les emissions de GEH s'haurien de determinar i s'haurien d'implementar estratègies de mitigació en les EDARs per tal de reduir el seu impacte.

En aquesta tesi s'han realitzat diferents estudis per a identificar alguns dels factors que desencadenen la producció de N_2O i NO durant la nitrificació i la desnitrificació en les aigües residuals. Es va estudiar la producció de N_2O usant diferents combinacions d'acceptors d'electrons durant la desnitrificació. En primer lloc es varen utilitzar tres fonts de carboni externes (acetat, etanol i metanol) en un cultiu mix desnitrificant. En segon lloc es va utilitzar un cultiu enriquit amb organismes desnitrificants acumuladors de fòsfor (dPAO) i un cultiu enriquit amb organismes desnitrificants acumuladors de glicogen (dGAO) per tal d'avaluar l'efecte d'utilitzar una font de carboni interna (polihidroxialcanoats, PHA) per la desnitrificació en la producció de N_2O. Els resultats van indicar que la competició d'electrons durant la reducció dels diferents òxids de nitrogen és un factor significatiu en els processos de desnitrificació heterotròfica ordinària utilitzant fonts de carboni externes com a donador d'electrons però no en els

processos de desnitrificació utilitzant PHA com a font de carboni interna com en els dPAO i dGAO. Els resultats també varen demostrar que generalment en els experiments realitzats amb els dGAOs es detectava una acumulació major de N_2O que en els experiments amb dPAOs, especialment quan s'utilitzava nitrit com a acceptor d'electrons. Per tant, es necessita una atenció especial en aquells sistemes en els que es promogui la via del nitrit ja que l'abundància dels dGAOs no només afectarà l'efectivitat del procés d'eliminació de fòsfor sinó que també probablement augmentarà les seves emissions globals de N_2O.

Més endavant es varen estudiar els factors que afecten la producció de N_2O i NO en la nitrificació parcial en un reactor discontinu seqüencial (SBR). Es va explorar l'efecte del oxigen dissolt (DO) a un nivell constant de pH i l'efecte del pH a un nivell constant de DO. També es va estudiar la relació entre la producció de NO i la velocitat d'oxidació d'amoni (AOR) així com la producció de N_2O i la AOR. Els resultats van mostrar que les relacions eren lineal i exponencial, respectivament. Aquesta investigació va destacar la importància de supervisar també les emissions de NO, ja que poden conduir a emissions de N_2O.

L'última investigació d'aquesta tesi va ser un estudi a l'EDAR de Girona per a avaluar la dinàmica d'emissions de N_2O i CH_4 en un dels seus reactors de flux pistó. Els resultats van mostrar les variacions estacionals i espacials en les emissions de N_2O en el reactor de flux pistó però només variacions espacials en les emissions de CH_4. La temperatura sembla que afecta el procés de nitrificació, originant zero emissions de N_2O a l'hivern i incrementant aquestes emissions durant la tardor i la primavera. Per altra banda, el metà estava dissolt en l'aigua residual de l'entrada de planta i en l'aigua de rebuig provinent del digestor anaerobi de l'EDAR. Una vegada l'aigua residual entra en les zones d'aireació del reactor de flux pistó, el CH_4 s'emet a l'atmosfera. Finalment, es van comparar les emissions directes amb les emissions de CO_2 indirectes causades pel consum d'energia i es va avaluar la petjada de carboni global del reactor flux pistó.

Resumen:

En las últimas décadas los gases de efecto invernadero (GEI) han aumentado debido a las actividades humanas. El cambio climático i la aceleración del calentamiento global son las consecuencias de este incremento. Últimamente se ha comunicado en los estudios científicos la relevancia de las emisiones de los GEI de las estaciones depuradoras de aguas residuales (EDAR). El óxido nitroso (N_2O) y el metano (CH_4) son los principales GEI emitidos directamente por este tipo de sistemas. El óxido nítrico (NO) también puede ser emitido durante el tratamiento de agua residual i es un potente compuesto destructor de la capa de ozono y tóxico para los organismos vivos. N_2O y CH_4 tienen un coeficiente de calentamiento global de 298 y 21 veces mayor que el dióxido de carbono (CO_2). Por lo tanto, incluso niveles bajos de emisiones de estos gases pueden ser importantes y aumentar la huella de carbono total de una EDAR.

La producción de N_2O y NO aún está en debate. Estos gases pueden ser emitidos a través de los procesos de nitrificación y desnitrificación. En el proceso de nitrificación se pueden producir cuando el amonio se oxida a nitrito por las bacterias amonio oxidantes (AOBs) y en el proceso de desnitrificación durante la reducción del nitrito a nitrógeno gas. Por otra parte, el metano se puede producir en condiciones anaerobias cuando la materia orgánica se degrada. Las emisiones de GEI deben ser determinadas y las estrategias de mitigación deben ser implementadas en las EDARs para reducir su impacto.

En esta tesis se han realizado diferentes estudios para identificar algunos de los factores que desencadenan la producción de N_2O y NO durante la nitrificación y desnitrificación en aguas residuales. Se estudió la producción de N_2O usando diferentes combinaciones de aceptores de electrones durante la desnitrificación. En primer lugar, se utilizaron tres fuentes de carbono externas (acetato, etanol y metanol) en un cultivo mixto desnitrificante. En segundo lugar, se utilizó un cultivo enriquecido con organismos desnitrificantes acumuladores de fósforo (dPAO) y un cultivo enriquecido con organismos desnitrificantes acumuladores de glicógeno (dGAO) para evaluar el efecto del uso de una fuente interna de carbono (polihidroxialcanoatos, PHA) para la desnitrificación en la producción de N_2O. Los resultados indicaron que la competición de electrones durante la reducción de los diferentes óxidos de nitrógeno es un factor

significativo en los procesos de desnitrificación heterótrofa ordinaria utilizando fuentes externas de carbono como donador de electrones, pero no en los procesos de desnitrificación que utilizan PHA como fuente de carbono interna como en los dPAO y dGAO. Los resultados también mostraron que generalmente en los experimentos realizados con los dGAO se detectaba una acumulación mayor de N_2O que en los experimentos con los dPAO, especialmente cuando se usaba nitrito como aceptor de electrones. Por lo tanto, se necesita una atención especial en aquellos sistemas en los que se promueva la vía del nitrito ya que la abundancia de los dGAO no solo afectará la efectividad del proceso de eliminación de fósforo, sino que también probablemente aumentará sus emisiones globales de N_2O.

Más adelante se estudiaron los factores que afectan la producción de N_2O i NO en la nitrificación parcial en un reactor discontinuo secuencial (SBR). Se exploraron el efecto del oxígeno disuelto (DO) a un nivel constante de pH y el efecto del pH a un nivel constante de DO. También se estudió la relación entre la producción de NO i la velocidad de oxidación del amonio (AOR) así como la producción de N_2O i la AOR. Los resultados mostraron que estas relaciones eran lineal y exponencial, respectivamente. Esta investigación destacó la importancia de supervisar también las emisiones de NO, ya que pueden conducir a emisiones de N_2O.

La última investigación de esta tesis fue un estudio en la EDAR de Girona para evaluar la dinámica de emisión de N_2O y CH_4 en uno de sus reactores de flujo pistón. Los resultados mostraron variaciones estacionales y espaciales en las emisiones de N_2O en el reactor de flujo pistón pero sólo variaciones espaciales en las emisiones de CH_4. La temperatura pareció afectar el proceso de nitrificación que condujo a cero emisiones de N_2O en invierno y un aumento de emisiones durante otoño y primavera. Por otro lado, el metano estaba disuelto en el agua residual i venía principalmente de la entrada de planta y del flujo de agua de rechazo. Una vez este entra en los tanques de aireación del reactor de flujo continuo es emitido a la atmosfera. Finalmente, las emisiones directas cuantificadas durante el estudio fueron comparadas con las emisiones de CO_2 indirectas debidas al consumo de energía y se evaluó la huella de carbono global del reactor de flujo continuo.

BLOCK I - LITERATURE REVIEW, AIMS AND RESEARCH APPROACH

Chapter 1

Chapter 1 General Introduction

1.1 Global warming

Global greenhouse gas (GHG) emissions from human activities have increased since pre-industrial times, and this increase is the main driving cause of climate change. Humans enhance the greenhouse effect directly by emitting large uncontrolled amounts of greenhouse gases into the atmosphere (IPCC, 2013). Different factors reflect how strongly greenhouse gases affect the climate of Earth such as, the time that the gas will remain in the atmosphere or its ability to absorb energy. Considering these factors, the global warming potential can be calculated as compared to an equivalent mass of carbon dioxide which is equal to 1 (US EPA, 2013a). The main GHG are detailed below:

- Carbon dioxide (CO_2) is the largest contributor to climate change and it is emitted primarily through the burning of fossil fuels (oil, natural gas, and coal), solid waste, trees and wood products. Changes in land use have also a contribution to the total emissions. Deforestation and soil degradation add CO_2 to the atmosphere, while forest regrowth captures CO_2 from the atmosphere.

- Methane (CH_4) emissions result from livestock and agricultural practices and from the anaerobic decay of organic waste in municipal solid waste landfills (US EPA, 2013a). CH_4 is also produced during anaerobic digestion of sludge produced in the treatment of wastewater. This CH_4 can be reclaimed and combusted to eliminate the greenhouse gas effect obtaining energy. Its global warming potential is 21 times higher than carbon dioxide (IPCC, 2014b).

- Nitrous oxide (N_2O) is produced by biological processes that occur in soil and water and by a variety of anthropogenic activities in the agricultural, energy-related, industrial, and waste management fields. N_2O is also produced and emitted from biological nutrient removal (BNR) systems during wastewater treatment as has been reported by different researchers (Hanaki et al., 1992; VonSchulthess et al., 1994). While total N_2O emissions are much lower than CO_2 emissions, N_2O is approximately 298 times more powerful than CO_2 at trapping heat in the atmosphere (IPCC, 2014b), constituting the third most important greenhouse gas after CO_2 and CH_4. It is also known to be involved in the depletion of the ozone layer together with nitric oxide (NO) (Ravishankara et al., 2009). Its high increase rate (0.73 ± 0.03 ppb/yr over the last three decades) has enhanced the attention on its sources and sinks to study its effects more in detail (IPCC, 2014b).

- Fluorinated gases are a group of gases that includes hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), and sulphur hexafluoride (SF_6), among other chemicals. These gases are emitted from a variety of industrial processes and commercial and household uses, and do not occur naturally. Sometimes are used as substitutes for ozone-depleting substances such as chlorofluorocarbons (CFCs). Their persistence into the atmosphere are from a few weeks to thousands of years and the global warming potential varies depending on each specific gas (US EPA, 2013a).

Figure 1.1 depicts the increase detected over the last 250 years on the emissions of CO_2, CH_4 and N_2O. The atmospheric abundance of CO_2 was 390.5 ± 0.2 ppm in 2011; this is 40% greater than before 1750. Atmospheric N_2O was 324.2 ± 0.2 ppb in 2011 and has increased by 20% since 1750. Indeed, concentrations of N_2O have continued to increase at a nearly constant rate since about 1970 (IPCC, 2013). Average annual increases in CO_2 and N_2O from 2005 to 2011 are comparable to those observed from 1996 to 2005. Atmospheric CH_4 was 1803.2 ± 2.0 ppb in 2011; this is 150% greater than before 1750. CH_4 began increasing in 2007 after remaining nearly constant from 1999 to 2006 (Figure 1.1). HFCs, PFCs, and SF_6 all continue to increase relatively rapidly, but their contributions to radiative forcing are less than 1% of the total greenhouse gases. Radiative forcing is a measure of the net change in the energy balance in response to an external perturbation. The drivers of changes in climate can include, for example, changes in the solar irradiance and changes in atmospheric trace gas and aerosol concentrations (IPCC, 2013). Therefore, unravelling the sources of the GHG and implementing strategy mitigations is of great importance.

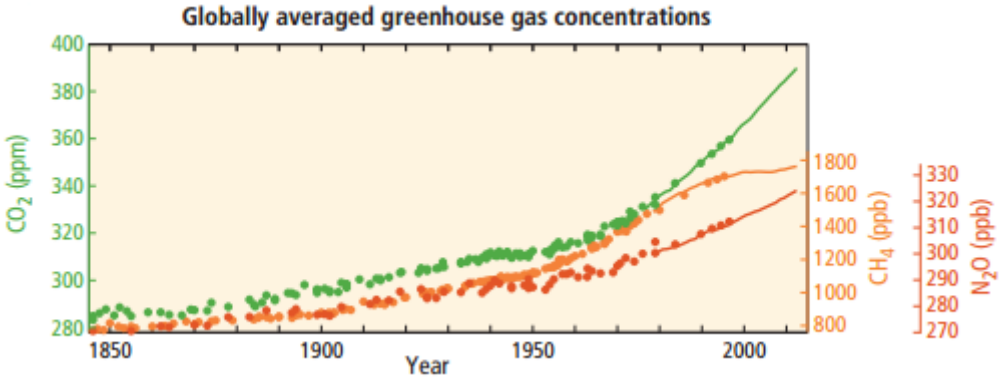

Figure 1.1: Atmospheric concentrations of CO_2, CH_4, and N_2O determined from air enclosed in ice core data (dots) and from direct atmospheric measurements (lines) (IPCC, 2014a).

1.2 Direct GHG emissions from wastewater treatment

The quantification of direct GHG emissions from wastewater treatment systems is currently based on the application of estimation methodologies that have been published by the Intergovernmental Panel on Climate Change (IPCC, 2013). These estimations, however, are considered highly uncertain for certain sectors such as the waste and wastewater treatment, being based in single-case studies were very limited data was provided.

When assessing direct GHG emissions from wastewater treatment systems, only CH_4 and N_2O are considered. CO_2 is produced indirectly as a result of fossil fuel combustion to generate the energy required for the operation of a wastewater treatment plant (WWTP), or it is produced directly during the respiration of organic matter. In the latter case it concerns short-cycle CO_2 that does not contribute to increased atmospheric CO_2 concentrations (Daelman et al., 2012). Also, CO_2 is assumed to originate from biogenic material and therefore it is excluded from greenhouse gas inventories (IPCC, 2013). International guidance on N_2O and CH_4 emissions from wastewater systems is presently inadequate for the advanced BNR process configurations being used in many developed countries. Furthermore, there is a lack of comprehensive studies on full- scale WWTPs that would allow for better characterization of the N_2O and CH_4 emissions potentially occurring under different physical configurations and treatment performance requirements (Foley et al., 2010a). Moreover, there is very limited data on CH_4 and N_2O emissions from full-scale wastewater treatment systems, and the process conditions that trigger gas production and emission in WWTPs are still under investigation (Rodriguez-Caballero et al., 2014).

Since 2006, the IPCC Guidelines include a N_2O estimation methodology to account for the direct emissions of this gas occurring within the ‘‘controlled nitrification and denitrification steps during wastewater treatment’’(IPCC, 2006). The proposed default emission factor is 0.0032 kgN_2O/person· year (uncertainty range: of 0.002–0.008) and is based on one full-scale study conducted by Czepiel et al., (1995) on a basic secondary treatment plant in New Hampshire, USA. However, the process description in this study is not sufficient to determine the extent of nitrification– denitrification activity (if any), nor does it seem reasonable to extrapolate the very low result from this one plant for use

as an international default N_2O emission factor for biological nutrient removal WWTPs (Foley et al., 2010b).

In the case for methane, the IPCC Guidelines comprises a CH_4 emission factor calculation based on the maximum CH_4 producing capacity (B_o) of the wastewater treatment system and a methane correction factor that depends on the treatment system. The good practice is to use country-specific data for B_o, where available, expressed in terms of kg CH_4/kg COD removed to be consistent with the activity data. If country-specific data are not available, a default value of 0.25 kg CH_4/kg COD can be used (Doorn et al., 2006). These values are based on a report by Doorn et al., (1997) that summarized the findings of field tests and provided emission factors for CH_4 and N_2O from wastewater treatment. It also included country-specific activity data on industrial and domestic wastewater which was used to develop country-specific emission estimates for CH_4 and N_2O.

Overall the GHG emissions from the waste and wastewater sector are estimated to be around 3% of the total estimated emissions (IPCC, 2014b). There are other sectors such as industry, or electricity and heat production that account for more GHG emissions compared to the wastewater sector (18% and 25%, respectively). Although it would seem that it is a tiny percentage from the waste and wastewater, these GHG emissions are not irrelevant considering that wastewater is increasingly being treated.

1.3 Mechanisms of N_2O production during wastewater treatment

Untreated wastewater consists of water with waste discharged from residential, commercial and industrial establishment. Raw wastewater contains pathogenic microorganisms, toxic compounds and has higher organic, nitrogen (N) and phosphorus (P) content as compared to natural waters (Metcalf and Eddy., 2003). Discharging the raw wastewater directly to water bodies will have a negative effect on environment and public health so an appropriate treatment is required.

Wastewater treatment objectives have been focusing on aesthetic and environmental concerns, targeting reduction in biological oxygen demand (BOD), total suspended solids (TSS) and essentially biological nutrient (N and P) removal. With impending vulnerability of fresh water resources, increasing effort has also been placed into

undertaking advanced treatment of wastewater for safe return to drinking water supplies.

The process of biological nitrogen removal aims to convert the influent nitrogen into harmless nitrogen gas which returns to the atmosphere. Biological nitrogen removal is achieved in three main steps, namely mineralization (complex organic nitrogen compounds converted to ammonia (NH_3)), nitrification (microbial oxidation of NH_3 to nitrate (NO_3^-) under aerobic conditions with nitrite (NO_2^-) as an intermediate) and denitrification (NO_3^- reduction to dinitrogen (N_2) gas under anoxic conditions with NO_2^-, NO and N_2O as intermediates). NO and N_2O may accumulate during both nitrification and denitrification, resulting in their emission to the atmosphere.

The most frequently used, economical and sustainable process to remove P from wastewater is the enhanced biological phosphorus removal (EBPR) process. EBPR takes place under anaerobic and aerobic conditions. During anaerobic conditions P is released to the environment and in the subsequent aerobic phase there is a P uptake. This P uptake can also take place under anoxic conditions. During this process, NO and N_2O can also be produced and emitted to the atmosphere as it will be explained in the following sections.

1.4 Denitrification

Denitrification is performed by a very diverse group of microorganisms which couple oxidation of organic or inorganic substrates to reduction of NO_3^-, NO_2^-, NO, N_2O and then to N_2 under anoxic conditions. Four different enzymes are involved in the process: nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NoR) and nitrous oxide reductase (NoS) (Figure 1.2) (Zumft, 1997). Each enzyme uses a redox active metal cofactor, such as molybdenum for NO_3^- reduction, iron or copper for NO_2^- reduction, iron for NO reduction, and copper for N_2O reduction (Richardson et al., 2009).

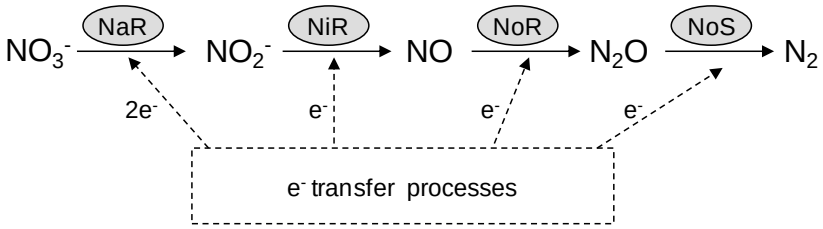

Figure 1.2: Nitrogen reduction steps and enzymes associated in denitrification.

The availability of electrons in organic carbon compounds is one of the most important factors controlling the activity of heterotrophs, which comprise the bulk of denitrifiers. In WWTP, when COD is limiting for complete N removal, methanol is commonly added as an external carbon source for denitrification mainly because of its cheap cost. However, other substrates such as ethanol or acetate are often added as electron donors in order to enhance the denitrification rates of the process. Constantine & Fick., (1997) found that the addition of acetic acid in a lab-scale denitrifying reactor resulted in higher denitrification rates as compared to when ethanol was added. Baytshtok et al., (2009) developed a denitrifying SBR with a methanol adapted culture and then switched the electron donor to ethanol. This study showed that the use of ethanol provided higher specific denitrification rates (SDNR) instead of methanol as carbon source for denitrification.

Denitrification can also be performed using internal storage compounds as carbon source. The EBPR process can perform denitrification under anoxic conditions. EBPR is mainly carried out by a group of bacteria known as polyphosphate accumulating organisms (PAO), and for the process to result in a net P removal, alternate anaerobic and aerobic/anoxic steps are needed. During anaerobic conditions PAO utilize an external carbon source to produce poly-β-hydroxyalkanoates (PHA) whilst hydrolyzing their intracellular poly-phosphate to obtain energy and releasing orthophosphates. In the aerobic phase PAO oxidize their stored PHA to generate the energy needed for orthophosphate uptake and to recover their intracellular poly-phosphate levels. The aerobic step can also be accomplished under anoxic conditions by a specific group of PAO, namely denitrifying PAO (dPAO), which can remove nitrogen and phosphorus simultaneously using NO_3^- or NO_2^- as electron acceptors (Figure 1.3a) (Kuba et al., 1996). This process can help to reduce the carbon requirements for nutrient removal and the energy consumption of WWTP. However, another group of bacteria, known as glycogen accumulating organisms (GAO), can also be found in this process. The

presence of GAO can lower the EBPR efficiency because they compete with PAO for the carbon substrates without performing phosphorus removal (Cech and Hartman., 1993; Whang and Park., 1999). GAO hydrolyze internal glycogen under anaerobic conditions to obtain energy for carbon uptake and storage as PHA. In aerobic conditions, GAO oxidize their internal PHA for cell growth and glycogen replenishment without phosphorus removal. Under anoxic conditions, the so-called denitrifying GAO (dGAO) can perform the same metabolism as in aerobic conditions but also achieving N removal through the denitrification process (Figure 1.3b).

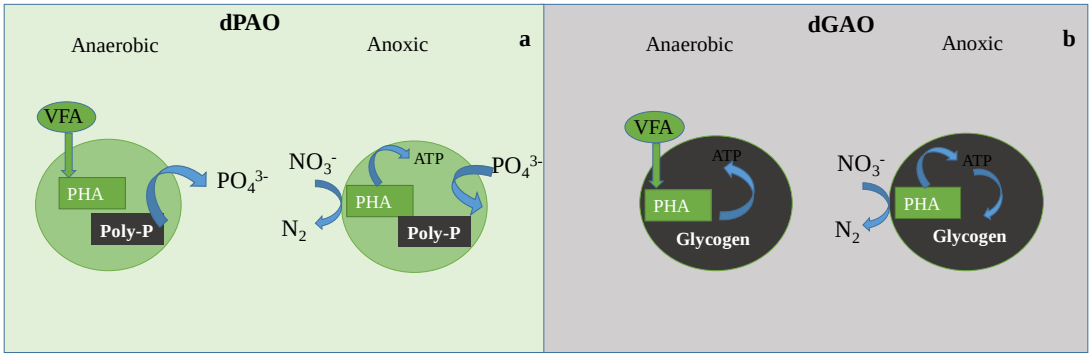

Figure 1.3: Metabolism of dPAO and dGAO.

N_2O is an intermediate compound in the denitrification process and its accumulation is strictly linked to the activity of the NoS enzyme. N_2O can accumulate due to two main reasons: i) when the majority of the denitrifying community does not possess the gene encoding for NoS, therefore having N_2O as the end product of denitrification; or ii) when nitrous oxide reduction rate is affected by a certain environmental or operational factor becoming lower than the nitrate or nitrite reduction rates. Several factors have been reported to lead to N_2O accumulation during denitrification to date:

- Oxygen (O_2): O_2 is known to inhibit both the synthesis and activity of denitrification enzymes (VonSchulthess et al., 1994). Also, it is known that NoS is more sensitive to oxygen than the other reductases. Although O_2 is not expected to be present in the anoxic parts of a WWTP, an over aeration on the aerobic tanks linked with a high internal recirculation might lead to the detection of certain concentrations of oxygen in the anoxic reactor, causing inhibition on the reduction of N_2O.

- pH: pH is known to have an effect on N_2O emissions. Hanaki et al., (1992) determined that N_2O accumulated at low pH in a lab-scale denitrifying culture using acetate and yeast extract as electron donors and NO_3^- as the final electron acceptor. N_2O production

at pH of 6.5 was significantly higher than that at pH of 7.5, although pH of 7.5 and 8.5 showed less difference. Later Thörn and Sörensson., (1996) determined an N_2O maximum production when the pH was between 5 and 6 in a pilot plant which was run as a nitrogen removal system with pre-denitrification in an anoxic basin followed by sedimentation. More recently, Pan et al., (2012) determined that substantial N_2O accumulation was observed at low pH levels (6.0-6.5) during denitrification likely due to electron competition among the four denitrification steps when electron supply from carbon oxidation was limited. Therefore the optimal pH range is considered to be 7.5-8.00.

- FNA/NO_2^-: Several studies have suggested that the presence of NO_2^- in the anoxic period could lead to N_2O accumulation. Zhou et al., (2008) demonstrated that free nitrous acid (FNA) rather than NO_2^- was the compound responsible for the inhibition detected in the N_2O reduction of an enriched dPAO culture. Pijuan and Yuan, (2010) also showed a higher accumulation of N_2O when NO_2^- rather that NO_3^- was present in the anoxic phase of an SBR reactor treating nutrient rich abattoir wastewater.

- Hydrogen sulphide or sulphide (H_2S): It is produced biologically in sewer pipes and could be introduced to the denitrification tank via the influent wastewater. H_2S is known to affect microbial activity in general since it is usually toxic to bacteria. Schönharting et al., (1998) suggested that H_2S in sewage could alter the activity of heterotrophic denitrification and lead to N_2O accumulation during biological wastewater treatment. Lately, Pan et al., (2013a) studied the potential inhibitory effects of H_2S on NO_3^-, NO_2^-, and N_2O reduction with a methanol-utilizing denitrifying culture. H_2S was found to be strongly inhibitory to N_2O reduction, with 50% inhibition. They also observed an N_2O accumulation during NO_3^- and NO_2^- reduction when concentrations were above 0.5 and 0.2 mg H_2S−S/L, respectively. Finally, they revealed that the protonated form of hydrogen sulphide (H_2S) was likely the true inhibitor of N_2O reduction, and the inhibitory effect was reversible.

- Copper: A deficiency of copper can lead to N_2O accumulation, since copper is necessary for the production of the enzyme Nos (Richardson et al., 2009). This has been reported for soils. Although there is not any study conducted in wastewater treatment systems it is not expected that copper limitation will play a major role on the N_2O

production of the plant since copper is one of the trace elements commonly found in wastewater.

- External carbon source: The type of carbon source can lead to differences in emissions as some studies have reported. Lu and Chandran., (2010) investigated the emissions of N_2O in two different denitrification reactors using methanol and ethanol respectively. They observed different emissions depending on the carbon source used and concluded that N_2O emissions could not be generalized for all carbon sources. Another study performed by Belmonte and co-workers, (2012) explored the N_2O emissions using acetate and swine wastewater as carbon sources during the denitrification process and the results showed different N_2O productions depending on the carbon source used having more emissions for the latter. However, it is still unclear if the type of electron donor (carbon source) can have an effect on the N_2O reduction rate.

- Internal carbon source: Internal storage compounds such as PHA can serve as carbon sources for denitrification in biological phosphorus removal process as it has been mentioned above. Schalk-Otte et al., (2000) observed that when external COD was limited and PHA served as the growth substrate, N_2O started to accumulate. PHA consumption is a rate-limiting step (Beun et al., 2002; Murnleitner et al., 1997), which may trigger competition for electrons between the denitrifying enzymes, and is a possible mechanism to explain N_2O emission by microorganisms growing on storage compounds. Previous studies have reported the accumulation of N_2O in those systems where denitrification was conducted using PHA, such as in biological reactors containing dPAO or dGAO (Wang et al., 2011; Zeng et al., 2003a).

- Electron competition: The negative effect of the simultaneous presence of different nitrogen oxides (NO_3^-, NO_2^- and N_2O) on their reduction rates during denitrification was first reported under low chemical oxygen demand per nitrogen (COD/N) ratios for ordinary heterotrophic denitrifiers that metabolized externally available carbon sources as the electron donor (VonSchulthess and Gujer, 1996). This concept, known as electron competition (Figure 1.4), was also reported when external carbon was available in excess in a denitrifying culture using methanol as the sole carbon source, and also when different COD loadings were applied in a study conducted with a methanol denitrifying culture (Pan et al., 2013a). They also reported that electron competition occurs not only under carbon limiting conditions but also under carbon abundant

conditions which in the latter could lead to N_2O accumulation. However, it is uncertain if this competition for the electrons in the reduction steps of denitrification could also be observed in other populations adapted to other substrates which can have higher denitrification rates than methanol, such as acetate or ethanol. Moreover, it is unclear whether using other substrates would affect this electron competition and lead to an accumulation of N_2O resulting in an incomplete denitrification with N_2O as the end-product of the process. It is also unclear if the denitrification process using PHA as the carbon source could affect the electron competition and the N_2O production and accumulation.

