Universitat de Girona

FROM INOCULA TO BIOLOGICAL REACTORS: MOLECULAR CHARACTERIZATION OF N-CYCLE BACTERIAL ASSEMBLAGES IN A PANAMMOX PROCESS

Alexandre Sànchez Melsió

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Doctoral thesis

From inocula to biological reactors: molecular characterization of N-cycle bacterial assemblages in a PANAMMOX® process

Alexandre Sànchez Melsió

2015

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Dr. Xavier Vila Portella

Dr. Jesús Colprim Galceran

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Universitat de Girona

El Dr. Xavier Vila Portella, professor titular del departament de Biologia de la Universitat de Girona, i el Dr. Jesús Colprim Galceran, professor titular del departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona,

DECLAREM:

Que el treball titulat "From inocula to biological reactors: molecular characterization of N-cycle bacterial assemblages in a PANAMMOX® process", que presenta l'Alexandre Sànchez Melsió per a l'obtenció de títol de doctor, ha estat realitzat sota la nostra direcció i que compleix els requeriments per poder optar a Menció Internacional.

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

Dr. Xavier Vila Portella   Dr. Jesús Colprim Galceran

Girona, 2015

They're Taking the Hobbits to Isengard!! They're Taking the Hobbits to Isengard…to Isengard!!

-Legolas-

DEDICATÒRIA

The end is the beginning is the end. .. Això cantaven els Smashing Pumpkins fa uns anyets! Una mica més dels que ha durat aquesta tesi… en fi… A falta d'uns pocs mesos per tenir llest el *manuscript* definitiu és ara quan em comencen a fallar les forces i per tant potser és el moment per donar-vos les gràcies. Sempre he pensat que una tesi és un viatge vital, en el qual t'emportes una mica de cada persona, així que és possible que bocins de tu que estàs llegint aquesta dedicatòria estiguin amagats darrera d'un títol, una figura o una lletra. Això va per vosaltres. El que llegireu és 100% Àlex.

Especialment dedicat a aquells que m'heu anat repetint "Què? Com? Encara no està?" "No deu ser pas tant difícil!" "Encara estàs estudiant no?" "T'ho juro que em demanaré festa el dia que defensis la tesi"... No puc posar per escrit el que m'hagués agradat fer amb el vostre cap.

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LLISTAT de PUBLICACIONS

Alexandre Sànchez-Melsió, Joan Cáliz, M. Dolors Balaguer, Jesús Colprim, Xavier Vila.

Development of batch-culture enrichment coupled to molecular detection for screening natural and man-made environments in search of anammox bacteria for N-removal bioreactors systems.

Chemosphere (2009). Volume 75 (2): 169-179

Índex d'impacte: 3.499

Primer quartil. Posició 32 de 216 en la categoria d'*Environmental Sciences*.

Ramon Ganigué, Jordi Gabarró, Alexandre Sànchez-Melsió, Maël Ruscalleda, Helio López, Xavier Vila, Jesús Colprim, M.Dolors Balaguer.

Long-term operation of a partial nitritation pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process.

Bioresource Technology (2009). Volume 100 (23): 5624-5632

Índex d'impacte: 5.039

Primer quartil. Posició 17 de 165 en la categoria *Biotechnology* & *Applied Microbiology*.

ABBREVIATIONS

Abbreviation	Description	Abbreviation	Description
AMO	Ammonia MonoOxygenase	**NOB**	Nitrite-Oxidizing Bacteria
AnAmmOx	Anaerobic Ammonium Oxidation	**NOS**	Nitrite Oxidizing System
ABF	Anaerobic Biological Filter	**NXR**	Nitrite OxidoReductase enzyme
AOB	Ammonium-Oxidizing Bacteria	**OLAND**	Oxygen-Limited Autotrophic Nitrification-Denitrification
AOR	Ammonia oxidation rate	**OMZ**	Oxygen-Minimum Zone
ATP	Adenosine TriPhosphate	**PBS**	Phosphate Buffer Saline
bp	Base Pair	**PCR**	Polymerase Chain Reaction
CANON	Completely Autotrophic Nitrogen removal Over Nitrite	**PMF**	Proton Motive Force
CLSM	Confocal Laser Scanning Microscopy	**PN**	Partial Nitritation
COD	Chemical Oxygen Demand	**qPCR**	Quantitative Polymerase Chain Reaction
cPCR	Competitive Polymerase Chain Reaction	**RBC**	Rotating Biological Contactor
CTAB	CetylTrimethylAmmonium Bromide	**RNA**	RiboNucleic Acid
DEAMOX	DEnitrifying Ammonium Oxidation	**SBR**	Sequencing Batch Reactor
DGGE	Denaturing Gradient Gel Electrophoresis	**SHARON**	Single reactor system for High rate Ammonium Removal Over Nitrite
DNA	DeoxyriboNucleic Acid	**SDS**	Sodium Dodecyl Sulphate
dsDNA	Double Stranded DNA	**TAE**	Tris Acetate EDTA
EDTA	EthyleneDiamineTetraacetic Acid	**tRNA**	Transfer RiboNucleic Acid
FISH	Fluorescence In Situ Hybridization	**UASB**	Upflow Anaerobic Sludge Bed
HAO	HydroxylAmine Oxidoreductase	**WWTP**	WasteWater Treatment Plant
HH	Hydrazine Hydrolase		
HZO	HydraZine-Oxidizing enzyme		
ICM	IntraCytoplasmic Membrane		
ISR	Intergenic Spacer Regions		
ITS	Internal Transcribed Spacer		
LB	Luria Bertani		
MPN	Most Probable Number		
NADH	Nicotinamide Adenine Dinucleotide Hydrogen (=NAD+)		
NGS	Next Gen Sequencing		
NirS	Nitrite::nitric oxide oxidoreductase		

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RESUM

Actualment, la combinació d'una nitrificació autotròfica seguida d'una desnitrificació heterotròfica és el procés més comú d'eliminació de N en les EDAR. Tot i això, quan la relació C:N és baixa aquest procés basat en dos compartiments no funciona correctament i s'ha d'afegir un font de C externa, el que comporta un increment en el cost total d'eliminació del N. Davant d'aquesta tessitura s'han desenvolupat nous processos tecnològics prometedors, basats en les activitats concretes d'alguns bacteris quimiolitotròfics. Els bacteris anammox duen a terme un d'aquests metabolismes quimiolitotròfics, en el que l'amoni s'oxida en condicions anaeròbiques utilitzant el nitrit com a acceptor d'electrons i produint N_2 com a resultat. No obstant, els bacteris anammox no poden tractar directament els efluents i lixiviats provinents de les EDAR ja que necessiten una relació adequada d'amoni/nitrit (1:1) i per tant és necessari un pas previ que l'han de dur a terme els bacteris oxidadors d'amoni (AOB).

Un nou procés tecnològic anomenat PANAMMOX®, que combina la Nitritació PArcial i l'ANAMMOX, permet tractar amb èxit altes concentracions de N sense requeriments externs de C. La caracterització dels grups de bacteris relacionats amb el cicle del N presents tant en els diferents compartiments del PANAMMOX® com en els inòculs obtinguts per a la posada en marxa del reactor anammox és el principal objectiu d'aquesta tesi. Per a la seva realització es van utilitzar mètodes moleculars basats en PCR, acoblada a tècniques de *fingerprinting*, qPCR i FISH.

Un dels aspectes importants del treball va ser la cerca d'inòculs adequats per a un reactor biològic anammox i la monitorització microbiològica durant el procés. Es van recol·lectar vàries mostres a partir de diferents ambients susceptibles de contenir poblacions de bacteris anammox i es van enriquir en cultius tancats sotmesos a condicions anaeròbiques durant un llarg període de temps. Es van utilitzar tècniques químiques i moleculars per tal de detectar l'activitat anammox i identificar els bacteris responsables, respectivament. *Ca.* Brocadia anammoxidans es va detectar en cinc de tretze cultius, enriquits a partir d'ambients naturals, modificats i creats per l'home.

Es van realitzar diversos tests per tal d'aconseguir la detecció primerenca dels bacteris anammox a l'ambient, abans de l'enriquiment. Es van provar dos jocs d'encebadors juntament amb diverses condicions de PCR, a partir de les extraccions d'ADN provinent dels

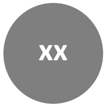
enriquiments d'anammox, per tal de rebaixar el límit de detecció de la PCR. El joc d'encebadors més utilitzat (Pla46F-Amx368R) no va aconseguir detectar bacteris anammox en les mostres amb baixa concentració. L'altra parella d'encebadors, Amx368-Amx820, va permetre recuperar algunes seqüències pertanyents a *Brocadiaceae*, que es podrien anomenar bacteris anammox "putatius" ja que no són suficientment properes filogenèticament als bacteris anammox coneguts. Aquestes seqüències es van detectar en els inòculs i estadis primerencs dels enriquiments amb bacteris anammox, de manera que la detecció d'aquests bacteris anammox "putatius" es podria considerar com un indicador de la presència de bacteris anammox i del desenvolupament potencial de les seves poblacions.

A dia d'avui encara no s'ha pogut aconseguir un cultiu pur de bacteris anammox i una de les raons pot ser la dependència d'aquests bacteris respecte altres espècies que haurien d'estar presents en la comunitat microbiana. Així doncs, els seus socis ecològics en poblacions ben desenvolupades de bacteris anammox i el seu possible paper en el procés de desenvolupament i enriquiment és un aspecte intrigant. Les anàlisis filogenètiques de les seqüències parcials del 16S rDNA que es van dur a terme a partir dels últims estadis dels enriquiments anammox van confirmar una composició de la comunitat bacteriana molt semblant a la descrita en altres casos estudiats i publicats. La comparació d'aquestes seqüències amb un gran nombre de seqüències relacionades, extretes de la base de dades de l'NCBI, suggereix que alguns membres de certs grups bacterians (principalment *Rhodocyclaceae*, *Chlorobi* i *Chloroflexi*) podrien jugar un paper clau en la presència i desenvolupament dels bacteris anammox.

Els bacteris nitrificants que estan directament o indirectament involucrats en la nitritació parcial (PN) del procés PANAMMOX® es van caracteritzar a través de tècniques moleculars. Es van estudiar els gèneres principals d'AOB i de bacteris oxidadors de nitrit (NOB) i es van determinar les seves dinàmiques poblacionals durant el temps de funcionament del PN. L'estudi dels AOB era especialment rellevant degut a que les elevades concentracions dels compostos nitrogenats a l'interior del reactor (fins a 3000 mg N L^{-1} d'amoni i nitrit) eren bastant excepcionals. En el cas dels NOB, la producció no desitjada de nitrat al llarg del funcionament del PN va revelar la presència residual de les seves poblacions tot i que les condicions d'operació del reactor els hauria de perjudicar. Les poblacions dominants de *Nitrosomonas* van canviar des d'espècies inicials de *Nitrosomonas* pertanyents al grup 6a (estrategues de la K) fins a espècies finals del grup 7 (estrategues de la r). En aquesta fase final, totes les seqüències recuperades estaven relacionades amb *Nitrosomonas* sp. IWT514, una soca d'AOB especialitzada a sobreviure en condicions extremes de compostos

nitrogenats. Per altra banda, les poblacions de *Nitrobacter* van resultar ostensiblement perjudicades al final de l'estudi tot i que una població de *Nitrospira* (només es va identificar un filotip, corresponent a *Ca*. Nitrospira defluvii) no només no va ser eliminada per les condicions de treball del reactor PN, sinó que va aconseguir trobar un nínxol ecològic per acabar-se imposant a les poblacions de *Nitrobacter*.

El desenvolupament metodològic d'aquest treball ha posat de manifest la importància de l'ús de les tècniques moleculars per escollir els inòculs adequats, combinat amb enriquiments previs de bacteris anammox en cultius tancats, podria prevenir retards en el desenvolupament de l'activitat d'eliminació del N. D'altra banda, un millor coneixement de la composició de les poblacions ha revelat la importància dels socis ecològics en la presència i desenvolupament dels bacteris anammox. A més, es va detectar una successió ecològica en el reactor PN, passant d'unes espècies inicials estrategues de la *K* que oxidaven altes concentracions d'amoni per cèl·lula fins a espècies estrategues de la *r* que treballaven per sota les seves capacitats. La informació aconseguida amb els dos temes (l'ús dels procediments metodològics i la composició dels grups de bacteris relacionats amb el cicle del N) hauria de millorar la posada en marxa i el funcionament de la tecnologia PANAMMOX® i tenir un millor control i eficiència en les diferents etapes del desenvolupament.



RESUMEN

Hoy en día la combinación de una nitrificación autotrófica seguida de una desnitrificación heterotrófica es el proceso más común para la eliminación de N de las EDAR. A pesar de esto, cuando la relación C:N es baja, este proceso de dos compartimentos no funciona correctamente y se debe añadir una fuente de C externa, comportando un aumento en el coste total de la eliminación del N. Ante esta tesitura se han desarrollado nuevos procesos tecnológicos prometedores, basados en las actividades concretas de algunas bacterias quimiolitotróficas. Las bacterias anammox llevan a cabo uno de estos metabolismos quimiolitotróficos, en el que el amonio se oxida en condiciones anaeróbicas utilizando el nitrito como aceptor de electrones y produciendo N_2 como resultado. Sin embargo, las bacterias anammox no pueden tratar directamente los efluentes y lixiviados provenientes de las EDAR ya que necesitan una relación adecuada de amonio/nitrito (1:1) y por lo tanto es necesario un paso previo que debe ser llevado a cabo por las bacterias oxidadoras de amonio (AOB).

Un nuevo proceso tecnológico llamado PANAMMOX®, que combina la Nitritación PArcial y ANAMMOX, permite tratar con éxito elevadas concentraciones de N sin requerimientos externos de C. La caracterización de los grupos de bacterias relacionadas con el ciclo del N presentes tanto en los diferentes compartimentos del PANAMMOX® como en los inóculos obtenidos para la puesta en marcha del reactor anammox es el principal objetivo de esta tesis. Para su realización se utilizaron métodos moleculares basados en PCR, acoplada a técnicas de *fingerprinting*, qPCR y FISH.

Uno de los aspectos importantes de este trabajo fue la búsqueda de inóculos adecuados para un reactor biológico anammox y la monitorización microbiológica del proceso. Se recolectaron varias muestras a partir de distintos ambientes susceptibles de contener poblaciones de bacterias anammox y se enriquecieron en cultivos cerrados en condiciones anaeróbicas durante un largo período de tiempo. Se utilizaron técnicas químicas y moleculares para detectar la actividad anammox y para identificar las bacterias responsables, respectivamente. *Ca.* Brocadia anammoxidans se detectó en cinco de trece cultivos, enriquecidos a partir de ambientes naturales, modificados y creados por el hombre.

Se realizaron varios tests para lograr la detección temprana de las bacterias anammox en el ambiente, antes del enriquecimiento. Se probaron dos parejas de cebadores junto con

distintas condiciones de PCR, a partir de las extracciones de ADN procedentes de los cultivos de anammox, con el fin de bajar el límite de detección de la PCR. La pareja de cebadores más utilizada (Pla46F-Amx368R) no logró detectar bacterias anammox en las muestras de baja concentración. La otra pareja de cebadores, Amx368-Amx820, permitió recuperar algunas secuencias que pertenecían a *Brocadiaceae*, que se podrían considerar como bacterias anammox "putativas" ya que no son suficientemente cercanas filogenéticamente a las bacterias anammox conocidas. Estas secuencias se detectaron en los inóculos y estadíos tempranos de los enriquecimientos con bacterias anammox, de modo que la detección de estas bacterias anammox "putativas" se podría considerar como un indicador de la presencia de bacterias anammox y del desarrollo potencial de sus poblaciones.

Hasta la actualidad, aún no se ha podido lograr un cultivo puro de bacterias anammox y una de las razones puede ser la dependencia de estas bacterias respecto de otras especies que deberían estar presentes en la comunidad microbiana. Así pues, sus socios ecológicos en poblaciones bien desarrolladas de bacterias anammox y su posible papel en el proceso de desarrollo y enriquecimiento es un aspecto intrigante. Los análisis filogenéticos de las secuencias parciales del 16S rDNA que se llevaron a cabo a partir de los últimos estadíos de los cultivos anammox confirmó una composición de la comunidad bacteriana muy parecida a la descrita en otros estudios publicados. La comparación de estas secuencias con un gran número de secuencias relacionadas, extraídas de la base de datos del NCBI, sugiere que algunos miembros de ciertos grupos bacterianos (principalmente *Rhodocyclaceae*, *Chlorobi* y *Chloroflexi*) podrían jugar un papel clave en la presencia y desarrollo de las bacterias anammox.

Las bacterias nitrificantes que están directamente o indirectamente involucradas en la nitritación parcial (PN) del proceso PANAMMOX® se caracterizaron mediante técnicas moleculares. Se estudiaron los principales géneros de AOB y de bacterias oxidadoras de nitrito (NOB) y se determinaron sus dinámicas poblacionales a lo largo del funcionamiento del PN. El estudio de las AOB fue especialmente relevante debido a que las altas concentraciones de los compuestos nitrogenados en el interior del reactor (hasta 3000 mg N L^{-1} de amonio y nitrito) eran bastante excepcionales. En el caso de las NOB, la producción no deseada de nitrato durante el funcionamiento del PN desveló la presencia residual de sus poblaciones a pesar de que las condiciones de operación del reactor deberían perjudicarlas. Las poblaciones dominantes de *Nitrosomonas* cambiaron des de las especies iniciales de *Nitrosomonas* pertenecientes al grupo 6a (estrategas de la K) hasta la especies

finales del grupo 7 (estrategas de la r). En estas fases finales, todas las secuencias recuperadas estaban relacionadas con *Nitrosomonas* sp. IWT514, una cepa de AOB especializada en sobrevivir en condiciones extremas de compuestos nitrogenados. Por otra parte, las poblaciones de *Nitrobacter* fueron ostensiblemente perjudicadas en la parte final del estudio aunque una población de *Nitrospira* (sólo se identificó un filotipo, correspondiente a *Ca*. Nitrospira defluvii) no sólo no fue eliminada por las condiciones de trabajo del reactor PN, sino que consiguió hallar un nicho ecológico para acabar imponiéndose a las poblaciones de *Nitrobacter*.

El desarrollo metodológico de este trabajo ha evidenciado la importancia del uso de las técnicas moleculares para escoger los inóculos adecuados, combinado con enriquecimientos previos de bacterias anammox en cultivos cerrados, podría prevenir retrasos en el desarrollo de la actividad de eliminación del N. Por otra parte, un mejor conocimiento de la composición de las poblaciones ha revelado la importancia de los socios ecológicos en la presencia y desarrollo de bacterias anammox. Además, se detectó una sucesión ecológica en el reactor PN, pasando de unas especies iniciales estrategas de la *K* que oxidaban altas concentraciones de amonio por célula a unas especies estrategas de la *r* que trabajaban por debajo de sus capacidades. La información conseguida con los dos temas (el uso de los procedimientos metodológicos y la composición de los grupos de bacterias relacionadas con el ciclo del N) debería mejorar la puesta en marcha y el funcionamiento de la tecnología PANAMMOX® y tener un mejor control y eficiencia en las diferentes etapas del desarrollo.

SUMMARY

Nowadays, the combination of the autotrophic nitrification together with heterotrophic denitrification is the most common process for N-removal in the WWTPs. However, when the C:N ratio is low, this two-step process does not properly work and an external C source must be added, causing an increase in the overall cost of N-removal. In front of this situation, some novel and promising technological processes have been proposed, based on specific activities of some chemolithotrophic bacteria. The anammox bacteria carry out one of these chemolithotrophic metabolisms, in which ammonia is oxidized under anaerobic conditions with nitrite as electron acceptor, producing N_2 as a result. However, anammox bacteria can not directly treat urban wastewater and leachate because they need an accurate ammonium/nitrite ratio (1:1), and therefore a previous step conducted by ammonium-oxidizing bacteria (AOB) is required.

A novel technological process called PANAMMOX®, combining PArtial Nitritation and ANAMMOX, can successfully remove high N loads without external C requirements. The characterization of the N-cycle bacterial assemblages present in the different PANAMMOX® components and the inocula achieved to start up the anammox reactor is the general aim of this thesis. Molecular methods based on PCR, coupled to fingerprinting methods, qPCR and FISH were carried out to fulfil this characterization.

The first topic of the present work was to search for suitable inocula for the anammox biological reactor and perform a microbiological monitoring of the process. Several samples were collected in environments susceptible to harbour anammox bacteria populations and they were enriched in batch cultures under anaerobic conditions during a long time period. Chemical and molecular techniques were used to detect the anammox activity and identify the responsible bacteria, respectively. *Ca*. Brocadia anammoxidans was detected in five of thirteen batch cultures, which were enriched from natural, modified and man-made environments.

Several tests were performed to achieve an early detection of the anammox bacteria in the environment, prior to the enrichment step. Two primer sets and different PCR conditions were tested, using the DNA isolations from the anammox enrichments, to lower the PCR detection limit. The most common primer set (Pla46F-Amx368R) failed in the detection of anammox bacteria in low-concentrated samples. The other primer set, Amx368-Amx820, allowed to retrieve some *Brocadiaceae* sequences, which could be named as “putative”

anammox bacteria since they are not phylogenetically close to any known anammox bacteria. These sequences were retrieved from inocula and early stages of the successful anammox enrichments, which lead to hypothesize that the detection of these "putative" anammox bacteria could be an indicator of anammox presence and the potential development of some anammox populations.

Any anammox pure culture has never been achieved until today and one reason could be the dependence of these bacteria from other species that should be present in the microbial community. Therefore, their ecological partners in well-developed anammox bacteria populations and the probable role of these species in the whole process of their enrichment and development is an intriguing matter. Phylogenetic analyses of the partial 16S rDNA sequences performed on the latest stages of the anammox enrichments confirmed a composition of the bacterial community similar to other cases reported in literature. The comparison of these sequences to a high number of related sequences from the NCBI database suggested that some members of particular bacterial groups (mainly *Rhodocyclaceae*, *Chlorobi* and *Chloroflexi*) may play key roles in the anammox bacteria development and occurrence.

Nitrifying bacteria that can be directly or indirectly involved in the partial nitritation (PN) of the PANAMMOX® process were characterized by molecular techniques. Main AOB and nitrite-oxidizing bacteria (NOB) genera were studied and their population dynamics along the PN reactor performance were also determined. AOB study was relevant since the N-compound concentration in the reactor (up to 3000 mg N/L of both ammonium and nitrite) were fairly outstanding. For NOB, the undesirable production of small amounts of nitrate along the PN performance disclosed the presence of residual NOB populations when PN operating conditions should have impaired them. Dominant *Nitrosomonas* populations changed from initial *Nitrosomonas* cluster 6a species (*K*-strategists) to later *Nitrosomonas* cluster 7 (*r*-strategists). At the end of the study, all the retrieved sequences were related with *Nitrosomonas* sp. IWT514, an AOB strain specialized in thriving at remarkably high N-compounds concentrations. On the other hand, *Nitrobacter* populations were ostensibly washed out at the end of the reactor performance but a *Nitrospira* population (only one phylotype was identified, corresponding to *Ca*. Nitrospira defluvii) not only was not removed by the conditions operating in the PN reactor, but it found an ecological niche to override *Nitrobacter* populations.

The methodological development of this work has revealed the importance of the use of molecular techniques for choosing appropriate seeds, combined to the previous enrichment of anammox bacteria in batch cultures, that could prevent delays on the development of N-removal activity. On the other hand, a better knowledge of the community composition has disclosed the importance of the ecological partners in the anammox bacteria occurrence and development. Besides, an ecological succession in the PN reactor was revealed, from initial *K*-strategists species oxidizing high concentrations of ammonium per cell to *r*-strategist species working under their capabilities. The accomplishment of both goals (the improvement of methodological procedures and the knowledge of the composition of N-cycle bacterial assemblages) can be useful to enhance the start-up and performance of the PANAMMOX® technology and have a better control and efficiency in its different stages of development.

PRESENTACIÓ

Aquesta memòria de Tesi Doctoral recull els principals resultats obtinguts en la recerca desenvolupada sobre els bacteris anammox, els bacteris oxidadors d'amoni (AOB) i els bacteris oxidadors de nitrit (NOB) en els diferents estadis de desenvolupament del procés PANAMMOX®, dissenyat per millorar els tractaments per a l'eliminació de N en residus líquids amb una elevada càrrega nitrogenada.

En la **introducció** s'intenta aprofundir, en primer lloc, en les característiques generals (amb especial èmfasi en el metabolisme), la classificació i els patrons ecològics dels 3 grups de bacteris quimiolitotròfics que han estat objecte d'aquest estudi. Posteriorment es comenten les principals tècniques moleculars que s'han utilitzat en la recerca científica relacionada amb aquests microorganismes i finalment, en l'últim apartat, es fa referència a l'aplicació d'aquests bacteris quimiolitotròfics en els nous processos de tractament d'aigües residuals. A continuació s'exposen els **objectius** generals de la tesi doctoral. L'apartat de **material i mètodes** està centrat en la definició i descripció detallada de tots els procediments metodològics, tècniques i protocols específics que s'han utilitzat en aquest treball, des de l'enriquiment i cultiu de bacteris anammox i els mètodes químics d'anàlisi a les tècniques moleculars aplicades.

La part central de la memòria és l'apartat de **resultats i discussió**, que es divideix en 4 capítols, cadascun dels quals inclou una secció de resultats i una altra de discussió. En el **primer capítol** de resultats (*Anammox bacteria enrichment in batch cultures*) es descriu com es van aconseguir els enriquiments necessaris per poder inocular un reactor biològic anammox i la identificació dels bacteris responsables d'aquesta activitat. En el **segon capítol** (*Optimization of molecular techniques for the detection of anammox bacteria*) s'exposa com es van provar diferents tipus de PCR per tal de rebaixar els límits de detecció i aconseguir diagnosticar la presència de bacteris anammox en mostres poc concentrades, abans del procés d'enriquiment o en les seves fases primerenques. El **tercer capítol** (*Bacterial diversity in anammox enrichments*) tracta sobre la identificació molecular, en comunitats microbianes que contenien poblacions ben desenvolupades de bacteris anammox, dels altres bacteris que s'hi van poder detectar i el seu possible rol en la presència i desenvolupament dels anammox. En el **quart capítol** (*AOB and NOB dynamics in high ammonium concentrated bioreactor*) es presenta la caracterització molecular i l'estudi de les dinàmiques dels AOB i NOB presents en un reactor de nitritació parcial que tractava

lixiviats amb concentracions molt altes d'amoni i nitrit com a pas previ al seu processament en un reactor anammox.