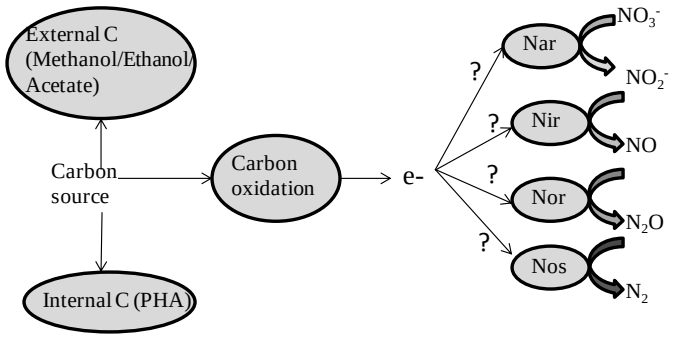

Figure 1.4: Possible electron competition occurring during denitrification (adapted from Pan et al, 2013a).

1.5 Nitrification

Nitrification is the oxidation of NH_3 to NO_3^- via NO_2^- using O_2 as the terminal electron acceptor. These reactions are carried out by two groups of autotrophic microorganisms. i) Ammonia oxidizing bacteria (AOB) oxidize NH_3 to NO_2^- via a two-step reaction: NH_3 is first oxidized to hydroxylamine (NH_2OH) which is further oxidized to NO_2^- in the second step. ii) Nitrite oxidizing bacteria (NOB) perform the oxidation of NO_2^- to NO_3^- (Figure 1.5). Although N_2O and NO are not intermediates in the catabolic pathway of nitrification, its production has been reported during the first step of nitrification where ammonia is oxidized to nitrite by AOB.

N_2O and NO can be produced through two different routes in AOB: (i) the hydroxylamine oxidation pathway: N_2O and NO are intermediates of the NH_2OH biological oxidation to a nitrosyl radical (NOH), followed by NO_2^- or produced by chemical decomposition of hydroxylamine (Law et al., 2012b) and (ii) the nitrifier

denitrification pathway: reduction of NO_2^- by AOBs under oxygen-limiting conditions or elevated NO_2^- concentrations (Wunderlin et al., 2012).

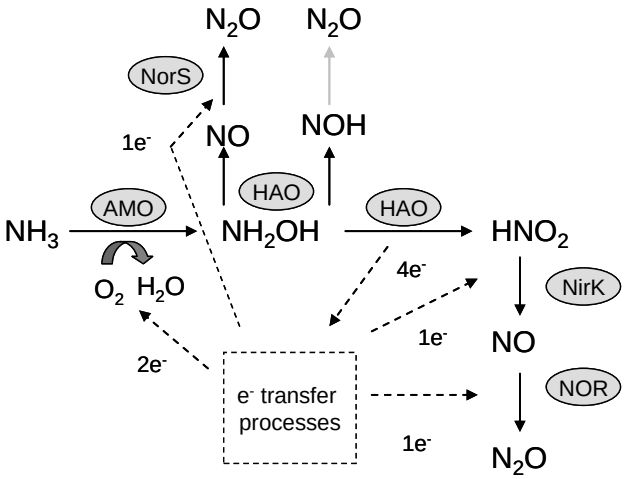

Figure 1.5: Possible nitrogen transformation pathways and enzymes involved in ammonia oxidizing bacteria (adapted from Kim et al., 2010). Black arrows represent biological processes; grey arrows represent chemical mediated processes. Dashed arrows represent electron fluxes.

Several factors have been reported to affect N_2O production in AOBs:

- $\underline{NO_2^-}$: NO_2^- is the toxic end product of aerobic NH_3 oxidation in AOB and it is considered as one of the key parameters affecting N_2O emissions in these bacteria, by increasing their nitrifier denitrification activity. Foley et al., (2010b) reported that higher N_2O generation was associated to higher NO_2^- concentrations in wastewater treatment systems. Later on, Law and co-workers., (2013) determined that the N_2O production rate was the highest at NO_2^- concentrations below 50 mg N/L using an enriched AOB biomass from a partial nitritation reactor treating synthetic reject wastewater. When NO_2^- was increased, N_2O production rate gradually decreased. In their study, higher NO_2^- concentrations resulted in lower N_2O emissions suggesting that exceedingly high NO_2^- concentrations in nitritation systems is not necessary related to an increase on N_2O production. However, this is contradictory to the results reported by Kampschreur et al., (2008a) who found higher N_2O production when adding NO_2^- in step wise mode (NO_2^- pulses of 5 and 15 mg N/L) during aerobic ammonium (NH_4^+) oxidation in a full nitrification system.

More recently, Castro-Barros et al., (2016) reported that nitrite pulses resulted in an increase in N_2O and NO emissions in a nitrifying lab-scale reactor fed with low strength ammonium wastewater. These emissions decreased to original levels when nitrite was

completely oxidized to nitrate. High nitrite concentrations showed an inhibitory effect on the nitrifying activity of non-adapted bacterial groups to nitrite, likely due to NO accumulation.

These differences could be related to the fact that different AOB strains possess different adaptation strategies to high NO_2^- environments. This was suggested by Cua and Stein., (2011). Therefore, it is possible that the same NO_2^- concentration triggers different N_2O production depending on the type of AOB. Another explanation could refer to the adaptation of AOB to different environments with different NO_2^- concentrations.

- NH_2OH: Hydroxylamine is one of the key intermediates in the catabolic metabolism of AOB. Also, NH_2OH is highly toxic for many bacteria and although AOB seem to be more tolerant to this compound than other microorganisms, its accumulation can cause a decrease on their NH_3 oxidation rate (Böttcher and Koops, 1994; Xu et al., 2012). NH_2OH accumulation enhances N_2O production via the hydroxylamine oxidation pathway. So, when NH_2OH is externally added in an AOB culture, N_2O production will be enhanced (Stein, 2011). Wunderlin et al., (2012) explored the effect of NH_2OH addition in a nitrifying culture. They observed that 6.9-8.5% of the oxidized NH_2OH was converted to N_2O, which was much higher than the N_2O emitted in those experiments where NH_3 instead of NH_2OH was added (1.3-3.8%). In another study, Rodriguez-Caballero and Pijuan, (2013) explored the N_2O emission dynamics of a nitritation SBR treating synthetic reject wastewater. They observed that the presence of only NH_2OH at the beginning of the settling phase, when DO concentration was zero, triggered production of N_2O which was emitted at the beginning of the subsequent cycle.

- Temperature: Temperature is a very important parameter during nitration having a direct effect on ammonia oxidation rate (AOR) (Guo et al., 2010; Kim et al., 2008) since AOBs grow better at a temperature around 30ºC. If the temperature differs it could also affect the enzymatic activities (i.e. NirK and NoR), which can be related to N_2O and NO emission.

-NH_4^+ concentration: NH_3/NH_4^+ has been reported as an important factor affecting N_2O and also NO emissions in AOB under aerobic and anaerobic conditions. The effect of pulse NH_4^+ additions on N_2O production under aerobic conditions was first reported by

Kampschreur et al., (2008a). An increase on N_2O emissions was found each time that NH_4^+ was added. NO was also emitted but only when NH_4^+ was present and was not affected by the concentration of NH_4^+. Later on, Wunderlin et al., (2012) observed N_2O production as soon as NH_4^+ was added in a batch test conducted with nitrifying sludge. They attributed this N_2O production to a shift in the AOB metabolism from a low specific activity (periods without NH_4^+) towards the maximum specific activity (after a pulse of NH_4^+). More recently the relationship between the AOR and the N_2O production rate was found to be linear in the pH range of 6.5-8 (Law et al., 2011). In another study, the same authors revealed that the relationship between N_2O production specific rate (N_2Osp) and ammonia oxidation specific rate (AORsp) was exponential in an enriched AOB culture (Law et al., 2012a).

- pH: An early study by Hynes and Knowles, (1984) reported that the rates of production of N_2O were changing when changing pH within the range of 5.4 to 9.5, having a maximum N_2O production at pH 8.5 in a pure culture of *Nitrosomonas europaea*. However, when changing pH, other parameters such as free ammonia concentration (FA) and FNA are also changing. Shiskowski and Mavinic, (2006) suggested that FNA rather than NO_2^- was the actual electron acceptor for the nitrifier denitrification pathway in AOB. They observed a reduction in N_2O production rate when pH was increased, which they attributed to the lower availability of FNA to AOB cells. More recently Law et al., (2011) studied the effect of pH on N_2O production and revealed that the N_2O production rate of an enriched AOB culture was dependent on the pH which in turn, affected the ammonia oxidation rate. They studied this effect on the range of 6-8.5.

- Dissolved oxygen (DO): DO is a parameter that can also affect N_2O production during nitrification. Peng et al., (2014) studied the effect of DO on N_2O production and their results showed that as DO increased the N_2O production rate also increased. Later on Peng et al., (2015) reported the combined effect of DO and NO_2^- concentrations on the N_2O production of a nitrifying culture. Results showed that at each DO level, as NO_2^- concentration increased so did the N_2O production rate. On the other hand, at each NO_2^- level, N_2O production rate decreased as DO concentrations increased. With this investigation, they showed the importance of studying and controlling two parameters at the same time.

On the other hand, reports on NO production have been very scarce. Rodriguez-Caballero and Pijuan, (2013) studied the N_2O and NO emissions in a partial nitrification reactor using different cycle configurations to minimize these emissions and concluded that NO should be also taken into account when implementing mitigation strategies to reduce N_2O, since some of these strategies might result in increased NO emissions. Yu et al., (2010) also studied the production of NO and N_2O under transient anoxic conditions in a pure culture of AOB and reported N_2O emissions during transient conditions (from anoxic to aerobic) when ammonia had been accumulated. However, NO was mainly produced during anoxic conditions. The relationship between the ammonia oxidation rate and the NO production rate was found to be linear for a pure culture of *Nitrosomonas europaea* using synthetic wastewater (Stüven and Bock., 2001). Kampschreur et al., (2008b) studied the NO and N_2O emissions in a full-scale WWTP treating reject wastewater in a two-reactor nitritation-anammox process. The NO emissions from the nitritation reactor were 0.2% of the N-load and nitrifier denitrification by AOBs was considered to be the most probable cause of NO and N_2O emission from the nitritation reactor.

1.6 CH_4 production

Methane is produced under anaerobic conditions. This process takes places in four different steps. In the first step (hydrolysis) complex organic polymers are hydrolysed into simpler soluble organic compounds. In this step, large quantities of hydrogen (H_2) are produced. In the second step (acidogenesis) volatile fatty acids (VFAs), alcohols, H_2 and CO_2 are produced. Then in the third step of the process (acetogenesis) acetate, H_2 and CO_2 are produced from the fermentation products such as lactate, butyrate and propionate. Also, acetate can be produced by H_2 and CO_2. In the fourth step (methanogenesis), CH_4 is produced by methanogenic population. There are two types of methanogens: hydrogenotrophic methanogens that produce CH_4 from H_2 and CO_2 and acetotrophic methanogens that use acetate to produce CH_4 and CO_2 (Mara and Horan., 2003). Figure 1.6 shows a scheme of the different steps of the process.

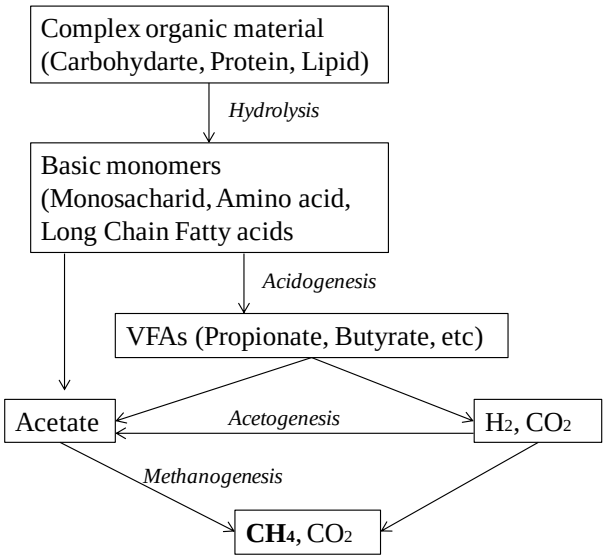

Figure 1.6: Production of methane adapted from Ersahin et al., (2011).

1.7 Direct GHG emissions from full scale WWTP

In the past few years the concern about the quantification and investigation of N_2O emissions from full-scale BNR processes has increased. However, results are variable and there is still not a consensus to explain the exact causes behind N_2O emissions. The reasons for that are that the studies reporting N_2O emissions from full-scale systems are based on different WWTP configurations and different biological treatments. Also, another factor affecting the differences in increasing the high variability of the emissions reported is the methodology used. Most of the studies are performed in short-term (days-weeks) showing only diurnal patterns of these emissions. The sampling strategy (grabbing samples or online monitoring) is also a factor that can lead to over or underestimation of the N_2O monitoring. In order to correlate the emissions with diurnal and seasonal variability, high frequency data of the parameters and emissions in long-term periods is needed. This can only be achieved with a long-term continuous online monitoring of the emissions of the WWTP. Continuous online monitoring is done by monitoring N_2O concentration and flow range of gases over the operational range of the BNR process using portable online equipment. Floating hoods are used to cover a small portion of the reactors surface to capture a representative grab sample in order to determine diurnal and long-term temporal dynamics in the N_2O emissions and provide a more reliable means to quantify them (Pan et al., 2016). Different studies have monitored N_2O emissions in WWTP in order to systematically quantify such emissions from full-scale BNR operations. Ahn et al., (2010b) studied 12 different WWTPs with

different configurations. Results showed an emission factor (amount of N_2O-N emitted relative to the nitrogen load) range of 0.01− 1.8% N_2O/Total Kjeldahl Nitrogen (TKN). In general, N_2O emissions were two to three orders of magnitude higher in aerated zones than in non-aerated zones. A high degree of diurnal variability in emission factors from the overall processes sampled was also observed and it was linked to diurnal variations in influent N-loading. This diurnal variability was corroborated by Aboobakar et al., (2013) in a study within the nitrifying line of an activated sludge process (ASP). Their results showed an average mass emission greater in the gaseous 0.036% of the influent total nitrogen than in the dissolved (0.01% of the influent total nitrogen) phase. More recently, Pan et al., (2016) studied the spatial variation of N_2O emissions in a full-scale step-feed plug-flow reactor. They used multiple gas collection hoods to simultaneously measure emissions along a plug-flow reactor. N_2O fluxes exhibited strong spatial-temporal variations along the reactor path, indicating that it is crucial to consider spatial variations of N_2O emissions when quantifying emissions factors from plug-flow reactors. Kosonen et al., (2016) studied the N_2O emissions in a long-term online monitoring campaign showing a diurnal variation of the N_2O that had a strong correlation with the alternation of the influent BOD and NH_4-N load to the aerated zones. They determined an annual emission factor of 1.9% of the influent nitrogen load which is in the high range values of long-term data reported in the literature.

The large variation in N_2O emissions among the investigated plants reported by the different studies was probably due to the different configurations and operational conditions applied. Additionally, different monitoring and quantification methods used could have been a contributing factor. The large variation also implies that N_2O emissions from a treatment plant can be reduced through proper plant design and operation. In order to find a right balance between operational efficiency and saving energy without increasing N_2O emissions, a clearer understanding of emissions obtained from real-time data is needed.

WWTPs also emit methane. Methane is emitted after it enters the plant via stripping from the incoming wastewater, or after it is formed at the plant itself. The influent of a WWTP can also contain dissolved methane formed in the sewer system. Also, significant dissolved methane concentrations are found in the reject wastewater stream

coming from the anaerobic digester which is normally recirculated to the inlet of the WWTP. Part of this CH_4 can be biologically oxidized in the bioreactor but some will be stripped to the atmosphere during aeration. Daelman et al., (2012) studied the methane emissions in a municipal wastewater treatment plant in the Netherlands and determined that 80% of the dissolved methane in the influent was oxidized in the plug-flow reactor. This could be exploited as a means to further decrease methane emissions from wastewater treatment. The methane emission related to the anaerobic digestion of primary and secondary sludge counted for about three quarters with respect to the WWTPs overall methane emission.

Many studies have focused on quantifying N_2O or CH_4 emissions separately and few have assess both GHG from the same WWTP over a long term period (12 months). Daelman et al., (2013) studied the CH_4 and N_2O emissions of a plug-flow and two carousel reactors that were covered over a period of 16 months and did not find a significant correlation between daily average methane emissions and atmospheric temperature (r^2=0.18). Also, any correlation was reported between the daily average nitrous oxide emissions and wastewater temperature. Daelman and co-workers (2013) showed the importance of long-term monitoring since the emissions factors determined in this study (28 g N-N_2O/kg $TKN_{influent}$) was 80 times higher than the proposed by the IPCC, (2006) (0.35g N-N_2O/kg $TKN_{influent}$). They also reported that both CH_4 and N_2O emissions exceeded the plant's indirect carbon dioxide emissions related to electricity consumption. Since the WWTP studied by Daelman et al., (2013) was fully covered (except for the secondary clarifiers) it was not possible to determine spatial GHG variations.

It is therefore important to have a clearer and more realistic perspective of the quantification of GHG emissions from WWTPs in order to reduce these emissions and identify ways to mitigate them.

Chapter 2

Chapter 2 Objectives

The experiments presented in this thesis were conducted to achieve two main objectives:

- Identifying operational factors triggering N_2O production during nitrification and denitrification in wastewater treatment systems.
- Identify the N_2O and CH_4 emission patterns of a plug-flow reactor located in a full-scale WWTP and their seasonal variations.

To achieve these goals, the following sub-objectives were pursued:

- Unravelling the effect of the competition for electrons on N_2O reduction rate in a denitrifying mixed microbial community using different external carbon sources.
    - To study the impact of organic carbon sources and the competition for electrons on the N_2O reduction rate with three carbon sources: acetate, ethanol and methanol.
    - To study the effect of each carbon source on the different nitrogen oxides reduction rates.
    - To determine the effect of carbon limitation on the nitrogen oxides reduction rates using ethanol and acetate.
- Exploring N_2O production in dPAO and dGAO cultures during denitrification under different electron acceptors using PHA as sole carbon source.
    - To explore the denitrification kinetics and the N_2O accumulation potential in two separate enriched cultures of dPAO and dGAO.
    - To assess the preference for a nitrogen oxide for each culture.
    - To determine the occurrence of electron competition in the different denitrification kinetics from the two cultures.
- Studying the relationship between NO and N_2O during nitritation. The effect of pH and DO on the emission of both gases was also investigated.
    - To study the effect of ammonia oxidation rate on NO and N_2O production
    - To assess the relationship between NO and N_2O production in partial nitrification
    - To determine the effect of pH and DO on the production of NO and N_2O in an enriched AOB culture.

- Long term simultaneous multiple sites monitoring of a full-scale plug-flow reactor treating domestic wastewater
    - To characterize the N_2O and CH_4 emission patterns in a plug-flow reactor.
    - To study the emissions in different aerobic compartments with multiple gas collection hoods simultaneously and identify the factors affecting these emissions.
    - To assess the C footprint of the plug-flow reactor

THESIS OUTLINE

According to these objectives the research work of this thesis has been distributed along the following 4 chapters of results:

CHAPTER 4: Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community.

CHAPTER 5: Distinctive denitrifying capabilities lead to differences in N_2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms.

CHAPTER 6: Distinctive NO and N_2O emission patterns in ammonia oxidizing bacteria: effect of ammonia oxidation rate, DO and pH.

CHAPTER 7: Direct GHG emissions from a full-scale plug-flow reactor: identifying temporal and spatial variations.

Chapter 3

Chapter 3 Methodology

3.1 Lab scale systems

4 different reactors were used in this thesis in lab-scale systems. These reactors were developed in order to enrich 4 different cultures of common denitrifying bacteria, denitrifying polyphosphate accumulating organisms (dPAOs), denitrifying glycogen accumulating organisms (dGAOs) and ammonia oxidizing bacteria (AOB). Moreover, a batch test reactor was used in order to assess the different batch tests performed on common denitrifiers, dPAOs and dGAOs, respectively. A more detailed explanation of how these reactors were developed is given in the following sections

3.1.1 Denitrifying mixed culture reactor

The lab-scale experiments were performed in cylindrical SBRs. In Chapter 4 a 6L SBR was used and inoculated with activated sludge from the WWTP of Girona (Spain) to develop a mixed denitrifying culture. It was operated at ICRA laboratories in 6h cycles, consisting of anoxic feed (5min) where 1L of synthetic wastewater was added, anoxic phase (5h), aerobic mix (15min), settling (20min) and withdrawal (20min). The synthetic wastewater had a concentration of 90 mg NO_3^--N/L and 300 mg COD/L and included 900 mL of solution A and 100 mL of solution B. The composition of solution A was (per L): 0.55 g $NaNO_3$, 0.33 g $MgSO_4 \cdot 2H_2O$, 0.033 g $CaCl_2 \cdot 2H_2O$, 0.145 g K_2HPO_4, 0.01g Allythiourea 96% (ATU), 0.27 g NH_4Cl and 220 mL of trace elements solution (per L): 1.5 g $FeCl_3 \cdot 6H2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.18g KI, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.15 g $CoCl_2 \cdot 6H_2O$ and 10 g EDTA). Solution B contained 2.13 g/L sodium acetate, 0.634 mL/L ethanol (96%) and 0.896 mL/L methanol (99.9%), resulting in 100 mg COD/L of each carbon added to the reactor. This solution was autoclaved to avoid any COD biodegradation.

The sludge retention time (SRT) was 20 days and the hydraulic residence time (HRT) was 36h. Nitrate and COD were completely removed at the end of the anoxic phase. The pH was controlled at 7.5 ± 0.4 using 0.6M hydrochloric acid (HCL). Dissolved oxygen (DO) concentration was also controlled with a programmable logic controller (PLC) between 2-2.5 mg O_2/L by supplying air at 5 L/min. Redox potential was also monitored.

3.1.2 Enriched denitrifying phosphorus accumulating reactor

In Chapter 5 a 2L SBR was used in order to enrich a dPAO culture (Figure 3.1). The dPAO reactor was inoculated with sludge from the WWTP of Beirolas (Portugal). The reactor was operated at the laboratories of the Chemistry Department at the Universidade Nova de Lisboa during a 4 months research stage. This SBR operated in 6h cycles consisting in: 5min feed-1; 102min anaerobic phase, 4min feed-2, 114min anoxic phase, 90min aerobic phase and 45min of settling and decant. The reactor was fed with synthetic wastewater with the following characteristics:

Feed-1 (950 mL added) consisted of 0.59 g NH_4Cl/L, 0.95 g $MgSO_4 \cdot 7H_2O$/L, 0.44 g $CaCl_2 \cdot 2H_2O$/L, 0.01 g ATU/L, 0.03 g EDTA/L, 1.91 g $C_2H_3O_2Na \cdot 3H_2O$/L, 0.2 mL $C_3H_6O_2$/L (200 mg COD/L in the reactor), 0.25 g K_2HPO_4/L, 0.15 g KH_2PO_4/L (30 mg P/L in the reactor) and 3.17 mL of trace element stock solution per liter of feed (Carvalheira et al., 2014a). Feed-2 (50 mL added) consisted of 6.07 g $NaNO_3$/L (25 mg $N\text{-}NO_3^-$/L in the reactor).

The pH was controlled at 7.5 ± 0.1 with 0.1M HCL. The SRT was 10 days and was maintained by wasting mixed liquor at the end of the aerobic phase. The HRT was maintained at 16h.

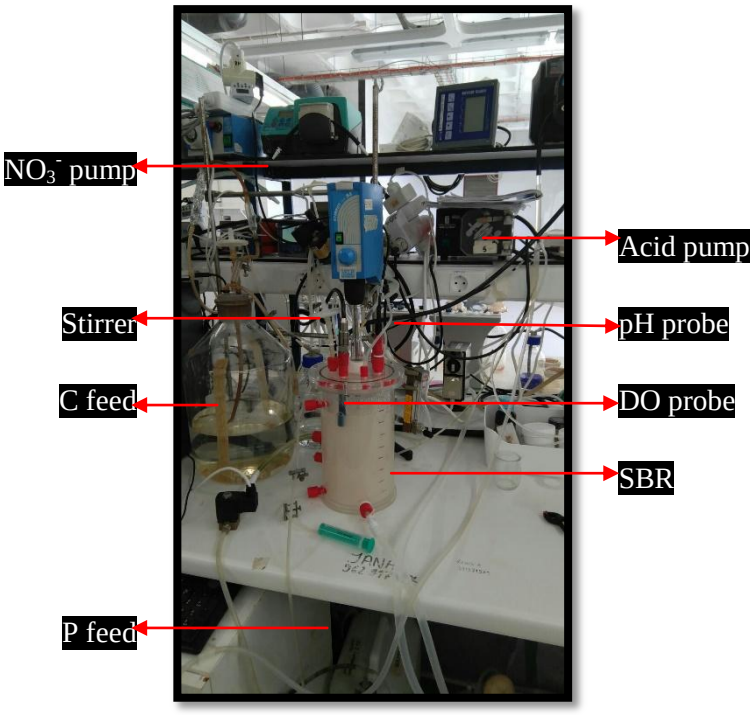

Figure 3.1: dPAO enriched-SBR operated at the Universidade Nova de Lisboa.

3.1.3 Enriched denitrifying glycogen accumulating reactor

In Chapter 5, a 6L SBR was inoculated with sludge from the WWTP of Girona (Spain) in order to enrich a dGAO culture (Figure 3.2). The reactor at ICRA laboratories operated in 6h cycles and consisted in the same phases as the dPAO reactor. The synthetic wastewater used consisted of two different feeds. Feed-1 (900 mL added) contained 0.03 g K_2HPO_4/L (0.7 mg P/L in the reactor), 0.13 g NH_4Cl/L, 0.89 g $MgSO_4 \cdot 7H_2O$/L, 0.41 g $CaCl_2 \cdot 2H_2O$/L, 0.2 g ATU/L, 0.03 g EDTA/L, 11.33 g $C_2H_3O_2Na$/L, 2.57 g $C_3H_5NaO_2$/L (200 mg COD/L in the reactor) and 2.97 mL of trace element stock solution per liter of feed. The trace element solution was the same as for the dPAO reactor. Feed-2 (100 mL added) consisted of 14.5 g $NaNO_3$/L (30 mg N-NO_3^-/L in the reactor).

pH was controlled at 7.5 ± 0.1 with 0.1M HCL. The SRT was 10 days and was maintained by wasting mixed liquor at the end of the aerobic phase. The HRT was kept at 16h.

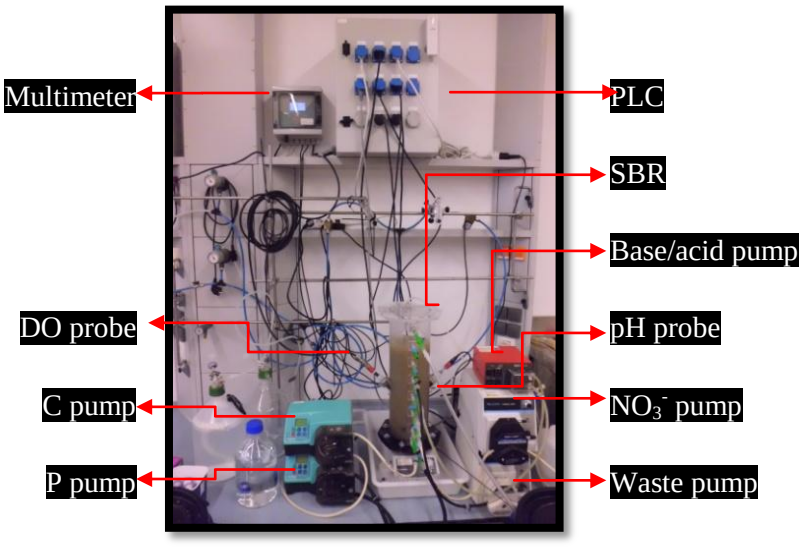

Figure 3.2: Typical SBR used at ICRA laboratories (the image in this case corresponds to the dGAO reactor).

3.1.4 Enriched ammonia oxidizing bacteria reactor

In Chapter 6, an 8L SBR was inoculated with activated sludge from the WWTP of Girona (Spain) and operated at ICRA laboratories to develop an enriched AOB culture, performing partial nitrification from a synthetic reject wastewater (1 g N-NH_4^+/L). The mixed liquor temperature was controlled at 30ºC using a water jacket, to mimic the common temperature conditions of reactors treating reject wastewater. The SBR was operated in cycles of 6h, consisting of feed-1 (2min), aeration-1 (105min), feed-2 (2min), aeration-2 (103min), settling (132min) and decanting (15min). 1L of synthetic wastewater was added in each feeding period, providing an HRT of 24h. DO was controlled with a PLC between 1.5-2.0 mg O_2/L by adding air or nitrogen gas at 5 L/min. The synthetic wastewater had the characteristics of a typical anaerobic digester liquor. The wastewater composition was modified from Kuai and Verstraete., (1998): 5.63 g/L of NH_4HCO_3 (1 g N-NH_4^+/L), 0.064 g/L of each KH_2PO_4 and K_2HPO_4 and 2 mL of trace element stock solution. The trace element solution included (g/L): 1.25 EDTA, 0.55 $ZnSO_4 \cdot 7H_2O$, 0.4 $CoCl_2 \cdot 6H_2O$, 1.27 $MnCl_2 \cdot 4H_2O$, 0.40 $CuSO_4 \cdot 5H_2O$, 0.05 $Na_2Mo_4 \cdot 2H_2O$, 1.37 $CaCl_2 \cdot 2H_2O$, 1.25 $FeCl_3 \cdot 6H_2O$ and 44.40 $MgSO_4 \cdot 7H_2O$. The feed had a pH of 8 and a molar ratio of ammonium to bicarbonate of 1:1. After feeding, the pH of the reactor increased to 7.5 and decreased afterwards due to the nitrification reaction. When pH reached 7, it was automatically controlled by adding 1M $NaHCO_3$ solution.

3.1.5 Batch tests reactor

The batch tests conducted in Chapters 4 and 5 were performed in a 330 mL batch reactor (Figure 3.3).

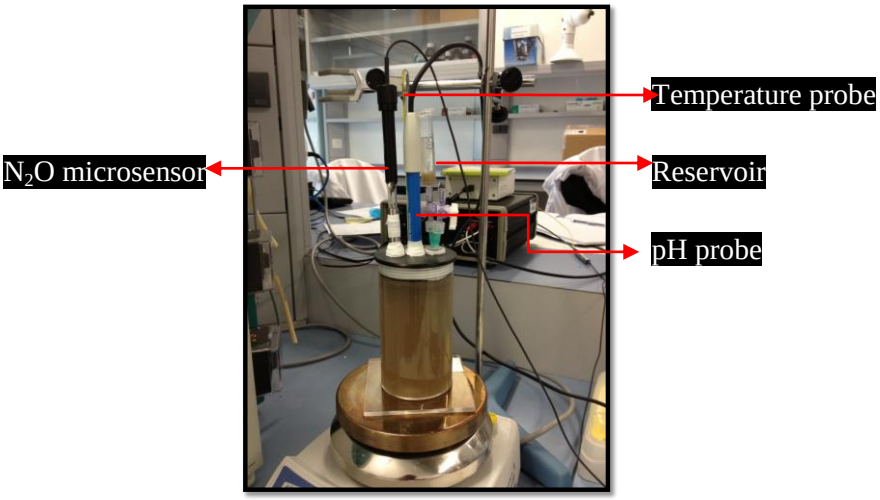

Figure 3.3: Batch reactor.

A 5 mL reservoir was connected to the lid of the reactor. The reservoir avoided the entrance of air into the batch reactor when liquid samples were taken during the batch test. All the batch tests were conducted taken the sludge from the SBR at the end of the anoxic phase to ensure that NO_3^-, NO_2^- and COD were removed completely. Since the reactor did not have head space and it was completely sealed, anoxic conditions were ensured during the whole experiment and no exchange of N_2O between liquid and gas phases occurred. pH was manually controlled during the experiment at 7.5 ± 0.1 by adding 0.6M HCL or 0.5M NaOH.

3.2 Full-scale monitoring

The full-scale monitoring campaign was conducted at the WWTP of Girona (Spain). This plant treats domestic wastewater of 275,000 population equivalents (PE) with a flow of 38,000 m^3/day. The plant configuration consists of a primary treatment followed by a primary settling and then the wastewater is treated biologically in two parallel and identical plug-flow reactors where nitrogen, phosphorus and COD removal are performed. The plant has the capacity to treat wastewater in three lines but at present only two lines are working. The plug-flow reactor consists of two anoxic zones followed by three aerobic zones, then wastewater flows to an anoxic zone and a final fourth aerobic zone. After the biological treatment, the treated water flows to the secondary settlers from where it is discharged into a river. The sludge is compressed in two thickeners and after is digested anaerobically. The reject water from both processes

is released into the inlet of the plant for its treatment. A scheme of the configuration of the plant can be seen below (Figure 3.4). N_2O and CH_4 emissions were monitored online using a multi-hood gas system. Three hoods were placed in the plug-flow reactor in the aerated zone 1, 2 and 3, respectively.

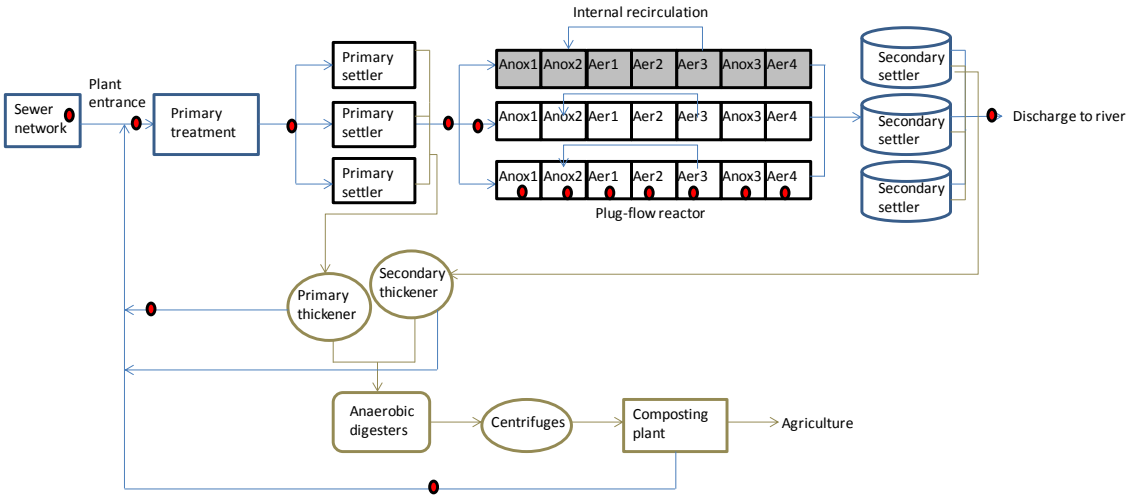

Figure 3.4: Scheme of the configuration of the WWTP of Girona (in grey is marked the plug-flow reactor line where the gas collection hoods were placed and the red dots represent the CH_4 dissolved grab samples).

3.3 Chemical analysis

Mixed liquor suspended solids (MLSS), volatile MLSS (MLVSS) and COD were analysed according to the standard methods (American Public Health Association, 1995).. NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} were analysed via ion chromatography (ICS5000, DIONEX.) at ICRA laboratories and through segmented flux analysis (Skalar 5100, Skalar Analytical, Netherlands) at UNL (Universidade Nova de Lisboa). This methodology was used in Chapters 4, 5, 6 and 7. VFAs were analysed via gas chromatography (Trace GC Ultra ThermoFisher Scientific) at ICRA laboratories (used in Chapter 4 and 5) and via liquid chromatography at high resolution using a Biorad Aminex precolumn and an HPX-87H column and a UV detector adjusted to 210nm. Sulfuric acid (0.01M) was used as eluent in a 0.6mL/min flow-rate and 50ºC of operating temperature at UNL (methodology used in Chapter 5). Analysis for the total phosphorus (TP), COD, BOD and TKN were performed according to the standard methods (American Public Health Association., 1995). Dissolved methane samples were filtered through a 0.22 µm Millipore filter and immediately injected into a vacuumed glass tube using a hypodermic needle attached to a plastic syringe. The tubes

were allowed to reach the gas–liquid equilibrium overnight. The gas phase was measured with a gas chromatograph (Thermofisher ScientificInc, USA) equipped with a flame ionization detector (FID). Additionally, the NH_4^+ concentration at the inlet of the bioreactor at Girona WWTP and in the second aerobic zone was continuously monitored utilizing two on-line ion-selective electrodes (ammo::lyser™) coupled to a monitoring station (S::CAN Messtechnik GmbH, Austria). These methodologies were used in Chapter 7.

3.4 N_2O dissolved measurements

N_2O microsensors were used to continuously monitor the dissolved N_2O in the liquid phase in Chapters 4 and 5. This type of microsensor is a miniaturized Clark-type sensor (Figure 3.5) with an internal reference and a guard cathode (N_2O-R), it has a detection limit of 0.1µM in water and a response time less than 1 sec (Unisense A/S, Arhus, Denmark).

Figure 3.5: Clark type N_2O microsensor.

3.5 N_2O, NO and CH_4 gas measurements

The N_2O, NO and CH_4 emissions were continuously analysed by commercial gas analysers in Chapter 6 and Chapter 7, respectively. NO was analysed via a chemiluminescence gas analyser CLD64 (Eco Physics, Dürten, Switzerland). N_2O and CH_4 were analysed with an infra-red gas analyser V-A 3000 (Horiba, Japan) equipped with a sample conditioning system (series CSS, M&C Tech group). Off gas was

collected continuously (at 0.5 L/min) from the reactor headspace in Chapter 6 and from three floating hoods located at the surface of the first, second and third aerated zones of the plug-flow reactor of the WWTP of Girona in Chapter 7. Concentration data was logged every 15s for the N_2O and CH_4, and every 5s for the NO concentration.

3.6 Multi-hood gas collection system

Gas measurements in Chapter 7 were done using a multi-hood gas collection system (Figure 3.6). Multiple sampling locations were chosen to investigate the spatial variation in N_2O and CH_4 emissions from different parts of the plug-flow reactor. The locations of the gas hoods were the aeration zone 1, 2 and 3, respectively. The gas hoods were not placed within the anoxic zones since there was no measurable gas flow and previous studies have shown that N_2O fluxes from un-aerated zones are negligible (Law et al., 2012b). The on-line gas-phase N_2O and CH_4 monitoring was conducted over a 5 months period. The off-gas collected from each of the three gas hoods was transferred to a central off-gas monitoring unit, via 12mm diameter polyamide gas tubing. Once the off-gas from each of the hoods reached the monitoring unit, gas temperature, pressure and flow rate were measured and recorded in real-time. After the flow meter, a small portion of the gas (0.5 L/min) was diverted and pumped to the gas conditioning unit (series CSS, M&C Tech group) and analyser (Horiba VA3000) (Figure 3.6 right). As the analyser can only measure one gas stream at a time, a software was used to control the cyclic opening and closing of solenoid valves to direct the gas captured from the individual hoods to the analyser at 20-minute intervals. This software contained all the necessary codes to operate a system of sensors connected to an Arduino, and controlled by a Raspberry Pi. The Arduino sensors were continuously reading and sending the data to the serial port (USB) of the Raspberry Pi. N_2O, CH_4 concentration (in ppmv), temperature, flow rate and pressure were logged at 15 seconds intervals. The analyser was serviced and calibrated on-site, according to manufacturer's instructions, using compressed air, nitrogen, 80 ppmv N_2O gas standard (Linde) and 160 ppmv CH_4 gas standard (Linde).

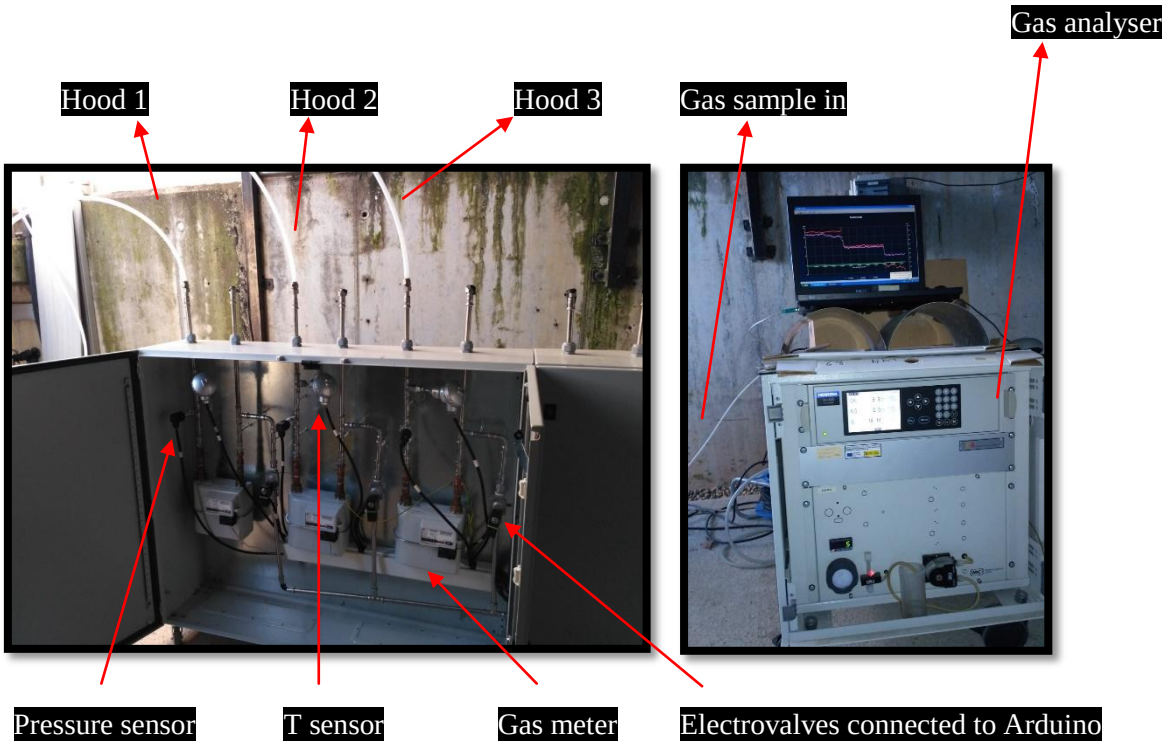

Figure 3.6: Multi-hood gas collection system.

3.7 Microbial analysis

Fluorescence *in situ* hybridization (FISH) was performed as described in Nielsen et al., (2009) in order to evaluate the microbial populations present in some of the reactors. In Chapters 5 and 6 a detailed explanation of the microbial analysis performed in each reactor is given.

3.8 Calculations

3.8.1 Specific NOx reduction rates

The measured maximum specific NO_3^-, NO_2^- and N_2O reduction rates presented in Chapters 4 and 5 ($r_{NO3-,m}$, $r_{NO2-,m}$ and $r_{N2O,m}$) were determined through linear regression of the NO_3^-, NO_2^- and N_2O profiles, respectively divided by the MLVSS concentration. The true reduction rate of each nitrogen oxide was calculated as follows:

$$r_{NO_3^-} = r_{NO_3^-,m} \quad \text{(Eq. 1)}$$

$$r_{NO_2^-} = r_{NO_3^-} - r_{NO_2^-,m} \quad \text{(Eq. 2)}$$

$$r_{NO} = r_{NO_2^-} \quad \text{(Eq. 3)}$$

$$r_{N_2O} = r_{NO_2^-} - r_{N_2O,m} \quad \text{(Eq. 4)}$$

N_2O production rate was considered to be equal to the nitrite reduction rate based on the assumption that NO did not accumulate. NO is a potent cytotoxin and its accumulation causes bacterial decay (De Boer et al., 1996). Also in order to prevent accumulation of cytotoxic vels, intracellular concentrations of nitric oxide are typically maintained at low nanomolar levels through synchronized regulation of Nir and Nor (Goretski et al., 1990). Therefore, the NO reduction reaction is prioritized and not the rate-limiting step of denitrification.

3.8.2 Specific electron consumption rates and electron distribution

The specific electron consumption rates for nitrate (Nar), nitrite (Nir), nitric oxide (Nor) and nitrous oxide (Nos) were calculated as follows:

$$r_{Nar,e} = \frac{r_{NO_3^-}}{14} \cdot 2 \quad \text{(Eq. 5)}$$

$$r_{Nir,e} = \frac{r_{NO_2^-}}{14} \cdot 1 \quad \text{(Eq. 6)}$$

$$r_{Nor,e} = \frac{r_{NO}}{14} \cdot 1 \quad \text{(Eq. 7)}$$

$$r_{Nos,e} = \frac{r_{N_2O}}{14} \cdot 1 \quad \text{(Eq. 8)}$$

Eq. 5-8 express the electron consumption of Nar, Nir, Nor and Nos respectively, in mmol e-/ gVSS·h. For the case of Nor, the reduction rate of NO was assumed to be equal to the NO_2^- reduction rate. Electron distribution was calculated as the ratio of electron consumption rate for each of the nitrogen oxide reductases to the total electron consumption rate, expressed as a percentage (Eq. 9):

$$Electron\ distribution\ (\%) = \frac{r_{NOx,e}}{r_{Nar,e}+r_{Nir,e}+r_{Nor,e}+r_{Nos,e}} \cdot 100 \quad \text{(Eq. 9)}$$

3.8.3 Specific N_2O and NO emission rates and emission factors

In order to calculate the N_2O and NO production rates in Chapter 6 equations 10 and 11 were used:

$$N_2O\ production\ rate\ (g\ N-N_2O/g\ VSS\cdot min)=\frac{\sum N_2O\ emitted\ (g)}{\Delta t(min)\cdot\frac{gVSS}{L}\cdot V(L)} \quad \text{(Eq. 10)}$$

$$NO\ production\ rate\ (g\ N-NO/g\ VSS\cdot min)=\frac{\sum NO\ emitted\ (g)}{\Delta t(min)\cdot\frac{gVSS}{L}\cdot V(L)} \quad \text{(Eq.11)}$$

Where V is the volume of the reactor at the moment that the MLVSS were taken.

Δt is the interval of time during which the N_2O or the NO production rates were calculated.

Ammonia oxidation specific rate was calculated as follows:

$$AOR_{sp}=\frac{N-NH4+\ consumed}{g\ VSS\cdot min} \quad \text{(Eq. 12)}$$

In order to calculate N_2O and NO emissions in Chapter 6 equation 13 was used.

$$N_2O\ emitted=\sum\left(C_{N-N_2O}\cdot Q_{gas}\cdot\Delta t\right) \quad \text{(Eq. 13)}$$

Where

$C_{N-N_2O}=C_{N-N_2O}$ (ppmv) · N_2O molar volume (0.0402 at 1atm and 25°C) · 10^{-6} · 28 $\left(g\ N-N_2O/_L\right)$

Q_{gas} is the gas flow rate (L/min)

Δt is the time interval by which the off-gas N_2O was recorded.

A homologous calculation was done for the NO emission but the concentration of NO (g NO/L) was multiplied by 14 g N /mol.

3.8.4 N_2O and CH_4 emission rates and emission factors

In order to calculate the N_2O and CH_4 emission factors in Chapter 7 equation 14, 15 and 16 were used:

$$N_2O\ emitted\ =\sum_i^n\left(\sum\left(C_{N-N_2O}\cdot Q_{gas}\cdot\Delta t\right)_{hood\ i}\right)\cdot\frac{A_{zone\ i}}{A_{hood\ i}} \quad \text{(Eq. 14)}$$

Where

$C_{N-N_2O}=C_{N-N_2O}$ (ppmv) · N_2O molar volume (0.0402 at 1atm and 25°C) · 10^{-6} · 28 $\left(g\ N-N_2O/_L\right)$

Q_{gas} is the gas flow rate (L/min)

Δt is the time interval by which the off-gas N_2O was recorded.

$A_{zone\ i}$ is the area of the zone of the plug-flow reactor where hood i was placed $A_{hood\ i}$ is the area of the hood which is 0.13 m^2.

$$Emission\ factor = \frac{N_2O\ emitted}{TKN\ load} * 2 \quad \text{(Eq. 15)}$$

Where

TKN load corresponds at the same time interval Δt.

Equation 15 is multiplied by 2 because it was assumed that both plug-flow reactors present in the plant presented the same emissions.

A homologous calculation was done for the CH_4 emission but the concentration of CH_4 (g CH_4/L) was multiplied by 16 g CH_4/mol.

BLOCK II - RESULTS

Chapter 4

Chapter 4 Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community

This article was published as:

Anna Ribera-Guardia, Elissavet Kassotaki, Oriol Gutierrez; Maite Pijuan. 2014. Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. Process Biochemistry 49 (12), 2228-2234.

4.1 Preliminary remarks

This study investigates the impact of organic carbon sources and the competition for electrons on the N_2O reduction rate in a denitrifying culture developed with three carbon sources: acetate, ethanol and methanol. The effect of each carbon source on the different nitrogen oxides reduction rates is assessed and compared. Finally, the effect of carbon limitation on the nitrogen oxides reduction rates is determined using ethanol and acetate.

4.2 Materials and methods

4.2.1 Bioreactor set-up and operation

A cylindrical 6L SBR was inoculated with activated sludge from Girona's wastewater treatment plant to develop a denitrifying culture. The operation and set-up of this reactor is explained in Chapter 3.

Cycle studies were carried out on a weekly basis to monitor the denitrification activity of the reactor. Samples for the analysis of nitrate, nitrite, COD and acetate were taken every 60 min and filtered with 0.22 µm Millipore filters. At the end of the aerobic phase mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were also analysed. Chemical analyses are detailed in Chapter 3.

4.2.2 Batch tests

Batch tests were carried out to study the effects of nitrate, nitrite and nitrous oxide on each other's reduction rates using three different substrates independently (acetate, ethanol and methanol) and a combination of the three.

Four sets of experiments were conducted. The first three were carried out using the three different carbon sources separately (acetate, ethanol and methanol) and in the last set a combination of the three was used. Seven types of batch tests were conducted using different electron acceptors for the first three sets of experiments. For the last set, when a mix of the 3 carbon sources was added, only batch tests A to E were carried out (Table 4.1). All the batch tests were conducted in triplicate.

Table 4.1. Batch tests conducted for each set of experiments.

Batch test type	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Electron acceptors used	NO_3^-	NO_2^-	N_2O	NO_3^- N_2O	NO_2^- N_2O	NO_3^- NO_2^-	NO_3^- NO_2^- N_2O

4.2.3 Batch reactors set-up and operation

A batch reactor was used to carry out the batch tests (the description is in Chapter 3). Liquid samples were taken during the batch tests for the analysis of nitrate, nitrite and acetate. Mixed liquor samples were taken using a syringe and immediately filtered through disposable Millipore filter units (0.22 µm pore size) and analysed. N_2O was continuously monitored with an N_2O microsensor (Unisense A/S, Arhus, Denmark). The sludge was pretreated with one hour aeration to oxidize any internal COD that could be present, half an hour bubbled with nitrogen gas to ensure anoxic conditions and finally washed with a phosphate buffer solution (PBS) previously sparged with nitrogen and placed in the batch reactor.

4.2.4 Experiments

The batch started by adding the electron acceptor at a concentration of 20 mg N/L (of each of the electrons acceptors used depending on the batch test), followed by the addition of the carbon source at a concentration of 100 mg COD/L. In the last set of experiments, the three carbon sources were added simultaneously at a concentration of 100 mg COD/L (equally divided between acetate, ethanol and methanol). In the cases where nitrous oxide was used as the electron acceptor (batches C, D, E and G), it was added first in order to have a stable signal of the microsensor and see clearly the changes on its production or consumption. After the N_2O addition, the carbon source and the other nitrogen oxides (depending on the test) were added simultaneously. An example of batch test E using ethanol as the carbon source is shown in Figure 4.1 where 20 mg $N\text{-}N_2O$/L were added at minute 5 and 100 mg COD/L (ethanol) and 20 mg $N\text{-}NO_2^-$/L were added at minute 10.

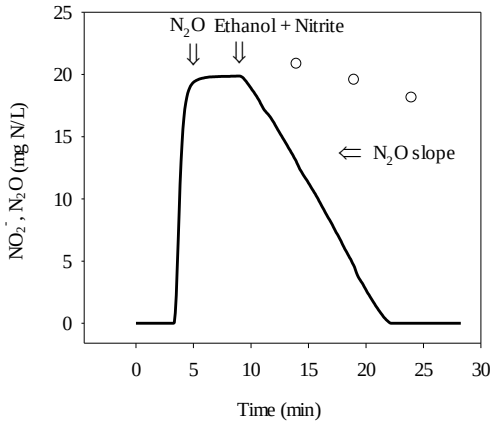

Figure 4.1: Nitrous oxide and nitrite profile using ethanol as the sole substrate and nitrite and nitrous oxide as electron acceptors (○ NO_2^-, and -N_2O).

The calculations of the maximum specific NO_3^-, NO_2^- and N_2O reduction rates for this study are shown in Chapter 3.

4.3 Results and discussion

4.3.1 Effect of substrate on nitrogen oxides reduction

Figure 4.2 shows an example of the nitrate, nitrite and nitrous oxide profiles within batches A, B and C using acetate, ethanol or methanol.

The reduction rates obtained for each nitrogen oxide differed depending on the carbon source added. The lowest rates were obtained when methanol was used as the only carbon source. These results are in agreement with the work of Mokhayeri and co-workers., (2008) who showed significantly higher nitrate reduction rates with acetate and ethanol than with methanol. Nitrate reduction rate was similar when acetate and ethanol were used independently. Interestingly, in the case of ethanol, some nitrite accumulated during nitrate reduction due to the lower nitrite reduction rate compared with the rate for nitrate reduction (Figure 4.2b). In the case of the tests where acetate was used as the sole substrate (Figure 4.2a, d, g), nitrate reduction was the slowest of all the rates, being followed by the nitrite and nitrous oxide reduction rate.

N_2O reduction presented the highest rate when ethanol was used as the only substrate (Figure 4.2h). These differences are likely due to the mixed microbial community present in the SBR. Although the same COD concentration was added for each of the substrates, it is possible that acetate and ethanol were preferred against methanol, growing in the SBR more microorganisms able to consume these two substrates. Also, there is the possibility that each carbon source helps to grow a specific microbial community which might have differences in terms of affinity for different electron acceptors or even have different amounts of genes encoding for each of the enzymes involved in the reduction of the nitrogen oxides. It was reported that the growth yields for ethanol and acetate are very similar and higher than methanol (US EPA, 2013b) corroborating the fact that more microorganisms consuming acetate and ethanol might be present compared to the methanol consuming microorganisms. Indeed, at the end of the batch tests carried out with methanol there were 30 mg COD/L still remaining, indicating a slower consumption of this substrate probably due to a smaller fraction of the biomass being able to consume it.

It is also important to remark that the reduction of nitrate was not complete when acetate was used as the carbon source (Figure 4.2a), although sufficient acetate (100 mg COD/L) was added at the beginning of the test. This fact suggests that not all the acetate was being used for denitrification purposes. Since no other electron acceptor was available, it is hypothesized that some of this substrate was stored in the form of an intracellular polymer, such as PHA. In the batch test presented in Figure 4.2a, 11.3 mg of $N\text{-}NO_3^-$ were reduced to nitrogen gas, with a theoretical COD requirement in form of acetate of 32 mg COD/L. However, if we take into account the bacterial growth the theoretical COD requirements will increase to 79 mg COD/L. But 91 mg COD/L were consumed which suggest that part of this COD was stored probably as PHA inside the cells. Many microorganisms have been shown to store acetate into PHA under anaerobic conditions (van Rijn et al., 2006; Zeng et al., 2003a) or even when oxygen is present as an electron acceptor in a process that has been called “feast/famine” (Coats et al., 2007; Dionisi et al., 2004). Unfortunately, PHA could not be analysed at the time of the study and this hypothesis could not be corroborated.

With all the substrates, nitrous oxide reduction rate was always higher than nitrate or nitrite reduction rates when a single electron acceptor was used (Figure 4.2g, h, i)

indicating that each denitrifying microbial group carried out full denitrification and N_2O never accumulated under normal operational conditions. This was indeed a corroboration of the cycle studies results performed in the denitrifying SBR where the nitrate added was always completely reduced without N_2O being detected.