Seguidament, el bloc de **continguts generals** conté dos grans apartats on es destaquen i es relacionen entre si els principals aspectes abordats pels diferents capítols de la tesi. Per una banda, un apartat metodològic on es discuteix sobre l'ús d'enriquiments davant altres mètodes d'enriquiment (com podrien ser els reactors biològics) i els efectes de l'elecció d'encebadors per a la detecció de bacteris anammox, com a mecanismes per optimitzar el procés d'obtenció d'inòculs per als reactors biològics. Per altra banda, en un segon apartat es valora la informació obtinguda a partir de la detecció i caracterització dels diferents grups de bacteris quimiolitotròfics en els diferents compartiments estudiats i la seva importància per al coneixement i millora dels processos que es donen en els diferents estadis de desenvolupament de la tecnologia PANAMMOX®.

Per últim, es presenten les **conclusions** generals del treball i l'apartat bibliogràfic amb totes les **referències** esmentades al llarg de la memòria de la tesi doctoral.

OUTLINE

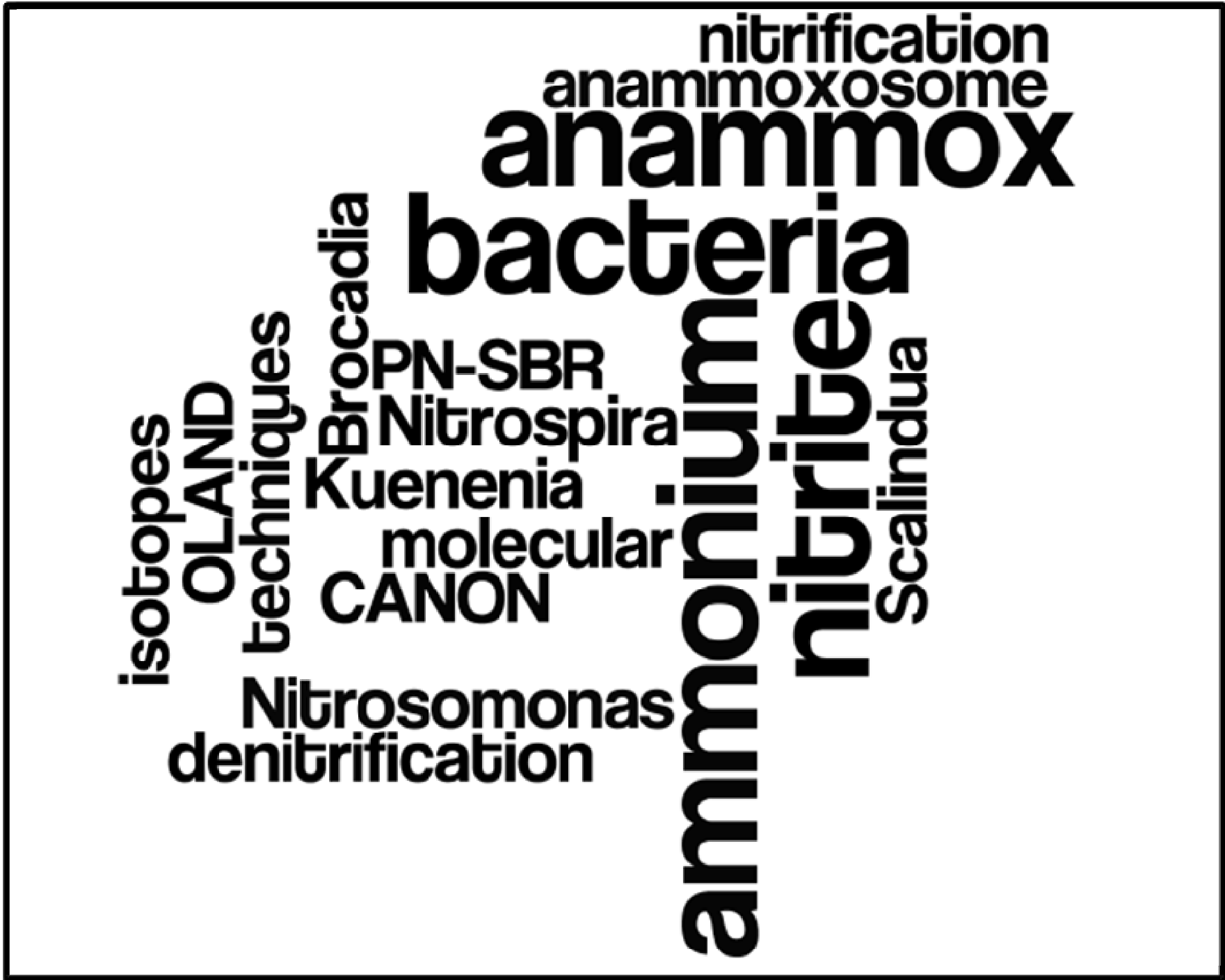
This Doctoral Thesis compiles the main results obtained in the research performed about anammox bacteria, ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the different development stages of the PANAMMOX® process, designed to improve the N-removal treatments in liquid wastes with high nitrogen loads.

In the **introduction** are explained in depth, in first term, the main features (with special emphasis in the metabolism), classification and ecological patterns of the 3 chemo-lithotrophic bacterial groups under study. Afterwards, the main molecular techniques performed in the scientific research related to these microorganisms are discussed and, finally, the last section is devoted to the application of the chemolithotrophic bacteria in the novel wastewater treatment process. The general **objectives** of the doctoral thesis are defined below. The **material and methods** section is focused on the definition and detailed description of all the methodological procedures, techniques and specific protocols used in this work: anammox bacteria enrichment and culture, chemical analyses and molecular techniques.

The central part of this thesis is the **results and discussion** section, split into 4 chapters, each one including their own results and discussion. In the **first chapter** of results (Anammox bacteria enrichment in batch cultures), it is described how anammox enrichments were achieved, in order to be further used as inocula for an anammox biological reactor, and the identification of the responsible bacteria for this activity. In the **second chapter** (Optimization of molecular techniques for the detection of anammox bacteria), it is explained how different PCR approaches were tested to lower the PCR sensitivity limits and to achieve anammox bacteria detection in low-concentrated samples, prior to the enrichment process. The **third chapter** (Bacterial diversity in anammox enrichments) deals about the molecular characterization of the ecological partners found in communities harbouring well-developed anammox bacteria populations and their probable role in anammox presence and development. In the **fourth** chapter (AOB and NOB dynamics in high ammonium concentrated bioreactor), it is described the molecular characterization and dynamics of the AOB and NOB populations present in a partial nitritation reactor treating high ammonium and nitrite concentrations as a previous step to anammox reactor.

Next, the **global remarks** section has two main subdivisions where the main aspects addressed by the different chapters of the thesis are highlighted and related among them. In one hand, a methodological discussion about the use of enrichment procedures in batch cultures in comparison to other methodologies (such as biological reactors) and the effects of the primer set selection for anammox bacteria detection as a tool for inocula achievement optimization. In the other hand, the information obtained from the detection and characterization of the different chemolithotrophic bacterial groups and their relevance in the knowledge and improvement of the processes carried out in the different development stages of the PANAMMOX® technology.

At last, the global **conclusions** of the work and the literature section with all the **references** mentioned throughout the doctoral thesis are stated.



INTRODUCTION

1.1 BIOLOGICAL AMMONIUM and NITRITE OXIDATION for NITROGEN REMOVAL

Fighting against nitrogen pollution in aquatic systems is one of the main environmental challenges at the beginning of XXIth century. Ammonium (NH_4^+) and nitrate (NO_3^-) are the most common ionic (reactive) forms of dissolved inorganic nitrogen in aquatic ecosystems. In contrast, nitrite is usually found in trace amounts in aerobic habitats and only accumulates at low oxygen partial pressure (Dworkin and Falkow, 2006). Due to human activities, the nitrogen equilibrium has changed and, thus, these nutrients can cause several environmental problems (mainly related to eutrophication) such as toxic algal blooms, loss of oxygen, fish kills, pollution of groundwater and health risk from public water supply, loss of biodiversity (including important species for commerce and recreation), loss of aquatic plant beds and coral reefs, among other problems (Carpenter et al., 1998).

Wastewater from anthropogenic origin requires to be treated before returned to the environment, to avoid ammonia or nitrate toxicity. The excessive load on the environment and the more stringent legislations on wastewater discharges have made the removal of nitrogen from wastewater an important part of the general water treatment process (Windey et al., 2005).

Two different wastewater treatment concepts can be performed: either the separation of impurities from water, or the partial or complete mineralization of impurities. Separation processes are based on fluid mechanics (sedimentation, centrifugation, filtration and flotation) or on synthetic membranes. Additionally, physical-chemical processes can be used to separate dissolved or emulsified compounds from water. Impurities can be mineralized by biological and chemical processes. The chemical oxidation advantages are: no sludge production, mineralization of non-biodegradable compounds and smaller reactor volumes. On the other hand the main advantages of biological processes in comparison to chemical oxidation are: no need to separate

colloids (microscopic particles that do not settle if they do not receive a previously coagulation) and disperse solid particles before treatment, lower energy consumption, lower costs due the use of open reactors and no need for waste gas treatment. Because biological nitrogen removal is effective and less expensive, it has been widely adopted instead of the physical and chemical processes (EPA, 1993). The most common biological method for nitrogen removal in wastewater treatment plants (WWTPs) is based on the combination of two processes, autotrophic nitrification and heterotrophic denitrification. The former aerobically oxidizes ammonium to nitrate via nitrite and the latter consists on the anaerobic reduction of nitrate to dinitrogen gas (N_2). When both metabolisms are coupled, the whole process promotes a net loss of nitrogen from the system (Bernhard et al., 2005). The main focus of this work is to improve the knowledge of the biological processes involved in aerobic and anaerobic ammonium oxidation in nitrogen removal systems.

Nitrification is a two-step process involving microorganisms from two phylogenetically-unrelated groups of chemolithotrophic bacteria. First, ammonium is oxidized to nitrite by ammonium-oxidizing bacteria (AOB), and subsequentially the nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (fig. 1.1). Up to date, considerable attention has been devoted to investigate the ecology and physiology of AOB, more than NOB for two main reasons (Bothe et al., 2000; Kowalchuk and Stephen, 2001). Firstly, because the oxidation of ammonium is the rate-limiting step in the whole process of nitrification and also in the biological nitrogen removal and because it has some important drawbacks, such as slow growth rate and a high sensitivity to environmental factors such as pH and temperature shifts. Due to AOB sensitivity to disturbances, breakdown of the nitrification process is frequently reported from municipal and industrial WWTPs (Wagner et al., 2002). Secondly, all AOB found in WWTPs belong to *Betaprotebacteria* subdivision, meaning that studies based on molecular methods can use a single primer set in Polymerase Chain Reaction (PCR) amplification for almost all AOB species. This methodological advantage does not happen with NOB, a polyphyletic group (NOB species are spread through *Nitrospirae* and *Chloroflexi* phyla and three *Proteobacteria* subdivisions) for which a wide-range primer set for all species cannot

be described and several specific primer combinations are required to analyze the whole group.

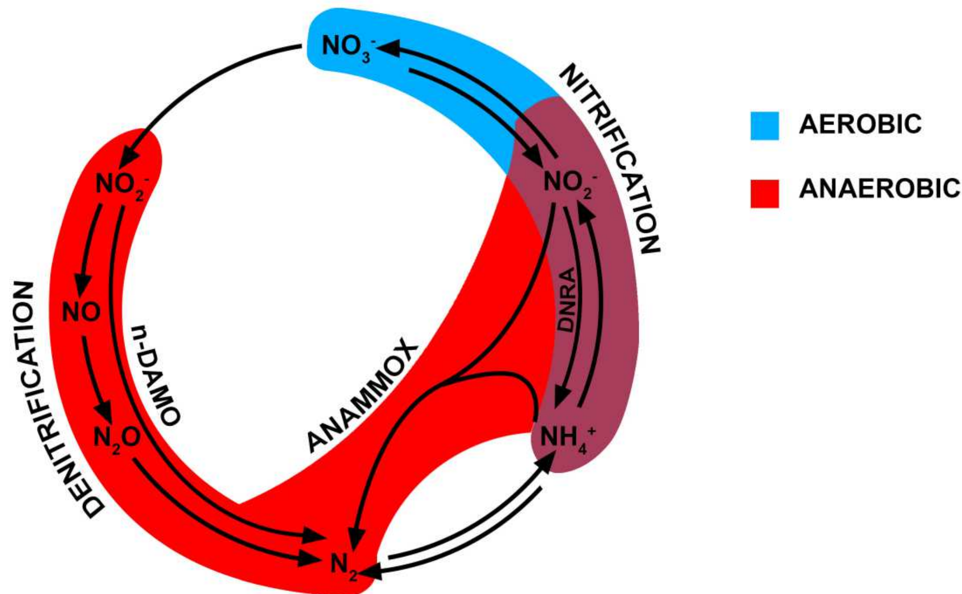


Figure 1.1: Simplified Nitrogen cycle, highlighting the aerobic and anaerobic processes and the different N forms involved.

Although AOB and NOB have been traditionally considered as the main microbial groups responsible of the nitrification processes, current studies recently introduced archaea capable of chemolithotrophic ammonia oxidation to nitrite (Konneke et al., 2005; Kim et al., 2011a). The genome sequence of ammonia-oxidizing archaeon showed that they contain some key genes involved in the ammonia oxidation (ammonia monooxygenase, *amo*), similar to those present in AOB, but lack in other ones also present in aerobic ammonia oxidation (hydroxylaminase oxidoreductase, *hao*) (Walker et al., 2010; Kim et al., 2011a). Besides, to date, any archaea has been described to be able to oxidize nitrite to nitrate (Schleper and Nicol, 2010). Additionally, some heterotrophic bacteria and fungi can also oxidize ammonium and/or reduce nitrogen from organic compounds to one of the nitrification intermediates: hydroxylamine (NH_2OH), nitrite and nitrate. In contrast to AOB, oxidation of ammonium by heterotrophic nitrifiers has not found to be linked to cellular growth (De Boer and Kowalchuk, 2001). In the same study it was also reported that nitrification can be performed using both inorganic and organic substrates for

growth, depending on the conditions. For example, *Thiosphaera pantotropha* under low-oxygen concentrations combines the nitrifying activity with the aerobic denitrification to dissipate reducing equivalents (NADH).

Despite these interesting variants of the ammonium oxidation, the most promising advances in nitrogen removal biotechnology during the past decade were related to a shortcut of the nitrogen cycle: the ANaerobic AMMonium OXidation (ANAMMOX) (Mulder et al., 1995). In this biological process, mediated by bacteria belonging to the order *Planctomycetales*, ammonium is oxidized under anaerobic conditions with nitrite as electron acceptor, giving N_2 as a result (fig. 1.1). This novel metabolism combines the anaerobic requirements and the final products of denitrification with the chemolithotrophic features of nitrification, delivering an outstanding resource for ammonia removal.

1.2 AMMONIUM-OXIDIZING BACTERIA (AOB)

AOB were first discovered by S.N. Winogradsky at the end of the 19th century, identifying the genera *Nitrosomonas* (*N. europaea*), *Nitrosospira* (*N. briensis; N. antartica*) and *Nitrosococcus* (*N. nitrosus*). Although Winogradsky postulated the existence of a great diversity of species capable of growing upon ammonium oxidation, it was not until the late 60s of 20th century when new AOB species were isolated (Watson, 1965; 1971).

The aerobic oxidation of ammonium to nitrite, conducted by AOB, is the first step of nitrification and the overall stoichiometric equation goes as it follows:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

AOB have their optimal activity at mesophilic temperatures and neutral pH (7.5 to 7.8). These aerobic gram-negative bacteria, as strict chemolithotrophs, gain energy by oxidizing ammonium as their sole source of energy and electrons and produce organic molecules by reducing CO_2 via Calvin cycle. The complete genome sequence of

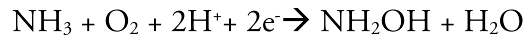
Nitrosomonas europaea and *Nitrosococcus oceani* allowed to identify the genes and the encoding proteins necessary for the function of the lithotrophy and autotrophy but also demonstrated the presence of genes for organic compounds catabolism in both species (Chain et al., 2003; Klotz et al., 2006). Some AOB (*Nitrosococcus, Nitrosolobus,* and *Nitrosomonas*) have intracytoplasmic membrane (ICM) systems which may serve for energy conservation through the generation of a protomotive gradient, since this is the location of Adenosine TriPhosphatase (ATPase) and the key enzyme in ammonia oxidation, ammonia monooxygenase (AMO). In *Nitrosomonas eutropha*, the arrangement of the ICMs is dependent on the physiological state of the cells (Schmidt et al., 2001).

AOB are a small group of bacteria that play a central role in the nitrogen cycle in both terrestrial and aquatic ecosystems. From an anthropogenic point of view, the activity of AOB can be detrimental and beneficial. The emission of gaseous N oxides (nitrous oxide (N_2O) and nitric oxide (NO)) during the oxidation of ammonium has a relevant environmental impact on the chemistry of the atmosphere (Shaw et al., 2006). Both NO and N_2O are among the atmospheric trace gases involved in the greenhouse effect. Moreover, they are implicated in the destruction of ozone in the troposphere and the oxidation of NO causes the presence of nitric and nitrous acids, main components of acidic precipitation. On the other hand, AOB activity is encouraged in order to reduce the ammonia content of WWTPs' sewage before discharge into receiving waters (Arp et al., 2002) or for the bioremediation of sites contaminated with chlorinated aliphatic hydrocarbons (Chain et al., 2003).

METABOLISM (CATABOLISM) and ITS GENETIC REGULATION

In autotrophic ammonia oxidizers, two key enzymes are necessary for energy conservation during the oxidation process: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (fig. 1.2).

AMO catalyzes the oxidation of ammonia to hydroxylamine:



$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$$

HAO catalyzes the oxidation of hydroxylamine to nitrite:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

AMO and HAO are co-dependent, because they generate the substrate and electrons for each other, respectively. The electrons extracted in this oxidation process are proposed to follow a redox cascade via the two tetraheme cytochromes *c*554 and c_M552 to the electron chain at the level of ubiquinone. However, two electrons must be directed towards AMO for the oxidation of NH_3 (through the ubiquinone pool) and only the remaining two follow the electron transport chain for ATP generation (Arp et al., 2002) (fig. 1.2).

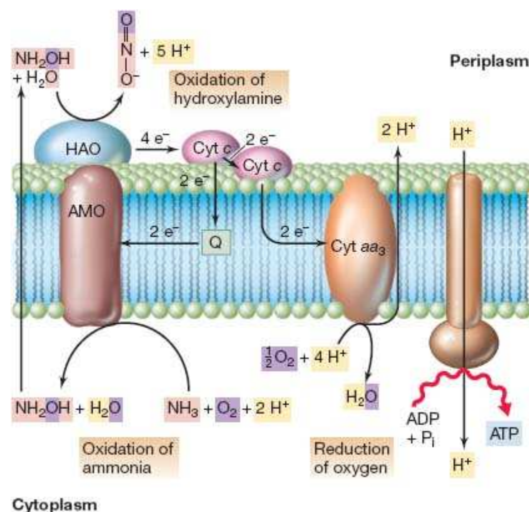


Figure 1.2: Schematic representation of ammonia oxidation catabolism and the enzymes linked to this process in AOB. The location of the products and the reactants, as well as the enzymes and the cytochromes, are highlighted. AMO: ammonia monooxygenase; HAO: hydroxylamine oxido-reductase (extracted from Madigan and Brock (2009)).

AMO is the membrane-bound enzyme that catalyzes the oxidation of ammonia to hydroxylamine. Specific AMO content seems to be regulated by ammonium concentration, because the amount of AMO enzyme in the cell is higher at ammonium limiting conditions than at high ammonium concentrations (Pinck et al., 2001). This enzyme was initially proposed to consist of two subunits (AmoA, AmoB) but the

presence of a third one, AmoC, has been lately confirmed (Klotz et al., 1997; Alzerreca et al., 1999). The genes coding for the proteins (*amoA*, *amoB*, *amoC*) are located in the *amo* operon (Arp et al., 2007). The DNA sequence of all three genes has been determined in both *Nitrosomonas europaea* and *Nitrosospira* sp. NpAV (Norton et al., 1996; Hommes et al., 1998). Multiple copies of the *amo* operon have been found in most studies of AOB belonging to *Betaproteobacteria*: two copies in *Nitrosomonas europaea* (Hommes et al., 1998) and three copies in *Nitrosospira* sp. NpAV (Norton et al., 1996). On the opposite, *Nitrosococcus oceani* has only one copy (Alzerreca et al., 1999; Klotz et al., 2006). The degree of similarity between the duplicated genes is remarkable, differing by only one or two nucleotides (Norton et al., 1996). At present, it is unknown why multiple copies of the *amo* gene exist, but there are indications that the different copies of the gene can be differently expressed and support growth under different conditions.

HAO catalyzes the oxidation of hydroxylamine to nitrite (fig. 1.2), being the only reaction deriving energy from redox reactions: four electrons are achieved from the oxidation and two are used to generate ATP in the electron chain transport. HAO is located as a soluble enzyme in the periplasmic space, but it is anchored in the cytoplasmic membrane (Bothe et al., 2000). It is an unusual enzyme with a highly complex structure, containing eight *c*-type hemes. Seven of the hemes are each covalently bound to a protein and the eighth heme has an additional covalent bond, designated P460, that it is thought to be the active site (Arp et al., 2002). The cytochrome P460 is encoded by a gene separated from those coding for HAO (McTavish et al., 1993). Few things are known about *hao* copy numbers in the different AOB, but it was reported that the genes encoding for HAO in *Nitrosomonas europaea* had three copies (Hommes et al., 1998). However, it is not yet known whether these three copies are expressed differentially.

AOB PHYLOGENY and DIVERSITY

Analysis of 16S rRNA gene sequences provides evidences that AOB split into two phylogenetic groups, one within the *Betaproteobacteria* and the other one within the *Gammaproteobacteria* subdivisions (Purkhold et al., 2000). However, the majority of the AOB currently identified belong to the β subdivision of the *Proteobacteria*, and only two species belonging to the genera *Nitrosococcus* (*N. halophilus* and *N. oceani*) are included within the *Gammaproteobacteria* subdivision (Bothe et al., 2000) (fig. 1.3).

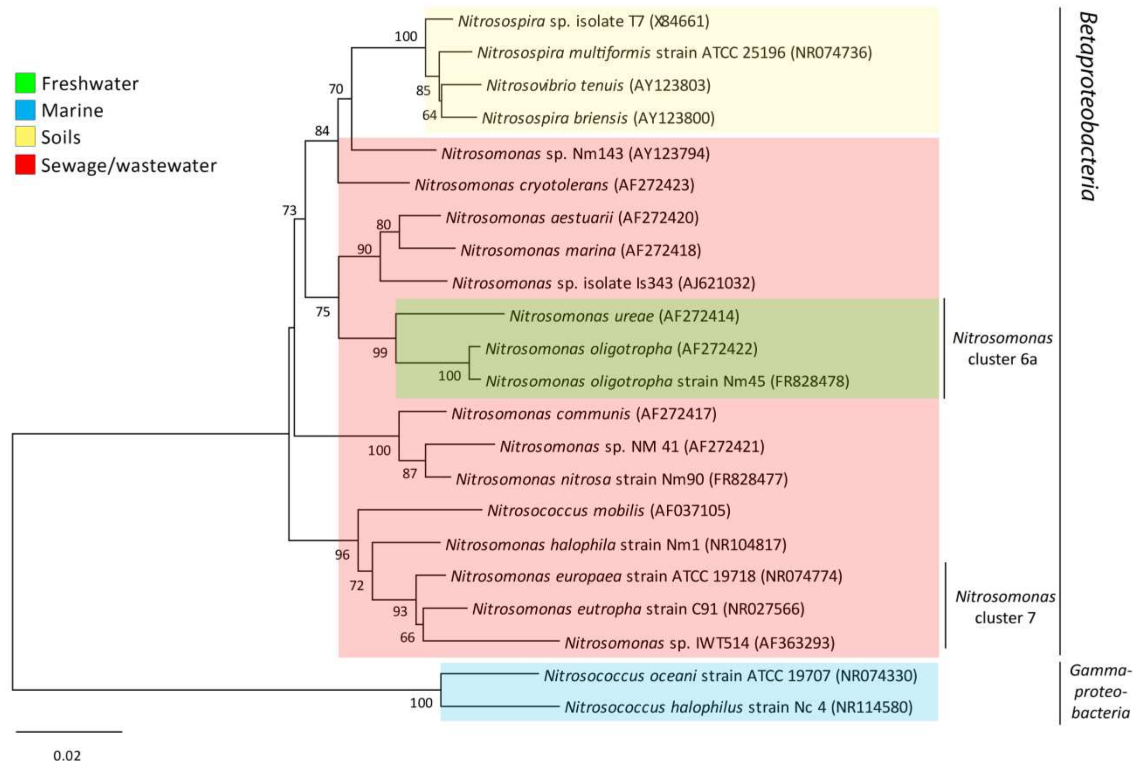


Figure 1.3: Maximum-likelihood phylogenetic tree calculated with β and γ AOB 16S rRNA gene sequences obtained from National Center for Biotechnology Information (NCBI) database. The four AOB major ecotypes are approximately indicated. *Nitrosomonas oligotropha-ureae* (cluster 6a) and *Nitrosomonas europaea-eutropha* cluster (cluster 7) were highlighted. The resulting bootstrap values higher than 50% are displayed in nodes of the tree. Accession numbers of 16S rRNA gene sequences are given within brackets. The bar represents 2% estimated sequence divergence.