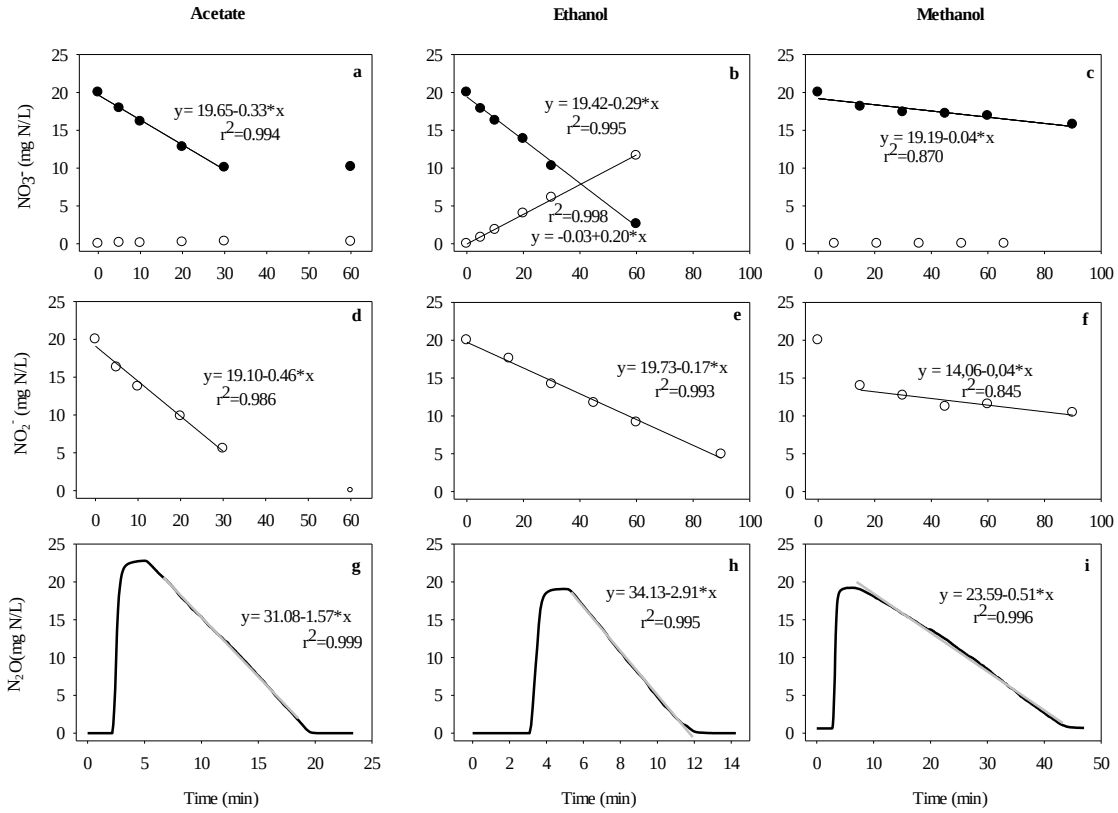

Figure 4.2: Nitrate, nitrite and nitrous oxide profiles with their correspondent regression lines for acetate, ethanol and methanol with nitrate, nitrite and nitrous oxide as single electron acceptors respectively (● NO_3^-, ○NO_2^-, and - N_2O).* Notice the different scale of the x-axis. It is due to the velocity of the reduction of each of the electron acceptors added depending on the batch tests.

4.3.2 Effect of electron competition on nitrogen oxides reduction and N_2O accumulation

A flow of electrons is required during the denitrification process for all the reductive steps which are provided from the oxidation of carbon via the electron transport processes. The general understanding was that this electron supply was never a limiting step for heterotrophic denitrification if the organic carbon was in excess. However, a study by Pan et al., (2013a) strongly suggested that electron consumption rates were limited by the upstream electron supply from the carbon oxidation and electron transport processes. These authors concluded that all the denitrification enzymes (NaR,

NiR, NoR and NoS) competed for electrons coming from a common electron supply system, and this competition occurred even when carbon was in excess. Since the electron supply would depend on how fast a substrate can be oxidized, it is possible that the so called "electron competition" might depend on the type of substrate used. To investigate the extend of the electron competition in a denitrifying culture adapted to three different substrates, seven different tests with different combinations of electron acceptors were carried out for each of the substrates (Table 4.1). As mentioned above, when added as a single electron acceptor, N_2O reduction rate was always significantly higher than nitrate or nitrite reduction rates for the three substrates tested. However, this rate was the most decreased when more than one electron acceptor was added (Figure 4.3, tests D, E, F, and G). Interestingly, in these tests, the reduction observed for nitrate and nitrite reduction rates was less dramatic. This observation not only suggests the occurrence of a competition for electrons but also a preference in electron flow when this competition occurs, giving priority to the reduction of nitrate/nitrite rather than N_2O. This prioritization on the reduction of different electron acceptors can be related to the bioenergetics of denitrifying bacteria that will give priority to those processes with more energy production. If we take into account that 80% of the ATP is generated during the reduction of nitrate to nitrous oxide, the lack of further reducing the N_2O to N_2 will make very little difference to the overall energy production on this group of microorganisms, originating a net accumulation of N_2O under certain conditions (Richardson et al., 2009).

When methanol was used as the only carbon source, nitrate and nitrite were reduced constantly and slowly and there was not an accumulation of nitrite in any of the batch tests conducted (Figure 4.3c, test F). However, these results are opposite to the ones obtained by Lu and Chandran, (2010) where they achieved a near complete nitrite reduction in the ethanol-fed SBR but not in the methanol-fed sequential batch reactor when nitrite and nitrate were added. The differences in the accumulation of nitrite in this study are possibly due to the mixed culture of the biomass used since these authors had two different specialized cultures because there was only one carbon source added in each of the SBRs.

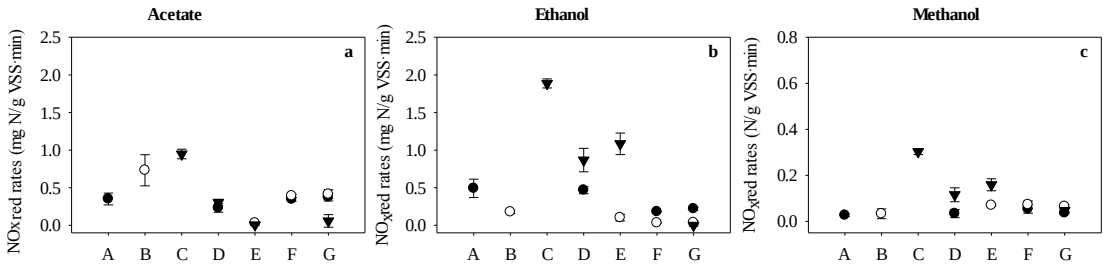

Figure 4.3: Nitrate, nitrite and nitrous oxide specific reduction rates using acetate (a), ethanol (b) and methanol (c) respectively, with error bars showing the standard deviation associated (● NO_3^-, ○NO_2^-, and ▼N_2O).*Notice the different scale of the y-axis in the case of methanol.

Nitrous oxide accumulated in some cases, being its reduction rate lower than nitrite reduction rate. This accumulation can be observed in the F type of batch tests with acetate and ethanol (Figure 4.4) but not when methanol was used. When acetate was used (Figure 4.4a) nitrous oxide started to accumulate as soon as the substrate and electron acceptors were added to the batch reactor (Nitrite reduction rate was 0.393 ± 0.02 mg N/g VSS·L and the nitrous oxide reduction rate was 0.106 ± 0.031 mg N/ g VSS·L). In this case the production of N_2O was higher than its consumption. In the case of ethanol, the majority of nitrate was accumulated as nitrite and the small amount of nitrite reduced resulted in nitrous oxide which was not further reduced (Figure 4.4b; being the nitrite reduction rate 0.032 ± 0.003 mg N/g VSS·L and the nitrous oxide reduction rate was 0.002 ± 0.003 mg N/ g VSS·L).

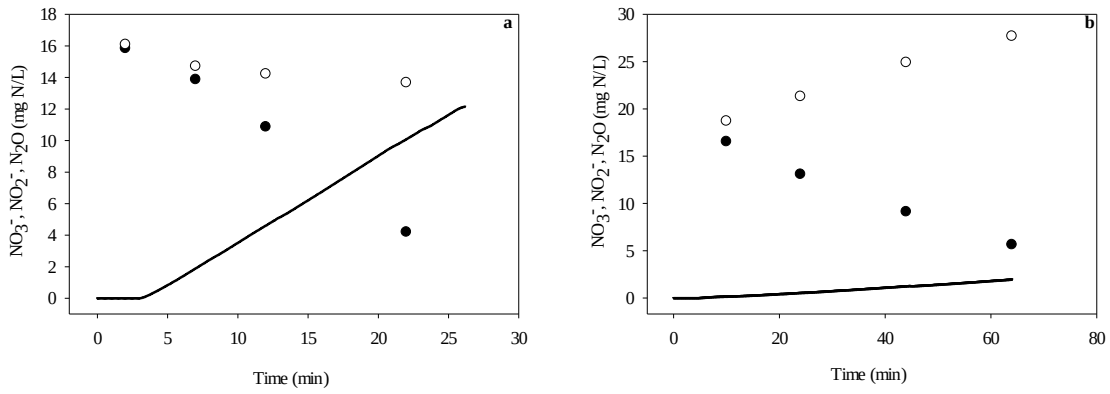

Figure 4.4: Nitrate, nitrite and nitrous oxide profiles for batch tests type F using acetate (a) and ethanol (b) respectively (● NO_3^-, ○NO_2^-, and -N_2O).

Figure 4.5 shows the reduction rates of the nitrogen oxides when a combination of the three carbon sources was used (100 mg COD/L divided equally between acetate, ethanol and methanol). In the work conducted by Pan et al., (2013a) they reported that the true reduction rates for all the added nitrogen oxides decreased when there was more

than one electron acceptor added in comparison to the tests with a single electron acceptor added. This is also the case in this study; nitrate and nitrite reduction rates were reduced when there was more than a single electron acceptor added. Nitrous oxide reduction rate was also reduced due to electron competition (batches D and E). However, there was not N_2O accumulation in any of the batch tests. These experiments are in agreement with the behaviour inside the SBR where N_2O accumulation was never detected.

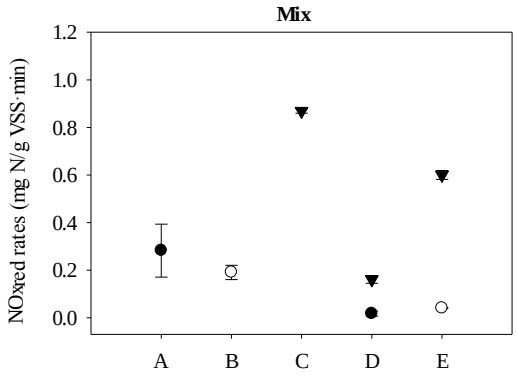

Figure 4.5: Nitrate, nitrite and nitrous oxide specific reduction rates using a combination of the three carbon sources with error bars showing the standard deviation associated (● NO_3^-, ○ NO_2^-, and ▼ N_2O).

4.3.3 Effect of substrate limitation on N_2O reduction

Figure 4.6 shows the nitrate, nitrite, nitrous oxide and COD profiles for the batch tests type D using acetate and ethanol. In these tests, two zones could be differentiated, A and B, marking the period when external substrate was available (zone A) and the period when no external substrate was remaining (zone B).

In the case of acetate, nitrate reduction almost stopped at around 40 minutes. However, when ethanol was used, nitrate reduction was drastically reduced at around 20 minutes and nitrous oxide started to be accumulated.

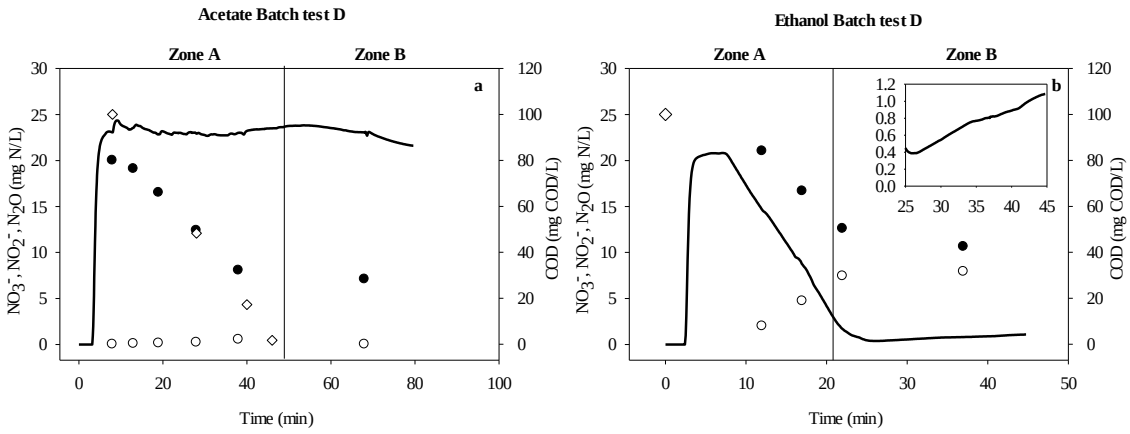

Figure 4.6: Nitrate, nitrite, nitrous oxide and COD profiles for batch type D using acetate (a) and ethanol (b) (● NO_3^-, ○NO_2^-, -N_2O and ◊ COD). Zone A refers to the time when external carbon is available and zone B refers to the time when external carbon is limited. In the case of ethanol there is a zoom for the N_2O accumulation.

Our hypothesis is that in Zone B there was a limitation in the supply of electrons due to a limitation on the external carbon. This limitation caused a reduction in all the reduction rates (Table 4.2) but this reduction differed depending on the substrate. When acetate was available (Figure 4.6, zone A) the N_2O reduction rate was 31% higher than the nitrite reduction rate and it did not accumulate. In the case of ethanol, the N_2O reduction rate was 323% higher than the nitrite reduction rate in zone A. On the other hand, when external substrate was depleted (zone B), both rates decreased one order of magnitude.

More experiments are needed to clarify the effect of substrate limitation on the N_2O reduction rate and also to elucidate if storage polymers might play a role on the reduction of this rate**.**

Table 4.2. Nitrate, nitrite and nitrous oxide reduction rates using acetate and ethanol when there is external carbon availability (zone A) and when there is external carbon depletion (zone B).

	Acetate			**Ethanol**		
	NO_3^- reduction	NO_2^- reduction	N_2O reduction	NO_3^- reduction	NO_2^- reduction	N_2O reduction
	mg N/g VSS·min			mg N/g VSS·min		
Zone A	0.231±0.055	0.224±0.052	0.294±0.041	0.468±0.047	0.205±0.068	0.868±0.154
Zone B	0.020±0.001	0.029±0.001	0.040±0.014	0.081±0.011	0.055±0.013	0.050±0.038

4.4 Implications of the study

In municipal wastewater treatment facilities, the majority of denitrification occurs using the easily biodegradable COD present in the wastewater. However, and due to the increase on the demands of nitrogen removal in recent years, the addition of external carbon is required to achieve the desired nitrogen effluent concentrations. Although a wide range of carbon sources have been studied as a source of electrons for denitrification (Aravinthan et al., 2001; Hallin and Pell., 1998; Purtschert et al., 1996; Tam et al., 1994), the most commonly used in full-scale applications are acetate, ethanol and methanol. It has been reported that acetate causes an immediate increase on the denitrification rate after its addition while ethanol and methanol require a longer period for the activated sludge to achieve its maximum denitrification rates (Aesoy et al., 1998; Hallin and Pell., 1998). The reason behind this is the fact that these three carbon sources are oxidized via different metabolic pathways within denitrifying bacteria. The oxidation of acetate for instance, occurs via the tricarboxylic acid cycle (TCA cycle) which is a common pathway in denitrifying bacteria (Gottschalk., 1986; White., 2000). On the other hand, the oxidation of methanol cannot be conducted through the TCA pathway and requires the activation of two other metabolic pathways with the need of specific enzymes for methanol degradation (Hallin and Pell., 1998) which seem to be present in a small fraction of the denitrifiers present in wastewater treatment plants. This would explain the higher denitrification rates detected when acetate was individually added in our study compared with the rates obtained with methanol. Finally, for the case of ethanol, its oxidation requires two specific enzymes to convert it to acetate which is further oxidized via the TCA cycle. Ethanol oxidizers are expected to be present in activated sludge and it is proven that ethanol addition significantly increases denitrification rates. However, lab-scale studies reported that their yield is three times greater than the one obtained with acetate (Constantine & Fick., 1997) and therefore, more sludge is expected to be produced when used in full-scale, increasing its treatment costs.

Our study compares the denitrification capabilities of a mixed microbial denitrifying population developed with these three carbon sources, when these ones are added independently or as a mix. Special emphasis is given to the nitrous oxide reduction observed with each carbon source. Results obtained suggest that ethanol would be the carbon source with lower nitrous oxide emission potential, since its N_2O reduction rate

is the highest compared with the other carbon sources under the different electron competition scenarios tested. However, an integrated assessment taking into account the aspects previously mentioned should be conducted before selecting one substrate or another one.

Chapter 5

Chapter 5 Distinctive denitrifying capabilities lead to differences in N_2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms

This article was published as:

Anna Ribera-Guardia, Ricardo Marques, Corrado Arangio, Monica Carvalheira, Adrian Oehmen, Maite Pijuan. 2016. Distinctive denitrifying capabilities lead to differences in N_2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms. Bioresource Technology 219, 106-113.

5.1 Preliminary remarks

This study explores the denitrification kinetics from two separate enriched cultures of dPAO and dGAO and compares their N_2O accumulation potential under different conditions. Two sequencing batch reactors were inoculated to develop dPAO and dGAO enriched microbial communities separately. Seven batch tests with different combinations of electron acceptors (nitrate, nitrite and/or nitrous oxide) were carried out with the enriched biomass from both reactors. Additionally, the effect of the simultaneous presence of several electron acceptors in the reduction rates of the different nitrogen oxides was assessed in dPAOs and dGAOs.

5.2 Materials and methods

5.2.1 Bioreactors set-up and operation

Two lab-scale sequential batch reactors (SBRs) were operated to develop a dPAO and a dGAO enriched culture, respectively. Both reactors were operated as described in Chapter 3.

Cycle study analyses were weekly performed. Samples were taken during each phase to analyse nitrate, nitrite, phosphate, ammonia and VFAs. Samples were filtered through 0.22µm Millipore filters. At the end of the cycle samples for MLSS and MLVSS were also taken.

5.2.2 Batch tests experiments

Both reactors were in steady state conditions and displaying typical dPAO and dGAO phenotypes when the batch tests were conducted.

7 different batch tests (A-G, Table 5.1) with different combinations of electron acceptors (NO_3^-, NO_2^-, N_2O) were carried out in a sealed batch reactor with no head-space (in order not to have N_2O stripping) with enriched dPAO or dGAO sludge withdrawn from the end of the anaerobic phase of the parent SBR, respectively. Nitrogen gas was sparged into the batch reactor (explained in detail in Chapter 3) to ensure anoxic conditions during the batch tests. In each batch, a concentration of 20 mg NOx-N/L of each nitrogen oxide indicated in Table 5.1 was initially added as a pulse.

Dissolved N_2O concentration was continuously monitored with an online N_2O microsensor (Unisense A/S, Denmark; Ribera-Guardia et al., 2014; Wang et al., 2015) and samples for the analysis of nitrate, nitrite, phosphate, ammonia and VFAs were taken along the experiment. All the experiments were carried out in duplicates. Biomass concentration was also analysed at the end of each test to calculate the specific reduction rates. Batch tests for both cultures were conducted over a period of 2 months.

Table 5.1. Batch tests conducted for each set of experiments.

Batch test type	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Electron acceptors used	NO_3^-	NO_2^-	N_2O	NO_3^- N_2O	NO_2^- N_2O	NO_3^- NO_2^-	NO_3^- NO_2^- N_2O

5.2.3 Chemical and microbial analysis

Chemical analysis for the dGAO and dPAO reactors were performed as described in detail in Chapter 3.

FISH was also performed at the end of the anaerobic and aerobic phases using the following oligonucleotide probes: EUB338, EUB338II, and EUB338III were applied together (EUBMIX), for most *Bacteria* (Daims et al., 1999); as well as PAO651, PAO462 and PAO846, (PAOMIX) which refer to most of the members of *Accumulibacter* group , Acc-I-444 which refers to Type I of PAOs (able to denitrify from nitrate and nitrite), Acc-II-444 which refers to Type II of PAOs (able to denitrify from nitrite only) (Flowers et al., 2009), GAOQ989, GAOQ431 and GB_G2 (GAOMIX) which refer to the *Candidatus Competibacter phosphatis* (able to denitrify from nitrate and nitrite) (Crocetti et al., 2000); TFO_DF218 and TFO_DF618, (DFImix) for Cluster I of *Defluviicoccus*-related GAOs (able to denitrify from nitrate but not from nitrite); DEF988 and DEF1020 with helpers H966 and H1038, (DFIImix) for Cluster II of *Defluviicoccus*-related GAOs (not able to denitrify); DF198 for Clusters III of *Defluviicoccus*-related GAOs (DFIII) and DF181A and DF181B for Cluster IV of *Defluviicoccus*-related GAOs (DFIV). FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63 x oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The

area containing Cyt-3 labelled specific probe (PAOMIX, PAOI, PAOII, GAOMIX, DEFIMIX, DEFIIMIX, DEFIII and DEFIV, respectively) cells was quantified as percentage of the Cyt-5 labelled bacteria probe (EUBMIX) within each image using the ImageJ and Pixel Counting programs.

An N_2O microsensor was used to monitor continuously the dissolved N_2O in the liquid phase.

The calculations of the measured maximum specific NO_3^-, NO_2^- and N_2O reduction rates and the specific electron consumption rates are shown in Chapter 3.

5.3 Results and discussion

5.3.1 Reactor performance and microbial community characterization

After 5 months of operation, stable nitrogen and phosphorus removal was achieved in the dPAO SBR. The reactor was operating with 100% volatile fatty acids removal, 72% phosphorus removal and 93% nitrate removal, with no nitrite accumulation. During the anaerobic phase acetate and propionate were completely consumed, releasing phosphorus into the liquid phase. During the following anoxic phase nitrate added was almost completely removed with a simultaneous phosphorus uptake. No nitrite accumulation was detected. Finally, during the aerobic phase, the remaining phosphate was taken up (Figure 5.1a). The P release/VFA uptake ratio was 0.44 ± 0.07 Pmol/Cmol. This P/C ratio agrees well with the one obtained by Carvalheira et al., (2014b) using an enriched PAO culture fed with the same combination of acetate-propionate, suggesting that the activity observed in the bioreactor resulted mainly by dPAO rather than dGAO.

The dGAO reactor was operated for half a year before the experiments were conducted. Figure 5.1b shows a typical cycle study profile. All VFAs were consumed during the anaerobic period. During the following anoxic phase, the nitrate added was completely consumed and nitrite accumulated while nitrate was present. Afterwards, nitrite was also consumed. Phosphate concentration did not change and remained very low during the whole cycle (<1 mg P/L).

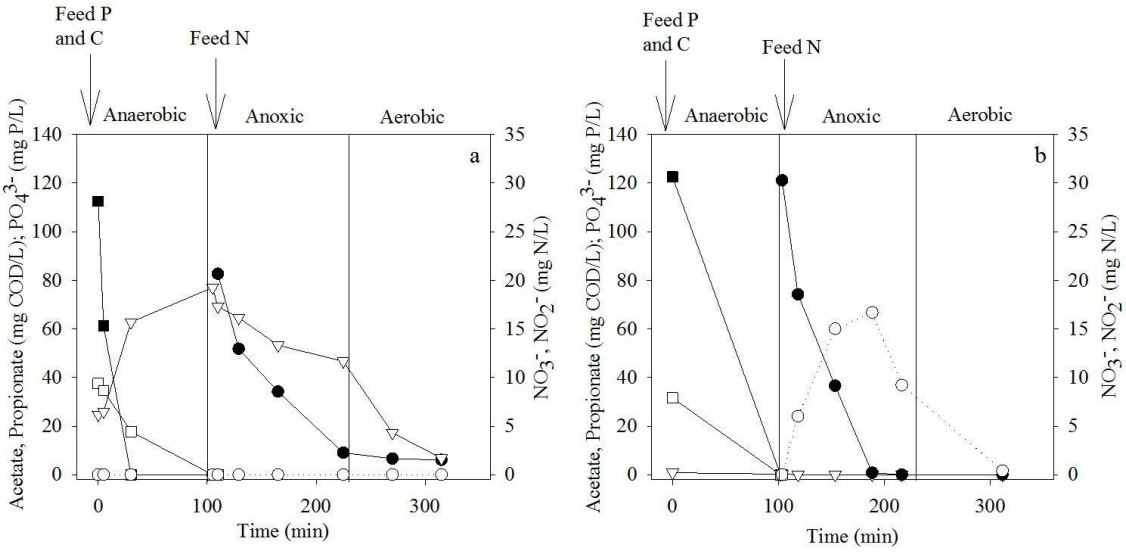

Figure 5.1: Experimental acetate (■), propionate (□), phosphate (▽), nitrate (●), and nitrite (○) profiles analyzed during a typical cycle study conducted in the dPAO (a) and dGAO (b) reactors.

Microbial analysis were conducted in each SBR at the time when the batch tests were carried out. Table 5.2 shows the quantification of each microbial community through the FISH technique.

Table 5.2. FISH quantification of the dPAO and dGAO SBR cultures used in the batch tests.

FISH PROBES	Relative abundance
dPAO-SBR	
PAO I	26.03 ± 4.75 %
PAO II	15.42 ± 2.82 %
PAOMIX	42.40 ± 8.32 %
GAOMIX	22.93 ± 4.41 %
DFImix, DFIImix and DFIII	4.17 ± 0.16 %
dGAO-SBR	
GAOMIX	55.60 ± 1.86 %
DFImix, DFIII and DFIV	6.33 ± 0.24 %
DFIImix	13.20 ± 0.88 %
PAOMIX	14.30 ± 1.52 %

42% of the bacterial community present in the dPAO-SBR was targeted by PAOMIX (comprising the microorganisms belonging to the *Accumulibacter*-PAO group), with 26% being type PAO I (able to denitrify from nitrate and nitrite) and 15% being type PAO II (only able to denitrify from nitrite). Also, GAOs were detected in this biomass

with 23% of the bacterial community belonging to the *Competibacter*-GAO group and 4% belonging to the *Defluviicoccus*-GAO group. The fact of having dGAOs in the dPAO reactor could affect the availability of VFAs for the dPAO microorganisms since dGAOs compete with dPAOs for the same organic carbon source. However, as it was mentioned before the behaviour of the dPAO reactor showed an activity of an enriched dPAO culture.

For the case of dGAO-SBR, *Competibacter* (targeted by GAOMIX) and *Defluviicoccus-GAO* comprised around 75% of the microbial population while around 14% of the bacterial population belonged to the *Accumulibacter*-PAO group. In this case a low percentage of dPAOs was found in the dGAO reactor. dPAO could affect on the uptake of VFAs in the anaerobic phase and also on the nitrogen removal in the anoxic phase but since there is no P feed in the dGAO reactor no P removal is observed. Moreover since only 14% of the bacterial population were dPAOs, the dGAO reactor had a good behaviour.

An example of two images from the FISH quantification of the PAOmix for the dPAO culture and of the GAOmix for the dGAO culture is shown in Figure 5.2

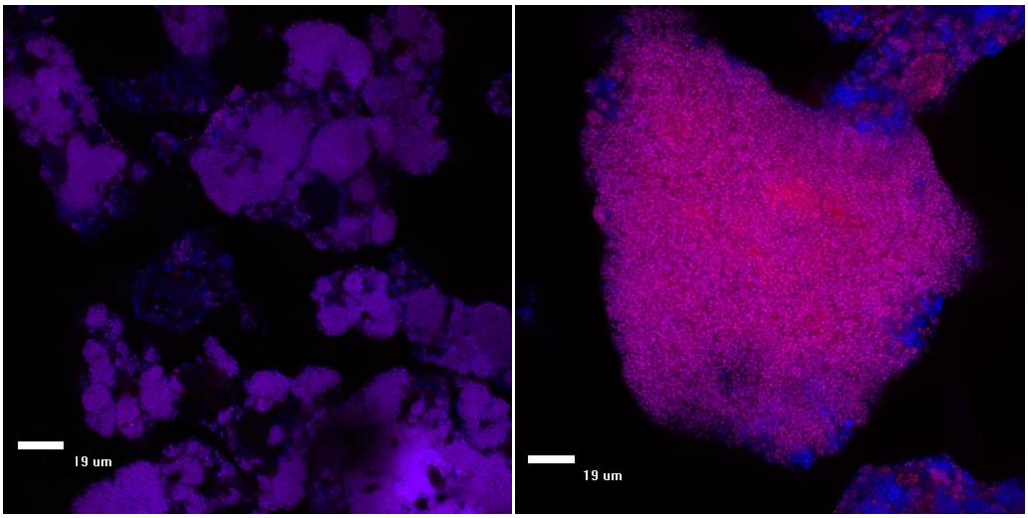

Figure 5.2: FISH images of the enriched dGAO biomass (left) and enriched dPAO biomass (right) used in the batch tests. In blue is shown EUBMIX (all bacteria) and in magenta is shown GAOMIX and PAOMIX.

5.3.2 Distinctive denitrification kinetics of dPAO and dGAO cultures with different electron acceptors

Figure 5.3 shows the experimental profiles obtained in the batch tests conducted with one electron acceptor (tests A-C, see Table 5.1) for the dPAO and the dGAO cultures respectively.

In tests A and B, N_2O accumulated in both cultures, since its reduction rate was slower than the nitrite reduction rate. For the case of dGAOs, nitrite also accumulated (batch test A) indicating that dGAO had a preference to consume nitrate against nitrite. That was not the case for dPAOs where nitrite did not accumulate in any of the cases. The nitrite reduction rate in dPAOs was around 2 times higher than that of dGAOs in test B (21.24 ± 3.96 mg N/g VSS·h and 9.96 ± 1.44 mg N/g VSS·h, respectively). Therefore, nitrate reduction can be considered as the rate-limiting step for dPAO.

Nitrite addition caused an important increase on N_2O accumulation in the case of dGAOs (Figure 5.3b-right). dPAOs, had a higher nitrous oxide reduction rate than dGAOs, especially when nitrite was added as the sole NOx in test B (18.9 ± 4.62 mg N/g VSS·h and 1.63 ± 0.71 mg N/g VSS·h, respectively), suggesting a possible inhibitory effect of nitrite on the nitrous oxide reductase for dGAOs, which was not observed for dPAOs.

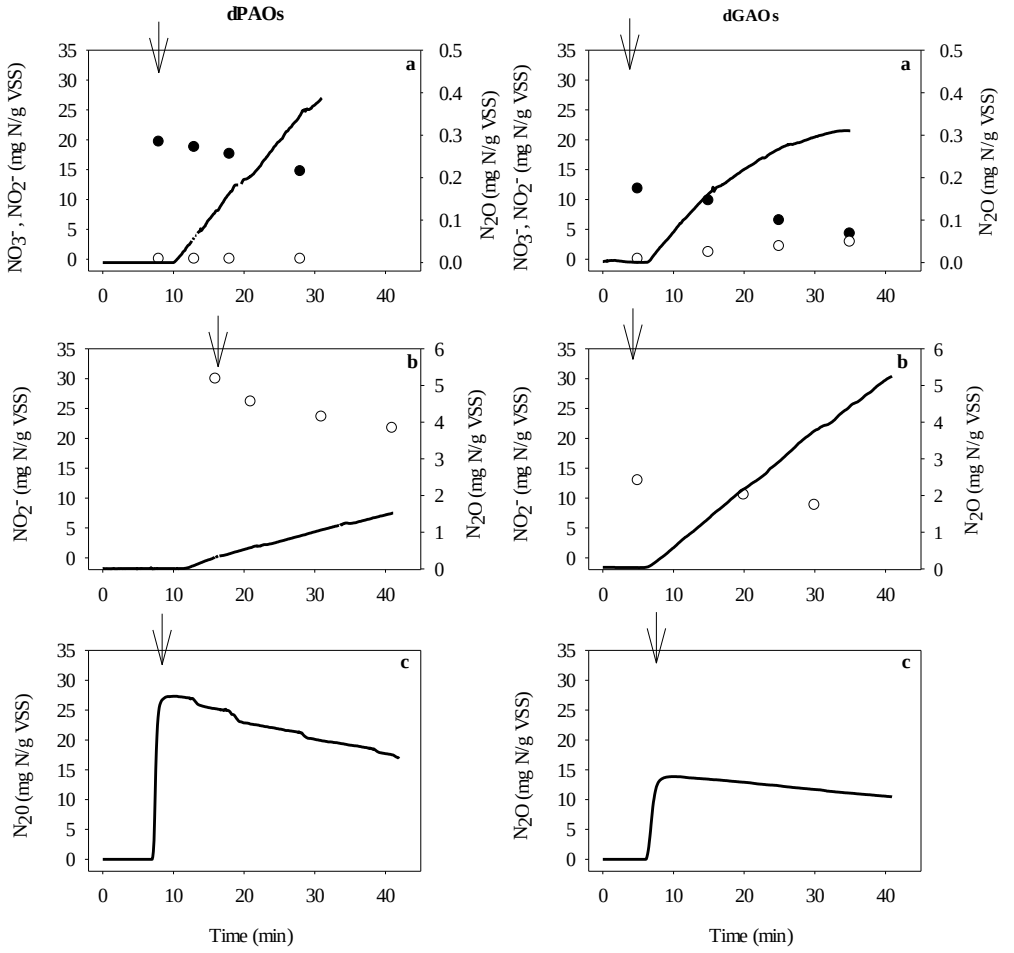

Figure 5.3: Nitrate (●), nitrite (○), and N_2O (-) profiles for batch tests A, B and C for dPAO (left) and dGAO (right) cultures. The arrows represent the moment when NOx was added. Notice the different N_2O axis scale in Tests B (b) compared with Tests A (a).

Figure 5.4 shows the specific reduction rates for each electron acceptor added in each type of batch test conducted. When comparing both cultures, dPAOs had higher reduction rates in general compared with dGAOs, showing higher denitrifying capacity.

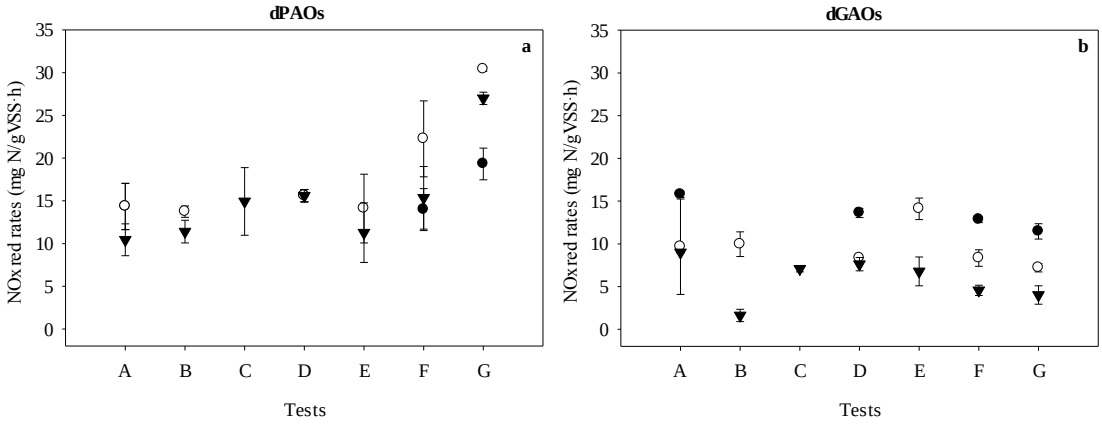

Figure 5.4: Nitrogen oxides reduction rates (● NO_3^-, ○NO_2^-, and ▼N_2O) for dPAOs (a) and dGAOs (b) cultures.

In the case of dPAOs, nitrate reduction rate was relatively constant across the different tests. Interestingly, nitrite reduction rate significantly increased in those tests where it was added simultaneously with nitrate (Tests F & G). It was found that dPAOs had a preference for nitrite as electron acceptor, presenting the highest N reduction rates in all the tests where nitrite was added. N_2O reduction rate was slightly lower than nitrite reduction rate in the majority of the tests, resulting in some N_2O accumulation (see Table 5.3).

On the other hand, the dGAO population presented a preference for nitrate, having higher nitrate reduction rates than those of nitrite. Also, the rate of nitrate reduction was relatively constant in all tests where nitrate was added, independently if it was added alone (test A) or in combination with other electron acceptors (Tests D, F & G). An important reduction on the nitrous oxide reduction rate was observed in those tests where nitrite was added (tests B, E, F & G), suggesting an inhibitory effect of nitrite towards the last step of denitrification in dGAOs.

Denitrification kinetics for both cultures differ depending on the electron acceptors used. When using nitrate, whether as a sole electron acceptor or in combination with nitrite and/or nitrous oxide, NO_3^- reduction rates for dPAO and dGAO cultures are similar (around 15.88 ± 2.40 mg N/g VSS·h and 13.43 ± 1.80 mg N/g VSS·h, respectively) in all the scenarios tested. However, nitrite reduction rates are only similar when nitrate is not present (batches B & E; around 13.92 ± 0.22 mg N/g VSS·h for dPAOs and 12.01 ± 2.94 mg N/g VSS·h for dGAOs). In the cases where nitrate is present (batches A, D, F and G) nitrite reduction rate decreases significantly in the case of dGAOs compared to dPAOs (around 20.73 ± 7.30 mg N/g VSS·h in the dPAO culture and 8.39 ± 1.00 mg N/g VSS·h in the dGAO culture), which might be due to a preference to reduce nitrate over nitrite. In the study of Zeng et al., (2003b) it was reported that when adding nitrate as the electron acceptor there was accumulation of nitrite and N_2O for a dGAO culture which agrees well with the results in this study. They postulated that this accumulation could be due to different dGAO populations mediating the different steps in denitrification. McIlroy et al., (2014) found that subgroup 1 of *Competibacter*-related GAOs called "*Candidatus Competibacter denitrificans*" was able to denitrify from nitrate to nitrite, from nitrate to nitrogen gas and also from nitrite to nitrogen gas whereas another subgroup (subgroup 5) of *Competibacter*-related GAOs called "*Candidatus Contendobacter odensis*" was only

able to denitrify from nitrate to nitrite. In our study it was not possible to determine the different sub-groups of *Competibacter* present in the SBR, but the results obtained are consistent with this reasoning behind the preference of dGAOs towards nitrate.

Table 5.3 shows the percentage of N_2O produced per nitrogen reduced for all the tests conducted.

Table 5.3. Percentage of N_2O accumulated per N-reduced for both cultures.

Batch test type*	**N_2O accumulation per N-reduced (%)**	
	dPAOs	**dGAOs**
A	8.72 ± 0.20 %	7.12 ± 2.16 %
B	17.40 ± 5.90 %	83.95 ± 4.79 %
D	0.00	13.71 ± 5.81 %
E	20.11 ± 1.90 %	56.90 ± 4.92 %
F	31.20 ± 2.70 %	45.45 ± 0.89 %
G	11.30 ± 3.10 %	48.45 ± 5.94 %

*Test C is not presented since only N_2O was added.

In almost all cases, dGAOs presented higher N_2O accumulation per N-reduced than dPAOs. The percentage of accumulation was very high for the test where nitrite was added alone, around 80%. In general, the N_2O accumulation levels in dPAOs were lower than those found in dGAOs, with the highest being 31% for test F. These values indicate that high N_2O emissions are very likely to occur in those systems where denitrification is carried out by dGAOs, and/or where nitrite accumulates. Gao et al., (2017) reported N_2O production in a system with simultaneously P and N removal using PHA as the carbon source for the denitrification process with a high abundance of *Accumulibacter*. They reported that when external carbon was not available, PHA was used as carbon source for denitrification and this led to N_2O accumulation. However, Ge et al., (2017) studied the N_2O emissions from an (anaerobic/oxic/anoxic) AOA sequencing biofilm batch reactor with dPAOs and it reported that at the highest C/N ratio, the synthesis of intracellular carbon was increased and it reduced the N_2O emissions. This is similar to our results since dPAOs show lower N_2O emissions than dGAOs. Nitrous oxide reduction rates were lower for dGAOs than dPAOs in all the scenarios tested. Therefore, there was more N_2O accumulation in the dGAO culture. Lemaire and co-workers., (2006) reported that the net N_2O production from

denitrification was linked to dGAOs, which were responsible for denitrification in a simultaneous nitrification, denitrification and phosphorus removal reactor. Also, Zeng et al., (2003b) and Zhu and Chen., (2011) reported that dGAOs were the major contributor to N_2O production in their study.

NO_2^- did not accumulate in any of the batch tests for the dPAO culture, which is in agreement with the results found by Carvalho et al., (2007), who found no accumulation of nitrite in a dPAO reactor fed with propionate as the sole carbon source. However, nitrite accumulated in all the batch tests with the dGAO culture, which can be explained by two possible reasons: i) the microbial population characteristics, with a predominant dGAO group only being able to conduct the first step of denitrification; ii) an inhibition by nitrite/free nitrous acid (FNA) on the reduction step of this compound. The concentration of FNA in the batch tests where nitrite was accumulated ranged from 0.31- 1.92 µg HNO_2-N/L. This concentration is similar to the one reported by Semerci and Hasılcı., (2016) (0.01-2.27 µg HNO_2^-N/L) in a dPAO and dGAO culture, who found an increase of dGAOs over dPAOs under these FNA levels . Also, Ye and co-workers., (2013) studied the effect of FNA on the anaerobic and aerobic metabolism of GAOs and found that PAOs were more affected by FNA than GAOs under the same FNA concentrations. The fact that nitrite reduction was not affected in dPAOs under the same FNA/nitrite concentrations as in the dGAO culture suggests that FNA inhibition did not play an important role in the accumulation of nitrite in dGAOs. We hypothesize that nitrite accumulation was due to the microbial composition within the dGAO culture. Indeed, Tayà et al., (2013) showed that nitrite was more readily utilized by dPAO than *Defluviicoccus* GAO when propionate was fed as the C source, which corroborates our results. Overall, the fact that a wider diversity of *Accumulibacter* PAO sub-groups seem to be capable of nitrite reduction as compared to the diversity of GAO sub-groups (Oehmen et al., 2010) could explain why PAOs were more able than GAOs to denitrify the ~20 mg NO_2^--N/L fed during the batch tests.

The highest denitrification rate within the dPAO culture was obtained when nitrite was used as electron acceptor. Also, when using nitrate as the electron acceptor, all the nitrogen oxide reduction rates decreased. This suggests the presence of two different types of dPAO groups, one able to denitrify from nitrate and another able to denitrify from nitrite. This hypothesis is consistent with previous reports from Oehmen et al., (2010) and would imply that the denitrification rates are lower in the case of nitrate

since only one of the dPAO groups is able to reduce nitrate, while both are able to reduce nitrite. This hypothesis is corroborated with the quantification of the microbial community, being 26% of the dPAO culture from *Accumulibacter* group Type PAO I and 15% from *Accumulibacter* group Type PAO II. Therefore, as there were more dPAOs able to denitrify nitrite than to denitrify nitrate, nitrite reduction rates were higher than nitrate in all cases for the dPAO culture. This hypothesis would also explain the fact that in the cases where nitrate and nitrite was added (batches F & G), nitrite reduction rate was higher than when adding nitrate (batches A & D). When there was addition of NO_3^-, PAO Type I were responsible for reducing it to NO_2^-, making it the rate limiting step for nitrite reduction by both PAO Types, while in batches F and G both sub-groups of PAOs could reduce NO_3^- and NO_2^- simultaneously at their maximum rates, due to the higher simultaneous abundance of both nitrogen oxides, thereby reducing nitrite faster.

5.3.3 Electron competition and distribution when using PHA as carbon source for denitrification

Tests D-G were carried out in order to see if there was a competition for electrons when several electron acceptors were present simultaneously. Figure 5.5 shows the electron consumption rates of all the nitrogen oxides reductases for all the batch tests in both dPAO (a) and dGAO (b) cultures and the electron distribution for dPAOs (c) and dGAOs (d) respectively.

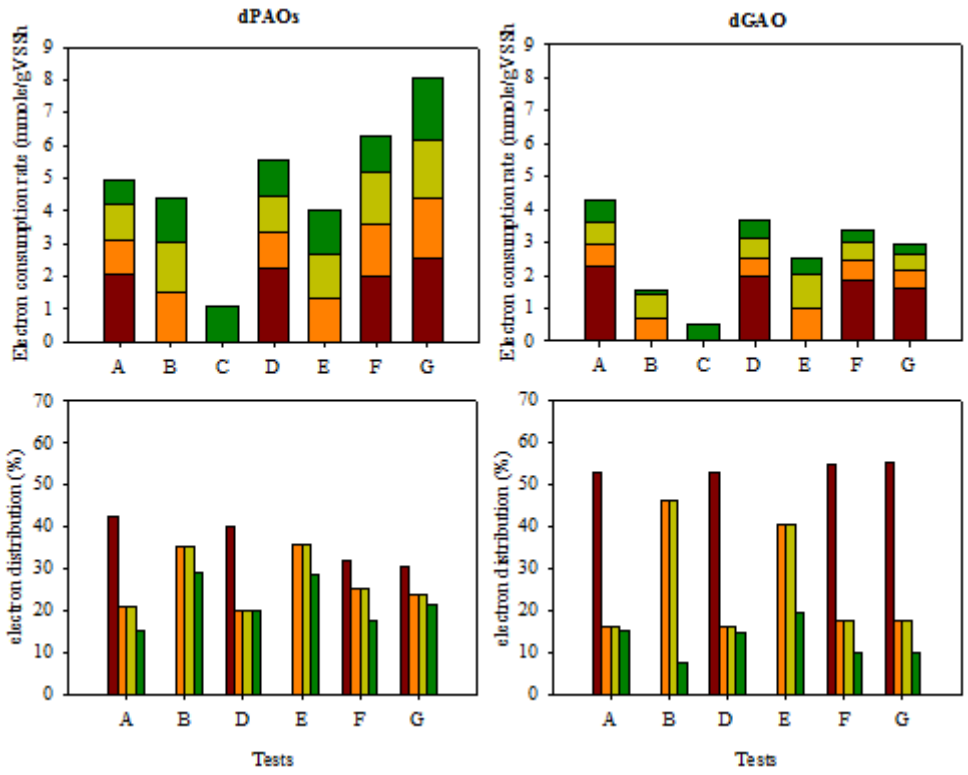

Figure 5.5: Electron consumption rates (a and b) and electron distribution (c and d) for nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NoR) and nitrous oxide reductase (NoS) for dPAO (left) and dGAO (right) cultures.

The electron consumption rates by NaR, NiR, NoR and NoS were very similar in all the experiments independently of the electron acceptor addition scheme. This suggests that there was not competition for electrons in either dPAOs or dGAOs. Higher electron consumption rates were obtained in the dPAO tests due to the fact that this culture had higher denitrification rates. The maximum electron consumption rate in the dPAO culture was obtained in experiment G, where all the electron acceptors were added simultaneously.

The electrons were distributed depending on the electron acceptors added in each test. For example, in the case of Test A in dPAOs, the expected electron distribution was found, with around 40% of the electrons going to NaR and the remaining 60% almost evenly distributed among the other reductases. This was also the case for Test D, where nitrate was added together with N_2O. Interestingly, in those tests where nitrate was added together with nitrite, the percentage of electrons distributed to NaR decreased, increasing the fraction diverted to the other reductases. This suggests the activation of another microbial group which denitrifies from nitrite.

In the case of dGAOs, the clear preference for nitrate is highlighted in Figure 5.5. Between 50 to 60% of electrons were derived to NaR, with the remaining evenly distributed among the other reductases when nitrate was added alone or in combination with nitrous oxide (tests A & D). The addition of nitrite (tests B, F & G) caused a clear decrease on the electrons diverted to NoS.

It is likely that electron competition was not significant due to the different subgroups of PAO and GAO organisms present in the SBRs and their preferences for utilising nitrate or nitrite, which were activated depending on the electron acceptors added. Since there were different groups of microorganisms performing the different steps of the denitrification process, electron competition between NaR and the other reductases was not detected. This is expected since the electron supply system from the different subgroups of dPAOs and dGAOs is independent of each other.

Accumulibacter, *Competibacter* and *Defluviicoccus* (Cluster I) have been found to possess different mechanisms for anaerobic acetate uptake (Burow et al., 2008; Saunders et al., 2007). Wei et al., (2014) showed that the electron consumption rate of NiR and NoS descended with the PHA degradation rate in a dPAO culture. Accordingly, electron competition between nitrite reductase and nitrous oxide reductase did not get intensified when carbon was degraded more slowly in denitrification with PHA. These findings are in agreement with our results. Overall, it appears that electron competition during the reduction of different nitrogen oxides is a significant factor in ordinary heterotrophic denitrification processes based on external carbon sources as the electron donor, and not in PHA-driven denitrification processes by PAOs or GAOs.

5.4 Implication of the study

N_2O is an intermediate compound in the denitrification process and its accumulation is strictly linked to the activity of the NoS enzyme. N_2O can accumulate due to two main reasons: i) when the majority of the denitrifying community does not possess the gene encoding for NoS, therefore having nitrous oxide as the end product of denitrification; ii) when nitrous oxide reduction rate is affected by a certain environmental or operational factor becoming lower than the nitrate or nitrite reduction rates. Several environmental factors have been reported to lead to N_2O accumulation during denitrification such as the effect of electron acceptors (oxygen, nitrite/FNA or nitric

oxide), pH, electron donors (type of organic carbon used for denitrification or internal storage compounds such as PHA) or the relationship between COD/N in the wastewater (Alinsafi et al., 2008; Du et al., 2016; Lu and Chandran, 2010; Park et al., 2000; Zhou et al., 2008). In this study, the N_2O emissions of the denitrification process using PHA as the carbon source has been investigated using a dPAO and a dGAO enriched cultures, respectively. Results showed that generally, higher N_2O accumulation was detected in the tests conducted with dGAOs than those conducted with dPAOs. This accumulation becomes critical when nitrite is present, substantially inhibiting the last step of denitrification in dGAOs. This inhibition does not seem to occur in dPAOs (at least at the concentration range tested in this study). Special attention needs to be paid on those systems where nitrite pathway is promoted since the abundance of dGAOs will not only affect the effectiveness of the P removal process of the plant but also will most likely increase its overall N_2O emissions.

Chapter 6

Chapter 6 Distinctive NO and N_2O emission patterns in ammonia oxidizing bacteria: Effect of ammonia oxidation rate, DO and pH

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6.1 Preliminary remarks

This study explores the relationship between NO and N_2O production rates with the ammonia oxidation specific rate (AORsp) in an enriched AOB culture. Different concentrations of ammonia were applied in a SBR performing partial nitritation in order to determine the effect of AORsp on N_2O and NO production rates. The effect of changes on the DO concentration on the overall NO and N_2O emissions was assessed by increasing and decreasing the DO maintaining a constant pH at 7. Finally, the effect of pH on N_2O and NO was also tested by maintaining the DO at 1.5-2 mg O_2/L while pH was gradually decreased from 8 to 6.5.

6.2 Materials and methods

6.2.1 Bioreactor set-up and operation

A cylindrical 8L SBR was inoculated with activated sludge from a local domestic WWTP located in Girona (Spain). The description of the operation of the SBR is detailed in Chapter 3.

Cycle studies were carried out on a weekly basis to monitor the nitrification activity of the reactor. Samples for the analysis of ammonia, nitrate and nitrite were taken along the cycle and filtered with 0.22 µm Millipore filters. At the end of the cycle MLSS and MLVSS were also analysed.

6.2.2 Batch tests

Batch tests were conducted in the same parent reactor. Three sets of experiments were carried out (Table 6.1) . The first set consisted on adding a continuous feed (6.57 mg N-NH_4^+/min) followed with different ammonia concentration pulses to see the effect of the AOR on the N_2O and NO production. The DO and pH were controlled at the same values as in the parent reactor. Samples were taken every 30 minutes to analyse ammonia and nitrite.

The second set of experiments was conducted to explore the effect of DO on N_2O and NO emissions. Three different batch tests were conducted in this set of experiments. In the first batch (2.1) pH was maintained constant at 7 while DO was increased every 15

minutes from 0.5 to 3 mg O_2/L in a stepwise manner. The DO increased from 0.5-1mg O_2/L to 1-2.5 mg O_2/L and 2.5-3 mg O_2/L. The second batch (2.2) mimicked the first but with DO decreasing every 15 min from 3 to 0.5 mg O_2/L in a stepwise mode. In this case the DO decreased in the ranges of 3-2.5, 2-1.5 and 1-0.5mg O_2/L. A pulse of NH_4Cl (50 NH_4^+-N/L) followed by a continuous feed (6.57 mg NH_4^+-N/min) was added in the reactor. In the third batch test (2.3), DO was set at 0 mg O_2/L and pH was maintained at 7 to see the effect of anoxic conditions on the N_2O and NO emissions. No NH_4^+ was added in this test.

The third set of experiments consisted on exploring the effect of pH on N_2O and NO emissions. Five different batch tests were conducted (3.1-3.5). In the first batch (3.1), DO was maintained constant at 1.5-2 mg O_2/L while pH was gradually decreased 0.5 units every 15 minutes from 8 to 6.5. The other batch tests were conducted under the same conditions as batch 3.1. Batch test 3.2 was conducted without addition of ammonia. Batch test 3.3 was carried out without biomass and without the addition of ammonia. In the fourth batch test (3.4) RO water was used without biomass but with the addition of ammonia in the reactor. In batch test 3.5 NaOH was added. All the experiments lasted between 60 and 120 minutes.

Table 6.1. Description of the batch tests conducted.

Set	**Parameters**		
	NH4+	**DO**	**pH**
1	Continuous feed	1.5-2 mg O_2/L	7-7.3
2	143 mg N-NH_4^+/L after feeding	2.1) ↑0.5-3 mg O_2/L	7
		2.2) ↓3 to 0.5 mg O_2/L	7
		2.3) 0 mg O_2/L	7
3	143 mg N-NH_4^+/L after feeding	1.5-2 mg O_2/L	3.1) ↓ 8-6.5 + NH_4^+ + Biomass
			3.2) ↓ 8-6.5 + Biomass
			3.3) ↓ 8-6.5
			3.4) ↓ 8-6.5 + NH_4^+ + RO water
			3.5) add NaOH+ NH_4^+

*↑ means increased and ↓ means decreased

Samples for NH_4^+ and NO_2^- were taken every 15 minutes and filtered through 0.22 µm Millipore filters. At the end of each test samples for MLSS and MLVSS were taken in order to calculate the N_2O and NO production specific rates and the ammonia oxidation specific rate.

6.2.3 Chemical and Microbial analyses

Chemical analyses were performed as detailed in Chapter 3.

FISH was performed as described in Nielsen et al., (2009) using Cy5-labelled EUBMIX (for all bacteria) and Cy3-labelled AOBMIX (for AOBs) comprising equal amounts of oligonucleotide probes Nso1225, NEU and NmV. FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63 x oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The area containing Cy3-labelled specific probe (AOBMIX) cells was quantified as a percentage of the area of Cy5-labelled bacteria probe (EUBMIX) within each image using pixel counting program.

4-amino-5-methylamino-2′,7-difluorofluorescein diacetate (DAF-FM DA) (Kojima et al., 1998) was used for a visual qualitative assessment of the cellular NO production (Namin et al., 2013). In the same procedure DAPI was used for the qualitative assessment of all bacteria. Cell suspension was diluted with 20µM DAF-FM DA solution and incubated for 60 minutes at room temperature and dark conditions. After a 50µg/mL DAPI solution was added to the cell suspension and DAF-FM DA solution and it was kept 15 minutes at 4ºC protected from the light. Then it was centrifuged and washed with a 0.5M TrisHCl solution and incubated for 30 minutes at room temperature in dark conditions before being visualized with an epifluorescence microscope.

The calculations of the specific N_2O and NO emission rates and emission factors are detailed in Chapter 3.

The N_2O and NO emissions were analysed by commercial gas analysers described in detail in Chapter 3.

6.3 Results

6.3.1 Reactor performance

After 1 year of operation, stable nitrogen removal was achieved in the AOB-SBR. The reactor was operating with a 91% of ammonia converted to nitrite and nitrate was not accumulated in the effluent, achieving a complete nitritation process. Quantification of the AOB abundance in the biomass through the FISH technique showed that 79.3 ± 3.6 % of the bacterial community was targeted with the AOBMIX probe.

Ammonia was consumed and nitrite was produced in both aerobic phases. There was a peak of N_2O and NO at the beginning of the cycle (Figure 6.1). These emissions were produced during the first 5 minutes of the cycle and then decreased very quickly. The peak of N_2O was much higher than the one of NO (500ppmv and 6ppmv, respectively).

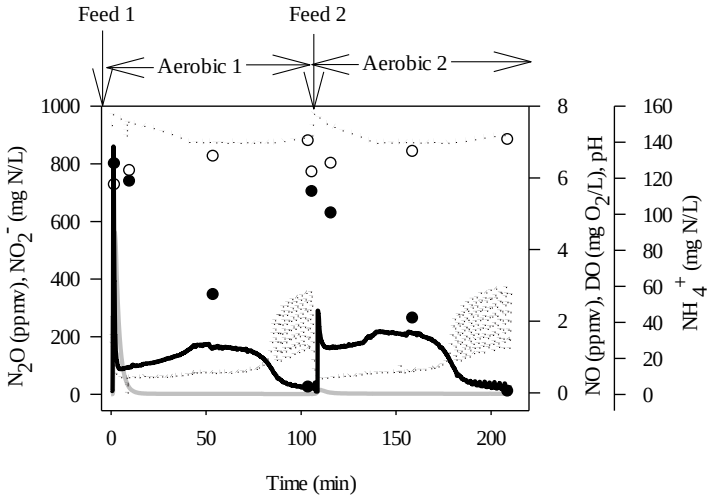

Figure 6.1: Experimental profiles of N_2O (—), NH_4^+ (●), NO_2^- (○), NO (—), DO (···) and pH (···) during a typical cycle study of the AOB reactor. Nitrate was not detected in any of the samples taken.

The peak of N_2O decreased sharply after the addition of ammonia but the production level of NO showed a gradual increase possibly corresponding to the increase on the nitrite concentration. Also, the NO concentrations decreased when DO increased. When ammonium was almost depleted NO decreased to nearly zero. After the second feed, there was another peak of NO which was lower than the one observed during the first 5 minutes of the cycle which can be related to the concentration of ammonia. The pattern of NO in the second aerobic phase was similar to the one in the first aerobic phase showing a gradual increase likely due to an increase on the nitrite concentration and a decrease when DO was decreased. However, N_2O did not show the same pattern on the

second aerobic phase since after the second feeding phase, there was a much lower peak of N_2O than in the first feeding phase. This is due to the fact that the production of N_2O also occurred during the settling phase and was emitted during the first 5 minutes of the cycle due to stripping when aeration started (Rodriguez-Caballero and Pijuan, 2013).