Based mainly on cell morphology, *Betaproteobacteria* AOB were traditionally classified into five genera: *Nitrosomonas, Nitrosococcus*, *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* (Koops and Möller, 1992). More recently (Koops and Pommerening-Röser, 2001), two phylogenetic clusters were defined inside *Betaproteobacteria* on the

basis of 16S rDNA sequence homology, separating the overall species inside *Nitrosomonas* (including *Nitrosococcus mobilis*, which is phylogenetically closely related to nitrosomonads) from a re-defined genus *Nitrosospira*. It has been suggested that the latter could include all species belonging to *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* (Purkhold et al., 2003) since they are very closely related to each other. Concerning *Nitrosomonas*, this cluster contains at least six distinct lineages (Purkhold et al., 2003): *N.eutropha / N.europaea*, *N.oligotropha / N.ureae*, *N.communis*, *N.marina, N.cryotolerans* and *Nitrosomonas* sp. Nm143 (fig. 1.3).

AOB ECOLOGICAL PATTERNS

AOB are ubiquitous in soils, freshwater and marine aerobic environments where ammonia is available, and ammonia is available virtually everywhere. The ecophysiological relationships among species enclosed in the *Nitrosomonas* cluster are considerably clear, in contrast to *Nitrosospira* cluster, which ecophysiological characteristics are not known enough to discriminate among species contained within. The performance of molecular surveys on *Nitrosospira* cluster is not easy due to the very close relationships among their species (Koops and Pommerening-Röser, 2001).

Although AOB can be phylogenetically organized into two big groups, the ecophysiological and genetic data support the hypothesis (Arp et al., 2007) that AOB can be split into four major ecotypes (fig. 1.3): (a) sewage/wastewater, (b) freshwater sediments, (c) soils and (d) marine and high salt environments.

SEWAGE / WASTEWATER

Nitrosomonas species seem to be the dominant ammonium oxidizer in activated sludge from WWTPs and bioreactors, environments usually featuring high nitrogen and dissolved oxygen concentrations (fig. 1.3). This premise is supported by a large number of studies (Purkhold et al., 2000; Satoh et al., 2004; Mota et al., 2005; Lim et al., 2008; Montràs et al., 2008; Ganigué et al., 2009). Moreover, some diversity is observed in *Nitrosomonas* species distribution due to ammonium concentration. Nowadays it is

accepted that *N. oligotropha/ureae* lineages, also called *Nitrosomonas* cluster 6a (fig. 1.3), are the dominant AOB in environments with low ammonium concentrations (Bollmann and Laanbroek, 2001). On the other hand, high ammonium loads were found to be a selective parameter for growth of *N. eutropha / europaea* lineage, also called *Nitrosomonas* cluster 7 (fig. 1.3) (Whitby et al., 1999; Otawa et al., 2006). *Nitrosomonas* was not only found in conventional sewage disposals, but also *N. marina* and *N. ureae* were present in constructed wetlands linked with rhizosphere and bulk sediment (Ruiz-Rueda et al., 2009).

FRESHWATER SEDIMENTS

Nitrosomonas cluster 6a also contains the dominant AOB representatives in natural freshwater habitats (Bollmann and Laanbroek, 2001; Koops and Pommerening-Röser, 2001) and estuaries (Stehr et al., 1995; Bernhard et al., 2005) (fig. 1.3). These bacteria may have low K_s values (Koops and Pommerening-Röser, 2001) and the capacity for microaerophilic and anaerobic respiration, with nitrite or hydrazine as electron acceptors (Schmidt and Bock, 1998; Schmidt et al., 2001; Arp et al., 2007).

SOILS

Nitrosospira spp. seem to be the dominant ammonium oxidizers in most soil environments (Hiorns et al., 1995; Kowalchuk et al., 1997; Mendum et al., 1999) (fig. 1.3). The ecological distribution of AOB appears to be ruled by the pH values in the soils. *Nitrosospira* and *Nitrosovibrio* strains have been isolated from acidic soils whereas *Nitrosolobus* spp. have been isolated from neutral soils. In addition, members of the *Nitrosomonas* cluster 6a were also detected from a moderately acidic soil (Koops and Pommerening-Röser, 2001).

MARINE and HIGH SALT ENVIRONMENTS

Seawater and high salt environments contain a wide range of AOB diversity without a clear dominant species. Some *Nitrosomonas* species have been found in marine

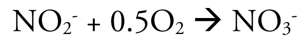
environments, from *N. europaea* (Hovanec and DeLong, 1996), *N. marina* (Grommen et al., 2005) and *N. aestuarii* (Itoi et al., 2006) to the more recent *Nitrosomonas* sp. Nm143 (Foesel et al., 2008). In addition, one strain of *N. cryotolerans* has also been detected in marine samples (Jones et al., 1988). The two known representatives of the genus *Nitrosococcus* (*N. oceani* and *N. halophilus*) have relatively strong salt requirements. In fact, the distribution of *N. oceani* seems to be restricted to marine environments (Koops and Pommerening-Röser, 2001) (fig. 1.3), while strains of *N. halophilus* were isolated from sediment samples of a salt lake in Saudi Arabia and a salt lagoon in the Mediterranean Sea (Koops and Pommerening-Röser, 2001). *Nitrosospira* was also detected in the marine environments (Bano and Hollibaugh, 2000; Hollibaugh et al., 2002).

AOB identification in extreme environments with high salt conditions have only been accomplished by molecular techniques. Initially, it was thought that AOB relatively low energy efficiency should cause difficulties for surviving in energetically expensive extreme conditions, limiting their growth in such extreme conditions (Oren, 1999; Sorokin et al., 2001). However, *Nitrosomonas* has been detected in a hypersaline lake in California (Ward et al., 2000), in sediments of a polluted marine fish farm (McCaig et al., 1999) and in an alkaline lake in Mongolia where they did not have obligate salt requirements, but revealed strong salt tolerance (Sorokin et al., 2001).

1.3 NITRITE-OXIDIZING BACTERIA (NOB)

The aerobic oxidation of nitrite to nitrate, conducted by the NOB, is the second step of the nitrification process (Yamanaka and Fukumori, 1988) and is necessary to fully complete the whole nitrification process. Historically, *Nitrobacter* was the first NOB discovered and described by Sergei and Helene Winogradsky. One of its species was lately named *Nitrobacter winogradskyi* (ATCC 24391) after him.

The overall stoichiometric equation of the nitrite oxidation goes as it follows:



$$NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

NOB are mainly considered chemolithotrophic organisms, which conserve energy from the oxidation of nitrite and reduce CO_2 as a carbon source (Bock, 1976; Bock et al., 1991) via the Calvin-Benson-Basham pathway (Starkenburg et al., 2006). Despite of these general features some studies confirmed that *Nitrobacter* is a facultative lithoautotrophic, being able to grow aerobically with nitrite as electron donor or by oxidation of simple organic compounds, such as pyruvate, acetate, α-ketoglutarate and glycerol (Bock, 1976; Freitag et al., 1987; Starkenburg et al., 2008), as organoheterotrophic. *Nitrospira*-like bacteria have been also reported (Daims et al., 2001) to grow mixotrophically (they can simultaneously incorporate organic and inorganic carbon sources) using pyruvate but not acetate, butyrate or propionate.

NOB optimal pH range for growth is 7.5 to 8.0 and their temperature range is 5-39°C, with an optimum between 28 and 30°C (except for *Nitrospira moscoviensis*, which its optimal temperature is 39°C (Ehrich et al., 1995; Alawi et al., 2007)). Some NOB contain internal structures, such as cytoplasmic inclusions of glycogen and polyphosphates in *Nitrobacter*, or carboxysomes with enzymes and components required for respiration in most but not all species of *Nitrobacter* and *Nitrococcus* (van Niftrik et al., 2004).

NOB activity, as well as AOB, has also beneficial and detrimental consequences. As detrimental aspects, the final conversion of ammonium into nitrate, which is very mobile and can readily leach from soils, eventually can contribute to eutrophication of aquatic ecosystems, groundwater pollution and N shortage in soils (Tortoso and Hutchinson, 1990). As beneficial aspects, NOB can remove toxic nitrite from the environment (Philips et al., 2002a), and increase N availability to plants, only limited by denitrification or high nitrate leaching.

METABOLISM (CATABOLISM) and ITS GENETIC REGULATION

Nearly all the biochemical investigations have been focused on the genus *Nitrobacter*. The oxidation of nitrite to nitrate is performed in *Nitrobacter* by only one enzyme, the nitrite oxidoreductase (NXR) (Starkenburg et al., 2006). *Nitrobacter* employs NXR to oxidize nitrite to nitrate, with electrons travelling a very short electron transport chain (because of the high redox potential of the NO_3^-/NO_2^- couple) to the terminal oxidase of the *a*- and *c*-cytochromes. The activity of the cytochromes aa_3 generates a proton motive force for ATP generation. The reduction of cytochrome *c* is a thermodinamically unfavorable step and therefore only small amounts of energy are available from this reaction. Thus, growth yields of nitrifying bacteria (cells biomass produced per oxidized substrate) are low (Madigan and Brock, 2009). Nitrite also plays as electron donor for the reduction of nicotinamide adenine dinucleotide (NAD^+) via reverse electron flow as well as for the generation of ATP by oxidative phosphorilation (Freitag and Bock, 1990). In *Nitrobacter* the NXR enzyme is located in the inner cell membrane and at the intracytoplasmic membranes (ICM) side of the cytoplasmic membranes (Lucker et al., 2010) (fig. 1.4).

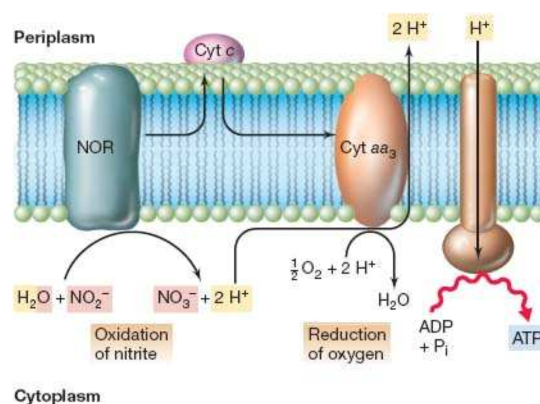


Figure 1.4: Oxidation of nitrite to nitrate by NOB. NOR (nowadays renamed as NXR): nitrite oxidoreductase (extracted from Madigan and Brock (2009)).

The catalitically active NXR enzyme consists of two subunits, encoded by *nxrA* and *nxrB*, respectively. Kirstein and Bock (1993) showed that a third gene (*nxrX*), which is

located between *nxrA* and *nxrB* (denominated as gene *nxrAXB* cluster), may assist in the folding of the NXR. The complete genome sequence revealed that *Nitrobacter winogradskyi* Nb-255 contains, besides the *nxrA* and *nxrB* genes, additional *nxrAB* genes (Starkenburg et al., 2006). In *Nitrobacter*, the concentration of NXR varies depending on growth conditions. Synthesis of the enzyme is induced by nitrite, and the enzyme is the major constituent of nitrite-oxidizing membranes (Bock et al., 1991).

Initial biochemical studies of *Nitrospira* revealed several significant differences in the genetic regulation between *Nitrospira* and *Nitrobacter* (Watson et al., 1986). Immunological biochemical analyses showed that the nitrite oxidizing system (NOS) from *Nitrospira* and *Nitrospina* differs from the NXR enzyme of *Nitrobacter* (Spieck et al., 1998; Bartosch et al., 1999). In *Nitrospira* cells, which do not possess intracytoplasmic membranes, the nitrite-oxidizing system is found in the perisplasmic space and is associated with the outer surface of the cell membrane (Spieck et al., 1998).

NOB PHYLOGENY and DIVERSITY

There are six phylogenetically-unrelated genera of NOB: *Nitrobacter* (*Alphaproteobacteria*), *Nitrospira* (*Nitrospirae*), *Nitrococcus* (*Gammaproteobacteria*), *Nitrospina* (*Deltaproteobacteria*), *Nitrolancea (Chloroflexi)* and *Candidatus* Nitrotoga[1] (*Betaproteobacteria*) (fig. 1.5). *Nitrobacter* is a member of the *Bradhyrhizobiaceae* and it is closely related (97-98% 16S rDNA identity) to *Bradhyrhizobium* and *Rhodopseudomonas* (Starkenburg et al., 2008). It is a phylogenetically recent genera with four species: *N. winogradskyi*, *N. hamburgensis*, *N. vulgaris*, and *N. alkalicus* (Orso et al., 1994; Vanparys et al., 2007). Navarro and collaborators (1992a) suggested that the species previously known as *Nitrobacter agilis* might be a subspecies of *Nitrobacter winogradskyi*. *Nitrococcus*, *Nitrospira* and *Nitrolancea* genera only have one species each, *Nitrococcus mobilis*, *Nitrospina gracilis* and *Nitrolancea hollandica* respectively (Watson and Waterbury, 1971; Sorokin et al., 2012).

[1] *Candidatus* is a taxonomical category used for describing prokaryotic entities for which more than a mere sequence is available but for which characteristics required for description according to the *International Code of Nomenclature of Bacteria* are lacking (Murray & Stackebrandt, 1995).

The genus *Nitrospira* is more distantly related to the other known NOB, because it is part of the deep-branching bacterial phylum *Nitrospirae*, and consists in four species. Two of them (*N. moscoviensis, N. marina*) have been cultured (Watson et al., 1986; Ehrich et al., 1995) and two (*Ca.* N. bockiana, *Ca*. N. defluvii) are still uncultured (Spieck et al., 1996; Lebedeva et al., 2008).

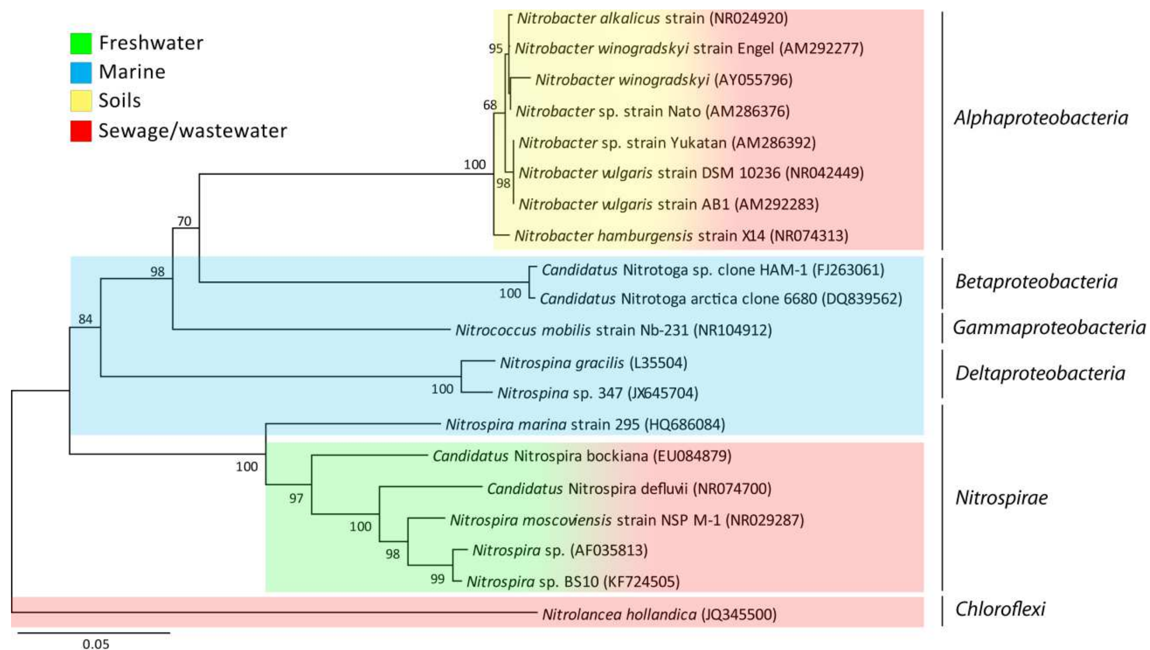


Figure 1.5: Maximum-likelihood phylogenetic tree calculated with NOB 16S rRNA gene sequences obtained from NCBI database. The four NOB major ecotypes are approximately indicated. The resulting bootstrap values higher than 50% are displayed in nodes of the tree. Accession numbers of 16S rRNA gene sequences are given within brackets. The bar represents 5% estimated sequence divergence.

NOB ECOLOGICAL PATTERNS

NOB species have been less studied than AOB and therefore little is known about their ecophysiology. Historically, members of the *Nitrobacter* genera have been used as the primary model organism for studying nitrite oxidation (Starkenburg et al., 2006) since nearly the overall of the cultured strains retrieved were related to this genus (Bartosch et al., 2002). Traditional culture-based techniques overlooked *Nitrospira* due to the difficulties for achieving cultures. However, the application of non-cultured methods revealed the presence of *Nitrospira* in most of the locations where it was though that *Nitrobacter* was the dominant nitrite oxidizer (Daims et al., 2001). NOB are widely

distributed and they can also be split into four ecotypes (fig. 1.5): a) sewage/ wastewater, b) freshwater sediments, c) soils d) marine (Spieck and Bock, 2005).

SEWAGE / WASTEWATER

Traditionally *Nitrobacter* genera was considered to be the dominant NOB in WWTPs (fig. 1.5), but the recent detection of *Nitrospira*-like sequences and cells in various environments (Ehrich et al., 1995; Burrell et al., 1998; Hovanec et al., 1998; Juretschko et al., 1998) and the absence of *Nitrobacter* spp. in similar habitats (Hovanec and DeLong, 1996; Schramm et al., 1996; Wagner et al., 1996) might therefore indicate a competitive advantage of *Nitrospira* spp. in these environments. Nowadays it is accepted that *Nitrospira*-like nitrite oxidizers are *K*-strategist and they are well-adapted to low nitrite and oxygen concentrations. On the other hand, *Nitrobacter* spp. are thought to be relatively fast-growing *r*-strategists with low affinities to nitrite and oxygen (Schramm et al., 1999). Since nitrite-concentrations in most WWTPs are low, *Nitrospira* would outcompete *Nitrobacter* in these systems (fig. 1.5). In treatment plants with temporally or spatially elevated nitrite concentrations (such as nitrifying SBR), both nitrite-oxidizers should be able to coexist (Wagner et al., 2002).

FRESHWATER SEDIMENTS

Nitrospira-like species have been reported from freshwater marshes (Watson et al., 1986), freshwater aquaria (Hovanec et al., 1998) and natural human-impacted hydrosystems (Cébron and Garnier, 2005a) (fig. 1.5). In the latter environments, some nuances are required. *Nitrospira* spp. was found to be the dominant NOB in most of the lower Seine River but *Nitrobacter* species took relevance in the effluent of the WWTPs, where nitrite was in high concentrations and thus favoured the growth of these *r*-strategists (Cébron and Garnier, 2005a).

SOILS

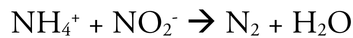
Nitrobacter is considered to be the dominant species in soil systems (Degrange and Bardin, 1995; Degrange et al., 1998) (fig. 1.5) and consequently in hydrosystems formed by soil erosion and leaching. *Nitrobacter* spp. has been detected in both acidic (Hankinson and Schmidt, 1988; De Boer et al., 1991) and basic soils (Sorokin et al., 1998). However, the application of molecular techniques also allowed *Nitrospira* spp. detection in several terrestrial habitats (Smit et al., 2001; Bartosch et al., 2002). Alawi and collaborators (2007) firstly described *Ca*. Nitrotoga artica enriched from permafrost-affected soils of the Siberian Arctic. According to its psicrophilic status, this NOB is able to oxidize nitrite at 4°C and no activity was observed at 25°C.

SEAWATER

To date, all the members of *Nitrococcus* and *Nitrospina* genera are considered to be obligated halophilic bacteria and they seem to be restricted to marine environments (Koops and Pommerening-Röser, 2001) (fig. 1.5). Their optimal growth was obtained in 70-100% seawater-based media. Neither of them were able to grow in the laboratory with freshwater-based mineral media, even if NaCl was added (Watson and Waterbury, 1971). It probably means that some essential components of seawater, such as trace elements, should be required for their growth. *Nitrospira marina* was found to be an obligated halophilic species and also to grow in a media with 70-100% seawater (Watson et al., 1986).

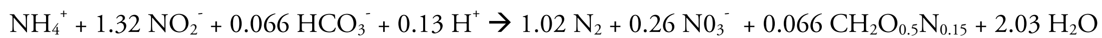
1.4 ANAMMOX BACTERIA

The anaerobic ammonium oxidation (anammox) is a biological process in which ammonium is oxidized under anaerobic conditions, using nitrite as the electron acceptor, to dinitrogen gas according to the stoichiometry described by Strous and collaborators (1998):



$$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O$$

It has been suggested that bicarbonate can be uncoupled from the catabolic reaction (Sliekers et al., 2003), as far as small amounts of NO_3^- are produced (Strous et al., 1999b). Therefore the whole stoichiometric reaction should be:



$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ N0_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

In fact, as early as 1965, oceanographers already noticed that the amount of ammonium accumulating in an anoxic fjord was far less than that expected for inert ammonium under anoxic conditions (Richards, 1965), suggesting that ammonium could be oxidized in absence of oxygen. Later, Broda (1977) described the potential existence of chemolithotrophic bacteria able to oxidize ammonia to N_2 with nitrate as oxidant. These predictions were based on thermodynamic calculations, but the existence of the microorganisms still had not been demonstrated. It was not until 1995 when the anammox process was experimentally discovered and documented in a denitrifying pilot plant at Gist-Brocades, Delft, the Netherlands (Mulder et al., 1995). Therefore, denitrification could not longer be considered as the only significant metabolic pathway of N_2 production, useful for nitrogen removal.

Initially, anammox metabolism was thought to be performed by nitrifying bacteria which were able to manage under anaerobic conditions, using nitrate instead of oxygen as electron acceptor (Schmidt and Bock, 1997). Later, the bacterium responsible of the anammox reaction was identified by molecular techniques (Strous et al., 1999a), branching off deep in the order *Planctomycetales*, and named as *Ca.* Brocadia anammoxidans. Physically separated cells of *Ca*. B. anammoxidans were shown to oxidize ammonium with nitrite to N_2 under strictly anaerobic conditions (Strous et al., 1999a). However, it still has been impossible to date to achieve a pure culture of anammox bacteria (Jetten et al., 2005a).

Physiological features of anammox bacteria have been relatively well characterized. They have optimal pH and temperature ranges of 6.7-8.3 and 20-43°C, respectively (Strous et al., 1999b). Anammox bacteria are inhibited by one of their substrates, N-NO_2^-, but there is a wide divergence in the literature about the threshold levels of N-

NO_2^-, ranging from 100 mg L^{-1} to 750 mg L^{-1} (Strous et al., 1999b; Kimura et al., 2010). Carvajal-Arroyo and collaborators (2014b) suggested that this wide range of nitrite toxicity could be attributed to the physiological status of the anammox cells. Moreover, the same authors highlighted the importance of ammonium as a protection for nitrite inhibition (Carvajal-Arroyo et al., 2014a), showing that cells exposed to nitrite are more sensitive than the cells that were simultaneously exposed to nitrite and ammonium. Nitrite inhibition could be overcome by addition of trace amounts of hydrazine and hydroxylamine (Strous et al., 1999b). Moreover, anammox bacteria are reversibly inhibited by very low levels of O_2 (>1 µM) (Jetten et al., 2005a).

Anammox bacteria are slow-growing microorganisms with typical doubling times reported of 15-30 days (Strous et al., 1998; Fux et al., 2004; van der Star et al., 2008) . However, there have been some reports on fast growing anammox bacteria. Japanese researchers working in anaerobic biological filtrated bed and using *Fluorescence In Situ Hybridization* (FISH) direct counting method reported a shorter doubling time of 1.8 days (Isaka et al., 2006). Tsushima and collaborators (2007a) enriched anammox bacteria in semi-batch cultures and by qPCR estimated their doubling time of 3.6-5.4 days. One of the last contribution to this issue (Lotti et al., 2014) established a doubling time value of 3.3 days through kinetic characterization in a suspended cell anammox culture.

INTERNAL ORGANIZATION of ANAMMOX CELLS

Anammox bacteria (as most *Planctomycetales* do) present an internal compartmentalization based on membrane systems. Thus, the anammox cell presents three different compartments (fig. 1.6). The most external region of the cytoplasm, related with the cell wall and the cytoplasmic membrane, constitutes the paryphoplasm. The riboplasm, where the nucleoid is located, is placed in between the intracytoplasmic membrane and the anammoxosome membrane (van Niftrik et al., 2004). Finally, the anammoxosome is the third compartment, occupying most of the central part of the cell, being an unique structure for anammox bacteria (van Niftrik and Jetten, 2012). All

membranes in the anammox cells are composed nearly exclusively of unique ladderane lipids that have 3-5 linearly concatenated cyclobutane rings, a structure unique in the nature (Jetten et al., 2005b; Neumann et al., 2014). Anammox lipids contain a combination of ester-linked (typical of *Bacteria* and *Eukarya*) and ether-linked (typical *Archaea*) fatty acids (van Niftrik et al., 2004). It was proposed that anammox bacteria have evolved ladderane lipids as the major component of their biomembranes to avoid the diffusion of toxic metabolic intermediates (such as hydrazine). These lipids provide an unusual density (higher than a conventional membrane) and impermeability to the membrane because of their structural rigidity and size (van Niftrik et al., 2004). These unique lipids are used as biomarkers for detecting anammox bacteria in environmental samples.