6.3.2 Correlation of NO and N_2O with AOR

In order to identify the correlation between NO and N_2O production and the ammonia oxidation rate, different concentrations of ammonia were added to the reactor to achieve different ammonia oxidation rates. As it was mentioned in Chapter 1 NH_3/NH_4^+ has been reported as an important factor affecting N_2O and also NO production in AOB systems. Having higher N_2O and NO production when ammonia is added due to a shift on the activity of AOBs. Therefore, the AOR can be linked to N_2O and NO production in AOB systems. Figure 6.2 shows an example of the profiles of NO, N_2O and NH_4^+ obtained in the first set of experiments.

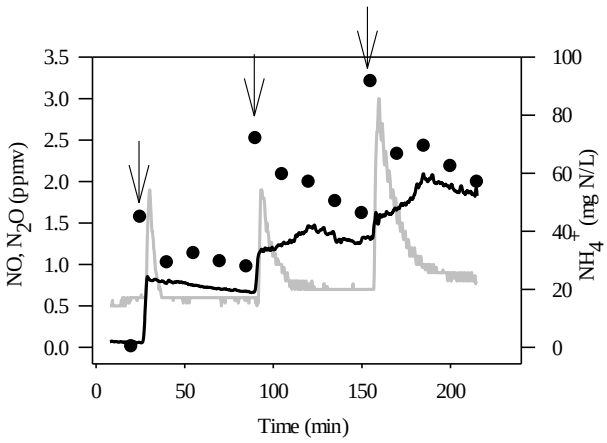

Figure 6.2: Experimental profiles of NO (—), N_2O (—) and NH_4^+ (●) at pH 7 and DO=1.5-2 mg O_2/L. The arrows represent the time when a pulse of ammonia was added. Nitrate was not detected in any of the samples taken.

Before the addition of ammonia there was no NO or N_2O emissions, indicating that the oxidation of ammonia by AOB had to be occurring to detect emissions. At minute 20, 50 mg NH_4^+-N/L were added as a pulse followed by a continuous addition of ammonia throughout all the experiment. At minute 90 and 155, two more pulses of 50 mg NH_4^+-N/L were added. After these pulses, a peak of N_2O was observed which decreased as ammonia was decreasing. On the other hand, NO presented a peak after each addition of ammonia. However, differing from the N_2O pattern, NO increased its baseline every time that ammonia was added suggesting an effect of the ammonia concentration on the

NO production. The ammonia oxidation rate was 0.70, 0.86 and 1.08 mg NH_4^+-N/g VSS·min, respectively after the addition of each pulse.

Figure 6.3 shows the results obtained in the first set of experiments that were conducted at DO=1.5-2 mg O_2/L and pH=7-7.3 which are the same parameters used in the parent SBR. The different concentrations of ammonia were added in pulses to study the effect of AORsp in NO (Figure 6.3a) and N_2O specific production rates (Figure 6.3b).

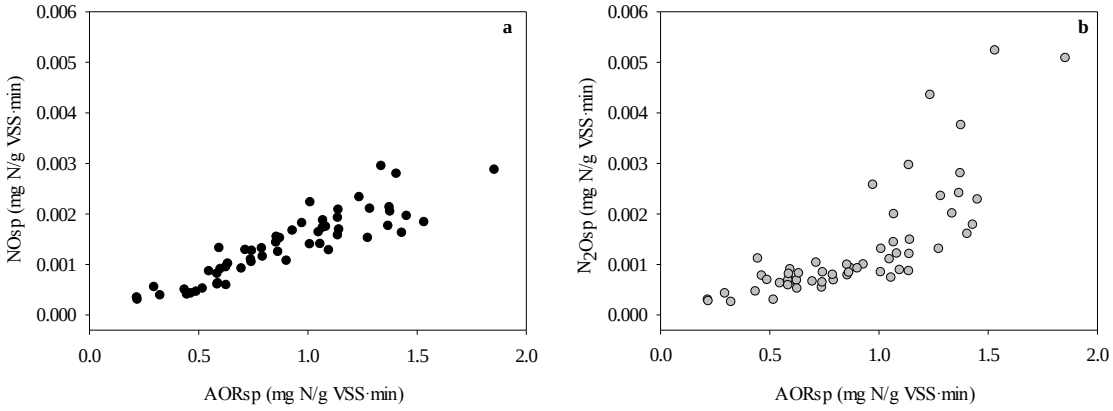

Figure 6.3: Correlation between the specific nitric oxide production rate (a) and the specific nitrous oxide production rate (b) with the specific ammonia oxidation rate.

Slightly higher NO than N_2O emissions were observed at the lower AORsp range (from 0 to 1 mg N/g VSS·min). At higher AORsp, N_2O emissions overcame the emissions from NO. The relationship between NO production rate and AORsp was lineal (r^2=0.81) whereas the relationship of the N_2O production and the ammonia oxidation rate was exponential (r^2=0.75. An r^2=0.6 was obtained when a linear relationship was fitted into the N_2O vs AOR data).

Linear correlations were found with the ammonium concentration (Figure SI.1, Annex). This is due to the fact that an increase on ammonia resulted in an increased AOR (Figure SI.2. Annex) which has been previously reported to be the true factor affecting N_2O emissions (Law et al., 2012a).

During these tests, some sludge samples were taken to conduct a chemical staining for NO. Figure 6.4 shows the presence of NO inside the biomass extracted from the test conducted at AORsp of 1.08 mg N/g VSS·min (Figure 6.3). Most of the biomass was targeted by the NO stain, indicating the biological origin of NO during these tests.

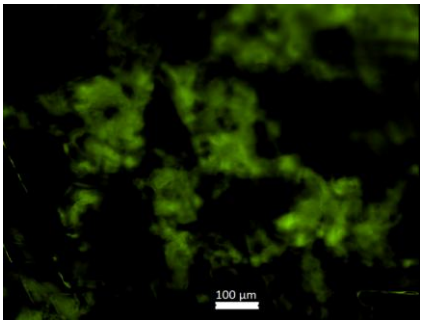

Figure 6.4: Biomass stained with the DAF-FM DA fluorescence probe.

6.3.3 Effect of DO on NO and N_2O emissions

The second set of experiments was conducted to assess the effect of DO and anoxic conditions on the overall NO and N_2O emissions. Figure 6.5 shows the profiles of NO, N_2O, NH_4^+, NO_2^-, pH and DO when DO was decreased (a) and increased (b) in a step-wise mode.

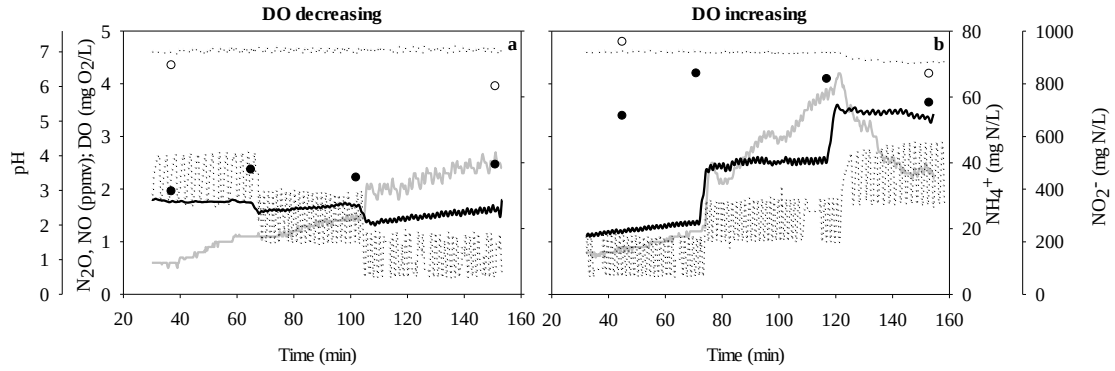

Figure 6.5: Experimental profiles of N_2O (···), NH_4^+ (●), NO_2^- (○), NO (—), DO (···) and pH(···) during set 2 of tests: DO decreasing from 3 to 0.5mg O2/L (a) and increasing from 0.5 to 3mg O2/L (b). Nitrate was not detected in any of the samples taken.

In the test were DO was decreased (Figure 6.5a), N_2O increased in a linear manner and only a small jump on the N_2O signal was observed when the DO was reduced to the lowest set point tested. On the other hand, the NO signal suffered a small decrease every time the DO set point was decreased but within the same DO range, the NO profile was relatively constant.

On the other hand, in the test where DO was increased from 0.5 to 3 mg O_2/L (Figure 6.5b), N_2O increased within the first two DO set-points and also a jump on the N_2O

concentration was detected when moving from the lowest DO to the intermediate set-point tested. Interestingly, the N_2O concentration started to decrease as soon as the DO set-point was increased to 2.5-3 mg O_2/L. NO had a similar pattern as in the other test. Its concentration remained stable under each DO set-point only increasing when the set-point was increased. Table 6.2 shows a comparison between the rates and ratios obtained during the different DO set-points in both experiments.

Table 6.2. N_2O and NO emission rates and ratios and AORsp at different DO levels and activity of the AOBs when DO was decreasing and increasing.

DO decreasing					
DO Range (mg O_2/L)	N_2O production rate (mg N/g VSS ·h)	N_2O produced/NH_4^+ consumed	NO production rate (mg N/g VSS ·h)	NO produced/NH_4^+ consumed	AORsp (mg N-NH_4^+/g VSS ·h)
2.50-3.00	0.06	0.08%	0.06	0.08%	73.65
1.50-2.00	0.08	0.09%	0.05	0.06%	88.59
0.50-1.00	0.14	0.18%	0.05	0.06%	77.18
DO increasing					
DO Range (mg O_2/L)	N_2O production rate (mg N/g VSS ·h)	N_2O produced/NH_4^+ consumed	NO production rate (mg N/g VSS ·h)	NO produced/NH_4^+ consumed	AOR (mg N-NH_4^+/g VSS ·h)
0.50-1.00	0.06	0.23%	0.08	0.29%	25.47
1.50-2.00	0.16	0.27%	0.14	0.23%	60.78
2.50-3.00	0.18	0.24%	0.20	0.27%	73.64

When comparing both experiments it was observed that both N_2O and NO production were higher in the experiment where the DO was increased from 0.5 to 3 mg O_2/L as compared with the test where the DO was decreased. This might be related to the different behaviour in terms of the AORsp detected between both tests (Table 6.1). In the test started with the lowest DO concentration range, the AORsp increased progressively when the DO was increased, indicating that the AOR was limited by the DO at the beginning of the test. Interestingly, in the batch started with the highest DO

range, the AOR remained relatively constant at high values and seemed not to be affected by the DO.

Another experiment was conducted under anoxic conditions to determine the possible effect of oxygen depletion on NO and N_2O emissions in AOB. Figure 6.6 shows the profiles of NO, N_2O, NO_2^-, pH and DO when DO was 0 mg O_2/L. Results show that as soon as DO was depleted from the mixed liquor, there was a peak of NO and a very low peak of N_2O suggesting that nitric oxide production was more affected by anoxic conditions than N_2O production. The production of NO was significant and after the peak it was slowly decreasing until reaching a stable value at around 15 ppmv. On the other hand, N_2O showed a low peak and afterwards it remained constant at around 5 ppmv, also indicating a continuous production of N_2O during anoxic conditions.

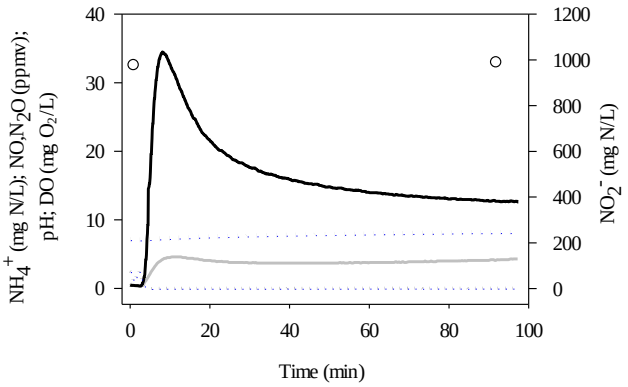

Figure 6.6: Experimental profiles of N_2O (—), NO_2^- (○), NO (—), DO (···) and pH ( ) of batch test 2.3: when DO was 0 mg O_2/L. Nitrate was not detected in any of the samples taken.

6.3.4 The effect of pH on N_2O and NO emissions

Figure 6.7 shows the effect of a step-wise pH decrease from 8 to 6.5 on N_2O and NO emissions. DO was kept constant at 1.5-2 mg O_2/L which are the same conditions as in the parent SBR.

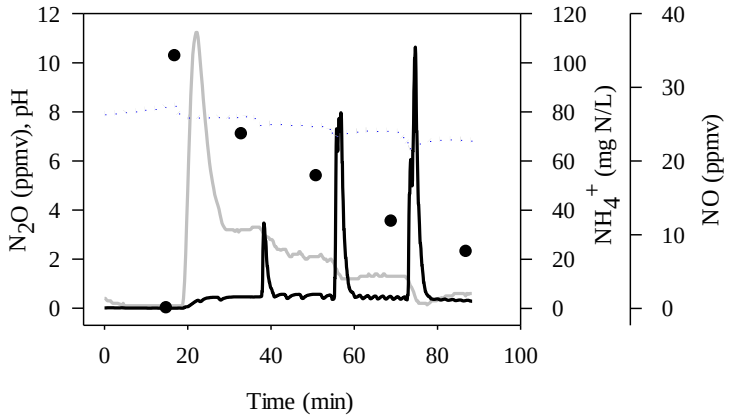

Figure 6.7: Experimental profiles of N_2O (—), NH_4^+ (●), NO (─), and pH (···) at DO=1.5-2 mg O_2/L in batch test 3.1: while pH is decreasing from 8 to 6.5. Nitrate was not detected in any of the samples taken.

Before ammonia addition, no emissions of NO or N_2O were detected. Around minute 20, ammonia was added which produced a peak on N_2O. This peak is associated to the activation of the ammonia oxidation by AOB and lasted for 10 min approximately, reaching a stable N_2O baseline after the decrease of the peak. Every time that pH was decreased, N_2O also decreased, reaching a new baseline. On the other hand, the NO emissions detected follow a complete different trend. NO increased to a baseline when ammonia was added. But each time the set point of pH was decreased 0.5 points by adding 0.6M HCL, NO increased in the form of a peak. The fact that NO showed a peak when HCL was added suggests a chemical formation of NO. To clarify this hypothesis batch tests 3.2-3.5 were conducted.

Figure 6.8 shows the results of batches 3.2 and 3.3 using biomass diluted with effluent water with high concentrations of nitrite and without ammonia (a) and without biomass neither ammonia but using the effluent water with high nitrite concentrations (b).

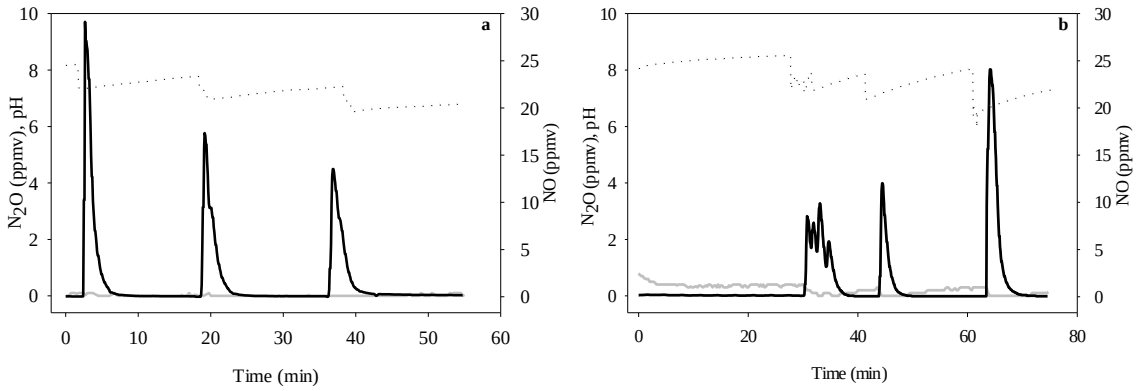

Figure 6.8: Experimental profiles of NO (─), N_2O (┄) and pH (···) at DO=1.5-2 mg O_2/L of batch tests 3.2 and 3.3: pH decreasing from 8 to 6.5 without ammonia but with biomass (a) and without biomass (b).

When ammonia was not added in the AOB culture (Figure 6.8a) the production of N_2O was negligible even when pH was changed. However, NO was produced each time HCL was added in a similar fashion as observed in Figure 6.7. In the case when AOB biomass was removed from the reactor (Figure 6.8b) N_2O was neither produced but the same pattern for NO was observed. This clearly indicates that NO was chemically produced due to the addition of HCL. Further experiments were conducted with RO water that did not contain nitrite (Figure SI.3, batch test 3.4). In this case NO emissions were not detected indicating that nitrite was the precursor of the chemical production of NO. Also, a test was conducted with RO water to assess the effect of increasing the pH with NaOH (Figure SI.4) but no emissions were detected in that case.

6.4 Discussion

6.4.1 Correlation of NO and N_2O vs AORsp

Results showed that the correlation between N_2O and AORsp was exponential whereas the relationship between NO and AORsp was lineal. The exponential correlation between N_2O and AORsp was also found by Law et al., (2012) using an enriched AOB culture similar to the one used in this study. In their case the range of AORsp tested was wider (0-5.8 mg N/g VSS·min) than the one used in this study (0-2 mg N/g VSS·min). These authors also postulated that at high ammonia and nitrite concentrations (500 mg N/L) and low DO concentrations (0.5-0.8 mg O_2/L), the chemical breakdown of the nitrosyl radical (NOH), an intermediate in NH_2OH oxidation to nitrite could become

dominant for the production of N_2O. To avoid this increase on N_2O production, they suggested that AOR should be lower than its maximum level to minimize the N_2O production rate. Also, Schneider et al., (2013) reported that the N_2O specific production rate was positively correlated with the AORsp during stable nitritation reporting a linear correlation in their study.

Fewer studies have been focused on NO. Stüven and Bock., (2001) reported that for a pure culture of *Nitrosomonas europaea* in synthetic wastewater, NO production rate linearly correlated to its ammonia oxidation rate. They postulated that release of NO was due to an imbalanced ammonium oxidation in the oxidation of hydroxylamine. They also postulated that NO production is a side effect of a detoxification mechanism used by AOBs to eliminate the nitrite. This would explain the fact that ammonia oxidizers continuously produce relatively high amounts of NO and, occasionally, nitrogen dioxide (NO_2).

The linear relationship between NO production and the AORsp in this study suggests that the production of NO is higher than its reduction leading to the accumulation of this gas. This is in agreement with Kozlowski et al., (2016) who found that a pure culture of *N. multiformis* (AOB) had a linear rate of oxygen consumption during ammonia oxidation and this oxygen consumption led to a production of NO till a maximum and then when half of the available oxygen was consumed, NO started being consumed. A possible mitigation strategy would be reducing the AOR and trying to reach the point where AOR is equal or lower than the nitric oxide reduction rate. At the same time, this would also reduce the N_2O emissions. This is in agreement with Kozlowski and co-workers., (2014) who suggested that the absence of NorB expression alone in *N. europaea* had no effect on growth or substrate oxidation rates or on NH_2OH accumulation but did result in diminished N_2O production in comparison to that of the wild type.

These results highlight the importance of also monitoring NO emissions on those systems where AOB are dominant.

6.4.2 The effect of changing DO

Higher N_2O and NO emissions were detected in the test with increasing DO. This could be due to the difference on the activity of AOBs. From the results reported in this paper, AOB activity and its emissions seem to be influenced not only by the DO applied but also by the conditions that AOB have been previously exposed to since interestingly, in the batch started with the highest DO range, the AOR remained relatively constant at high values and seemed not to be affected by the DO.

The fact that N_2O emissions decreased when DO increased could be due to a change on the contribution pathway for N_2O production. This was reported by Peng et al., (2014) who studied the effect of DO on a nitrifying culture and determined that as DO increased the contribution of the nitrifier denitrification pathway decreased while the contribution of the hydroxylamine oxidation pathway increased. However, later on Peng et al., (2015) suggested that nitrifier denitrification was the dominant contribution pathway of N_2O production in an enriched nitrifying sludge with AOBs and NOBs in a wide range of DO and nitrite concentrations. They reported that the hydroxylamine oxidation pathway was only active when DO was high and nitrite was low which is not the case here.

When anoxic conditions were applied in the reactor, an immediate production of NO and N_2O was observed. The production of NO was 7 times higher than that of N_2O. Anoxic conditions in AOB have been suggested to cause an over expression of the nitrite reductase gene and an under-expression of the genes encoding for ammonia oxidation, hydroxylamine oxidation and nitric oxide reduction leading to NO accumulation (Kampschreur et al., 2008a; Kester et al., 1997). Yu et al., (2010) reported that under anoxic or anaerobic conditions, AOBs can utilize alternate electron acceptors such as nitrite, dimeric nitrogen oxide (N_2O_4) and produce N_2O and NO. They showed a production of NO under strict anoxic conditions which correlates with our results but no N_2O production was reported. Also, Kampschreur et al., (2007) reported that oxygen depletion during ammonia oxidation clearly increased NO emissions in an enriched nitrifying culture. However, Law et al., (2011) showed that NO was produced under anoxic conditions but N_2O was produced in the transient from anoxic to aerobic. In our

study, N_2O was produced under anoxic conditions (Figure 6.6). Schmidt, (2008) reported that the oxidation of hydroxylamine does not depend on oxygen and it is catalyzed by hydroxylamine oxidoreductase (HAO) under both oxic and anoxic conditions which could explain the production of N_2O when DO is zero. This would suggest that N_2O emitted under anoxic conditions would be produced through the hydroxylamine pathway.

6.4.3 The effect of pH

The results of the third set of experiments conducted decreasing the pH revealed that N_2O was produced biologically when ammonia was present and that each time the set point of pH was decreased, N_2O decreased to a new baseline. These results agree with the ones obtained by Law et al., (2011) who reported an immediate change on the N_2O production when pH was changed from 7 to 8 till reaching a new baseline in a partial nitritation reactor. They also showed a negligible production of N_2O when ammonia was not present but there was nitrite and pH was changed which corroborates with our results (Figure 6.8a). On the other hand, NO was produced chemically in the tests. Each time HCL was added, there was a peak of NO that decreased sharply after the addition. This production could be due to the deprotonation of HNO_2 (Eq. 16), since the pka value of the NO_2^-/HNO_2 couple is 3.29 and therefore under acidic conditions NO will be formed (Schreiber et al., 2012; Udert et al., 2005). The fact that there is a NO peak every time that HCL is added might indicate that there is a sudden local pH drop to values lower than the pH setpoint, originating the NO peaks detected. After the water volume is homogenized the NO returns to its baseline level, that is attributed to that pH.

$$2HNO_2 \leftrightarrow NO + NO_2 + H_2O \qquad \text{(Eq. 16)}$$

The results from this study highlight the importance of monitoring NO in addition to N_2O. In order to assess operational strategies to mitigate N_2O emissions, NO emissions being controlled could help to diminish N_2O emissions.

Chapter 7

Chapter 7 Direct GHG emissions from a full-scale plug-flow reactor: identifying temporal and spatial variations

This study has been submitted to the Water Research as:

Anna Ribera-Guardia, Lluís Bosch, Lluís Corominas and Maite Pijuan.2017. Direct GHG emission from a full-scale plug-flow reactor: identifying temporal and spatial variations.

7.1 Preliminary remarks

Nitrous oxide and methane emissions of a plug-flow reactor treating domestic wastewater from the municipality of Girona were studied during 5 months from November till March. A multiple gas hood collection system was used to simultaneously monitor the first 3 aerated zones of the plug-flow reactor. The temporal and spatial variations were studied for both GHG in the three aerobic zones monitored. Also, a comparison between the direct and indirect emissions (related to electricity consumption from the plug-flow reactor) was conducted. The C footprint of the plug-flow reactor was also determined.

7.2 Materials and methods

The description of the monitoring site is detailed in Chapter 3.

The gas monitoring was conducted in one of the plug-flow reactors. It consists of two anoxic zones followed by three aerobic zones, then wastewater flows to an anoxic zone and a final fourth aerobic zone. There is an internal recirculation from the third aerobic zone to the second anoxic zone. Three gas hoods were placed in the first three aerobic zones of the plug-flow reactor to measure the N_2O and CH_4 emissions (Figure 7.1).

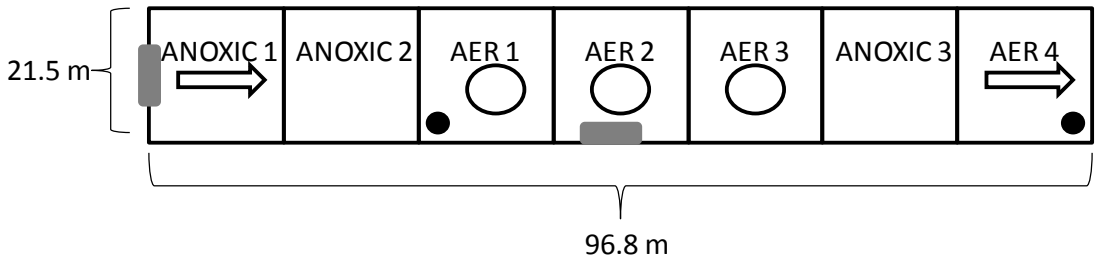

Figure 7.1: Plug-flow reactor configuration and zone of study. The black dots represent the plant DO sensors located in aeration zone 1 and aeration zone 4. The squares represent the online ammonia sensors at the inlet of the plug-flow reactor and in aeration zone 2. The white dots represent the place where the gas hoods were placed. The arrows represent the direction of the wastewater flow.

Chemical analysis of TP, TKN, BOD, COD, nitrate, nitrite, ammonia and phosphate are explained in Chapter 3.

Calculations of the N_2O and CH_4 emission factors are detailed in Chapter 3.

Software SPSS 21.0 was used for all statistical analyses with two-tailed Pearson's correlations test (r). Its probability value, *p*, was considered as significant if $p < 0.05$ and strongly significant if $p < 0.01$.

7.3 Results

7.3.1 Process performance

The WWTP of Girona presented a 91 ± 6 % COD removal, 87 ± 5% TKN removal and 98 ± 1% P removal. The plug-flow reactor operated correctly following regular patterns. The main characteristics of the influent wastewater and treated effluent as well as some process parameters are summarised in Table 7.1.

Table 7.1. Influent and effluent characteristics and process parameters of the WWTP of Girona

Influent wastewater	
Flow (m^3/day)	42801.26 ± 1361.87
COD (mg COD/L)	411.58 ± 39.42
TKN (mg N/L)	44.05 ± 2.24
PO_4^{3-}-P (mg P/L)	5.20 ± 0.27
pH	7.71 ± 0.18
Plug- flow reactor	
MLSS (mg/L)	3813.89 ± 207.1
MLVSS/MLSS (%)	75.06 ± 1.87
HRT (h)	15.88 ± 0.75
SRT (days)	19.27 ± 0.62
Treated Effluent	
COD (mg COD/L)	25.84 ± 2.21
TN (mg N/L)	8.28 ± 1.16
PO_4^{3-}-P (mg P/L)	0.13 ± 0.03

Data provided by plant operators. Data corresponds to average 151 values obtained from samples distributed across the experimental period.

7.3.2 Spatial and temporal N_2O and CH_4 emission patterns

N_2O and CH_4 emissions were monitored across the first 3 aerobic sections of the plug-flow reactor from November 2016 till March 2017. To ease the comparison of the data collected, this was grouped in 6-7 day periods and total emissions as well as emission factors were calculated for each one of these periods. Figure 7.2 shows an example of 3 different periods distributed across the monitored months.