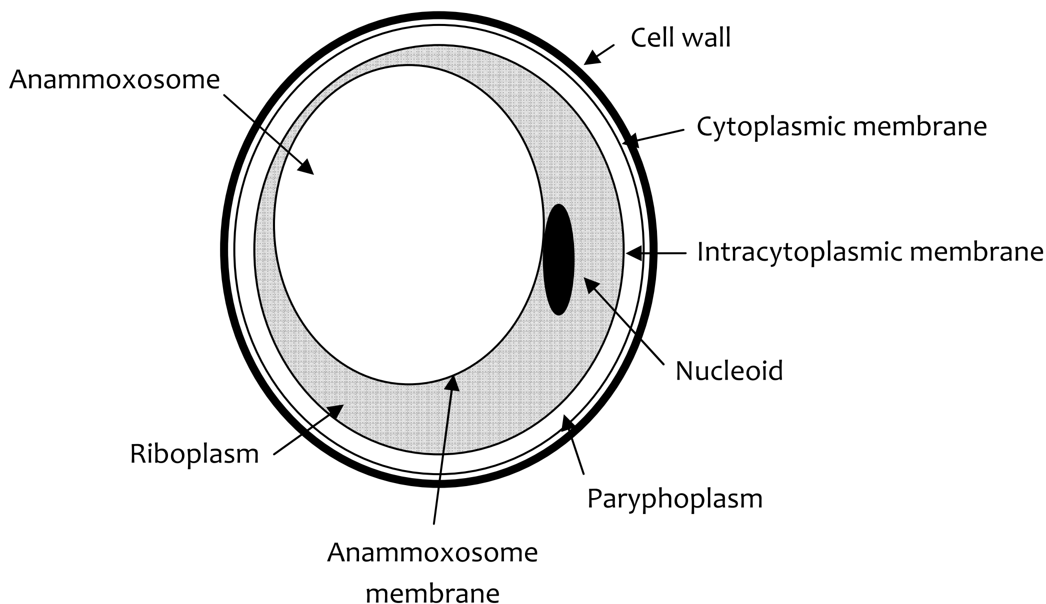


Figure 1.6: Schematic drawing of the anammox cell internal structure.

METABOLISM (CATABOLISM) and ITS GENETIC REGULATION

Some hypothesis were previously formulated to discern the anammox metabolism, but it was not after the completion of the *Ca*. Kuenenia stuttgartiensis genome (Strous et al., 2006) that it began to be clarified. It was observed that certain coding gene clusters

were missing (such as hydroxylamine oxidase) and instead, some new others were found, such as nitrite reductase (NirS). This finding suggested the role of the nitric oxide (NO) rather than hydroxylamine as an intermediate in the anammox metabolism. From here on, metabolic aspects start to get hardly complicated and different explanations arose. To avoid going deeper into all of them, only one of the last and more accepted hypothesis will be summarized.

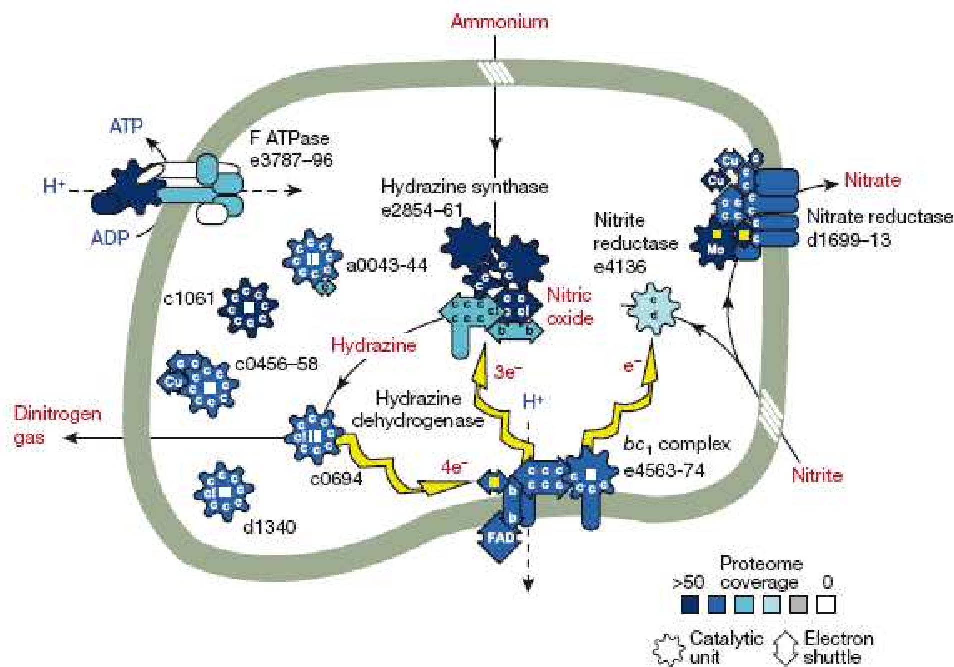


Figure 1.7: Biochemical pathway and chemical engineering of *Ca*. Kuenenia stuttgartiensis involving the anammoxosome (extracted from Kartal et al. (2011)). Hydrazine synthase is a non-membrane associated enzyme. Yellow arrows indicate electron flow; yellow square, iron-sulfur clusters; b, haem b; c, haem c; d haem d; Mo, molybdopterin.

Kartal and collaborators (2011) figure out the anammox metabolic pathway (fig. 1.7) from the metagenomic information and some *in vivo* tests that were carried out to confirm the NO importance and specify the role of the anammoxosome in these processes. Nitrite and ammonium would diffuse through the anammoxosome membrane and nitrite would be reduced inside the anammoxosome by the nitrite reductase (NirS) to the very reactive free-radical NO. Loosely membrane associated hydrazine synthase would convert ammonium and NO to hydrazine. Afterwards, hydrazine would be oxidized to dinitrogen gas, which will diffuse through the anammoxosome membrane. Anammox catabolism and energy growth could be attributed to ATPase bounded in this membrane. The *bc1* complex would be involved

in the electron transport from hydrazine oxidation to both nitrite and NO reduction and in the flow of protons to the external side of the anammoxosome.

ANAMMOX PHYLOGENY and DIVERSITY

Anammox bacteria are deep-branched members of the phylum *Planctomycetes*, belonging to the order *Planctomycetales*. This phylum is also included in a major group of *Planctomycetes*, *Verrucomicrobia* and *Chlamydia*, known as PVC superphylum (Wagner and Horn, 2006), although new phyla, such as *Lentisphaerae* and OP3, were recently proposed to be included (Fuerst, 2013). *Planctomycetes* is one of the major divisions of the domain *Bacteria* and is considered a morphologically and phylogenetically distinct group of prokaryotes. Typically, *Planctomycetes* are aerobic chemoorganoheterotrophs, present budding reproduction (Lee et al., 2009) and contain internal compartmentalization linked to cellular functions. For years, the presence of membrane-surrounded structures inside the cytoplasm, such as the nucleus and several organules, has been considered as a major distinguishing feature between eukaryotic and prokaryotic cells (Lindsay et al., 1997). Therefore, it is notably singular that some prokaryotic microorganisms within phylum *Planctomycetes* contain true membrane-surrounded organules, such as the anammoxosome present in anammox bacteria. From an ecophysiological outlook, the discovery of new *Planctomycetes* species in new ecological niches highlights the fact that it is a highly diverse and ubiquous phylum within the domain *Bacteria*.

Since the discovery of the first anammox bacterium, five genera have been described and the group has been split into two phylogenetic branches (fig. 1.8). The first group encloses: *Ca*. Kuenenia, including *Ca.* Kuenenia stuttgartiensis (Schmid et al., 2000); *Ca.* Brocadia, including *Ca.* B. fulgida (Kartal et al., 2008), *Ca*. B. sinica (Hu et al., 2010) and *Ca*. B. anammoxidans, the first-known anammox bacterium (Strous et al., 1999a); and *Ca*. Anammoxoglobus, including *Ca.* A. propionicus (Kartal et al., 2006).

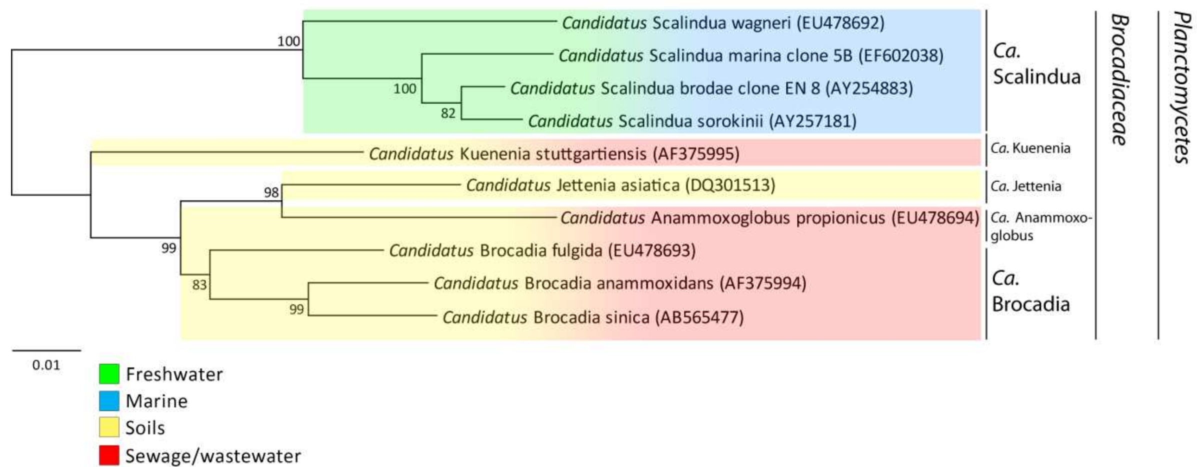


Figure 1.8: Maximum-likelihood phylogenetic tree calculated with anammox bacteria 16S rRNA gene sequences obtained from NCBI database. The four anammox major ecotypes are approximately indicated. The resulting bootstrap values higher than 50% are displayed in nodes of the tree. Accession numbers of 16S rRNA gene sequences are given within brackets. The bar represents 1% estimated sequence divergence.

Furthermore, there is still a novel phylotype, named *Ca*. Jettenia asiatica, which has been detected in sludge from a bioreactor by 16S rRNA gene analyses (Quan et al., 2008) and it has been included in the first group. *Ca*. Scalindua constitutes the second group, including *Ca.* S. wagneri, *Ca*. S. marina (Brandsma et al., 2011), *Ca.* S. brodae (Schmid et al., 2003) and *Ca.* S. sorokinii (Kuypers et al., 2003).

ANAMMOX BACTERIA ECOLOGICAL PATTERNS

Although anammox bacteria were discovered in WWTPs nowadays almost all anammox species have been detect in nature (Humbert et al., 2009; Moore et al., 2011; Zhu et al., 2011b; Russ et al., 2013). However, little is known about the factors that control their spatial and temporal distribution (Hamersley et al., 2009). Some studies have examined different parameters such as nitrate availability, organic content and salinity (Dalsgaard et al., 2005; Zhang et al., 2008), but they have been inconclusive. The general conviction is that *Ca*. Brocadia, *Ca*. Kuenenia and *Ca*. Anammoxoglobus are commonly found in non-saline environments and man-made systems (Schmid et al., 2005; Hu et al., 2013) while *Ca*. Scalindua-like species are mostly present in natural saline ecosystems (Penton et al., 2006; Schubert et al., 2006;

Villanueva et al., 2014). The distribution of anammox bacteria has been studied by culture-independent approaches in four major ecotypes: (a) sewage/wastewater, (b) freshwater and estuarine sediments, (c) soils and (d) marine environments.

SEWAGE/WASTEWATER

Ca. Brocadia and *Ca*. Kuenenia spp. are the dominant anammox bacteria in activated wastewater sludge (Isaka et al., 2006; Pathak et al., 2006; Chamchoi and Nitisoravut, 2007; Date et al., 2009) (fig. 1.8). Different species belonging to *Ca*. Brocadia genera (Strous et al., 1999a; Kartal et al., 2008), and even the novel anammox bacteria *Ca*. Jettenia asiatica were identified in activated sludge originally retrieved from WWTPs (Zu et al., 2008; Zhu et al., 2011a). Moreover, although *Ca.* Scalindua was today considered as a freshwater and marine genera, it was originally identified and described from an activated sludge (Schmid et al., 2003). These findings prove the great anammox bacteria diversity that can harbour these ecosystems. After the whole genomic sequence of *Ca.* Kuenenia stuttgartiensis was known (Strous et al., 2006), a lot of genes not directly related with N-removal were identified proving the great versatility of anammox bacteria, initially considered as specialized bacteria. One example of this genetic versatility was found in a new species described in 2006 (Kartal et al., 2006), *Ca.* Anammoxoglobus propionicus, which was discovered when some activated sludge that were suspicious to contain anammox bacteria species were amended with organic matter, such as propionate. Under these conditions, *Ca.* A. propionicus was detected because it was able to outcompete the rest of anammox bacteria present in the sample.

FRESHWATER and ESTUARINE SEDIMENTS

Freshwater and brackish systems appear to harbour a great diversity of anammox bacteria (fig. 1.8). Until recent dates, less attention had been made about the importance and distribution of anammox in freshwater environments although the first hint of anammox activity was discovered by Goering and collaborators (1966) who detected the production of labelled N_2 from a $^{15}NO_3^-$ tracer in subartic lake water.

Several years later Penton and collaborators (2006) performed broad-range investigations identifying *Ca.* Scalindua's 16S rRNA genes in several distinct freshwater environments. Besides, *Ca.* Scalindua spp. were identified in the anoxic water column of a deep, permanently stratified tropical meromictic lake (Schubert et al., 2006), in a Chinese river sediment (Zhang et al., 2007) and in the anoxic water column of a restored mining pit lake (Hamersley et al., 2009).

Ca. Scalindua spp. has been also detected in estuarine sediments (Meyer et al., 2005; Amano et al., 2007). *Ca.* Brocadia and *Ca.* Kuenenia-like species were detected in river (Zhang et al., 2007), in the sediments of an eutrophic Japanese lake (Yoshinaga et al., 2011), in estuarine sediments (Amano et al., 2007; Dale et al., 2009), in enrichments from brackish coastal lagoon sediments (Sànchez-Melsió et al., 2009) and in an eutrophic freshwater lake (Yoshinaga et al., 2011).

SOILS

The distribution, diversity and activity of anammox bacteria in terrestrial ecosystems have been even less studied than freshwater environments. Theoretically the concomitant presence of ammonium and nitrite in the oxic/anoxic interface could provide a suitable habitat for anammox bacteria. Recent studies have shown that terrestrial ecosystems can harbour more anammox diversity, rather than homogenous marine water columns, maybe due to the variety of anammox niches offered in soils.

Firstly, 16S rRNA gene sequences from *Ca.* Kuenenia, *Ca.* Brocadia, *Ca.* Scalindua and *Ca.* Jettenia spp. were detected in samples associated with water and/or high nitrogen contents, being *Ca.* Kuenenia and *Ca.* Brocadia the dominant genera (Humbert et al., 2009) (fig. 1.8). Sequences close to *Ca.* Kuenenia and *Ca.* Brocadia spp. were detected from soils unaffected by any human activity unlike their detection in the rest of the environments.

Remarkable was the finding of Zhu and collaborators (2011b), not only identifying diverse anammox species in paddy soil but clearly finding 16S rDNA sequences close to *Ca.* Jettenia asiatica and *Ca.* Kuenenia stuttgartiensis in the soil surface whereas *Ca.*

Brocadia-related sequences dominate in the soil depth. Furthermore, two previously unknown species (phylogenetically close to *Ca.* Jettenia asiatica and *Ca.* Brocadia fulgida), were discovered from a peat soil (Hu et al., 2011). It is likely that new anammox species would be discovered from the high diversity of soil niches, helping to better understand the role of anammox bacteria to Nitrogen cycle in terrestrial ecosystems.

MARINE ENVIRONMENTS

Only bacteria from the genus *Ca.* Scalindua have been identified in tropical, temperate, and arctic anoxic marine ecosystems (fig. 1.8). *Ca.* S. brodae/sorokinii or close 16S rRNA gene sequences have been detected in several locations such as Black Sea (Kuypers et al., 2003; Kirkpatrick et al., 2006), Benguela upwelling system (Kuypers et al., 2005), Peruvian oxygen minimum zone (OMZ) (Hamersley et al., 2007), and other marine sampling sites around the world (Schmid et al., 2007). In marine environments, the anammox diversity is rather low in regards to the rest of ecotypes and even comparing to marine AOB and NOB bacterial diversity.

GLOBAL IMPORTANCE

Anammox bacteria play a significant role in the transformation of fixed N. After their first detection in activated sludge from WWTPs, several anoxic environments have been screened for the detection of this group. Consequently, a massive detection of different anammox species or anammox 16S rDNA sequences has been produced almost elsewhere the main conditions of anoxia and presence of the appropriate nitrogen forms were accomplished. The more profuse were their findings, the more evident became their ecological role and quantitative importance in the N-transformations occurring in different kinds of natural and artificial environments.

Globally, 30-50% of the total N losses occur in oxygen-minimum zones (OMZs) (Kuypers et al., 2005), where heterotrophic denitrification was traditionally recognized as the only significant process converting fixed nitrogen to gaseous N_2 (Gruber and

Sarmiento, 1997). This statement had to be reconsidered after the quantification of anammox activity in marine sediments (Dalsgaard et al., 2003). In the Benguela upwelling, one of the most important site for primary production of the Atlantic ocean, nutrient profiles confirmed that anammox bacteria are responsible for massive losses of fixed nitrogen as gaseous N_2 (Kuypers et al., 2005). The respective contributions of anammox and denitrifying bacteria to marine N-losses can be distinguished via incubation of sediments or water sealed containers amended with excess ^{15}N-ammonium and ^{14}N-nitrite (Thamdrup and Dalsgaard, 2002; Kuypers et al., 2005). In anaerobic conditions only anammox species can provide unique $^{14}N^{15}N$ from labelled ammonia and unlabelled nitrite. Moreover, in most of these OMZs 16S rDNA analyses confirmed the presence of *Ca.* Scalindua spp. (Kuypers *et al.*, 2005). Later *in situ* environmental studies in marine OMZs have demonstrated that anammox coexist with AOB or AOA (Lam et al., 2007; Lam et al., 2009), even though these physiological groups compete for ammonium as a substrate (Yan et al., 2010).

Anammox global contribution to the fixed nitrogen loss is not only a marine environment issue, their contribution in lacustrine systems seem to have at least the same importance than in marine environments. Anammox activity was *in situ* measured in the second largest lake in the world, Lake Tanganyika, and the rates of anaerobic ammonium oxidation were similar to those measured in the Benguela upwelling system (Schubert et al., 2006). Hence, anammox in freshwater environments, such as big lakes, not only contribute to fixed nitrogen loss at a local scale but they can also can have a high relevance in this process at a global scale.

1.5 MOLECULAR BIOLOGICAL METHODS

Historically, studies of microbial communities have often depended on the culturability of the microorganisms. Unfortunately, AOB, NOB and anammox bacteria are chemolithotrophic microorganisms with several features that make them difficult to be cultured. Thus, the traditional cultivation methods are not convenient for these bacteria and they remained unknown when studies were based on them.

Lately, the assessment of molecular techniques in microbiological studies has become widely spread. Most of these techniques use gene targeting for specific detection with labelled (probes) or unlabelled (primers) oligonucleotides, through probing or sequencing approaches mainly based on FISH and PCR analyses, respectively. Their use has greatly contributed to discover a large AOB and NOB diversity, impossible to accomplish with traditional methods. Concerning anammox bacteria, and due to their more recent discovery, almost all research has based on molecular approaches, including also studies on isotopes and lipids (Schmid et al., 2005; Sinninghe Damste et al., 2005).

To date, most of the molecular biological studies based on prokaryote phylogeny have relied on detecting and sequencing the most useful molecular chronometer, the 16S rRNA gene. It shows a high degree of functionally constancy, which assures relatively good clocklike behaviour. It occurs in all organisms, but it has different sequences as a consequence of evolutionary changes. Different positions in their sequences have changed at different rates, allowing most phylogenetic relationships (including the most distant) to be determined (Woese, 1987). Moreover, its length (about 1500 bp) is shorter enough to allow an easy whole sequencing and longer enough to generate a high diversity of sequences. The knowledge of 16S rDNA sequences allows different approaches to study the composition of bacterial assemblages, mostly based on PCR and FISH analyses.

BIOMARKERS for DIVERSITY CHARACTERIZATION of BACTERIAL ASSEMBLAGES

16S rDNA/16S rRNA

PCR-based methods: anammox and most AOB cluster in single phylogenetic groups and therefore unlabelled oligonucleotides (primers) targeting 16S rDNA of both groups are available for PCR performance. Concerning AOB, the most usual oligo-nucleotides are the CTO primer set, targeting all the AOB within *Betaproteobacteria* subdivision (Kowalchuk et al., 1998; Purkhold et al., 2003; Satoh et al., 2004; Wang et al., 2007). Essentially, most of the PCR-based studies for anammox bacteria detection are based on the combination of a *Planctomycetes* (Pla46F) and an anammox (Amx368R) specific primer. It has proven that this primer set amplifies DNA of all the known anammox bacteria (Schmid et al., 2005).

Molecular analysis based on the PCR amplification of the 16S rRNA gene has been also the main assay for the NOB detection and identification in several environments (Dionisi et al., 2002; Cébron and Garnier, 2005a; Maixner et al., 2006; Alawi et al., 2007). However, because they are a polyphyletic group, it is not possible to develop a single primer set for the whole NOB assemblage based on 16S rDNA sequences. The most common solution for NOB molecular detection using 16S rRNA gene is the combination of several primer sets, each one targeting a different phylogenetic group. In this case, previous ecological information can be appropriate to avoid performing a high amount of tests with different primer sets. Although 16S rDNA operon is still the main tool for NOB phylogenetic analyses, some studies also pointed out its lack of discriminatory power within this group (Orso et al., 1994; Starkenburg et al., 2008).

FISH: Fluorescence *in situ* hybridization (FISH) with rRNA-targeted labelled probes is a widely used technique that enables to analyze the spatial organization, the phylogenetic affiliation and even to quantify the microbial populations. FISH can be

performed without any previous PCR but it is usually carried out after species identification by PCR and sequencing analyses. This technique is widely used for *Betaproteobacteria* AOB (Mota et al., 2005; Montràs et al., 2008). Up to now, the probes Nso190 and Nso1225 have shown the broadest specificity and therefore they are the most suitable for this approach. Few probes have also been described for *Gammaproteobacteria* AOB (Juretschko, 2000). In NOB studies, FISH analyses are frequently performed in parallel to PCR (Wagner et al., 2002; Maixner et al., 2006; Alawi et al., 2007; Blackburne et al., 2007; Montràs et al., 2008), but specific probes for each phylogenetic group are required because of the NOB polyphyletic nature (like on PCR-based approaches). Nit3 and Ntspa663 are the most common probes for *Nitrobacter* and *Nitrospira*, respectively (Dionisi et al., 2002; Spieck et al., 2006a).

Most of the initial probes designed for the detection of anammox organisms targeted either *Ca.* Brocadia and/or *Ca.* Kuenenia (Strous et al., 1998; Schmid et al., 2000; Schmid et al., 2001), being Amx820 the most widely used. This probe has been mainly applied to the detection of *Ca.* Brocadia and *Ca.* Kuenenia genus in sewage disposals and anammox bioreactors samples (Toh and Ashbolt, 2002; Isaka et al., 2006; Tsushima et al., 2007a; Tsushima et al., 2007b).

Although the wide implementation of FISH, it also has some disadvantages: (i) when a large number of microbial populations have to be followed simultaneously, the requirement of a similar stringency to perform multiple hybridization can limit its application; (ii) the description of bacterial assemblages with probes targeting large phylogenetic groups, like *Betaproteobacteria*, is rather uninformative at the level of microbial community activities; (iii) permeabilization of cells to the probes is different among species or groups, such as Gram-positive in respect to Gram-negative bacteria; and (iv) FISH techniques are unable to detect cells containing small numbers of ribosomes, generally associated with a low level of metabolic activity (Dabert et al., 2002).

FUNCTIONAL GENE MARKERS

Although the wide use of the 16S rRNA sequences as targets for phylogenetic AOB studies, there are other non-16SrRNA gene-based approaches that can allow specific studies. One option is to perform PCR with primers designed to target the *amo*A gene, especially the region encoding the C-terminus. It can be considered a suitable target site for fine-scale resolution among *Beta*- and *Gamma*- AOB *Proteobacteria* (Alzerreca et al., 1999; Bothe et al., 2000), a discrimination that is not always completely achieved with 16S rDNA primers. Another approach for AOB phylogenetic studies is based on the 16S-23S internal transcribed spacer (ITS). It is a region located between 16S and 23S rRNA genes that shows highly conserved sequences encompassing transfer RNA (tRNA) genes and relatively stable DNA regions (Mora et al., 2003). ITS sequence analyses have been applied successfully in AOB phylogenetic studies helping to discriminate among *Beta*- and *Gammaproteobacteria* (Aakra et al., 2001).

It was confirmed that 16S rRNA gene is indeed conserved in *Nitrobacter*, and one misidentified base pair during sequencing could result in a completely different position of the strain in a 16S rRNA gene dendogram. One solution could be targeting genes that encode for NOB key enzymes the, such as NXR, as biomarkers with a higher discriminatory power (Starkenburg et al., 2008). However, little methodological research has still been done to date in this field (Vanparys et al., 2007).

METHODS for BACTERIAL QUANTIFICATION

Traditionally, nitrifying bacteria have been quantified in natural samples by culture-dependent microbiological methods, such as the most probable number (MPN) technique (Matulewich et al., 1975; Belser and Schmidt, 1978; Suwa et al., 1994). However, the use of this technique has several disadvantages: (i) MPN is more time-consuming for the researcher than molecular techniques; (ii) the presence of unculturable species would also lead to underestimation of the total counting (Koops and Pommerening-Röser, 2001); (iii) MPN would give underestimation of the cell

counts, since nitrifying bacteria often occur as cell aggregates and a sufficient homogenization seems to be nearly impossible (Bartosch et al., 2002). Several DNA/RNA-based methodological approaches are possible to overcome these limitations.

QUANTITATIVE FISH

Nowadays it is common to quantify bacterial populations by counting the cells from several microscope fields after FISH staining, so-called quantitative FISH (Zhang et al., 2007). Quantitative FISH has been successfully performed with AOB (Schramm et al., 1999; Egli et al., 2003), NOB (Schramm et al., 1999; Egli et al., 2003; Mota et al., 2005) and anammox bacteria (Tsushima et al., 2007a). Although FISH analysis solves some problems derived from culture-dependent methods, it still has some inconveniences (some of them already aforementioned when describing 16S rRNA gene-based methods). It is specially difficult to obtain trustful results from bacteria that organize in aggregates: some probes find difficulties to penetrate in the aggregates and to distribute homogenously, and a confocal laser scanning microscopy (CSLM) together with considerable amount of time are required to obtain a complete study of such structure.