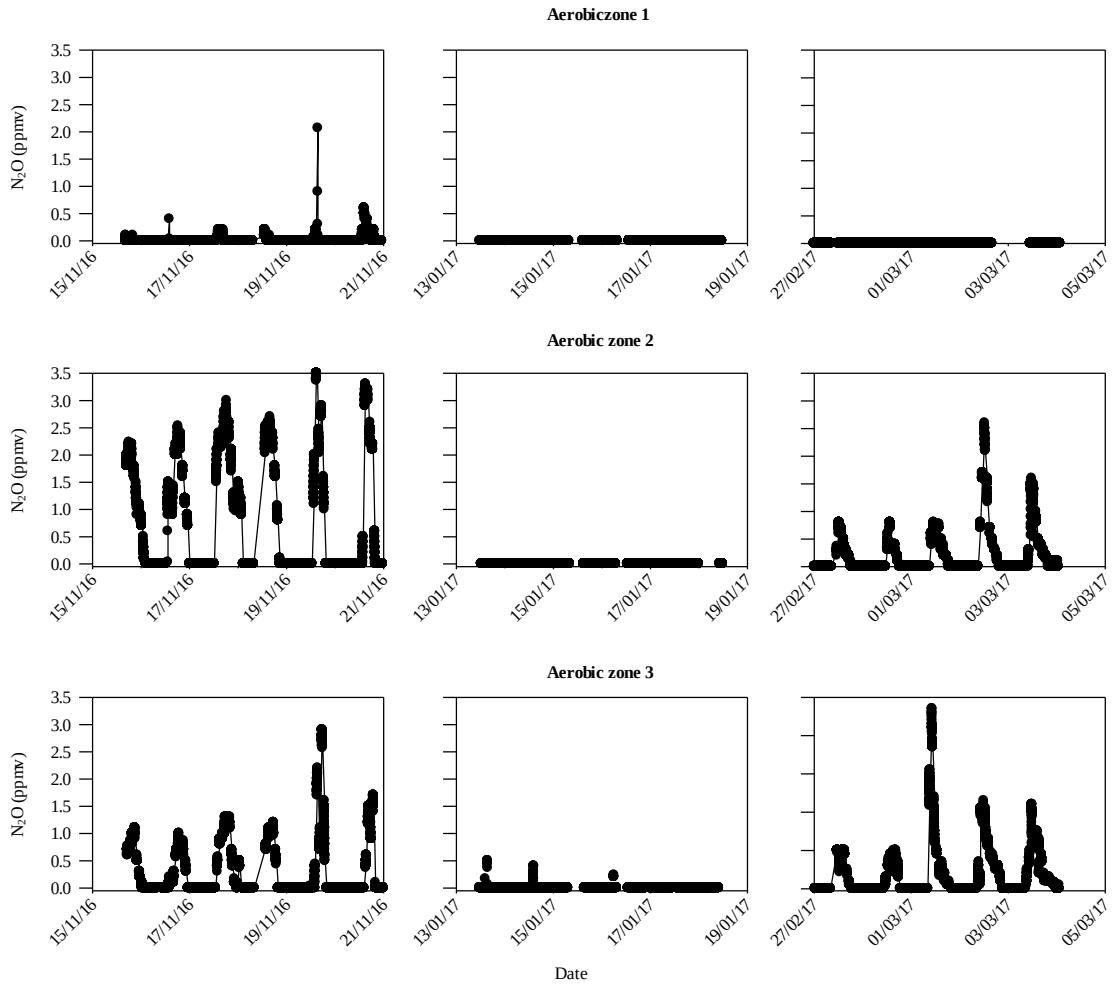

Figure 7.2: N_2O emissions from aerobic zones 1, 2 and 3 of the plug-flow reactor in November (left), January (center) and March (right).

N_2O emissions displayed a different pattern among the three zones. While hardly any N_2O was emitted from the first aerobic zone during the whole monitoring period, aerobic zones 2 and 3 presented similar emissions profiles, with peaks of N_2O occurring in a daily basis. Also, differential temporal emissions were found, with emissions detected in November, a decrease on emissions occurring beginning of December till

reaching a no emission period that lasted till end of February. Emissions started again at the end of February and kept increasing till the end of the monitoring period.

The external disturbances to which a WWTP is subjected are composition, flow rate and temperature of the incoming wastewater. A statistical analysis using Pearson's correlation coefficients was conducted between these variables and the total N_2O and CH_4 emissions and results are given in Table 7.2.

Table 7.2. Pearson's correlation coefficient r and *p*-value between the plug-flow N_2O and CH_4 emission's factor and external disturbance variables.

	Units	**r**	***p*-value**
		N_2O	
Influent flow rate	m^3/day	0.245	0.087
Temperature wastewater	ºC	0.583	0.003
TKN load of the reactor	kg N/L	-0.166	0.499
		CH_4	
Influent flow rate	m^3/day	-0.172	0.234
Temperature wastewater	ºC	0.118	0.593
COD load of the reactor	kg COD/L	0.149	0.356

There is a significant correlation between the N_2O emission and the temperature of the wastewater (p=0.003). The coldest wastewater temperature (Figure SI.5) was reached in the months with no N_2O emissions (December and January). However, all the other investigated parameters didn't have a strong correlation with N_2O emissions. For the case of CH_4 emissions the correlation with all the parameters assessed was very weak.

In table 7.3 is summarised the amount of N_2O emitted from each hood for 8 periods distributed between November and March. The fact that no emissions were detected in aerobic zone 1 indicates that there was no significant N_2O being accumulated in the previous anoxic zones. The highest emissions were found in aerobic zone 2 during all the monitoring period.

Table 7.3. N_2O and CH_4 production in aerobic zones 1, 2 and 3 from different periods comprised between November and March.

	kg N_2O-N produced/day			kg CH_4 produced/day		
Date	Aerobic zone 1	Aerobic zone 2	Aerobic zone 3	Aerobic zone 1	Aerobic zone 2	Aerobic zone 3
15/11/2016-22/11/2016	0.02 ± 0.02	0.96 ± 0.35	0.11 ± 0.04	21.88 ± 8.78	15.05 ± 3.96	1.48 ± 0.30
25/11/2016-1/12/2016	0.02 ± 0.02	0.30 ± 0.11	0.01 ± 0.01	22.76 ± 3.62	7.56 ± 0.59	2.02 ± 0.20
15/12/2016-22/12/2016	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00	21.54 ± 2.71	6.95 ± 1.34	1.93 ± 0.42
13/01/2017-19/01/2017	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	27.76 ± 2.71	5.97 ± 1.80	1.94 ± 0.45
15/02/2017-22/02/2017	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.03	25.63 ± 5.77	7.16 ± 4.39	3.03 ± 1.68
26/02/2017-05/03/2017	0.00 ± 0.00	0.11 ± 0.08	0.06 ± 0.04	19.32 ± 5.87	11.49 ± 1.46	3.69 ± 0.72
09/03/2017-16/03/2017	0.00 ± 0.00	0.36 ± 0.12	0.26 ± 0.12	32.92 ± 6.46	6.65 ± 3.00	3.42 ± 0.54

On the other hand, methane emissions were similar across the monitoring period and they did not present a clear daily pattern (Figure 7.3 and Table 7.3). CH_4 decreased along the plug-flow showing higher emissions in the first aerated zone than in the second and in the third.

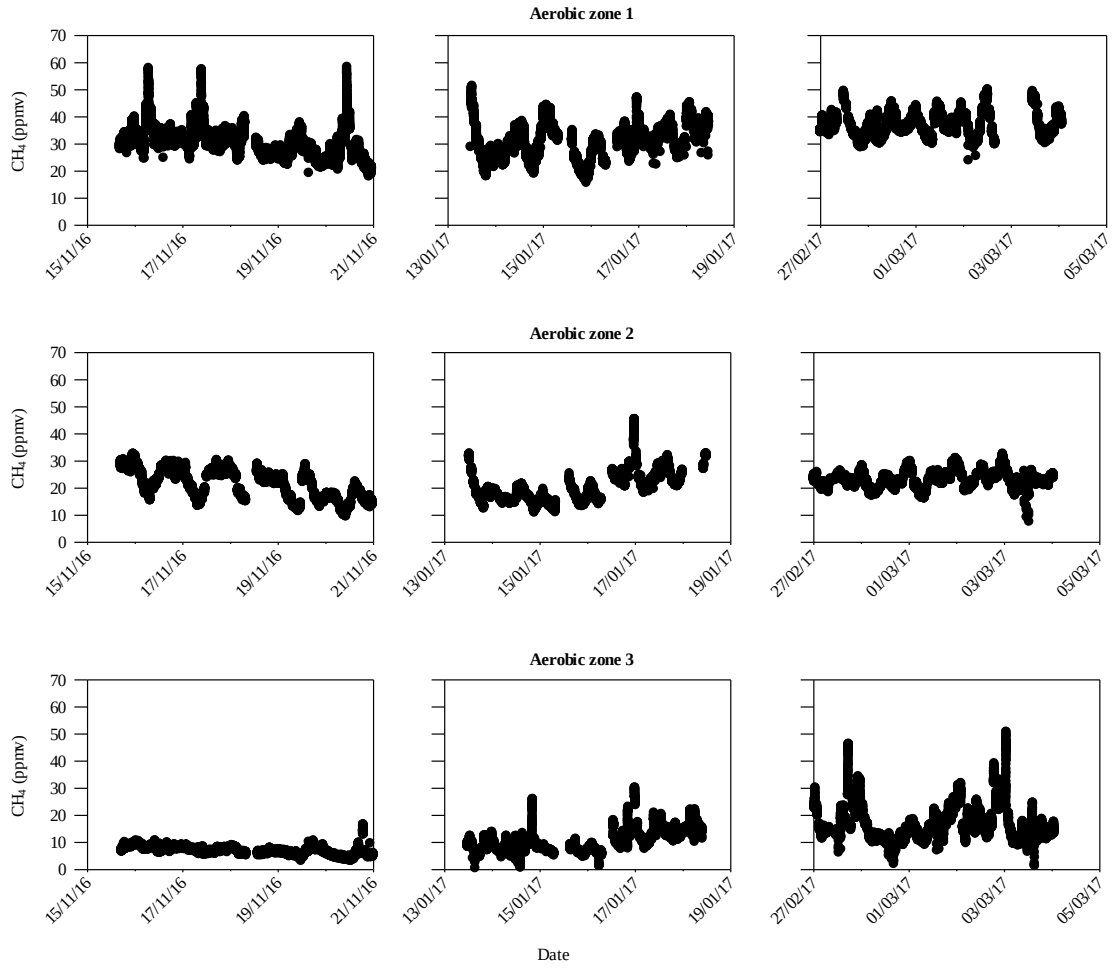

Figure 7.3: CH_4 emissions from aeration zones 1, 2 and 3 from the plug-flow reactor in November (left), January (center) and March (right).

To unravel the origin of this CH_4, dissolved methane samples were taken from different locations at the WWTP (locations indicated in Figure 3.4 in Chapter 3). The highest dissolved CH_4 values were found in the reject wastewater stream coming from the anaerobic digesters (0.52 ± 0.22 mg COD/L) and in the wastewater arriving to the plant from the sewer network (0.55 ± 0.19 mg COD/L). Before entering the plug-flow reactor the dissolved methane was 0.45 ± 0.05 mg COD/L decreasing to 0.13 mg COD/L in anoxic zone 2. These values were even lower when entering the aerobic zones of the plug-flow reactor (0.04, 0.02 and below detection limit in aerobic zones 1, 2 and 3, respectively) showing the same spatial variation pattern that was observed in Figure 7.3.

The N_2O and CH_4 emission factors were also calculated for each period and are shown in Table 7.4.

Table 7.4. N_2O and CH_4 emitted per TKN and COD load respectively for the different periods from November to March.

Date	kg N_2O-N/kg TKN influent	kg CH_4/kg COD influent
15/11/2016-22/11/2016	0.13% ± 0.04%	0.46% ± 0.12%
25/11/2016-01/12/2016	0.03% ± 0.01%	0.38% ± 0.04%
15/12/2016-22/12/2016	0.00% ± 0.00%	0.28% ± 0.03%
13/01/2017-19/01/2017	0.00% ± 0.00%	0.36% ± 0.03%
15/02/2017-22/02/2017	0.00% ± 0.00%	0.43% ± 0.09%
26/02/2017-05/03/2017	0.02% ± 0.01%	0.46% ± 0.08%
09/03/2017-16/03/2017	0.08% ± 0.02%	0.49% ± 0.08%

The N_2O emission factor ranged from 0-0.13% of the TKN load but presented a high fluctuation, decreasing to 0 during the months of December and January. On the other hand, the CH_4 emission factor was maintained relatively constant ranging between 0.28% (during the coldest months) and 0.49%.

7.3.3 Distinctive N_2O daily emission patterns across the monitoring period

Two slightly different N_2O emission patterns were found during the monitoring period. Figure 7.4 presents two 6-day period profiles where N_2O emissions are depicted together with the ammonium concentration profile obtained from aerobic zone 2.

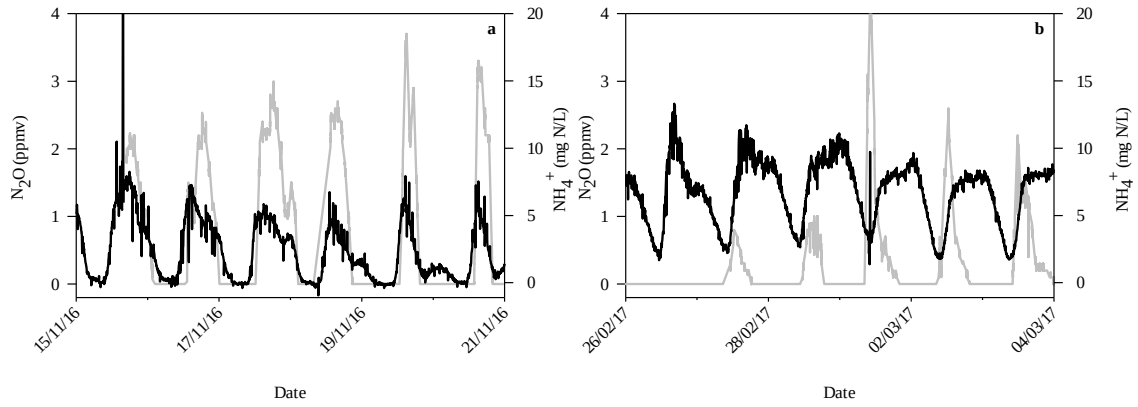

Figure 7.4: Typical ammonium (─) and N_2O patterns ( ) in the aerobic zone 2 of the plug-flow reactor found during the monitoring period of November (a) and the monitoring period of March (b).

The emissions profile found in November (Figure 7.4a) shows a strong significant correlation between the ammonium concentration profile and the N_2O emission profile

(Pearson correlation r=0.80, *p*=0.029). When ammonium started increasing there was an immediate increase on N_2O in the form of a peak which decreased to undetectable levels when ammonia was depleted. However, this pattern changed in March, when N_2O emissions started again in aeration zone 2 after a period without emissions (Figure 7.4b). The N_2O peaks were lower and the peaks started with an increase of ammonia but decreased before ammonia was depleted. The correlation between the ammonia and the N_2O emissions was not significant (r=0.326, *p*=0.475). The reason behind the differences in the N_2O emission patterns when comparing emissions from November and from March are unknown. The emissions from November are correlated with the presence of ammonium in the monitored zone, suggesting that N_2O is produced during nitrification of the ammonium. On the other hand, emissions from March only occur when ammonium arrives in the monitored zone, decreasing sharply much before this ammonium is depleted. In this case the N_2O peak emission could be more linked to the transient conditions rather to nitrification.

In order to further explore the correlation of this N_2O peak with not only ammonium but other dissolved nitrogen compounds, a 24-hour grab sampling study was conducted in the aerobic zone 2 during this last period (March). Results are presented in Figure 7.5.

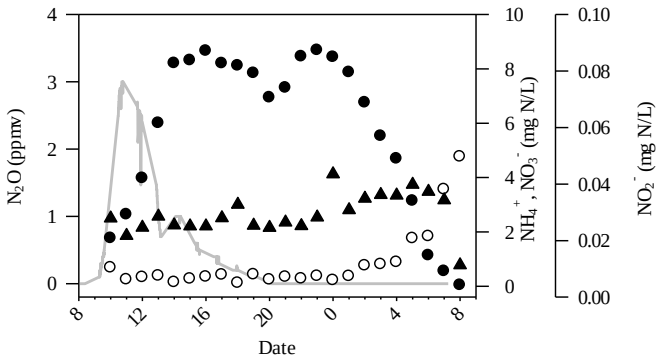

Figure 7.5: Daily N_2O (—), ammonium (●), nitrite (▲) and nitrate (○) concentration profiles measured in aerobic zone 2 measured in the 7th and 8th of March.

Ammonia concentration started increasing at around 9am until it reached a concentration of 8.5 mg N/L around 3pm. It was maintained at this level until it started decreasing at 12am reaching its lowest levels around 8am. Nitrate remained stable at very low levels till 12am that increased coinciding with ammonium decrease. Nitrite levels were very low at all times <0.04 mg $N0_2^-$-N/L). Interestingly, N_2O increased sharply as soon as ammonium increased but this increase only lasted for 2 hours,

starting to decrease afterwards till reaching negligible emissions around 6pm. Nitrite concentration remained stable at a very low level (0.02-0.04 mg NO_2^--N/L). Similar profiles were observed in other 24h intensive monitoring samplings in this zone and in aerobic zone 3 (Figure SI.6, supplementary information).

7.3.4 C footprint of the plug-flow reactor

The C footprint of a WWTP can be calculated taking into account the CO_2 emissions from the plant. These emissions can be direct from the biological processes taking place in the WWTP or indirect from the electricity consumption of the plant. In this thesis, the monitoring site were the plug-flow reactors of the WWTP of Girona, therefore the study of the C footprint was performed only for this site and it is explained below.

The electricity consumption of the two plug-flow reactors operating in the plant, including the electricity needed for aeration, was relatively constant during all the monitoring period as shown in Figure 7.6a. A part of the economic costs associated, the electricity consumed can be linked to indirect CO_2 emissions. For this calculation, the standard conversion factor of 0.308 kg CO_2/kWh was used which is the amount of CO_2 emitted during energy generation for 2016 in Spain according to the Catalan Office for Climate Change (OCCC) (Oficina Catalana del Canvi Climàtic, 2017). Figure 7.6b shows a comparison of the direct CO_2 emissions (attributed to N_2O and CH_4) from the plug-flow reactors and the indirect CO_2 emissions.

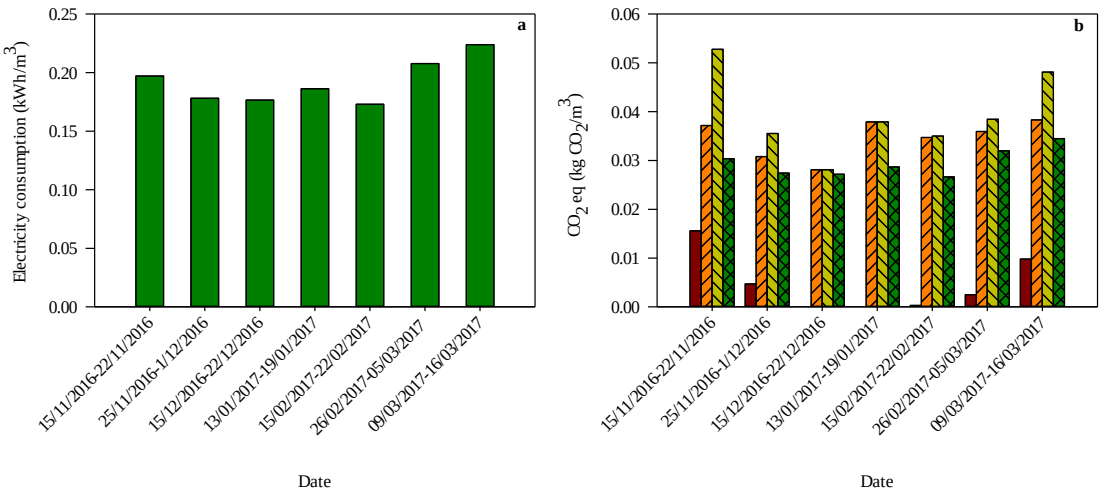

Figure 7.6: Electricity consumption (a) and direct (N_2O , CH_4 and total direct emissions) and indirect CO_2 emissions ( )b) from the plug-flow reactor of the WWTP along the monitoring period.

During all the monitoring period, direct CO_2 emissions were responsible for most of the C-footprint of the bioreactor. CH_4 was the major contributor to direct emissions compared to N_2O. Therefore, methane is the dominant gas to the C-footprint. CH_4 from the direct emissions represents between 45% and 57% of the total emissions. N_2O accounted for 15 % of the total emissions in November and March but was almost negligible in the other months.

7.4 Discussion

7.4.1 Quantifying GHG direct emissions

Many monitoring campaigns to quantify N_2O emissions have been conducted in the last decade, initially mainly focused on obtaining an emission factor and later also trying to unravel the factors affecting these emissions (Ahn et al., 2010b; Butler et al., 2009; Kampschreur et al., 2008b; Rodriguez-Caballero et al., 2015). In some of the initial studies, a grab sample approach was used (Czepiel et al., 1995; Foley et al., 2010b; Ye et al., 2014) but online monitoring revealed large variations on N_2O which could not be captured by the grab sample methodology. Thanks to all these studies it has been identified an emission range for N_2O which for most of the domestic WWTPs stays between 0-2.5% of the N-load. Despite this progress, it is still difficult to assess what the causes of detected variations on N_2O are and it is very challenging to extrapolate the findings from one plant to another, making the design and implementation of mitigation strategies case specific. Table 7.5 summarises some of the full-scale monitoring campaigns conducted worldwide in domestic WWTP with different configurations. Only studies using online monitoring have been considered.

Most of the monitoring campaigns conducted up to date only describe emissions over a relatively short period of time ranging from 1-2 days to 2 months. One of the few long term studies conducted by Daelman et al., (2015) in a WWTP from the Netherlands during 16 months showed significant differences on N_2O emissions across the year obtaining the highest emissions in April-May while hardly any emission was detected in November-December. Our results also show high temporal variations among the 5 months monitored highlighting the importance of long term monitoring campaigns to reliable identify the N_2O emission patterns from one plant. Having said that, the

implementation of long term monitoring campaigns at full-scale can be more challenging and definitely more costly than short term campaigns. Our data however shows high repeatability in the daily profiles for a short period of time (2-3 weeks). Therefore, long term monitoring could be simplified by monitoring 1week per month which would provide sufficient data to accurately estimate the temporal variations.

Also, the monitoring methodology can influence the emission data obtained. The majority of the studies use 1 floating hood placed in the surface of the bioreactor connected to an online analyser to quantify emissions (Aboobakar et al., 2013; Ahn et al., 2010a; Rodriguez-Caballero et al., 2015, 2014). However, gradients in concentrations of nitrogen species, dissolved oxygen, concentration of solids, etc. can be found in some reactor configurations such as plug-flow systems, widely used for domestic wastewater treatment (Pan et al., 2016; Rodriguez-Caballero et al., 2014). In these systems, strong spatial variations in N_2O emissions have been reported which difficult the accurate quantification of these emissions. Ahn et al., (2010a) found spatial N_2O variations in two different plug-flow reactors. N_2O was higher in the second aeration zone than in the first one, in the presence of non-limiting ammonia and dissolved oxygen concentrations. Also Aboobakar et al., (2013) reported a spatial variation on N_2O emissions. However, in that case, the first aerated zone (closer to the anoxic zone) was the one presenting higher N_2O emissions. They assumed that N_2O generation in the immediately preceding anoxic phase was due to incomplete denitrification. Rodriguez-Caballero et al., (2014) reported a spatial variation on a plug-flow reactor. In that study there were higher N_2O emissions in the first two aerobic zones compared to the third one. All these studies were conducted with one hood that was placed at different locations of the plug-flow on different days. To improve this monitoring approach, Pan et al., (2016) developed a multiple gas collection hood system to simultaneously measure N_2O emissions along the length of a step-feed plug-flow reactor. 3 different locations along the plug-flow were simultaneously studied and the highest N_2O emissions were recorded 50 meters from the beginning of the aeration zone in the 1st step feed and at the beginning of the aerated zone in the 2nd step feed. Using the multiple hood approach, we found the highest N_2O emissions in the second aerobic zone, indicating that N_2O was produced in this compartment during nitrification and not in the anoxic zone. Once again, these reports show differential emission hotspots for

plug-flow systems, stressing the need of monitoring at multiple sites to identify where the majority of the emissions come from.

Much less information is available regarding CH_4 emissions from wastewater treatment despite being also a strong greenhouse gas which can be produced in the sewer network (Auguet et al., 2015) and in WWTP where anaerobic processes take place. In 2012, Daelman and co-workers published the most comprehensive study on CH_4 emissions quantification from a WWTP treating domestic wastewater and having an anaerobic digester. This study lasted for 1 year and they monitored emissions from the whole plant. They found that the main source of methane was coming from the anaerobic digester and accounted for three quarters of the overall methane emission from the plant. In their study they also reported diurnal variability which was linked to the diurnal pattern of the influent flow and seasonal variability that showed a correlation with the average sludge content in the dewatered sludge storage tank. No spatial variability could be reported since the bioreactors were covered in that plant. Rodriguez-Caballero et al., (2014) also monitored CH_4 emissions from a plug-flow reactor from a WWTP with an anaerobic digester during 10 weeks. They found strong spatial variability, with the first aerobic zone emitting most of the methane. Daily CH_4 peak emissions were detected in the bioreactor and were related to the influent wastewater flow dynamics and a peak detected overnight in some days was related to the release of the reject wastewater stream into the influent. In our study, spatial variation was observed along the plug-flow reactor with higher CH_4 emissions in the first aerated zone and diminishing through the second and the third aerations zones. Results did not show any seasonal variations along the months of the monitoring campaign. Also, no diurnal pattern could be observed for the CH_4 emissions.

7.4.2 Diurnal variability on N_2O emissions

Nitrous oxide showed a diurnal pattern increasing as soon as ammonium was entering the compartment monitored at around 9am but decreasing at 11am reaching negligible values at 6pm. Aboobakar et al., (2013) also showed a diurnal variability. However, they reported a peak of gaseous emissions between midnight and 8:00 in the morning. This study was performed during eight weeks from August to October 2011. There was a significant correlation with ammonia loading into the nitrifying lane (p=0.029), thus supporting the theory that N_2O emissions are more likely to occur during higher

nitrification rates (Kampschreur et al., 2008b).This is contradictory to the findings of this study where there was a weak correlation between N_2O emissions and the TKN load entering the plug-flow reactor. Temporal variability as a function of ammonia loadings at full-scale has been suggested by other researchers (Ahn et al., 2010b). Diurnal N_2O emission profiles were reported by Pan et al., (2016) that conducted a seven-week period on-line gas-phase N_2O monitoring at six monitored locations across a two-step feed plug-flow reactor. The profiles generally followed a pattern with an "N_2O emission valley" in the morning and an "N_2O emission peak" after 18:00 pm.

A strong significant correlation (p=0.003) was found in this study between the N_2O emissions and the temperature of the wastewater corroborating the results linked to season variability in the plug-flow reactor. Daelman et al., (2015) also showed a seasonal variation on N_2O emissions in a study conducted in a WWTP in the Netherlands, but they didn't find any correlation between the emissions of N_2O and the mixed liquor temperature of the plant. However, similar findings were reported by Ahn et al, (2010b) who expected the emission of nitrous oxide from plants that are designed for complete nitrogen removal to be higher at higher temperatures because of the higher overall kinetics of the nitrogen transformations.

There is a lot of variability in all the studies of GHG emissions in full-scale WWTP therefore more research is needed in order to establish the main production parameters of N_2O and CH_4.

Table 7.5. Literature review of the online monitoring campaigns.

Process	Emission factors		Monitoring Methodology	Length of the study	Contribution to total C-footprint	Reference
	N_2O	CH_4				
Banderpho BNR	0.16±0.1%	N.Q.	1 floating gas hood	24 h (winter)	N.Q.	(Ahn et al., 2010a)
Plug-flow	0.4±0.14%	N.Q.	1 floating gas hood	24 h (winter)	N.Q.	(Ahn et al., 2010a)
Step-feed	0.18±0.18%	N.Q.	1 floating gas hood	24 h (winter)	N.Q.	(Ahn et al., 2010a)
Carrussel+plug-flow (both covered)	N.Q.	1.13%	off-gas from reactors sent to continuous analyser	11 moths	64% from CH_4	(Daelman et al., 2012)
Carrussel+plug-flow (both covered)	2.8%	N.Q.	off-gas from reactors sent to continuous analyser	16 months	N.Q.	(Daelman et al., 2015)
Plug-flow	0.036%	N.Q.	1 floating gas hood	2 months (August-Oct)	N.Q.	(Aboobakar et al., 2013)
Plug-flow	0.116%	0.016%	1 floating gas hood	10 weeks (June-Oct)	N.Q.	(Rodriguez-Caballero et al., 2014)

Oxidation ditch with surface aerators	0.52±0.16%	N.Q.	Online monitoring, offline sampling, mathematical modelling and oxygen balance	1 month (Oct-Nov)	N.Q.	(Ye et al., 2014)
SBR	6.8%	0.02%	1 floating gas hood	1 month (Feb-March)	60% from N_2O	(Rodriguez-Caballero et al., 2015)
Plug-flow	1.9±0.25%	N.Q.	3 floating gas hoods	7 weeks	N.Q.	(Pan et al., 2016)
A2O	1.29±1.07%	N.Q.	2 floating gas hoods	12 months	N.Q.	(Wang et al., 2016b)
Aerated filter	0.017-1.261%	N.Q.	2 floating gas hoods	12 months	N.Q.	(Wang et al., 2016a)
Nitrifying biofilter	2.26±0.46%	N.Q.	1 floating gas hood	1 week summer 2 weeks winter	N.Q.	(Bollon et al., 2016)
Plug-flow	0-0.13%	0.40±0.18%	3 floating gas hoods	5 months (Nov-March)	45-57% from CH_4	This study

BLOCK III - FINAL REMARKS

Chapter 8

Chapter 8 General discussion

GHG emissions have increased exponentially since pre-industrial times due to human activities causing climate change. During the last decades scientists have been studying different ways to mitigate these emissions in different sectors. A lot of effort has been made on understanding the production of N_2O and CH_4 in wastewater systems. Wastewater treatment systems remove pollutants from wastewater so it can be discharged to the aquatic environments without producing any harm. However, during the biological removal of these main pollutants (nitrogen and organic matter) CH_4 and N_2O can be produced, being emitted into the atmosphere.