COMPETITIVE PCR (cPCR)

The cPCR assay is based on competitive coamplification of a specific target sequence together with an internal standard whose concentration is known (Dionisi et al., 2002). This approach has excellent sensitivity and quantitative accuracy and it has the additional advantage of using a stringent internal control, because of the competitive principle on which is based. It has been used to enumerate AOB populations in environmental samples by targeting *amoA* and 16S rRNA gene, although its use has been very limited (Felske et al., 1998; Mendum et al., 1999). The cPCR technique based on 16S rDNA operon has also been performed to quantify NOB in sludge from several environments (Dionisi et al., 2002; Cébron and Garnier, 2005a).

QUANTITATIVE PCR (qPCR)

Nowadays, the use of qPCR for the quantification of unculturable and culturable bacteria is becoming fairly widespread (Lim et al., 2008). The qPCR has some advantages in front of cPCR since it is based on continuous monitoring of the fluorescence intensity throughout the performance of the PCR reaction, while keeping a quantitative correspondence with the initial amount of DNA. Moreover, it does not require either a competitive molecule or an endpoint-PCR manipulation. It is considered to be a fast, reliable, sensitive and convenient technique when used to enumerate uncultured bacteria (Limpiyakorn et al., 2006) and it is also faster and easier to be performed than hybridization techniques (Lim et al., 2008).

Although qPCR allows a very reliable quantification of the bacterial populations, it still presents some problems. The qPCR final result offers the number of gene copies of the sample, thus meaning that it is necessary a previous knowledge of the amount of gene copies in the bacterial genome to obtain reliable cell quantification. Fortunately, it has been reported that AOB and NOB species only have one 16S rRNA operon per genome (Navarro et al., 1992a; Aakra et al., 1999; Dionisi et al., 2002). Concerning anammox bacteria, it is reported that *Ca.* Kuenenia stuttgartiensis has only one 16S rRNA operon copy in its genome (Strous et al., 2006).

Several studies has been published by using qPCR for the quantification of nitrifying and anammox bacteria (Hermansson and Lindgren, 2001; Tsushima et al., 2007a; Hu et al., 2010; Kim et al., 2011b; Yao et al., 2011). Most of them are mainly focused on their quantification in engineered systems.

1.6 APPLICATION of ANAMMOX BACTERIA to N-REMOVAL TECHNOLOGIES

The combination of autotrophic nitrification with heterotrophic denitrification has traditionally been the most widely used method for nitrogen removal in biological wastewater treatments. The nitrification-denitrification sequential process performs the aerobic conversion of ammonium to nitrite and further to nitrate, which is finally converted to N_2 in anoxic conditions using a variety of electron donors, including methanol, acetate, ethanol, etc. As nitrification and denitrification have different requirements, besides N-compounds (oxygen for the former and organic carbon for the latter), and are conducted by different microorganisms (AOB/NOB and denitrifiers, respectively) these processes have to be separated in time or space (Khin and Annachhatre, 2004).

Many of the existing activated sludge systems in WWTPs are not properly designed for denitrification or even overloaded with nitrogen, especially in densely populated urban areas or under low organic matter situations (Kalyuzhnyi et al., 2006b). Actually, the presence of natural organic carbon in the wastewater after the aerobic phase, when denitrification is expected to occur, is so limited that the complete removal of nitrogen from WWTPs containing high concentrations of N-compounds requires a large amount of carbon source to be added to allow an effective denitrification procedure (van Dongen et al., 2001). The addition of electron donors such as methanol makes full-scale denitrification quite expensive, particularly when the wastewater contains only small amounts of biologically degradable carbon compounds (van Hulle et al., 2003). In addition, the WWTPs have to deal with another important inconvenience: they have to meet new requirements since the effluent discharge standards have become more stringent (Khin and Annachhatre, 2004; Zhu et al., 2008) with very strict ammonia limits for discharge of non-domestic effluents into the sewerage (Kalyuzhnyi et al., 2006b).

Nowadays, there are new technological developments to shorten the way for complete N removal without the above-mentioned problems. These processes are mainly based on the activity of chemolithotrophic bacteria, with anammox as the main N_2-producing metabolism, and focused on saving energy and economic costs (Strous et al., 1998; Kartal et al., 2004). Currently, anammox-based technologies are considered as one of the most pioneering systems for N removal in wastewater treatment. However, some requirements need to be fulfilled to get a complete performance of the anammox metabolism, especially to avoid the anammox inhibition by nitrite. These bacteria must be provided with nitrite and ammonium in a specific stoichiometry ratio (Strous et al., 1999b), thus requiring the previous ammonium to nitrite partial oxidation by AOB and/or AOA. Therefore, most of the novel technological developments are based on a combination of partial nitritation coupled with anammox, although their physiological requirements are rather different and the processes usually have to attempt to solve several shortcomings. Nowadays, practically 100 full-scale installations based on partial nitritation-anammox have been successfully developed worldwide (Lackner et al., 2014).

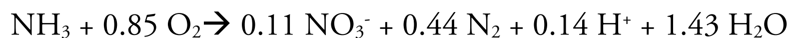
OLAND (OXYGEN-LIMITED AUTOTROPHIC NITRIFICATION-DENITRIFICATION)

As it has been previously stated, the nitrification and denitrification processes must be performed separately either in time or space. However, several studies showed some nitrogen removal under non-conventional conditions (in one reactor with aerobic conditions) and different hypotheses were pointed out: (i) aerobic denitrification carried out by a heterotrophic microorganism, *Thiosphaera pantotropha* (Robertson and Kuenen, 1984); (ii) oxidation of ammonium with nitrite to dinitrogen gas under oxygen-limited conditions conducted by AOB (Schmidt and Bock, 1997).

In this context, Siegrist and collaborators (1997) started up an autotrophic Rotating Biological Contactor (RBC) reactor based on biofilm structures capable of producing N_2 from ammonium in one reactor under oxygen-limited conditions, which was named

OLAND. The ammonium was partly nitrified to nitrite by AOB in the aerobic biofilm layer, close to the surface. Then, ammonium and nitrite diffused into the deeper anoxic layer of the biofilm and nitrite could be used as electron acceptor for the anammox reaction. This hypothesis about the anammox activity in the anoxic layer was confirmed by Wyffles and collaborators (2003) through ^{15}N tracer techniques. Later, molecular studies based on FISH analyses detected the presence of *Ca.* Brocadia anammoxidans or *Ca.* Kuenenia stuttgartiensis (only Amx820 probe that hybridizes both species was used) in an OLAND reactor treating black water (Vlaeminck et al., 2009b).

Thus, OLAND reactor contains AOB oxidizing ammonium to nitrite with oxygen as the electron acceptor and anammox bacteria combining this nitrite with residual ammonium to produce N_2 and some nitrate under oxygen-limited conditions (Vlaeminck et al., 2009b). The overall N removal stoichiometry goes as it follows:



$$NH_3 + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$$

Some challenges have to be solved in order to fully develop this technology, always based on RBC reactors. First, the OLAND start-up period must be considerably long to allow the full development of the biofilm, due to the inherent properties of this kind of microbial communities coupled to the slow growth of anammox bacteria. Secondly, high nitrogen removal efficiency relies on limited nitrite accumulation because its toxicity, requiring that AOB activity does not exceed anammox activity. From an economical point of view, OLAND can save 30-40% of the overall costs as compared to conventional nitrification-denitrification sequential process, because of the lower aeration requirement, sludge production and organic carbon addition.

DEAMOX (DENITRIFYING AMMONIUM OXIDATION)

Kalyuzhnyi and collaborators (2006b) proposed a new technology named DEAMOX, which does not require a separate production of nitrite and combines the anammox reaction with the autotrophic denitrifying conditions. In this system, sulfide is used as

an electron donor for the generation of nitrite from nitrate within an anaerobic biofilm (Kalyuzhnyi et al., 2006a) (table 1.1). After a pre-treatment step, which can be performed in different types of anaerobic reactors, an effluent rich in ammonia and sulfide is obtained and partially divided. One part (the aerobic flow) goes to a nitrifying reactor to generate mainly nitrate and low amounts of nitrite, using both AOB and NOB. The rest (the anaerobic flow) directly goes to the DEAMOX reactor, where it is mixed again with the other part, once oxidized (fig. 1.9). In the DEAMOX reactor, the conversion of nitrate and sulfide to nitrite and sulfuric acid is produced, and then ammonium is oxidized to N_2 by anammox bacteria using nitrite as electron acceptor (Kalyuzhnyi et al., 2006b). The theoretically required H_2S/NO_3^- ratio in the DEAMOX reactor is 1:4.

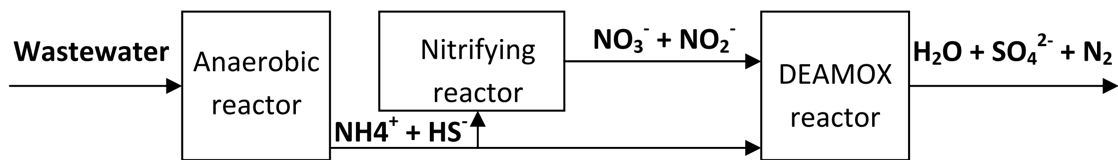


Figure 1.9: Flow diagram of the DEAMOX process (modified from Kalyuzhnyi et al., (2006b)).

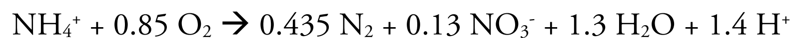
Few molecular studies based on PCR analyses were carried out on this kind of reactors, and they only focused on the identification of the anammox species, determining that neither *Ca.* Brocadia anammoxidans nor *Ca.* Kuenenia stuttgartiensis were responsible of the anammox activity inside the DEAMOX reactor (Kalyuzhnyi et al., 2006a). Even though it seems to possess important advantages in front of other technologies, few studies have been published to date about DEAMOX. However, the process has still several drawbacks, such as the involvement of too many reactors and the presence of sulfide, which can inhibit the anammox population in the DEAMOX reactor in the case of a sulfate-rich raw wastewater (Jin et al., 2013; Russ et al., 2014).

Table 1.1: A summary of the overall N removal stoichiometry in the DEAMOX process, split between the reactors where the reactions occur.

Location	Reaction
Anaerobic reactor	$N\text{-organic} + SO_4^{2-} \rightarrow NH_4^+ + HCO_3^- + CH_4 + HS^-$
Nitrifying reactor	$NH_4^+ + O_2 \rightarrow NO_3^- + NO_2^-$
DEAMOX	$4NO_3^- + HS^- \rightarrow 4NO_2^- + SO_4^{2-}$
	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$

CANON (COMPLETELY AUTOTROPHIC NITROGEN REMOVAL OVER NITRITE)

CANON is another single-reactor process (Dijkman and Strous, 1999; Sliekers et al., 2002), which also upholds partial ammonium oxidation to nitrite by AOB to allow anammox bacteria to use part of the remaining ammonium and nitrite for N_2 production, operating at low oxygen concentrations (Hao et al., 2002; Pynaert et al., 2002a; Pynaert et al., 2002b; Sliekers et al., 2002; Nielsen et al., 2005). The interaction between AOB and NOB under oxygen–limited conditions (<0.5% air saturation) results in a complete conversion of ammonium to N_2 in a single autotrophic reactor. The overall N removal stoichiometry goes as it follows (Khin and Annachhatre, 2004):



$$NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+$$

FISH studies by Sliekers and collaborators (2002), working on an SBR configuration for CANON technology, revealed that *Nitrosomonas* and anammox bacteria counted for 45% and 40% of the total amount of cells, respectively, while NOB could not be detected. The latter are not favoured by the CANON features, because they have to compete with the AOB for the oxygen and with the anammox bacteria for the nitrite. Furthermore, heterotrophic denitrifiers did not seem to be active. The situation is quite different from RBC-based systems, where the supply of ammonium and oxygen are more difficult to control. In these reactors, the three kinds of chemolithotrophic

bacteria related to N transformations can coexist simultaneously (Kartal et al., 2004), in a similar way than the OLAND process.

CANON is particularly suitable for the removal of ammonia from wastewater with low Chemical Oxygen Demand (COD). It saves 63% oxygen and 100% carbon sources in comparison with the traditional nitrification-denitrification process (Third et al., 2005). However, it requires an advanced and expensive process control which may represent a burden for its application (Kalyuzhnyi et al., 2006b).

PARTIAL NITRITATION (PN) - ANAMMOX

A two-step process based on the combination of a partial nitritation (PN) and anammox has also been described and tested for nitrogen removal (van Dongen et al., 2001; Ganigué et al., 2007). Stable PN in a first aerobic reactor can be sequentially combined with anammox process in a second anaerobic tank to ensure total nitrogen removal throughout autotrophic processes. This two-step approach has been successfully applied in the wastewater treatment and showed its feasibility in several full-scale installations worldwide (Lackner et al., 2014).

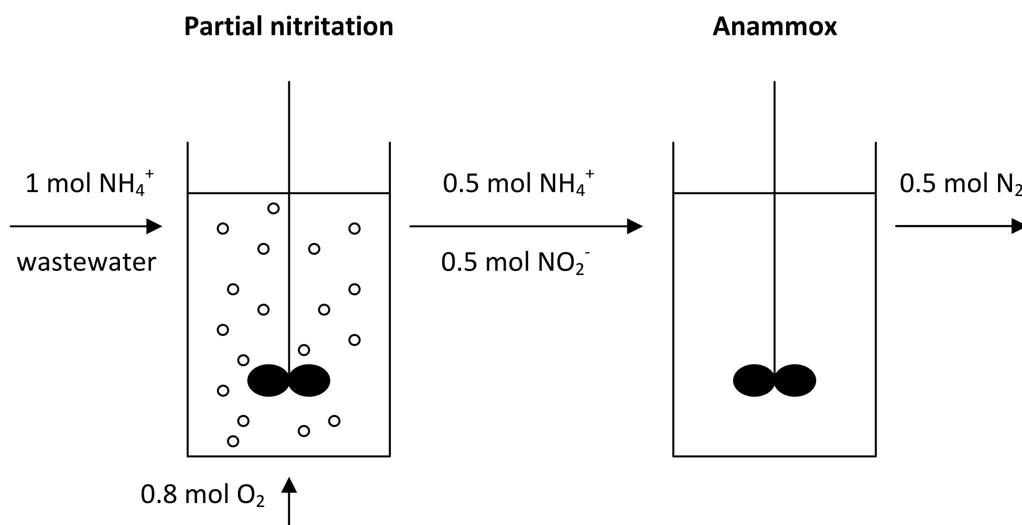


Figure 1.10: (modified from Volcke et al. (2006)) Schematic vision of the two-step configuration for partial nitritation (left) and anammox (right).

PN step must be performed previously to fulfil the stoichiometric requirements of anammox bacteria to completely remove N-compounds. In the PN reactor, ammonium is partially oxidized to nitrite by AOB, avoiding the final nitrite oxidation to nitrate (conducted by NOB), before feeding into the anammox reactor with a suitable influent in a 1:1 ammonium to nitrite molar ratio (Ganigué et al., 2007). The produced nitrite together with the remaining ammonium is then converted to N_2 in the anammox process (fig. 1.10). The aerobic oxidation of the ammonium in this reactor is generally conducted by *Nitrosomonas* spp. rather than *Nitrosospira* spp (Ganigué et al., 2009). On the other hand, *Ca.* Brocadia anammoxidans and *Ca.* Kuenenia stuttgartiensis are the most common species present in the anammox (Hwang et al., 2005; Ganigué et al., 2007; Okabe et al., 2011) reactor.

Different operational conditions can be applied in the PN reactor to benefit AOB in front of NOB:

(i) Temperature: The growth rate of AOB are usually higher at elevated temperatures whereas NOB definitively remained more and longer active at lower temperatures (Hellinga et al., 1998; Bae et al., 2001; Lackner et al., 2014).

(ii) Dissolved oxygen (DO): It is widely accepted that low DO operation favours AOB over NOB (Sin et al., 2008). However, recent studies conducted by Regmi and collaborators (2014) indicated the opposite when *Nitrospira* sp., rather than *Nitrobacter* sp., is present in the reactor. *Nitrospira* showed a higher substrate affinity, but it was not evident since most of the previous studies have completely overlooked *Nitrospira*. (Garrido et al., 1997; Bernet et al., 2001).

(iii) Influent alkalinity/ammonium: Ammonium oxidation is an alkali-consuming reaction. 1 mol alkali per mol ammonium can be used to assure a proper ammonium/ nitrite ratio (Anthonisen et al., 1976; Ganigué et al., 2007). This relationship has found to be suitable for the PN linked to anammox (van Dongen et al., 2001).

(iv) pH: This parameter directly influences the growth rates of both bacterial groups and it is closely related to the available substrate forms, according to a pH-dependent equilibrium between the concentrations of ammonia and ammonium. Increasing pH

leads to increase ammonia:ammonium ratio, thus promoting AOB over NOB (Ganigué et al., 2009). If the pH drops below 6.5, the ammonium oxidation will no longer take place.

(v) Sludge residence time (SRT): A PN reactor can also work without any biomass retention, meaning that the sludge age is equal than SRT. This variation of the PN process, which is usually performed at 35^oC, is called SHARON (Single reactor system for High rate Ammonium Removal Over Nitrite) (Hellinga *et al.,* 1998). By working at these conditions the SHARON can easily washout slow-growing NOB populations (35^oC is far from their optimal temperature) while keeping the faster-growing AOB populations.

The anammox reactor does not require such special operational conditions in its configuration, as compared to PN reactor. The stringent conditions inside the anammox reactor are able to outcompete most of the species (except for denitrifying bacteria if the effluent from PN reactor contains COD levels). However, one of the main drawbacks common to application of the anammox process is the requirement of a long start-up period, mainly due to slow growth rates of anammox bacteria (Tsushima et al., 2007b). The anammox reactor should operate to guarantee almost complete biomass retention inside the system (Dapena-Mora et al., 2004).

Strous and collaborators (1998) showed that the SBR is a suitable system to enrich slow-growing anammox bacteria. However, other studies also achieved successful anammox growing with biofilm attachment systems, such as fixed-bed reactors (Kindaichi et al., 2007; Tsushima et al., 2007b), fluidized bed reactors (Mulder et al., 1995), gas-lift reactors (Dapena-Mora et al., 2004), up-flow anaerobic sludge blanket reactors (Ahn et al., 2004), nonwoven biomass carriers (Fujii et al., 2002; Furukawa, 2003; Isaka et al., 2006) and membrane-sequencing batch reactors (Trigo et al., 2006) among others.

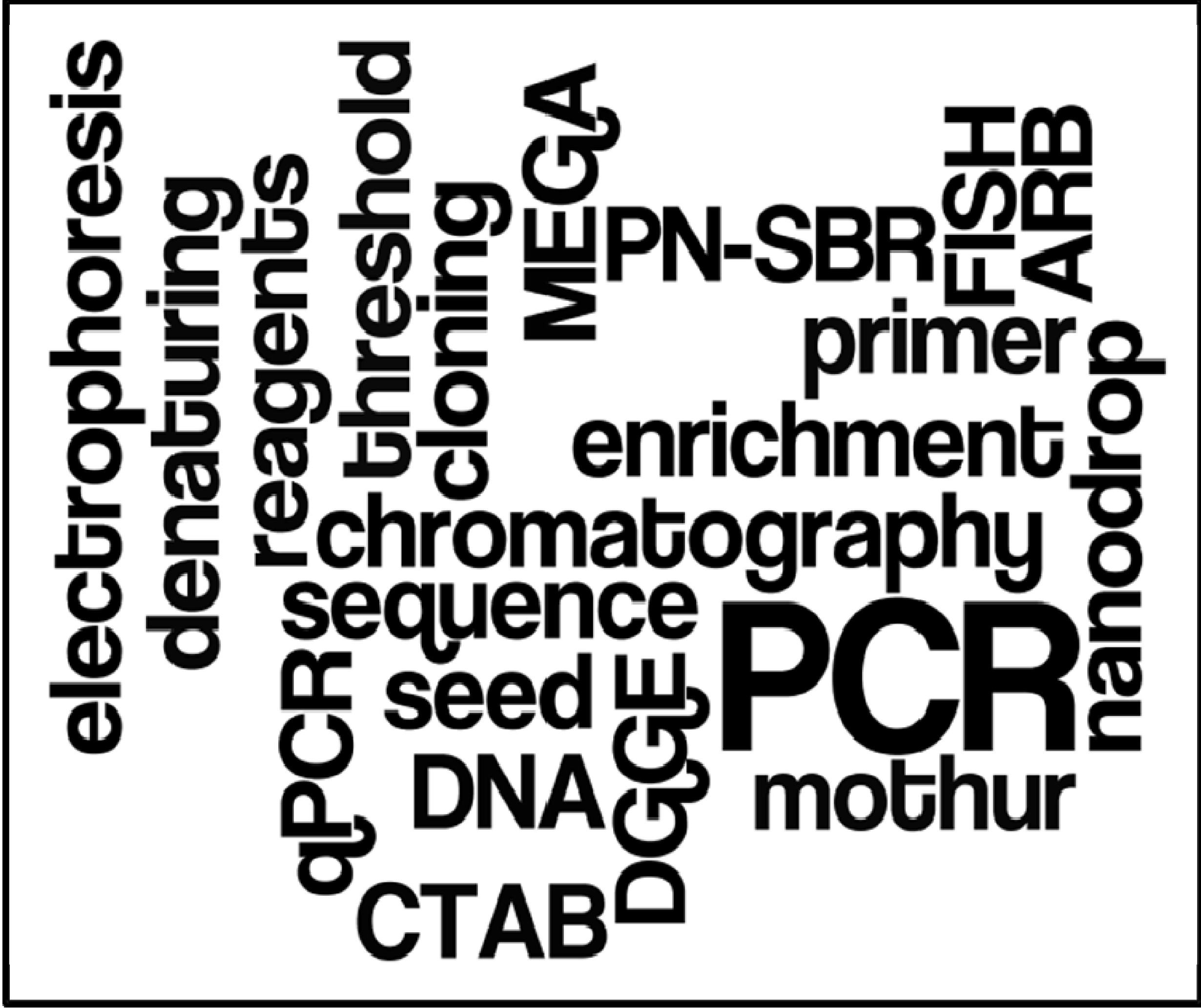
PANAMMOX® technology, combining Partial Nitritation (working under SBR configuration) and ANAMMOX reactor, has been the main N-removal technology analyzed, from a microbiological outlook in the development of this PhD thesis.

OBJECTIVES

The characterization of the N-cycle bacterial assemblages present in the different PANAMMOX® components and the inocula achieved to start-up the anammox reactor is the general aim of this thesis. Molecular methods based on PCR, coupled to fingerprinting methods, qPCR and FISH were carried out to fulfil this characterization.

Specific objectives:

1. To obtain anammox enrichments, useful as inocula for starting-up an anammox bioreactor.
2. To assess a molecular method based on PCR for early anammox detection in combination with batch culture enrichment. It would allow to select biomass sources to be potential anammox inocula.
3. To identify the microbial populations potentially coupled to anammox bacteria in the enrichments. The research on these ecological partners should be helpful for future optimization and increasing efficiency of the anammox process design and development.
4. To study the population dynamics of AOB and main NOB during the start-up and subsequent stable operation of a sequencing batch reactor designed to perform partial nitritation at remarkably high ammonium and nitrite concentration.



Material & methods

2.1 DNA SOURCES

ANAMMOX SEEDING SAMPLES

Several sampling sources were chosen to enrich anammox bacteria in batch cultures. The seeds were collected based on certain parameters (such as concomitant concentrations of ammonium and nitrite in anaerobic and/or microaerophilic conditions) that could lead into successful anammox enrichments. Three different sampling sources were selected to have wide-range seeds: natural, man-made systems and modified systems.

Table 2.1: Summary of the DNA seeds enriched in batch cultures. Enrichments are laid out depending on the nature of their seed.

System	Enrichment	Country	Source	Specifics
Natural	1	Murcia (Spain)	Marine sediment	Collected from la Manga del Mar Menor
	2	Salzkammergut (Austria)	Freshwater lake sediment	Collected from lake Toplitz
	5	Castelló d'Empúries (Catalonia)	Brackish coastal lagoon sediment	Collected from "la Massona" lake, in "Aiguamolls de l'Empordà" Natural Park.
Modified	6	Empuriabrava (Catalonia)	Wetland sediment	Collected from Empuriabrava artificial constructed wetland
Man-made	3	Girona (Catalonia)	Activated sludge	Collected from an anoxic SBR
	4	Sils-Vidreres WWTP (Catalonia)		Collected from an oxic/anoxic SBR
	3bis	Girona (Catalonia)		Collected from an anoxic SBR
	4bis	Girona (Catalonia)		Collected from an oxic/anoxic SBR
	10	Girona (Catalonia)		Collected from an anoxic SBR
	7	Taradell WWTP (Catalonia)		Collected from an anoxic reactor
	8	Orís WWTP (Catalonia)		Collected from an anoxic reactor
	11	Sils-Vidreres WWTP (Catalonia)		Collected from an anoxic reactor

Besides the information given in table 2.1 some considerations need to be done for the enrichment process in some batch cultures. Enrichments 3 and 3bis, and 4 an 4bis shared the same seed. However, sludge used for enrichments 3 and 4 were washed with anammox medium before starting-up the incubations, while running water was used to wash enrichment 3bis and 4bis. Enrichment 10 came from half of the volume of the enrichment 3 after 443 days of enrichment process.

Figure 2.1: Geographical locations of the sampling sites. The main area is enlarged to better point out the origin of each DNA seed used in this work.

ANAMMOX ENRICHMENTS

Each enrichment was prepared in Erlenmeyer flasks with butyl rubber stoppers using 100 mL of sample and 300 mL of mineral medium under anoxic conditions. The Erlenmeyers flasks were purged by two exits: one with a 0.2 µm filter for the entrance

and removal of N_2 and another for removing samples to analyze N-compounds (Toh and Ashbolt, 2002) (fig. 2.2 & 2.3).

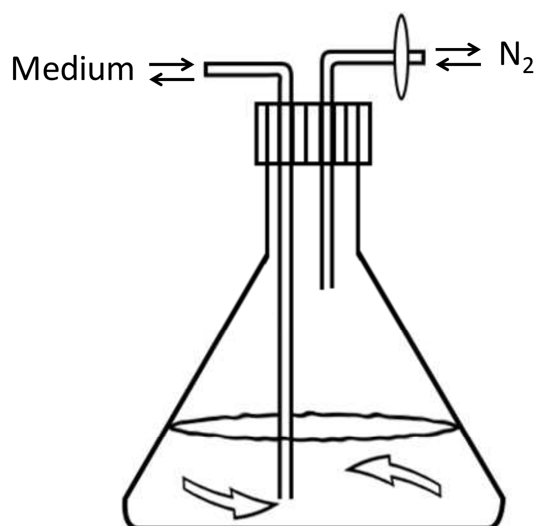


Figure 2.2: Schematic vision of the Erlenmeyer flask as an anammox batch culture. Both purge exits were indicated.

The medium was a modification from the one described by van de Graaf and collaborators (1996) (table 2.2). The medium and the trace elements were both prepared at pH 7.2-7.6. Both solutions were autoclaved separately and after cooling, 1.25 mL (per L of medium) of the trace elements were added aseptically to the autoclaved mineral medium. During the cooling process, N_2 was applied to avoid the entrance of O_2 in the mineral medium.

Table 2.2: Composition of the mineral medium and the trace elements used for the preparation of anammox medium.

Medium		Trace elements	
Element	**Concentration (g L^{-1})**	**Element**	**Concentration (g L^{-1})**
$NaHCO_3$	1.050	$EDTA.2H_2O$	15
KH_2PO4	0.025	$ZnSO_4.7H_2O$	0.43
$CaCl_2.2H_2O$	0.300	$CoCl_2.6H_2O$	0.24
$MgCl_2.6H_2O$	0.165	$MnCl_2.4H_2O$	0.99
$FeSO_4.7H_2O$	0.012	$CuSO_4.5H_2O$	0.25
$EDTA.2H_2O$	0.007	$NiCl_2.6H_2O$	0.19
		$Na_2SeO_3.5H_2O$	0.32
		H_3BO_3	0.014
		$NaMoO_4.2H_2O$	0.22

The enrichments were placed in a shaking incubator (Memmert) in the dark at 37°C. Their pH was kept in a range between 6.7 and 8.3 (Strous et al., 1999b) using 1 M HCl/NaOH solution. N-compound analyses were performed every 3 days. The medium without any biomass traces, required for the chemical analyses, was obtained by stopping the shaking during all night. However, a fully decantation was difficult in the enrichments inoculated with sediments or soils (such as enrichment 5 and 6). After the decantation, samples were collected by connecting one exit to a N_2 source. The medium was forced to exit by introducing N_2 and it was collected in a clean 15 mL tube. This procedure was carried out to assure the anoxic atmosphere inside the enrichments.

Figure 2.3: A single (right) and a wide (left) view of most of the anammox enrichments after decantation.

When ammonium or nitrite was consumed, $NH_4Cl/NaNO_2$ were supplied to maintain anammox activity. During the initial steps of the enrichment procedure, nitrate ($NaNO_3$) was also added to a final concentration of 10-30 mg L^{-1} to favour the elimination of degradable biomass by denitrifying bacteria and to avoid the production of H_2S by sulphate-reducing bacteria. Ammonium and nitrite supplies were added stepwise to increase their concentrations in the media throughout the enrichment period. Both N-compounds started with concentrations (after addition) of 20 mg L^{-1} and they were increased in 10 mg L^{-1} increments every step, once the batch cultures were able to remove them without inhibition problems. The medium was periodically

refreshed to avoid problems of accumulation or lack of substances because of the closed-system configuration of the batch cultures.

PN REACTOR (AOB-NOB)

A partial nitritation reactor working at remarkably high ammonium and nitrite concentration was the source to study the AOB and NOB diversity. The system under study was a pilot-scale PN working with Sequencing Batch Reactor (PN-SBR) conditions for the treatment of urban landfill leachate. This wastewater presented a high variability on its characteristics, with high ammonium concentrations (ranging from 2200 up to 5000 mg $N\text{-}NH_4^+$ L^{-1}) and low biodegradable organic matter content (about 800 mg O_2 L^{-1}). The reactor was operated at a temperature of 35 ± 1^oC and a dissolved oxygen (DO) set-point of 2 mg O_2 L^{-1}. The pH was controlled by the addition of HCl (1 M) to avoid values higher than 8.

It was initially seeded with a mixture of sludge coming from the wastewater treatment plant (WWTP) of Sils-Vidreres and the urban solids waste treatment plant of Orís. After a short start-up, the reactor was operated under a step-feed strategy with a total cycle length of 24 h (Ganigué et al., 2009). This strategy was based on 14 identical sub-cycles of 100 min, each consisting on 15 min of anoxic phase (feeding between minutes 4 to 14) followed by 85 min of aerobic reaction. The cycle ended-up on a settling and a draw phase, both of 20 min length. Thus, the cycle presented a predominance of aerobic phases (82.6%) over anoxic conditions (17.4%).

The process operation can be divided in 3 phases. Firstly, the reactor was operated without any bicarbonate adjustment. Since the low nitrite conversion rate, a $NaHCO_3$ solution started to be dosed on 59th day when pH decreased below 7.2. Nevertheless, solid $NaHCO_3$ started to be supplied on 220th day at the influent to reach a proper leachate composition because optimum results were still not achieved.

2.2 CHEMICAL TECHNIQUES

AMMONIUM CONCENTRATION QUANTIFICATION

The ammonium concentration was determined by distillation (B-324 distillation unit, BÜCHI) and subsequent valoration (Titrino 719S, Metrohm) with sulfuric acid. The basic principle of the distillation process is to convert ammonium to free ammonia by the action of a strong base (concentrated NaOH). This step allows the rising of the pH above 11. Ammonia diffuses to get caught in a solution of boric acid (H_3BO_3) in the form of ammonium. The concentration of ammonia distilled and trapped in this solution is calculated using sulfuric acid by a valoration with an endpoint (pH=4.65).

Few drops of sulfuric acid were added to the 15 mL tubes with the anammox medium when immediate ammonia analyses could not be performed. The step of ammonium conversion to ammonia gas was then avoided and the samples could be stored at 4°C for two days.

Protocol for ammonium analyses by distillation:

- *Warm the device and perform a cleaning step of the distillation unit (this step is essential if the previous samples contained high ammonia concentrations).*
- *Set blank through a preset program in the distillation unit.*
- *Add 10 mL of the sample in the specific tubes and put it in the device.*
- *Start distillation.*
- *Automatic valoration with sulfuric acid 0.1N.*

The ammonium concentration was quantified through the following formula:

$$N - NH_4^+ = \frac{(A - B)\ x\ 1000\ x\ 14}{V_{sample}}\ x\ NH_2SO_4$$

Where NH_2SO_4 = normality of sulfuric acid 0.1N; A = volume of sulfuric acid consumed in the valoration (mL); B = volume of sulfuric acid consumed in the blank valoration (mL) and V sample = volume of the sample (mL).

NITRITE and NITRATE CONCENTRATION QUANTIFICATION

The concentration of nitrite and nitrate concentration was performed by ionic chromatography (761 Compact IC, Metrohm). The samples were automatically injected by an automatic injector (831 Compact IC Autosampler, Metrohm). An anion column was used for the ion separation determination (Metrosep A Supp 5-250, Metrohm). Besides, a precolumn was used (Metrosep A Supp 5 Guard, Metrohm) to protect the IC anion column against contamination from the sample or the eluent.

Protocol for nitrite and nitrate analyses by distillation:

- *The samples must be filtered using 0.2 μM nylon filters. Once filtered, they have to be diluted (1/5) to avoid the collapse of the anion column.*
- *Preparation of the solutions (standards and buffer).*
- *A cleaning and a stabilization of the anion column must be performed by running the mobile phase for at least 30 min.*
- *Once the device is stabilized, the standards and subsequent samples were automatically injected. After the analysis of each sample, the results were obtained from a chromatogram (fig 2.4).*

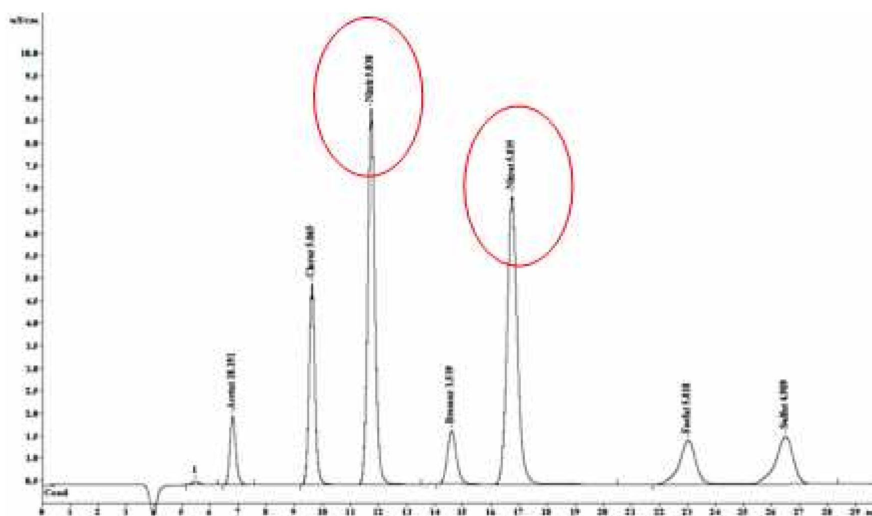


Figure 2.4: Chromatogram of a standard sample. The red circles show the nitrite and nitrate peaks.

Nitrite and nitrate calculations were automatically obtained by the interpolation in a standard curve.

2.3 MOLECULAR TECHNIQUES

DNA is the basis for the molecular characterization of the microbial communities in the samples used in this work (fig. 2.5). The bacterial genomic DNA, once extracted, is used as a template for the massive amplification of specific or general 16S rRNA gene fragments by PCR. Different techniques, such as DGGE and cloning, are carried out to separate the mixture of different PCR products to be sequenced and thus create a sequence database. This database allows the calculation of a phylogenetic tree showing the relationship among the phylotypes previously amplified by PCR. Besides, achieving DNA sequences and their affiliation in the phylogenetic tree can allow the design of new primers for better future characterization of the community. Moreover, the use of labelled probes for FISH analysis can be a complement for the detection and identification of species and showing their location in the environment. On the other hand, the PCR products can also be used to construct standard curves which are required for qPCR assays, allowing the quantification of the microbial populations present in the environmental samples.

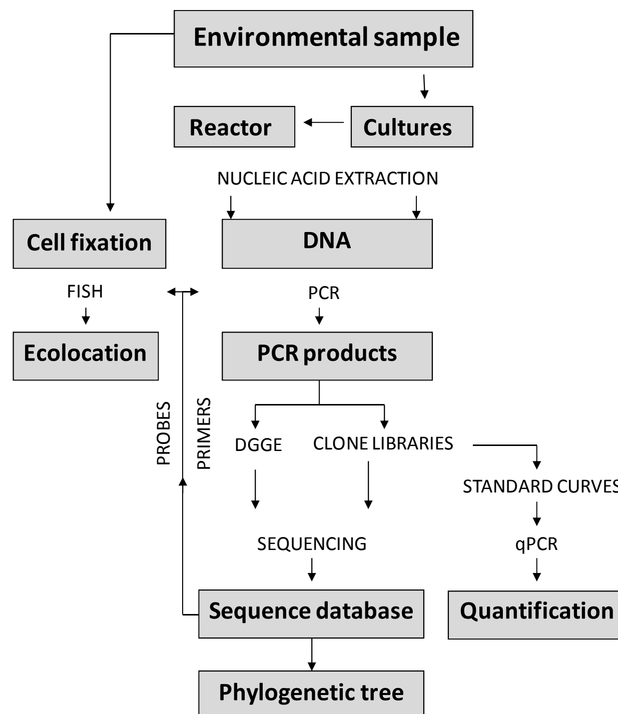


Figure 2.5: Flow chart of the different steps and the associated molecular techniques in the study of the structure and function of microbial communities.

Nowadays this scheme is changing especially in the methods for mixture DNA template separation. They are starting to be replaced for next-generation sequencing (NGS) procedures, such as 454, MiSeq or Ion Torrent, which can deliver thousands of sequences per sample. Unfortunately none of these techniques was applied in this work because all molecular analyses were carried out before NGS appearance.

DNA EXTRACTION

The use of molecular methods relies heavily on the isolation and purification of high quality nucleic acids. However, a standardized procedure for the extraction and purification of DNA is not possible, since the suitability of each method depends on the kind of sample. In the present work, two DNA extraction protocols were used: the CTAB method and a commercial kit DNeasy Blood&Tissue (Mobio).

CTAB (CETYLTRIMETHYLAMMONIUM BROMIDE) METHOD

Genomic DNA from the anammox enrichments was obtained using the CTAB method described by Lodhi (1994). As detailed in the protocol described hereby, this method is based on the application of the CTAB solution (detergent that solubilizes membranes to form a complex with DNA allowing selective precipitation) coupled with the use of the FastPrep-24 instrument (MP Biomedicals) to completely break the bacterial cells (physical treatment). The DNA isolations obtained with this method showed high DNA concentrations but the process presented a poor repeatability.

Protocol for DNA isolation with CTAB method:

- *Take 2 mL of the homogenized sample.*
- *Apply the protein removal treatment by adding twice 600 µL of chloroform:Isoamyl alcohol (CI).*
- *Precipitate DNA from the aqueous layer by adding 300 µL of NaCl (5 M) and 1.2 mL of ethanol 95% at −20°C.*
- *Maintain the sample for 2–3 h at −20°C.*
- *Centrifuge at 9000 g for 10 min.*
- *Wash the DNA pellet with 1 mL of 70% ethanol.*
- *Centrifuge at 9000 g for 10 min.*
- *Dry completely the samples and resuspend it in 50 µL of sterile MilliQ water.*

- *Centrifuge at 6000 g for 1 min.*
- *Transfer the resulting pellet a 1.5 mL tube containing 600 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl at pH 8.0 and 1.2 µL of β-mercaptoethanol).*
- *Lyse cells by a 45 s treatment in a Bead-Beater (Biospec Products).*
- *Add a solution of 20% polyvinylpolypyrrolidone (PVP) to remove the polyphenols.*
- *Incubate the mixture at 65°C for 1 h.*

DNEASY BLOOD & TISSUE

Genomic DNA from the PN bioreactor was obtained using the DNeasy Blood&Tissue commercial kit (Qiagen). Although some efficiency was lost as regards the CTAB method, the repeatability was one of their main features. Due to the posterior use for qPCR analysis, optimal subsample volume was determined from the relationship between sludge volume and DNA yield, in order to accomplish high DNA yield but avoiding saturation of the extraction process.

Protocol for DNA isolation with DNeasy Blood&Tissue commercial kit:

- *Harvest cells in a microcentrifuge tube by centrifuging at 5000 g for 10 min. Discard supernatant.*
- *Resuspend pellet in 180 µL buffer ATL.*
- *Add 20 µL proteinase K.*
- *Mix thoroughly by vortexing and incubate at 56°C until the tissue is completely lised. Vortex occasionally during incubation. If the sample is hard to lise, it can be incubated overnight.*
- *Vortex for 15 secs.*
- *Add 200 µL buffer AL to the sample and mix thoroughly by vortexing.*
- *Add 200 µL ethanol (96-100%) and mix again by vortexing.*
- *Transfer all the mixture into the DNeasy Mini spin column.*
- *Centrifuge at ≥ 6000 g for 1 min. Discard flow-through and the tube.*
- *Place the DNeasy Mini spin column in another tube and add 500 µL buffer AW1.*
- *Centrifuge at ≥ 6000 g for 1 min. Discard flow-through and the tube.*
- *Place the DNeasy Mini spin column in another tube and add 500 µL buffer AW2.*
- *Centrifuge at 20000 g for 3 min to dry the DNeasy membrane. Discard flow-through and collection tube.*
- *Place the DNeasy Mini spin column in a clean 1.5 mL tube and pipet 200 µL buffer AE directly onto the DNeasy membrane.*
- *Incubate at room temperature for 1 min, and then centrifuge at ≥ 6000 g for 1 min to elute.*

DNA QUANTIFICATION.

SPECTROPHOTOMETRIC DETERMINATION

Purity and concentration of DNA isolations were determined with the microvolume spectrophotometer Nanodrop UV/VIS N-1000 (Thermo), using 1 µL of sample. The use of the Nanodrop spectrophotometer was preferred rather than conventional spectrophotometer because of the small volumes obtained in DNA isolations (usually below 200 µL).

Nanodrop can work full-spectrum (200nm-750nm), but the absorbance is only measured at 230, 260 and 280nm (A230, A260 and A280, respectively) for acid nucleic quantification. Nanodrop quantifies the DNA concentration according to the correspondence between an A260 absorbance value of 1 and 50 µg mL^{-1} dsDNA (Sambrook et al., 1989). Moreover it also gives purity coefficients. The coefficient A260/A280 provides an analytical measure of the genomic DNA purity (Green and Sambrook, 2012). Thus, a DNA sample is considered to be pure when A260/A280 value ranges from 1.8 to 1.9 (Gallagher and Desjardins, 2008). Besides, a coefficient A260/A230 > 2.2 denotes a protein-free sample.

POLYMERASE CHAIN REACTION (PCR)

PCR is a quick procedure for *in vitro* amplification of specific DNA fragments (Mullis and Faloona, 1987). The PCR allows the specific and exponential synthesis of a predetermined DNA region via the use of two small, specifically designed fragments of DNA (so-called primers), which target the two termini of the nucleic acid molecule to be amplified.

PCR proceeds in three distinct steps governed by temperature and repeated during *n* cycles (fig. 2.6). In a single cycle, DNA template is firstly denatured to separate the complementary strands by applying temperatures close to 95°C. Then the reaction is cooled to an annealing temperature to allow the primers hybridize with the template. The annealing temperature depends on the nucleotide composition of the primers.

During this step the thermostable DNA polymerase starts to extend the primers as soon as they bind to the template. Finally, the reaction is heated to a higher temperature where the polymerase will extent the complementary strand. Hence, each template strand give rise to a new duplex at each cycle, doubling the number of copies of the target region.

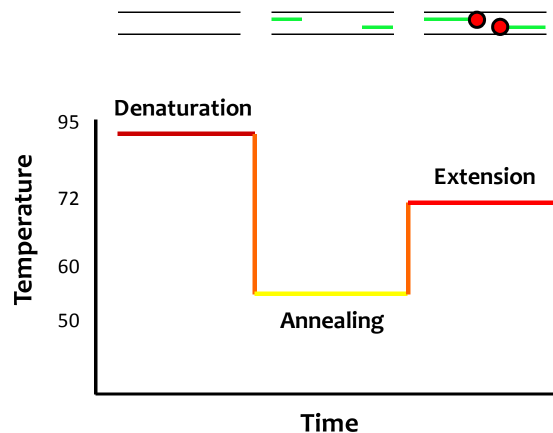


Figure 2.6: The PCR temperature cycle: (1) the temperature is raised to about 95°C to melt the dsDNA, (2) the temperature is lowered to let primers anneal, (3) the temperature is set to 72°C to let the polymerase extend the primers. Adapted from Kubista and collaborators (2006).

The advent of PCR has greatly accelerated the progress of studies on the genomic structure of various organisms, and any region in even highly complex genomes can be specifically amplified in a few hours by the technique, if the flanking nucleotide sequences are known (Saiki et al., 1988).

PCR REAGENTS

Buffer: It controls the pH during the PCR performance, not to vary from 6.8 to 8.3. Most suppliers provide 5x-10x reaction buffer with their own *Taq* DNA polymerase.

dNTPs: Nucleotides are essential for the extension of the new complementary strands to produce new duplex in each PCR cycle. It is important for successful PCR that the four dNTPs are present in equimolar concentrations, otherwise the fidelity of the PCR can be affected. Lower concentrations of dNTPs minimize the binding of the primer to an unintended template which in turn increase specificity and fidelity of PCR amplifications (McPherson and Moller, 2000).

Primers: They are the responsible of the specificity of the PCR. The optimal primer size is usually between 18 and 28 bases. Shorter primers are generally less specific but may result in more efficient PCR, whereas longer primers improve specificity yet can be less efficient. Melting temperatures (T_m) for both primers usually should not differ more than 5°C of each other (Grunenwald, 2003).

Taq DNA polymerase: The most common enzyme used for PCR amplification because of its thermostability. The half-life of *Taq* DNA polymerase is 40 min at 95°C, which is sufficient to remain active over 30 or more cycles. Recommended concentration for *Taq* DNA polymerase ranges between 1 and 2.5 units per 100 µL of PCR. Increasing this value can sometimes increase the yield of non-specific PCR products (Grunenwald, 2003).

$MgCl_2$: Magnesium is one of the most critical components in the PCR, as its concentration can affect the specificity and efficiency of the reaction. With an excess of $MgCl_2$ *Taq* polymerase is more error prone than a lower concentrations. Standard buffers can contain 1.5 mM $MgCl_2$ (McPherson and Moller, 2000).

H_2O (molecular grade): It is recommended the use of sterile water without any contaminants such as DNA or RNA. Although this water is usually provided completely sterilized, is recommended a previous autoclave step.

PRIMERS

Several primer sets were used in the present work. Primer sets targeting 16S rRNA gene are detailed in this section altogether with a brief explanation about their use (detection or quantification) and their specificity. In some cases different primer sets were used for detection or quantification of a same group of bacteria, mainly because of the length of the PCR product: phylogenetic analyses reclaim larger PCR products than qPCR analyses.

All the primers used in this work were synthesized and purified by reverse phase chromatography by Roche Diagnostics© GmbH.

Bacteria (detection)

Primer	Specificity	Sequence (5'-3')	Reference
27F	Universal	AGAGTTTGATCCTGGCTCAG	Lane (1991)
1492R	Universal	AAGTCGTAACAAGGTAACCGT	Lane (1991)

Primer set used to amplify the 16S rRNA gene of *Bacteria*. The PCR product is very suitable for complete phylogenetic analyses by cloning procedures because it contains almost the whole 16S rRNA entire gene (Ca. 1465 bp).

Bacteria (detection)

Primer	Specificity	Sequence (5'-3')	Reference
357F	*Bacteria*	CCTACGGGAGGCAGCAG	Muyzer et al. (1993)
907R	*Bacteria*	AAACTTAAAGGAATTGACGG	Muyzer et al., (1993)

Primer set used to amplify the 16S rRNA gene of *Bacteria*. The use of this primer set provides an amplicon of 550 bp, which has a suitable size for subsequent DGGE analyses. With this purpose, a 40-bp-long GC clamp was added at the 5' end of the forward primer.

AOB (detection)

Primer	Specificity	Sequence (5'-3')	Reference
CTO189F A/B	*Betaproteobacteria* AOB	GGAGRAAAGCAGGGGATCG	Kowalchuk et al., (1997)
CTO189F C	*Betaproteobacteria* AOB	GGAGGAAAGTAGGGGATCG	Kowalchuk et al., (1997)
CTO654R	*Betaproteobacteria* AOB	CTAGCYTTGTAGTTTCAAACGC	Kowalchuk et al., (1997)

Primer set used to specifically amplify 16S rRNA gene of the AOB belonging to the *Betaproteobacteria* subdivision. The CTO forward primer set worked as a 2:1 mixture of A/B and C primers. The resultant PCR products (Ca. 465 bp) were suitable for subsequent DGGE and cloning analyses. With this purpose, a 40-bp-long GC clamp was added at the 5' end of the forward primers.

AOB (quantification)

Primer	Specificity	Sequence (5'-3')	Reference
CTO189F A/B	*Betaproteobacteria* AOB	GGAGRAAAGCAGGGGATCG	Kowalchuk et al., (1997)
CTO189F C	*Betaproteobacteria* AOB	GGAGGAAAGTAGGGGATCG	Kowalchuk et al., (1997)
RT1R	AOB	CGTCCTCTCAGACCARCTACTG	Hermansson & Lindgren (2001)

Primer set used to amplify the 16S rRNA gene of the AOB belonging to the *Betaproteobacteria* subdivision. CTO forward primer set works as a 2:1 mixture of A/B and C primers. The use of these primers provides a 116bp-PCR-product, a suitable size for qPCR analyses.

Planctomycetes-Anammox (detection)

Primer	Specificity	Sequence (5'-3')	Reference
Pla46F	*Planctomycetes*	GGATTAGGCATGCAAGTC	Neef et al., (1998)
Amx368R	Anammox bacteria	CCTTTCGGGCATTGCGAA	Schmid et al., (2003)

Primer set used to semi-specifically amplify the 16S rRNA gene of the anammox bacteria. Amx368R primer amplifies all the known anammox bacteria whereas Pla46F amplifies all *Planctomycetes*. This primer set is mostly used in the detection of anammox bacteria in environmental samples. The resultant PCR products from this primer set had a size of Ca. 322 bp, suitable for subsequent DGGE analyses. With this purpose, a 40-bp-long GC clamp was added at the 5' end of the forward primer.

Anammox bacteria (detection)

Primer	Specificity	Sequence (5'-3')	Reference
Amx368F	Anammox bacteria	TTCGCAATGCCCGAAAGG	Schmid et al., (2003)
Amx820R	*Ca.* Brocadia and *Ca.* Kuenenia	GGGCACTAAGTAGAGGGGTTTT	Schmid et al., (2003)

Primer set used to specifically amplify the 16S rRNA gene of *Ca.* Brocadia anammoxidans and *Ca.* Kuenenia stuttgartiensis populations. The resultant PCR products from this primer set was Ca. 452 bp, suitable for subsequent DGGE analyses. With this purpose, a 40-bp-long GC clamp was added at the 5' end of the forward primer.