In order to mitigate these emissions from full-scale WWTP it is necessary to understand the main factors affecting these emissions as well as identifying where the production and emissions hotspots are. There have been many publications focusing on the study of different environmental and operational factors affecting N_2O production in laboratory-scale fully controlled reactors. These systems allow the enrichment of a particular group of microorganisms linked to a specific process occurring in a WWTP and therefore have been proved very useful when studying how a parameter affects the emissions from a particular group of microorganisms. With this approach the effect of pH, AOR, DO, COD, multiple electron acceptors, etc. on N_2O emissions from nitrifying and denitrifying bacteria has been established. However, it is still unclear why N_2O is produced in some cases and its link with NO, its precursor and a toxic gas.

Simultaneously, many monitoring campaigns have been conducted at full-scale WWTPs with the aim of unravelling the magnitude of these emissions and identifying a way of controlling them. The first attempts were conducted taking grab samples from different zones of the plant but sooner the methodology was improved with online monitoring with the use of hoods, which allowed identifying the high variability on emission dynamics. In recent studies, the monitoring of not only N_2O but also CH_4 has allowed to calculate the contribution of these direct emissions on the carbon footprint of the plant, showing their important role on the overall C emissions. Despite all these efforts there are still large variations among reported emissions from different WWTPs and it is still a challenge to implement effective mitigation strategies that can be extrapolated to other plants. More research is needed at full-scale, with better characterisation of these emissions over longer monitoring times.

Two chapters of this thesis were focused on exploring the effect of multiple electron acceptors on N_2O production from different denitrifying lab-scale reactors. Previous to this thesis, there was a publication suggesting that N_2O could be enhanced when multiple electron acceptors (nitrate, nitrite) were present simultaneously during denitrification. However, that was suggested using a very specific group of denitrifying bacteria, grown only with methanol as carbon source. This thesis further explored this hypothesis using 3 different microbial populations conducting denitrification using several substrates.

Another chapter inferred in the relationship between N_2O and NO emissions from ammonia oxidizing bacteria (AOB) using a laboratory scale reactor. While a lot of information has been acquired on how AOB produced N_2O in the last 10 years, its relationship (if any) with the emissions of NO remained hardly unknown. This chapter reported the results from specific experiments where the link between N_2O and NO was stablished at different ammonia oxidation rates.

Finally, the last chapter was based on the study of the different spatial and seasonal variations on N_2O and CH_4 emissions in the plug-flow reactor of a WWTP. Before this thesis there were many studies on monitoring N_2O emissions and some on the monitoring of CH_4 but few focused on monitoring the emissions of both gases. Moreover, these monitoring campaigns were performed placing a gas hood in different locations of a WWTP. In this thesis three different locations of a plug-flow reactor were monitored simultaneously using a multiple gas collection system with three gas hoods.

8.1 Occurrence of electron competition in different denitrifying populations

N_2O and NO can be produced through the denitrification process since these compounds are intermediates of the reduction of NO_3^- to N_2. Fluctuations in environmental conditions have been found to lead to inhibition of the N_2O reductase and accumulation of N_2O (Law et al., 2012b). The lack of biodegradable organic carbon is an important factor governing N_2O production during denitrification (Chung and Chung., 2000; Schalk-Otte et al., 2000). For complete denitrification, a COD to N ratio above 4 is required. Under conditions of limited carbon sources, the various denitrification enzymes compete for electrons, potentially resulting in incomplete

denitrification (Law et al., 2012b). The effect of this electron competition was studied in Chapters 4 and 5 of this thesis. Electron competition seemed to occur only when external carbon was added to the denitrification process in an enriched denitrifying SBR (Chapter 4). Nitrate and nitrite reduction rates were reduced when there was more than a single electron acceptor added. Nitrous oxide reduction rate was also reduced due to electron competition. These results corroborated the ones obtained by Pan et al., (2013a) who reported electron competition on a methanol-denitrifying enriched culture not only under carbon limiting but even when the carbon source was in excess. They also showed the electron distribution among the nitrogen oxide reductases which was affected by the carbon loading rate, with a lower fraction of electrons distributed to the N_2O reductase when the carbon loading rate was reduced. In Chapter 4 is shown that there was electron competition when using either of the three carbon sources (acetate, ethanol and methanol) but different nitrogen oxide reduction rates were obtained depending on the carbon source used. When using methanol, the N_2O reduction rate was the lowest compared to the one obtained with the other carbon sources while with ethanol it was found to be the highest. These was hypothesised to be due to the different microbial community present in the SBR, having different microorganisms able to utilize acetate and ethanol rather than methanol corroborating US EPA., (2013) that reported that ethanol and acetate had higher growth yields than methanol.

On the contrary, results presented in Chapter 5 show that there was not electron competition when using PHA as internal carbon source for denitrification. These experiments were conducted with dPAO and dGAO cultures. Results from FISH showed that there were different subgroups of microorganisms in both SBRs and these ones were the responsible of performing the different steps of the denitrification process. Therefore, electron competition between the reductases could not be distinguished because when different electron acceptors were added the different microorganisms (i.e. dPAO subgroup I was able to reduce nitrate and nitrite and dPAO subgroup II only able to denitrify from nitrite) were reducing them leading to differences on the nitrogen oxides reduction rates but showing no electron competition between the reductases. Also when comparing these results with the ones obtained for an enriched denitrifying culture in Chapter 4, in those batch tests the nitrogen oxides reduction rates (nitrate, nitrite and nitrous oxide) were reduced when there was more than a single electron acceptor added and this did not occur in the bath tests of Chapter

5. Therefore, it is plausible to suggest that electron competition occurred in a mixed denitrifying population receiving external carbon source but not in dPAO or dGAO enriched systems where PHA was the only carbon source available for denitrification.

Another important factor reported in Chapter 5 was that dGAOs accumulated more N_2O than dPAOs in all the batch tests conducted leading to the conclusion that dGAOs abundance should be controlled especially when promoting the nitrite pathway.

8.2 N_2O and NO emissions during ammonia oxidation

A description of the impact of DO and pH on the N_2O and NO emissions during ammonia oxidation is explained below.

8.2.1 Impact of DO

Transient changes in DO concentration have been shown to cause immediate increase in N_2O production by AOBs (Kampschreur et al., 2008a; Kester et al., 1997). It has been widely reported that N_2O production from nitrifying cultures is increased when DO is limited having maximum N_2O production rates under DO of 0.1 to 0.3 mg O_2/L (Goreau et al., 1980; Tallec et al., 2006). In contrast recovery from anoxia has been reported to be the cause for N_2O production by AOBs (Yu et al., 2010) which was also observed at a full-scale WWTP by Ahn et al., (2010). Various other studies also reported increased N_2O production during increased aeration rate. Kampschreur et al., (2008a) observed that N_2O production by AOB in a nitritation–anammox process decreased with decreased DO concentrations. However, the mechanisms leading to these observations were not identified (Law et al., 2012b). Due to these differences in N_2O production related to DO, in this thesis the impact of increasing and decreasing the DO level in an enriched AOB SBR was studied (Chapter 6). The relationship between AOR and N_2O and NO production rates was also explored. Results showed that as DO increased so did N_2O. However, when DO was decreased from the highest to the lowest concentration (from 3 to 0.5) N_2O increased in a linear manner. Results also showed that the AOR was kept constant during the whole experiment when DO was decreased. This should be further studied in order to reduce costs in WWTPs. Also maintaining the AOR would result in having a higher nitrification rate leading to the same effluent qualities in terms of nitrate. Moreover, the N_2O production when DO was decreasing

was much lower than when DO was increasing leading to overall lower N_2O emissions. The NO production when increasing and decreasing DO in Chapter 6 was also studied. In both experiments NO seemed to increase when DO was increasing and decrease when DO was decreased but its increase and decrease was in a constant manner. However, NO emissions were lower when DO was decreasing in a step-wise way. Therefore, it would be very interesting to reproduce these experiments in a real full-scale WWTP in order to observe whether decreasing the DO in a step-wise manner would reduce costs together with NO and N_2O emissions leading to a reduction on the C footprint of the plant.

The production pathway of NO and N_2O in this case might likely be the hydroxylamine pathway. In all the experiments conducted the NO_2^- concentration was between 700 and 1000 mg N-NO_2^-/L since they were performed in a partial nitrification reactor treating high concentrations of ammonium. It is known that the nitrifier denitrification pathway is not active at NO_2^- concentrations higher than 500 mg NO_2^--N/L (Law et al., 2011). It is also known that hydroxylamine oxidation pathway is active when the ammonia oxidation rate is increase (Wunderlin et al., 2012). Therefore, the results from this chapter would suggest that the hydroxylamine production pathway is more likely to be related to the production of NO and N_2O. However, further research could be performed using N-isotopes in order to clarify this hypothesis. Quantum cascade laser absorption spectroscopy (QCLAS) to analyze the site-specific isotope composition of N_2O in real time could be used. The net N_2O nitrogen isotopic signatures could be compared to published pure-culture investigations where the active pathways are known (Wunderlin et al., 2013).

8.2.2 Impact of pH

The effect of pH on N_2O has been investigated in several studies and also pH itself as a controlling factor for optimising the nitritation process in SBR systems (Fux et al., 2006; Ganigué et al., 2007; Gustavsson et al., 2008; Peng et al., 2004). pH has a direct effect on the AOB activity (Van Hulle et al., 2010), and also affects the concentrations of free ammonia (FA) and free nitrous acid (FNA). Increasing the pH shifts the equilibrium to FA, which is the true substrate of AOB (Suzuki and Kwok, 1974). The effect of changes in pH and the subsequent changes in FA and FNA concentrations could have a significant impact on N_2O production by AOB (Law et al., 2011). Hynes

and Knowles, (1984) reported that the optimum pH to produce NO_2^- and N_2O was approximately 8.5, in the investigated pH range of 5.4–9.5 in a fully aerobic *N. europaea* culture. Later Law et al., (2011) reported an increased N_2O production rate in an enriched AOB culture at pH 8.0 when compared with pH 6.0. The same was found in Chapter 6 of this thesis showing that N_2O was produced biologically when ammonia was present and that each time the set point of pH was decreased, N_2O decreased to a new baseline in the range of 6.5-8. Also, there was a negligible production of N_2O when ammonia was not present despite having nitrite and changing pH. For the case of NO it was only produced chemically when pH was decreased. This production could be due to the deprotonation of HNO_2 since when pH is lower than the pka of the reaction NO is formed. These results highlighted the importance of also monitoring NO in partial nitrification systems. In order to mitigate N_2O emissions it is also very important to control NO emissions and understand the factors behind its production.

8.3 N_2O and CH_4 production in full-scale systems

In full-scale WWTP, the N_2O emitted from activated sludge tanks is usually captured using a closed floating chamber. This technique was first used to measure N_2O flux from liquid surfaces in a municipal WWTP located in Durham, New Hampshire in USA (Czepiel et al., 1995). A similar approach was applied in full-scale studies of an intermittent activated sludge process in Japan (Kimochi et al., 1998). Although the emitted N_2O can be captured through the floating chamber, the off-line sampling do not capture the dynamic changes in the N_2O emission profiles. This can result in over- or underestimation of the N_2O emissions. Therefore, online, continuous monitoring of N_2O has been employed in recent years. In addition to temporal variations, spatial variations in N_2O emissions should be also considered, especially for continuous processes. Ideally, multiple hoods should be used to measure N_2O emissions from all zones simultaneously (Law et al., 2012b).

Methane emissions were first monitored in a study at the Dunham WWTP from the primary and secondary wastewater treatment processes. A closed-chamber technique was used to measure fluxes from non-aerated liquid surfaces and a bag technique was used to measure fluxes from aerated liquid surfaces (Czepiel et al., 1993). Later a report for the US EPA (Doorn et al., 1997) summarized the findings of field tests and provided

emission factors for CH_4 and N_2O from wastewater treatment. These data was the one used by the IPCC to determine a methane emission factor. This is not accurate since CH_4 emissions can vary from 0.08-1.20% kg CH_4/kg COD of the influent as it has been reported in many studies (Daelman et al., 2012). In Chapter 7 a multi hood collection system was used to monitor the N_2O and CH_4 emissions from one plug-flow reactor from the WWTP of Girona. Three different gas hoods were placed in three aerated zones of the reactor for 5 months to determine the seasonal and spatial variability of N_2O and CH_4 emissions. Results obtained showed a seasonal variation on N_2O emissions showing higher emissions in November 2016 then during December and January the emissions were down to zero but recovered in February and onwards. These emissions were correlated with the ammonia load entering the different aerated zones of the plug-flow reactor. Also, it was hypothesized that the temperature of the wastewater affected the nitrification rate (lower rates in the coldest months of December and January), leading to NH_4^+ accumulation in some of the monitored aerated zones of the plug-flow reactor. There was also a spatial variation on N_2O emissions. N_2O was negligible on the first aerated zone monitored and it was only observed in aerated zones 2 and 3 highlighting the importance of monitoring these emissions simultaneously. The N_2O emission factor recorded in this WWTP was 0-0.13% of the TKN load in the influent of the plant which is in the range of other studies such as Aboobakar et al., (2013) and Rodriguez-Caballero et al., (2014) who studied a plug- flow in a full- scale nitrifying ASP and a plug-flow reactor in the WWTP of Granollers (Spain), respectively.

The CH_4 emissions reported in Chapter 7 were 0.40 ± 0.18% kg CH_4 of the influent COD. This value is inside the varied range of CH_4 emissions reported by the literature. The differences in these values are due to different monitoring methodologies, different plant configurations and also the presence or absence of anaerobic digesters in the plants studied. In this thesis only gas emissions from the plug-flow reactor were studied but grab samples for dissolved CH_4 were taken along the WWTP. These samples corroborated the hypothesis of a spatial variation inside the plug-flow reactor since methane that is formed in the sewer network and in the anaerobic digesters is dissolved in the wastewater that flows to the plug-flow reactor and is stripped into the atmosphere when aeration starts. These CH_4 emissions did not show any significant seasonal variations during the 5 months-monitoring campaign.

In order to mitigate CH4 emissions we should not only focus on the bioreactor but apply some measures to the sewer network which is where methane is formed and enters the WWTP as adding nitrogen oxides like nitrite and nitrate to allow a good control on CH_4 emissions (Auguet et al., 2016, 2015). Another way of mitigating the CH_4 emissions would be to treat the reject wastewater that comes from the anaerobic digester via stripping through a controlled collection of the methane gas before the recirculation this water to the inlet of the plant.

On the other hand, for N_2O as it was explained in Chapter 7 sudden ammonia loadings in the bioreactor should be avoided after periods with no ammonia. Also, if there is simultaneous removal of P and N occurring in the bioreactor of the WWTP, the accumulation of nitrite should be avoided since it can lead to a major production of N_2O.

Also, in general in WWTPs a better design and good housekeeping can lead to a mitigation of N_2O and CH_4 emissions. For that it is necessary to determine previously these emissions from all the parts of a WWTP

This study at the authors' knowledge is the second reported on multi hood systems and the first with simultaneous monitoring of N_2O and CH_4 at different locations. It is indeed very important to measure the emissions at the different parts of the biological treatment of a wastewater treatment system simultaneously as well as to do a long-term monitoring in order to determine the seasonal variation of these emissions along the year.

Chapter 9

Chapter 9 Conclusions

The main conclusions of this thesis are:

Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community

- The N_2O reduction rate was the highest one compared to nitrate and nitrite reduction rates when a single nitrogen oxide is added in a mixed denitrifying community using three different external carbon sources (acetate, ethanol and methanol).
- Acetate and ethanol were the substrates providing higher reduction rates compared to methanol. This suggests the presence of different groups of bacteria specialized in consuming each of the substrates.
- The N_2O reduction rate was the most affected when there was competition for electrons. In some cases, this competition could increase the potential of nitrous oxide accumulation and lead to incomplete denitrification resulting in the release of N_2O as the end-product of the process.

Distinctive denitrifying capabilities lead to differences in N_2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms

- N_2O accumulation was higher in dGAOs compared to dPAOs. This accumulation was intensified in dGAOs when nitrite was added due to its inhibitory effect on N_2O reduction. Contrary, this effect was not observed in the dPAO biomass.
- No electron competition was detected in either of the two cultures when using PHA as the internal carbon for denitrification process. This was likely due to different sub-groups of PAO and GAO organisms and their preferences for reducing different nitrogen oxides.
- Favouring dPAOs over dGAOs can improve P removal efficiency in WWTPs and lead to lower levels of N_2O accumulation, particularly with nitrogen removal via the nitrite pathway.

Distinctive NO and N_2O emission patterns in ammonia oxidizing bacteria: Effect of ammonia oxidation rate, DO and pH

- NO linearly correlated with the ammonia oxidation rate whereas N_2O had an exponential correlation with the AOR in a partial nitrification SBR fed with high strength wastewater.
- NO and N_2O can be produced under anoxic conditions in a partial nitritation system, being the production of NO much higher than that of N_2O.
- NO was chemically produced when pH was decreased with HCl. N_2O was not affected by this addition and it was only produced when ammonia was present suggesting that its production was biological.
- NO emissions cannot be neglected in those reactors where AOB are predominant

Direct GHG emissions from a full-scale plug-flow reactor: identifying temporal and spatial variations

- N_2O emissions displayed strong temporal variations, with no emissions detected during December and January. On the other hand CH_4 emissions were relatively constant during the monitoring period from November 2016 till March 2017.
- Spatial variations were found for both gases across the aerated zones of the plug-flow reactor. CH_4 emissions decreased along the aeration path of the plug-flow reactor due to the stripping of the dissolved CH_4. On the other hand, the highest N_2O emissions were found in the second aerobic zone and were linked to nitrification.
- N_2O daily peak profiles were correlated with the arrival of ammonium in the monitored zone. Changes from no ammonium to an increase on its concentration caused a peak on N_2O.
- CH_4 emissions accounted for the majority of the C-footprint of the plug-flow reactor, overcoming the CO_2 indirect emissions associated to electricity consumption.

Chapter 10

Chapter 10 Future perspective

This thesis has been focused mainly in having a clearer understanding of the factors affecting N_2O and NO production during nitrification and denitrification using different microbial communities that are responsible for these processes at full-scale. The last part of the thesis has explored the long term N_2O and CH_4 emission dynamics and their spatial variability in a full-scale plug-flow reactor treating domestic wastewater.

Overall, the outcomes of this PhD thesis provide new knowledge and better understanding on GHG emissions from wastewater treatment but more research is still needed to tackle remaining research questions and implement robust and reliable mitigation strategies at full-scale.

10.1 N_2O and NO emissions from wastewater treatment processes

Different experiments studying the N_2O and NO production in different SBRs with ordinary heterotrophic denitrifiers, dPAOs and dGAOs respectively have been made during this thesis. While results have clarified the effect of the presence of multiple electron acceptors on N_2O reduction rate in different denitrifying communities it is still unclear the fundamental reasons behind the fact that heterotrophic denitrification processes based on external carbon sources present electron competition during the reduction of different nitrogen oxides while this does not occur in PHA-driven denitrification by dPAOs and dGAOs. Recently there have been some discoveries on the different PAO and GAO subgroups which seem to have different denitrifying capabilities. Conducting more studies with these specific subgroups would provide more information about their potential to accumulate N_2O and ultimately about the potential to conduct efficient denitrification.

The third chapter of results of this thesis has explored the relationship between N_2O and NO emissions in AOB and has shown how changes in AOR, pH and DO affects these emissions in a different way. However, the reasons behind the different behaviour of these two gases emitted by AOB is unknown. The use of isotopically labelled compounds could help to determine if N_2O and NO are emitted via the same or different pathways. Also, molecular tools could be used to assess the expression of the different genes involved in NO and N_2O production to really prove its biological or chemical origin under different conditions. The use of different mathematical models currently available for N_2O that include different N_2O production pathways could help to identify

if not only N_2O but also NO emissions can be predicted and which model provides a better fit.

10.2 N_2O and CH_4 emissions from full-scale wastewater treatment plants

The full-scale monitoring data presented in this thesis clearly shows the importance of the simultaneous monitoring at different locations to accurately determine the magnitude of the emissions. Also, temporal variations occur which highlights the need of long term monitoring data. Unfortunately, the campaign conducted in Chapter 7 could only be conducted for 5 months which happen to be the coldest months of the year. Further work should be conducted to monitor the warmest months of the year and clarify the effect of temperature on these emissions.

Also, the analysis of GHG emitted from other compartments of the WWTP such as the primary and secondary settlers would help to clarify the role of these parts on the overall C-footprint of the plant. In order to have a better understanding of the factors producing N_2O it is also highly recommended to take samples for the analysis of nutrients along the reactor in a long-term period to see if there is any correlation with the emissions as well as measuring the DO and the influent wastewater flow.

Another factor that would increase the knowledge of the N_2O emissions from a WWTP is to measure the dissolved N_2O along the different parts of the plant in order to understand how much N_2O is produced in total and how much is stripped to the atmosphere. On the other hand, it would also be interesting to study the NO emissions in full-scale as well as N_2O and CH_4. Since NO is an ozone-depleting compound and it can lead to N_2O emissions. Understanding the NO production pathways and find ways to mitigate them would also help to mitigate N_2O emissions.

Regarding CH_4, results provided in this thesis show the importance of its monitoring since important emissions of this gas can occur in WWTP especially those with anaerobic digesters. While the biological production of CH_4 is well understood, future research could be focus on exploring strategies of mitigation from full-scale facilities. Some studies have proven at lab-scale the ability of certain groups of microorganisms to consume methane, being some of these processes linked to denitrification. Investigating how dissolved methane could be used as a carbon source at full-scale and how to

promote its biological oxidation would be very useful to reduce these emissions. Additionally, avoiding the production of this gas in the sewer network that transports wastewater into the WWTP is crucial to avoid the uncontrolled release of this gas when reaching the plant. Results from this thesis show a significant amount of dissolved methane coming with the influent wastewater. Understanding the seasonal variability of this methane in the sewer wastewater would also help to decide when and how mitigation strategies need to be implemented in the sewer network.

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ANNEX

SUPPLEMENTARY INFORMATION

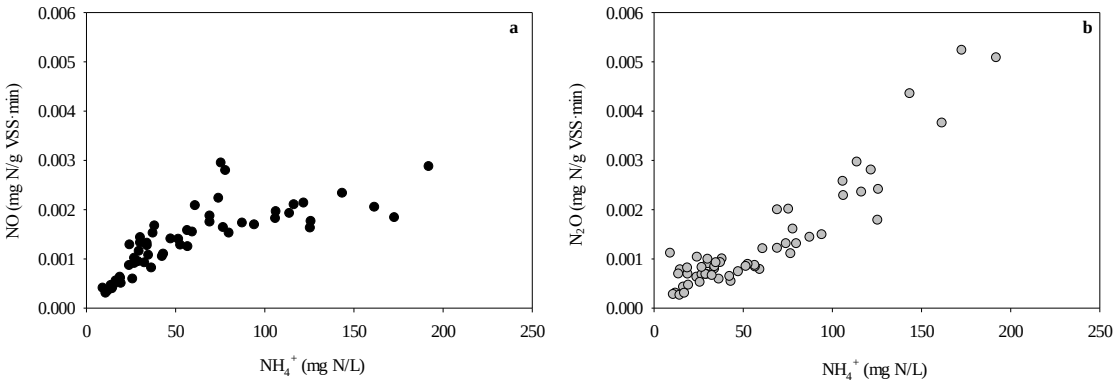

Figure SI.1: Correlation between the specific NO production rate and ammonia concentration (a) and the specific N_2O production rate and the ammonia concentration (b).

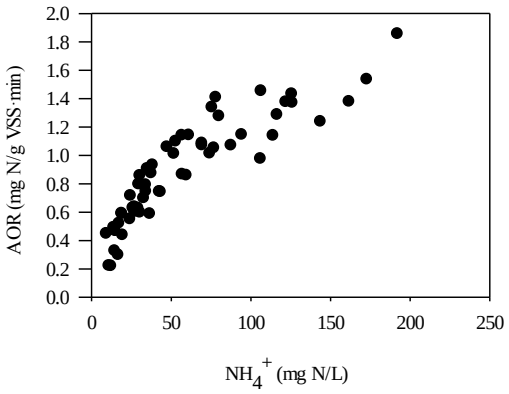

Figure SI.2: Correlation between ammonia oxidation rate and the different ammonia concentrations.

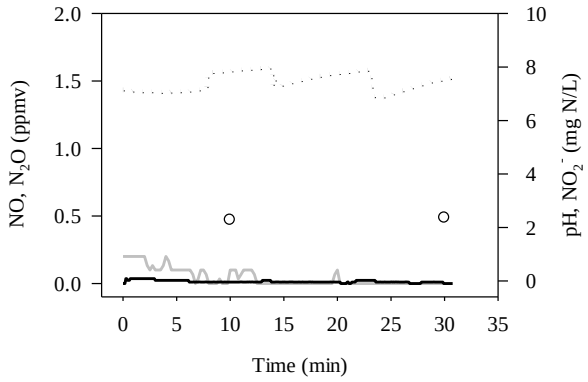

Figure SI.3: Experimental profiles of N_2O (—), NO_2^- (○), NO (—) and pH (···) of batch test 3.4: with distilled water and changing the pH set point from 8 to 7. Nitrate was not detected in any of the samples taken.

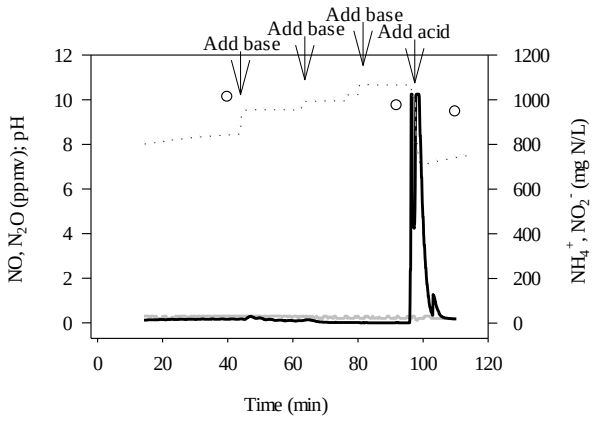

Figure SI.4: Experimental profiles of N_2O (—), NO_2^- (○), NO (─) and pH ( ) of batch test 3.5: without biomass and adding base (NaOH) and HCL to see the effect on NO production. Nitrate was not detected in any of the samples taken.

Supplementary information from Chapter 7

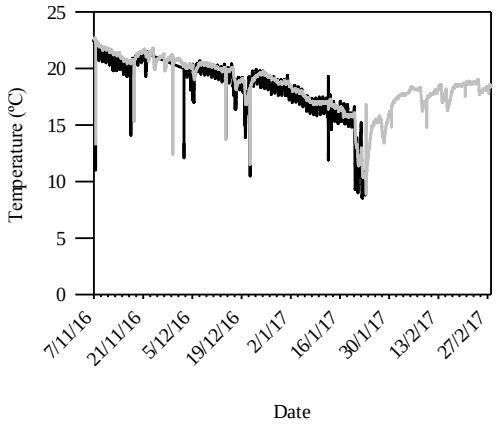

Figure SI.5: Profile of the temperature of the wastewater from November till late February of aeration zone 2 (—) and influent of the reactor (─).

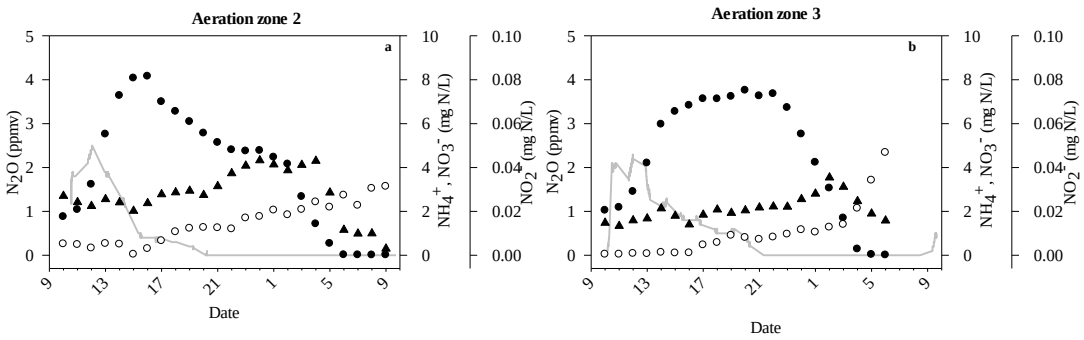

Figure SI.6: Daily pattern of N_2O (—), ammonium (●), nitrite (▲) and nitrate (○) concentration profiles measured in aerobic zone 2 measured in the 8th and 9th of March (a) and aeration zone 3 measured in the 2nd and 3rd of March (b).