Nitrobacter spp. (detection)

Primer	Specificity	Sequence (5'-3')	Reference
FGPS872F	*Nitrobacter*	CTAAAACTCAAAGGAATTGA	Degrange & Bardin, (1995)
FGPS1269R	*Nitrobacter*	TTTTTTGAGATTTGCTAG	Degrange & Bardin, (1995)

Primer set used to specifically amplify the 16S rRNA gene of *Nitrobacter* spp. for phylogenetic analyses. The resultant PCR products from this primer set was Ca. 392 bp, suitable for subsequent DGGE and cloning analyses. With this purpose, a 40bp-long GC clamp was added at the 5' end of the forward primer.

Nitrobacter spp. (quantification)

Primer	Specificity	Sequence (5'-3')	Reference
Nwi70F	*Nitrobacter*	GGCGTAGCAATACGTCAG	Montràs et al., (2008)
Nwi165R	*Nitrobacter*	ATCCGGTATTAGCCCAAG	Montràs et al., (2008)

Primer set used to specifically amplify the 16S rRNA gene of *Nitrobacter* spp. The resultant PCR products were suitable for qPCR analyses (Ca. 95 bp).

Nitrospira spp. (detection and quantification)

Primer	Specificity	Sequence (5'-3')	Reference
NSR1137F	*Nitrospira*	CCTGCTTTCAGTTGCTACCG	Dionisi et al., (2002)
NSR1269R	*Nitrospira*	GTTTGCAGCGCTTTGTACCG	Dionisi et al., (2002)

Primer set used to specifically amplify *Nitrospira* spp. for both phylogenetic and quantification analyses. PCR analyses provide a 151bp-PCR-product, suitable for qPCR analyses.

T3/T7 (detection)

Primer	Specificity	Sequence (5'-3')	Reference
T3		ATTAACCCTCACTAAAGGGA	Lane (1991)
T7		TAATACGACTCACTATAGGG	Lane (1991)

Primer set used for PCR amplification of vectors cloned inside plasmids. The plasmid used in this work (pUC19, Invitrogen) has the primer set binding site surrounding the insert area. The length of the PCR product depends on the insert used for cloning.

PCR PERFORMANCE

General conditions for PCR amplification were defined as described elsewhere (Innis et al., 1990; Giovannoni, 1991). PCR analyses were mainly carried out in total volumes of 50 µL in 200 µL thin wall polypropylene tubes (QSP). A common PCR reaction contained 0.8 mM premixed dNTPs (GeneAmp, Applied Biosystems), 1X Buffer, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Promega), and primers at 0.5 µM. 50 to 150 ng template DNA was used in each reaction, and a non-template control was carried out every time.

PCR mixes were prepared in a laminar flow cabinet AV-100 (Telstar) with an UV illumination for 15 min, previous to the PCR preparation. All the material was sterilized and exclusive for PCR performance. PCR analyses were performed in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems) using the thermal profiles defined in the references found in the primers section.

PCR EFFICIENCY

PCR amplification reactions are in general highly specific, and this specificity is determined by the hybridization primer-target gene. However, the efficiency of amplification can be altered by any parameter or reactive used in the reaction, such as: (i) purity of the reagents, (ii) distance, complementarity, length and sequence of the primers, (iii) quantity and purity of the DNA template, (iv) type of *Taq* DNA polymerase (v) $MgCl_2$ and dNTPs concentration and (vi) temperature and length of denaturing, annealing and extension steps.

Moreover, the efficiency of the PCR can also be improved by performing a nested-PCR. In a nested-PCR, two consecutive PCRs are performed with different primer sets. In a first PCR round, a wide-range primer set amplifies a large amplicon. Then a second PCR (second round) is carried out using this large amplicon as a template, but applying another primer set (commonly a more specific one) that produces a second amplicon shorter than the first one. Nested-PCR performance increases the sensitivity compared with conventional PCR, but the technique has some drawbacks. It increases the PCR

inherent biases, skewing the distribution of PCR products due to unequal amplification (more PCR products from the dominant bacterial species than the non-dominant), and besides, longer assay times are required since two PCRs are performed.

ELECTROPHORESIS METHODS

AGAROSE GELS

PCR products and DNA isolation efficiencies were checked with horizontal electrophoresis in agarose gels of different concentrations, depending on the experimental needs. Agarose concentrations in Tris Borate EDTA (TBE) 0.5x buffer ranged from 0.8% to 1.5% (w/v). The former concentration was used for checking DNA isolations and the latter for the PCR products.

After the electrophoresis, DNA has to be stained because it is a colorless molecule. Nowadays ethidium bromide (EtBr) is still the most commonly staining for DNA detection. This molecule is a DNA intercalator, inserting itself into the spaces between the base pairs of the double helix. EtBr has absorbance peaks at 300 and 360 nm and emits the yellow/orange fluorescence at 590 nm. EtBR is a potent mutagen, but nonetheless the sensitivity, simplicity and nondestructive nature of EtBr staining makes it the standard staining for dsDNA.

The procedure of horizontal electrophoresis is described below.

- *Melt the agarose in a microwave oven. Make sure the agarose is completely melted.*
- *Pour the melted agarose into casting gels containing a comb (or combs) to form the wells.*
- *Wait for some minutes to let the gel cool until the agarose is completely solid and the wells are formed. Carefully removed the comb by lifting it.*
- *Take the cooled gel and put it in a MINI-SUB CELL GT electrophoresis chamber (Bio-rad). Cover it completely with TBE 0.5X buffer.*
- *Load the samples mixed with loading buffer into the wells using a micropipette. Load the molecular weight ladder in a separated well. A 1 kb DNA ladder (Invitrogen) is usually used for checking the efficiency of DNA isolations (ranging from 250 bp to 10000 bp) and a 100 DNA bp ladder (Invitrogen) is used for checking PCR products (ranging from 100 to 1000 bp).*

- *Connect the electrophoresis chamber to a power supply POWER PAC 300 (Bio-rad) and apply 100 V for 30 min.*
- *Incubate the gel into 150 mL of TBE 0.5X buffer with 12 µL EtBr (10 mg mL^{-1}) (Bio-rad) for 20 min.*
- *Place the gel on a UV transilluminator to visualize DNA bands. The stained DNA with EtBr will appear bright orange on a pale orange background. Capture the DNA gel by digitalizing the image (Scion Image software, TDI).*

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE is an electrophoretic technique that can be used to achieve a genetic fingerprint that provides a pattern or profile of the genetic diversity for a specific gene in a microbial community (Muyzer and Smalla, 1998). This method allows to discriminate among DNA sequences having the same length but differing in base composition (Muyzer et al., 1993) and therefore it can be used to perform the electrophoretic separation and screening of heterogeneous PCR product mixtures. DGGE is adequate for analysis of a large number of samples but it provides less resolution of the microbial community diversity than cloning.

DGGE is based on the separation of the partially melted PCR products (not over 700 bp) in polyacrylamide gels containing linear gradient of DNA denaturants. This gradient forces the denaturing of the dsDNA to be partially denatured. Thus, the electro-phoretic mobility of DNA molecules drastically decreases because they change from a bind, helical structure to a partially melted molecule. DNA molecules with different sequences have different behaviours in front of a denaturing agent concentrations, so every sequence stops migrating at a specific position along the gel (Muyzer and Smalla, 1998).

One of the two primers has to contain a GC rich sequence, so-called GC-clamp, at the 5’ end. This clamp avoids the complete denaturing of the DNA molecule along the electrophoresis, which keeps the sample steady at a concrete position of the gel while the rest of samples are still migrating to achieve their final location. The composition of the GC-clamp used in this work was:

5’ CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C 3’

The denaturing gradient for each DGGE performance is a variable parameter which basically depends on the size and sequence of the fragments to be processed. Thus, wide denaturing ranges allow discriminating among fragments containing very different sequences, whereas narrow ranges are used to distinguish fragments whose sequences differ only in a few nitrogenated bases. With the use of the GC-clamp, DGGE may be able to detect nearly all possible nucleotide variations in any given sequences (Sheffield et al., 1989).

DGGE analyses were carried out in an INGENY PhorU2 System (INGENY) and different DGGE linear gradient of denaturing agents were used depending on the primer set (table 2.3).

Table 2.3: Summary of the denaturing gradients used depending on the primer set and the chapters of the result section where they carried out DGGE.

Primer set	Vertical denaturing gradient		Chapter
Pla46F - Amx368R (56°C)	30%	70%	II
Pla46F - Amx368R (64°C)	30%	70%	II
Amx368F - Amx820R	30%	70%	II
357F - 907R	40%	80%	III
CTO189F (A/B/C) – CTO654R	30%	70%	IV

DGGE performance is tough and the user should be skillful. The DGGE preparation is described below, divided in different sections.

Gel casting

- *Assemble the glasses with the spacers into the electrophoresis cassette structure.*
- *Prepare the acrylamide solution with the denaturing agents (table 2.4). Two solutions must be prepared to cast a 6% (w/v) polyacrylamide gel, combining the low denaturant concentrated (20 or 30%) with the high denaturant concentrated (70 or 80%).*
- *Add the ammonium persulfate and TEMED to both denaturant solutions in the last minute because they are the responsible of the quick polymerization of the acrylamide.*
- *Pour the two solutions in the gradient maker and then by the use of a peristaltic pump introduce the vertical gradient into the electrophoresis cassette. Stop the flow when the solutions are approximately 2 cm under the comb level.*
- *Let the gel polymerize for at least 1 h at room temperature.*

- *Place the comb and prepare the stacking solution. This solution has the same composition as the other solutions but without urea and formamide (table 2.4). Its function is to ensure that once the electrophoresis is running, the PCR products of different sizes enter at the same time into the denaturing gradient. Add 10 mL of the stacking solution until filling up the gel chamber.*

Table 2.4: *Concentration of the different solutions used for DGGE gel casting. All concentrations were calculated assuming a final volume of 25 mL.*

	0%	**20 %**	**30%**	**70%**	**80 %**
Urea (g)	0	2.10	3.15	7.35	8.40
Formamide (mL)	0	2	3	7	8
TAE 50x buffer pH 7.4 (mL)	0.5	0.5	0.5	0.5	0.5
Acrylamide/Bisacrylamide 40% (mL)	3.75	3.75	3.75	3.75	3.75
Ammonium persulphate 10% (µL)	150	150	150	150	150
TEMED (µL)	15	15	15	15	15
Milli-Q water (mL)	20.75	18.75	17.75	13.75	12.75

Sample loading and gel running

- *Put the electrophoresis cassette inside the electrophoresis tank with 17L of Tris Acetate EDTA (TAE) 1x buffer preheated to 60°C.*
- *Prepare the samples with loading buffer. Carefully remove the comb and load all the sample into the wells with special thin tips (Sorenson).*
- *Close the lid while paying attention to connect the negative and positive electrodes.*
- *Run the gel for 30 min with gentle TAE 1x buffer flow in the upper compartment. When the samples are all in the stacking gel then the TAE 1x buffer flow into the tank can be more vigorous.*
- *Run the gel for 14 h at 140 V.*

Gel staining

- *Incubate the gel in 500 mL of TAE 1x buffer with 1x SYBR Gold Nucleic Acid Gel Stain solution (Invitrogen) for 40 min under agitation.*
- *Place the gel on a UV transilluminator to visualize DNA bands. The stained DNA with SYBR Gold will appear bright orange without background. Capture the DNA gel by digitalizing the image (Scion Image software, TDI).*

DNA recovering

- *Assemble the XcitaBlue filter (Bio-rad) over the UV transilluminator to protect the user and the DNA. The user must also wear polarized goggles.*
- *Cut the desired band with a sterile scalpel.*

- *Place each acrylamide band in a 1.5 mL tube, add 50 µL of Milli-Q water and incubate overnight at 4°C or incubate at 65°C in a block heater (SBH130D Block Heater, Stuart) with periodical vortex steps.*

CLONING

Microbial communities can not always be properly characterized by DGGE procedure. In some cases, the whole 16S rDNA sequence of the microbial communities is required to provide robustness to the phylogenetic analyses. Therefore, DGGE can not be performed and cloning is recommended to screen the bacterial community. However, cloning is more time-consuming than DGGE and thus the analysis of larger sets of samples becomes more laborious; for example, when monitoring changes in natural microbial communities over time, particularly if multiple samples are required (Sanz and Köchling, 2007).

Previous PCR amplification of a specific gene is required for cloning. PCR product should be firstly inserted in a plasmid, and subsequently transfected into competent *E.coli* cells by using a thermal shock, which forces them to open their cell walls. The competent *E.coli* cells are grown in Petri dishes, yielding colonies that carry at the most one single plasmid. By selecting the colonies and growing them independently, every plasmid can be obtained individually and thus the mixture of phylotypes from the microbial community can be identified.

OneShot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen) together with the plasmid pUC19 (Invitrogen) were used in this work. This plasmid contains antibiotic resistance markers to help discriminating the transfected *E.coli* cells by growing them in ampicillin-containing media. The cloning procedure has different steps:

Cloning performance. Ligation

- *Insert the PCR product into the vector. Mix in a 0.2 mL tube the following reagents:*
    - *1 µL of salt solution.*
    - *1 µL of pUC19 plasmid.*
    - *1 µL of the PCR product.*
    - *2 µL of water.*

- *Incubate for 30 min at room temperature.*

Transformation

- *Briefly centrifuge the vial containing the ligation reaction and place on ice.*
- *Thaw the S.O.C. medium (Invitrogen) at room temperature.*
- *Thaw, on ice, one vial of the competent cells for each ligation for 30 min.*
- *Transfer 2 µL of the ligation reaction directly into vial of competent cells and mix gently.*
- *Incubate on ice for 20 min.*
- *Incubate for exactly 30 secs in a 42°C water bath. Do not mix or shake.*
- *Remove vial from the water bath and place it on ice for 2 min.*
- *Add 250 µL of pre-warmed S.O.C. medium (provided by the manufacturer).*
- *Incubate the vial at 37°C for 1 h in a shaking incubator at 225 rpm.*
- *Spread off 50 µL and 200 µL of the transformation product onto two separate Luria Bertani (LB) plates (Scharlab) and ampicillin (Sigma-Aldrich).*
- *Incubate the plates overnight at 37°C.*

Plasmid recovering

- *Prepare tubes of 5 mL of LB (Scharlab) with ampicillin (50µL mL^{-1}) (Sigma-Aldrich).*
- *Touch as much as colonies as required with sterile toothpicks, and put each one into separate tubes.*
- *Incubate the tubes at 37°C overnight in a shaking incubator at 225 rpm.*

Plasmid isolation and purification

The presence of the plasmid in the ampicillin-resistant *E.coli* cells was checked by PCR. The same primer set and PCR thermal profile were used with 1 µL of the homogenized culture as a template. The presence and the correct size of the plasmids was checked in a 1.5% (w/v) agarose electrophoresis. Although the PCR products obtained from the homogenized medium can be used for sequencing, it is recommended to isolate the whole plasmid from the *E.coli* genomic DNA. The commercial kit Ultraclean 6 minute Mini Plasmid Prep Kit (Mobio) was applied in this work to isolate plasmids in a quick and reliable assay.

The Ultraclean 6 minute Mini Plasmid Prep Kit protocol is described below.

- *Add up to 2 mL of liquid culture to a 2 mL tube and centrifuge for 1 min at 16000 g.*
- *Decant the supernatant by inverting the tube and centrifuge for an additional 5 secs at 16000 g.*
- *Remove any remaining liquid from tube with a pipette tip.*
- *Resuspend the cell pellet in 50 µL Solution 1 by bump vortexing 1 min.*
- *Add 100 µL of the Solution 2 to lyse the cells. Gently invert up to 8 times to mix.*
- *Add 325 µL of the Solution 3 to re-nature the vector to its double stranded form. Invert up to 8 times to mix.*
- *Centrifuge 1 min at 16000 g.*
- *Transfer supernatant to Spin Filter by pippeting and centrifuge 30 secs at 10000 g. Discard the flow-through.*
- *Add 300 µL of the Solution 4 to bind the DNA to the Spin Filter. Centrifuge 30 secs at 16000 g.*
- *Discard flow-through and centrifuge again for 30 secs.*
- *Transfer the Spin Filter to a new 2 mL tube. Add 50 µL of the Solution 5 to elute the plasmid.*
- *Centrifuge 30 secs at 10000 g.*

SEQUENCING and PHYLOGENETIC ANALYSIS

All the sequences recovered from DGGE and cloning procedures or from direct PCR amplification were purified and sequenced by external facilities (Macrogen Inc., www.macrogen.com). DNA recovered from DGGE bands was sequenced with the same primer sets used in their previous amplification (without the GC-clamp in the forward primer) whereas inserts from cloning were sequenced using T3 and T7 primers. A minimum volume of 30 µL and concentration of 100 ng μL^{-1} per sample were the requirements for an optimal sequencing.

ANALYSIS of DNA SEQUENCES

All the retrieved 16S rRNA sequences were firstly checked by Chromas 1.45 (McCarthy, 1998) and BioEdit Sequence Alignment Editor v7.0.5 (www.mbio.ncsu.edu/Bioedit/bioedit.html). Both software were helpful to check the quality of the sequences and to do a previous alignment. After alignment, sequences were trimmed off to create FASTA-formatted template files for subsequent analyses. Afterwards, all the retrieved

sequences were submitted and compared for closest relatives at the National Center for Biotechnology Information (NCBI) database using the BLASTn algorithm tool (Altschul et al., 1990).

Every FASTA-formatted template file was uploaded into MOTHUR v.1.29.2 (Schloss et al., 2009) and sequences were aligned using SILVA bacterial database (release 102) as reference alignment. After alignment, triangular distance-matrices were generated for 16S rDNA sequences to calculate the OTU clustering. The cutoff values of the sequences were determined by applying a 0.03 cutoff, meaning that the homology among them was less than 97%. Representative sequences were defined for each OTU with the tool implemented in MOTHUR. Besides, taxonomic identification was also defined using the SILVA bacterial database as template. The presence of chimera in all the sequences was checked with the specific tool included in this software.

Phylogenetic trees were mainly constructed with representatives of each OTU and with closely related sequences (predominately from culture members) that were recovered from NCBI database. Most of the phylogenetic trees were generated and represented by MEGA V5 package (Tamura et al., 2011). Tree topology and phylogenetic distances were computed using maximum likelihood method using Jukes-Cantor as a substitution model. Bootstrap analyses were performed with 1.000 repetitions, and only values higher than 50% are shown in the phylogenetic trees.

A single phylogenetic tree was calculated by ARB software package v.5.5 (www.arb-home.de) (Ludwig et al., 2004). In this case, the aligned FASTA-formatted template with the sequences gauged by MOTHUR was imported into the ARB software. The sequences were added to the backbone phylogenetic tree through the quick add marked tool to obtain their affiliation to the taxonomic groups defined in the ARB database.

SUBMISSION to ONLINE DATABASE

228 16S rRNA sequences were retrieved from the phylogenetic studies along this work. These sequences were published in GenBank, EMBL and DDBJ databases (table 2.5).

Table 2.5: Accession numbers for 16S rDNA sequences according to their definition and the chapter where they were retrieved.

Definition	Accession number From:	Accession number To:	Chapter
Uncultured bacterium partial 16S rRNA gene, isolate ANAMMOX (DGGE)	AM900561	AM900588	Chapter III
Uncultured bacterium partial 16S rRNA gene, isolate (DGGE)	AM905095	AM905116	Chapter II
Uncultured bacterium partial 16S rRNA gene, isolate band ANAMMOX (DGGE)	AM905117	AM905145	Chapter II
Uncultured *Nitrosomonas* sp. partial 16S rRNA gene (clone)	FM997762	FM997835	Chapter IV
Uncultured alpha proteobacterium partial 16S rRNA gene (clone)	FM998112	FM998169	Chapter IV

QUANTITATIVE PCR (qPCR)

Quantitative PCR (qPCR) allows the quantification of initial amounts of template in a PCR reaction by directly monitoring the synthesis of new amplicons. This technique requires a fluorescent reporter that binds to the product formed and at the same time reports its presence by emitting fluorescence. Two different approaches can be performed according to the kind of fluorescent reporter: a dye-labelled probe (e.g. Taqman) or dsDNA-binding dye (e.g. SYBR Green), which are considered as a specific label and a non-specific probe, respectively. SYBR Green was the fluorescent reported used in all the qPCR analyses carried out in this work. The basis of the SYBR Green dye performance is that it does not have virtually fluorescence when it is free in solution but it becomes brightly fluorescent when it binds to dsDNA (fig. 2.7).

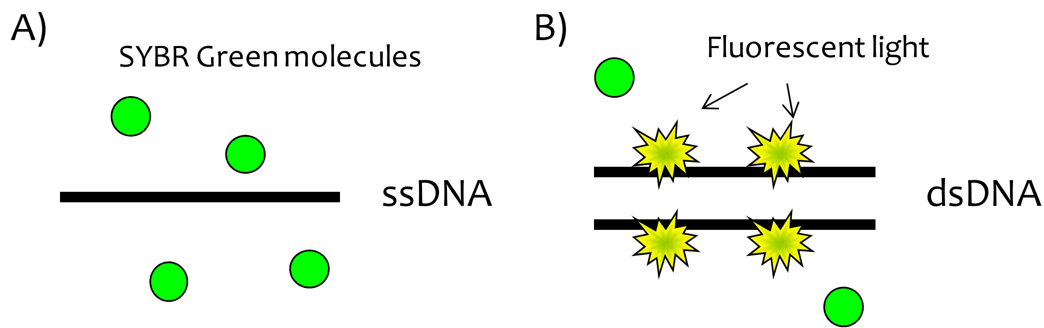


Figure 2.7: The SYBR Green molecule has high affinity to dsDNA. A) The dyes are free in the solution and do not emit fluorescence. B) SYBR Green fluorescence occurs as soon as it binds to dsDNA. Adapted from Kalland and collaborators (2009).

The fluorescence emission is proportional to the synthesized DNA, and it is collected by a photomultiplier tube (PMT) collector in the qPCR thermocycler. This fluorescence emission can be represented as an amplification curve showing three different phases (fig. 2.8):

- **Initiation phase**. It takes place during the first cycles of PCR, when the measured fluorescence does not surpass the baseline for its detection because the amount of DNA was too low.
- **Exponential phase**. An exponential increase of the emitted fluorescence can be observed, proportional to the amount of synthesized DNA.
- **Plateau phase**. In this last phase the signal levels off and saturates, indicating that DNA amplification has stopped.

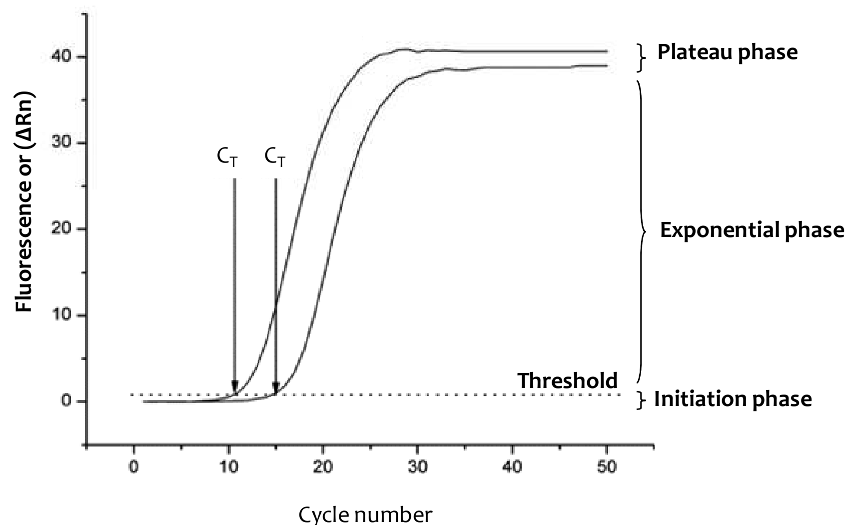


Figure 2.8: Model of a qPCR amplification curve showing the three phases. Adapted from Kubista and collaborators (2006).

The fluorescence values obtained during the initiation phase are used to calculate the threshold. The Threshold Cycle (C_T) corresponds to the first cycle at which the fluorescence can be detected, and thus it is the point to start the exponential phase. The increase in fluorescence (when represented in an exponential scale) is lineal and, therefore, proportional to the amount of DNA generated. As higher the initial DNA concentration of the target, as lower the C_T value.

The number of copies of the gene amplified from a sample can then be quantified by interpolating its C_T value in a linear standard curve of C_T values. This standard curve is previously built by performing the qPCR with 10-fold dilutions of quantified, known standards, and calculating a series of C_T values corresponding to different amounts of initial template DNA.

The fluorescence of the dye can include some sort of primer-dimer[2] artefacts, which interfere in the formation of specific amplicons. A control must be done with the addition of melting curve analysis after completing the qPCR assay, with the purpose of determining the maximum melting temperature of the amplicon and detecting the presence of possible primer-dimer artefacts. Since the latter are typically shorter than the targeted product, they melt at a lower temperature and their presence is easily recognized by melting curve analysis.

OBTENTION of STANDARD CURVES

Standard curve templates can be obtained from a variety of sources. In microbial ecology there are two common ways to obtain them. The first is to obtain genomic DNA containing the gene (standard) from a pure culture, obtained from environmental samples or from a culture collection (e.g. DSMZ). The second approach is to clone the gene into transformed *E.coli* competent cells, recover the plasmid (with the targeted gene) and quantify the number of gene copies by spectrophotometric methods.

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[2] Undesirable primer hybridization with other primer due to complementary bases, causing a loss of the efficiency of the PCR assay.

Since all the chemolithotrophic bacteria under study in this work are considered uncultured bacteria, the second strategy was found to be the most suitable. PCR products were obtained using the quantification primer sets (described in the primer set section) and they were cloned inside *E.coli* competent cells. 20 colonies were randomly selected from the clone library for each target and the plasmid was isolated by using the Ultraclean 6 minute Mini Plasmid Prep Kit (Mobio). After the isolation of the plasmids, they were sequenced with primers T3-T7 by Macrogen (Macrogen Ltd., Seoul Korea, www.macrogen.com) to obtain the sequences of the DNA fragments corresponding to the inserts. Primer sequences specific for quantification assays were compared to the respective target sequences in the inserts. This comparison was done to search clones without mismatches with the primer set sequence, since their presence could underestimate the quantification of the gene (Sipos et al., 2007). After checking the suitability of the clones, the concentration of the plasmids (containing the inserts) was quantified by Nanodrop UV/VIST N-1000 (Thermo). DNA concentrations obtained from spectrophotometric analyses were used to estimate the concentrations of molecules of each insert, according to Labrenz and collaborators (2004):

$$Copy\ number\ \mu L^{-1} = \frac{DNA\ concentration\ (g\ \mu L^{-1})}{(Weight\ of\ plasmid)\ x\ (Weight\ dsDNA\ bp)}\ x\ Avogadro\ number$$

Assuming that Avogadro number is 6.023E+23, and the weight of one bp of dsDNA is 660 Da. The weight of the plasmid is calculated including the insert.

Standard curves for each target were constructed from series of 10-fold dilutions of each insert until only less than 10 copies/well were added. Each standard curve was obtained from a minimum of 6 different concentrations of the insert to give consistence to the quantification. The PCR efficiency (*E*) of each standard curve was calculated according to $E = 10^{(-1/slope)}$ (Kubista et al., 2006).

qPCR PERFORMANCE

DNA template in the reaction mixtures was amplified and monitored with an ABI prism SDS 7300 system (Applied Biosystems). All the measurements (both standards and samples) were done in triplicate. Several 10-fold dilutions of each sample were quantified to assess for inhibition. Each PCR mixture (10.6 µL) was composed of 5.6 µL of 1x SYBR Green PCR master mix (Applied Biosystems), 0.3 µL of 10 µM forward and reverse primers and 4.4 µL of template, in MicroAmp Optical 96-well reaction plates (Applied Biosystems) with optical caps (Applied Biosystems). The qPCR thermal profile for AOB, *Nitrobacter* and *Nitrospira* were performed as it follows: 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. A dissociation stage was added at the end to check the specificity in the amplification of the primer set.

FLUORESCENCE *in situ* HYBRIDIZATION (FISH)

FISH was firstly developed by Amann and collaborators (1990) and it is based on the use of fluorescently-labelled probes targeting 16S rRNA gene and 23S rRNA gene sequences, thus allowing the identification, localization and quantification of microorganisms in almost every ecosystem. The 16S rRNA gene was chosen as almost a perfect target. All cells require ribosomes for translation and they are distributed homogenously in the cytoplasm. The probes are oligonucleotides that hybridize with a specific target due to complementarity of bases. They are also bound to fluorophores that emit fluorescence at a concrete wavelength when they are excited at a certain wavelength.

One of the main advantages of the FISH is based on the application of multiple probes to same target, giving a combination of colours, for accurate identification of the different target cells. For instance, if anammox bacteria is specifically hybridized with Cy3 (giving red coloration) and *Bacteria* are stained with Cy5 (giving a virtual blue coloration), the anammox bacteria will be depicted in rose, whereas the rest of bacteria will have blue staining.

The acquisition of the anammox bacteria with Amx820 probe (table 2.6) was done with two different microscopes. Observation of regular FISH analyses was performed in an Axioskop (Zeiss) epifluorescence microscope with ProgRes software (Jenoptik), located in the Laboratory of Molecular Microbial Ecology (Girona). The location of the anammox bacteria inside aggregates and their structure were studied with a Leica TCS 4D Confocal Scanning Laser Microscope (CSLM), located in the Microscopy Services of the Universitat Autònoma de Barcelona (UAB).

Table 2.6. Information of the anammox probe used for FISH analyses.

Probe	Fluorophore	Specificity	Sequence (5'-3')	% Form	Reference
Amx820	Cy3	*Ca.* Brocadia and *Ca. Kuenenia*	AAAACCCCTCTACTTAGTGCCC	40	Schmid et al. (2000)

FISH performance has different steps, described below.

Preparation of the fixation buffer

- *Heat 40 mL of Milli-Q water in the microwave oven until 55-65°C.*
- *Add 2 g of paraformaldehyde (PFA) and 150 µL of NaOH and stir until the PFA is completely dissolved.*
- *Add 5 mL of phosphate-buffered saline (PBS) 10x.*
- *Adjust pH to 7.2-7.4 with HCl.*
- *Adjust to a final volume of 50 mL with Milli-Q water.*
- *Store at 4°C for a immediate use.*

Sample fixation

- *Mix the sample with the fixation buffer (1:3 v/v)*
- *Incubate for 3-12 h at 4°C. Avoid prolonging incubation more than 12 h, since long incubation may produce sample autofluorescence.*
- *Centrifuge for 1 min at 8000 rpm and discard supernatant.*
- *Wash twice with cold PBS 1x and centrifuge 1 min at 8000 rpm.*
- *Discard the supernatant and resuspend the pellet in PBS 1x buffer:ethanol 96% solution (1:1).*
- *Store at -20°C.*

Slide preparation

- *Dispense 5 µL of the fixed sample in each slide well and let dry for 20 min at 46°C.*
- *Dehydrate the sample by following three consecutive steps with a series of alcohols: 50%, 80%, 96% for 3 min each.*
- *Dry the sample for 2 min at 46°C in a oven.*

Hybridization

- *Prepare hybridization buffer (over 1 mL):*
    - *180 µL NaCl 5M*
    - *Volume of formamide required for the probe (i.e 300 µL for a 30% of formamide)*
    - *20 µL Tris-HCl 1M at pH 7.4*
    - *(800 µL- µL of formamide) of Milli-Q water*
    - *1 µL of sodium dodecyl sulphate (SDS) 10%.*
- *Add 9 µL of hybridization buffer to each well, on the dehydrated sample.*
- *Add 25-50 ng of the probe over each slide well. Fluorochromes are photodegradable, so it is recommended to work in the dark.*
- *Put carefully the slide into the hybridization chamber and incubate for 2 h at 46°C. To avoid evaporation of the hybridization buffer, a soaked paper is added into the hybridization chamber.*

Washing and DAPI staining

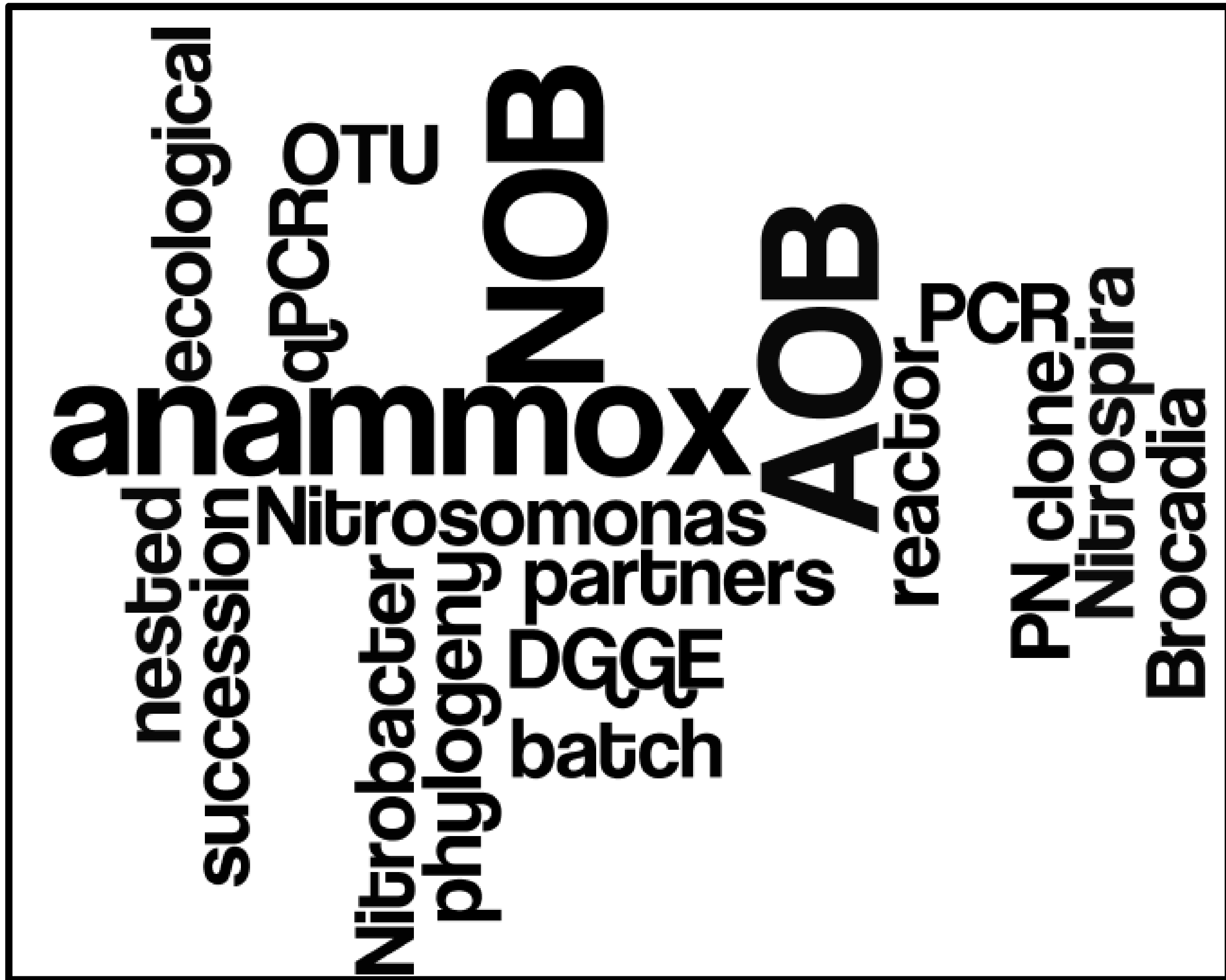
- *Prepare the washing buffer (over 50 mL):*
    - *x µL of NaCl (table 2.5).*
    - *1 mL of Tris-HCl 1M at pH 7.4.*
    - *0.5 mL of EDTA 0.5M at pH 8.0.*
    - *Adjust to a final volume of 50 mL with Milli-Q water.*
    - *50 µL SDS 10%.*

Table 2.7: *Summary of the NaCl requirements of the washing buffer in regards to the % of formamide.*

% formamide	[NaCl] mM	NaCl 5M (µL)
0	900	8900
5	636	6260
10	450	4400
15	318	3080
20	225	2150
25	159	1492
30	112	1020
35	80	700
40	56	460
45	40	300

50	28	180
55	20	100
60	14	40

- *Transfer the slide into a 50 mL tube containing preheated washing buffer and incubate for 10-15 min at 48°C.*
- *Remove the slide and wash it gently for 3 secs with cold Milli-Q water. Allow it to air-dry.*
- *Add 1 µL of 4',6'-diamidino-2-phenylindole (DAPI) 0.001% solution and let it for 30 min under dark conditions.*
- *Wash it gently with cold Milli-Q water.*



Results & Discussion

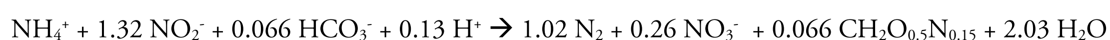
Chapter I

Anammox bacteria enrichment in batch cultures

3.1.1 BACKGROUND

Nitrogen is one of the major nutrients that wastewater biological treatments attempt to eliminate. Biological nitrogen removal is traditionally achieved by a combination of aerobic autotrophic nitrification and anaerobic heterotrophic denitrification processes (Metcalf&Eddy, 2004). Nevertheless much attention has been recently devoted to the anaerobic oxidation of the ammonium, where it is chemolito-trophically oxidized with nitrite as the electron acceptor, without requiring either organic matter or O_2, and both are finally converted into N_2.

The anaerobic ammonium oxidation was experimentally demonstrated and documented in a denitrifying pilot plant at Gist-Brocades (Delft, The Netherlands) by Mulder et al. (1995). Later, Strous and collaborators (1999a) firstly identified one of the bacteria responsible of the anammox activity (*Ca.* Brocadia anammoxidans), belonging to the order *Planctomycetales*, and described their physiological requirements (such as optimal temperature and pH), growth features (doubling time) and stoichiometry (Strous et al., 1999b), according to the following equation:



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

In the early stages of their study, the location of anammox bacteria populations in nature was barely known. Originally activated sludge from WWTPs were found to be suitable seeds that gathered all the conditions to find anammox bacteria (Strous et al., 1998; Egli et al., 2001). The continuous collection in the same environments usually lead to obtaining *Ca.* Brocadia anammoxidans and *Ca*. Kuenenia stuttgartiensis enrichments (Fujii et al., 2002; Chamchoi and Nitisoravut, 2007; Tsushima et al., 2007b). *Ca.* Scalindua wagneri was also initially found from an activated sludge (Schmid et al., 2003), but it was through following studies in marine environments (Kuypers et al., 2003; 2005) that novel species belonging to this genera (and related to other ones) were identified. Nowadays, their presence in the freshwater environments is also becoming remarkable (Schubert et al., 2006;

Hamersley et al., 2009). Thus, after more than ten years of investigation, the natural environments can probably be considered as the most important sites to retrieve different populations of anammox bacteria (Penton et al., 2006; Zhang et al., 2007; Hu et al., 2011).

The strategy used for the achievement of the enrichments is as important as the seed location. Different strategies can be used to enrich them, mainly involving pilot plants or bioreactors favouring anammox growth, which require great investments (Dijkman and Strous, 1999; van Dongen et al., 2001; Sliekers et al., 2002; Vlaeminck et al., 2009a), or using batch cultures, which can allow to screen multiple environments but are more time-consuming (Suneethi and Joseph, 2011).

The present chapter describes the successful anammox bacteria enrichment in batch cultures of sediments and sludge from different origins. The main aim of this work was to investigate the presence of anammox populations in different environmental samples (natural, man-made or modified systems) by chemical and molecular techniques. Furthermore, by achieving these anammox enrichments, they should be useful to be used as inocula for starting-up an anammox bioreactor, fed by a PN reactor (Ganigué et al., 2007) designed to remove ammonium from urban landfill leachates (López et al., 2008).

The methodological approach of this chapter consisted in collecting seeds from several locations and enriched them at 37°C, under anaerobic conditions, in Erlenmeyer flasks with a mineral medium modified from the one used by van de Graaf and collaborators (1996). Small volumes of the medium were extracted periodically from the batch cultures to monitor the nitrite, nitrate and ammonium by ionic chromatography and distillation. PCR and FISH analyses (with Pla46F-Amx36R primer set and Amx820 probe, respectively) allowed to identify the bacteria responsible of the anammox activity and to observe their *in situ* distribution in all the successful batch cultures. Most of the enrichments were maintained more than one year and some of them were ended after a 3 years-lifespan.

3.1.2 RESULTS

ENRICHMENT CULTURES

A wide range of environments was screened to look for the diversity of anammox species. Three different kinds of sources were defined as anammox bacteria suitable environments: natural environments, modified systems and man-made systems. From here on, several seeds from these sources were selected as potential environments although anammox bacteria activity was not previously evident. They should have specific physicochemical features such as concomitant concentrations of ammonium and nitrite in anaerobic and/or microaerophilic conditions. Besides, sediments, soils and activated sludge were preferred in front of water column samples or leachates because the former could perform a better sedimentation inside the enrichment. A proper sedimentation allowed a correct extraction of the liquid medium when necessary.

Samples from a freshwater lake (Toplitzsee), marine sediments (Manga del Mar Menor) and sediments from the deepest zone of a brackish coastal lagoon (La Massona) were used as natural environment sources. The diversity of natural seeding sources were intended to increase the probabilities to enrich the variety of known anammox bacteria, especially focused on the finding of *Ca.* Scalindua-like species. Sediments collected in an artificial constructed wetland (Empuriabrava) were included in the category of the modified systems. The vessels from this wetland were connected to the WWTP for the application of the tertiary treatment. Sludge from different WWTP digesters (Orís, Taradell and Sils-Vidreres) and anoxic experimental SBR systems from the Laboratory of Chemical and Environmental Engineering (LEQUIA) in the University of Girona (UdG) were also used, as man-made system samples. The latter sludge were presumably the most suitable source of *Ca.* Brocadia and *Ca.* Kuenenia-like species since some of them were already working at anoxic conditions and contained proper concentrations of different N-compounds.

Each batch culture was enriched with a single seed but some of them were seeded using the same inocula. Enrichment 3/4 and 3bis/4bis had the same seed but a washing step was performed in the latter prior to start-up the enrichment process. Additionally, the enrichment 3 (E3) was split after 443 days of incubation to ensure its viability, and the culture was re-named as enrichment 10 (E10).

CHEMICAL MONITORING

Periodical chemical analyses of nitrite, nitrate and ammonium were performed using small volumes of the mineral medium from the batch cultures. This screening of the N-compounds was carried out during more than one year for each batch culture. Different processes were detected throughout the enrichment period and it was necessary to pay attention to the behaviour of N-compounds to discriminate among them. Ammonium, nitrate and nitrite were measured along a large incubation period in all the batch cultures to be able to discern anammox activity in front of the other main processes involving N-compounds (nitrification and denitrification) (fig. 3.1.1).

Denitrification was expected to produce nitrite or nitrate consumption without any ammonium removal (fig. 3.1.1a) whereas nitrification involves a clear nitrate production coupled with ammonium and nitrite elimination (fig. 3.1.1b). In batch cultures with anammox activity, the nitrite consumption should be slightly higher than ammonium decrease and also a small nitrate formation was expected (fig. 3.1.1c).

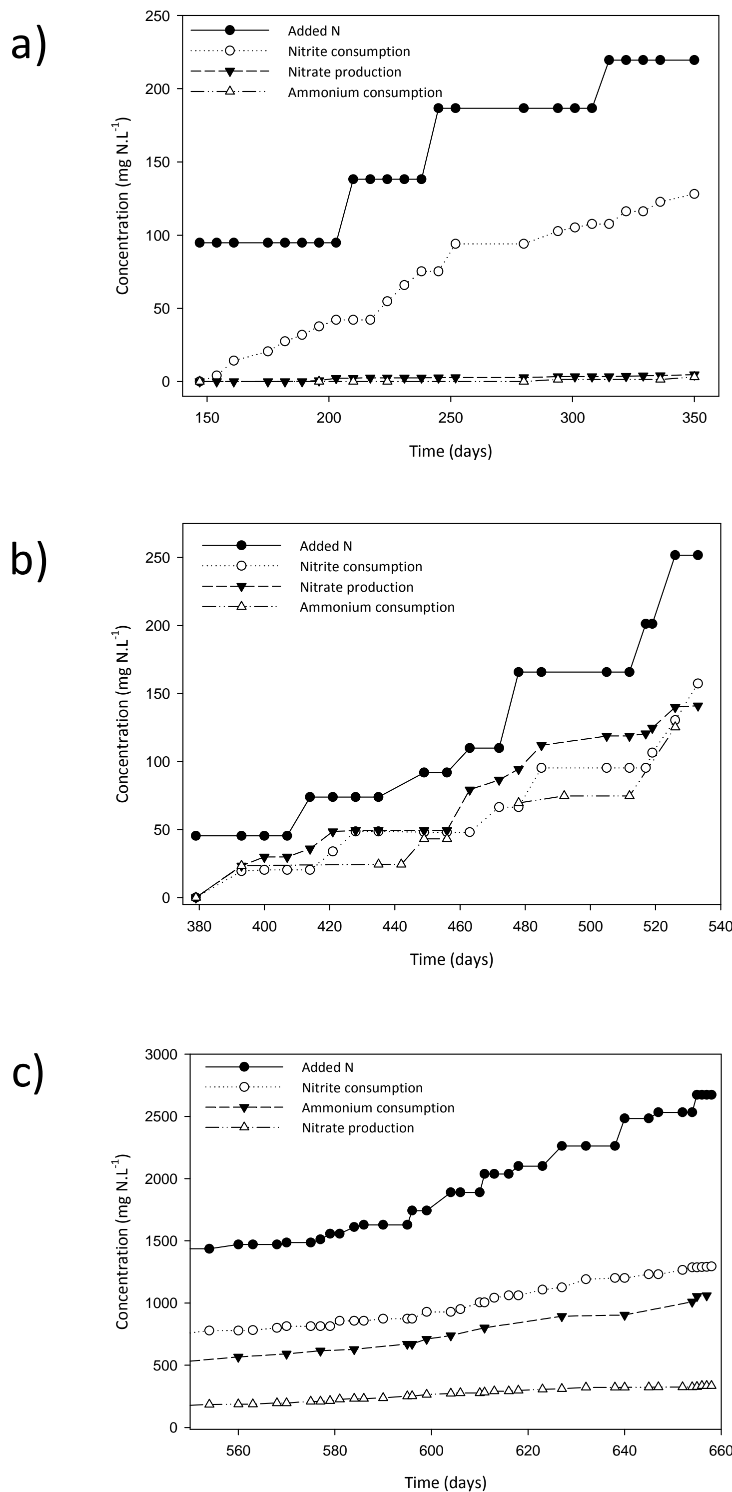


Figure 3.1.1: Progression of cumulative N-compounds in three different batch cultures that have been attributed respectively to (a) denitrification, (b) nitrification and (c) anammox processes. The overall N added (as NH_4Cl and $NaNO_2$ in different ratios, according to the limitations produced by their dynamics) was also showed.

After the long follow-up, five enrichments showed a clearly nitrite and ammonium consumption together with a continuous nitrate formation (table 3.1.1). The N-compounds relationship was calculated and agreed with the theoretical anammox stoichiometry described by Strous and collaborators (1999b), confirming their anammox activity. The enrichments came from different seeds: enrichment 5 (E5), from a natural environment (a brackish coastal lagoon); enrichment 6 (E6), from a modified system (a constructed wetland system); and the rest from man-made systems (an anoxic SBR and WWTPs).

Table 3.1.1: Metabolic activities detected in the enrichments from changes in N-compounds.

Enrichment	Origin	Ammonium	Nitrite	Nitrate	Metabolism
1	Harbour sediment	0	↓	↓	Denitrification
2	Alpine freshwater sediment	0	↓	0	Denitrification
3	SBR system A (washed with medium)	↓	↓	↑	Anammox
4	SBR system B (washed with medium)	↓	↑	0	Nitritation
3bis	SBR system A (washed with water)	↓	0	0	Sulphate reduction
4bis	SBR system B (washed with water)	↓	↑	↑	Nitrification
5	Brackish coastal lagoon	↓	↓	↑	Anammox
6	Constructed wetland system	↓	↓	↑	Anammox
7	Taradell WWTP sludge	0	↓	0	Denitrification
8	Orís WWTP sludge	↓	↓	↑	Anammox
9	SBR system	0	↓	0	Denitrification
10	Enrichment 3	↓	↓	↑	Anammox
11	Sils-Vidreres WWTP sludge	↓	↓	↑	Anammox

↓ = decrease, ↑ = increase, 0 = no changes detected

Anaerobic oxidation of the ammonium was not the only metabolism present in the batch cultures (table 3.1.1). Denitrification dominated in four enrichments (1,2,7,9) that came from natural and man-made environments. Denitrification also occurred prior to the unveiling of the potential anammox activity in all the successful batch cultures. Sulfate reduction was detected in the enrichment 3bis (E3bis) after 300 days (its seed belonged to a pilot plant working in anoxic conditions and it had been washed), which colour changed from original brownish to completely black, due to the production of sulfide. The enrichment medium was completely refreshed and it was monitored over a long time, but it never showed anammox activity. It was considered that anammox bacteria could have been eradicated by the presence of

sulfide, probably at a higher concentration than their half maximal inhibitory concentration (IC50) (Jin et al., 2013; Russ et al., 2014). Nitritation and nitrification were detected in enrichments 4 (E4) and 4bis (E4bis), respectively, as pointed out the removal of the ammonium in the former and a depletion of the ammonium coupled with nitrite formation in the latter. In both enrichments, seeds came from the same anoxic pilot plant.

Although five batch cultures developed anammox bacteria communities, some differences in the enrichment processes were observed regarding the date when the anammox activity was firstly detected. Thus, E3 clearly showed anammox activity after the third month (table 3.1.2), E8 and enrichment 11 (E11) took about 250 days, but more than one year was required to enrich enough anammox bacteria to detect their activity in E5 and E6.

Table 3.1.2: Summary of the enrichments lifespan showing the day of the first anammox activity detection. When no value is given, anammox culture was not detected.

Enrichment	Lifespan (days)	Anammox activity detection (days)
1	456	--
2	456	--
3	1029	180
4	555	--
3bis	548	--
4bis	554	--
5	900	521
6	919	413
7	392	--
8	443	280
9	443	--
11	350	250

MOLECULAR DETECTION

POLYMERASE CHAIN REACTION (PCR)

In parallel to activity measurements, anammox bacteria were also detected and identified by molecular analyses. Conventional PCR with the primer set Pla46F-Amx368R (Neef et al., 1998; Schmid et al., 2003) and a subsequent sequencing process allowed the identification of *Ca.* Brocadia anammoxidans in the five enrichments that showed anammox activity, with 98% nucleotide similarity to the respective sequence in the NCBI database (accession number: AF375994). However, it was not possible to obtain amplification by conventional PCR in the inocula and in the earlier stages of these enrichments.

FLUORESCENCE *in situ* HYBRIDIZATION (FISH)

Once *Ca.* Brocadia anammoxidans was identified in the five enrichments, periodical FISH analyses were performed to monitor them by using a specific probe (Amx820 Cy3-labelled). These assays were carried out to complement the information obtained from PCR and sequencing analyses.

Ca. Brocadia anammoxidans populations were hard to discriminate by FISH in the initial stages of the enrichments due to the presence of considerable background autofluorescence. The E3 was the first where it had been possible to distinguish single anammox cells in the earlier stages (fig. 3.1.2a). They showed the typical ring shape due to the absence of ribosomes in the central anammoxosome (Schmid et al., 2001). After a long enrichment period (fig. 3.1.2b), some simple cell-aggregated structures were seen, such as chain structures of anammox bacteria. In advanced stages of the same enrichment (after 823 days) clear anammox flocular aggregates were even observed (fig. 3.1.2c,d).

Epifluorescence images allowed a vague visualization of anammox cells and their structures. However, more precise analyses of the complex anammox aggregates

were required, and FISH preparations hybridized with Amx820 probe (Cy3-labelled) and DAPI (DNA staining) were observed using a confocal laser scanning microscope (CLSM).

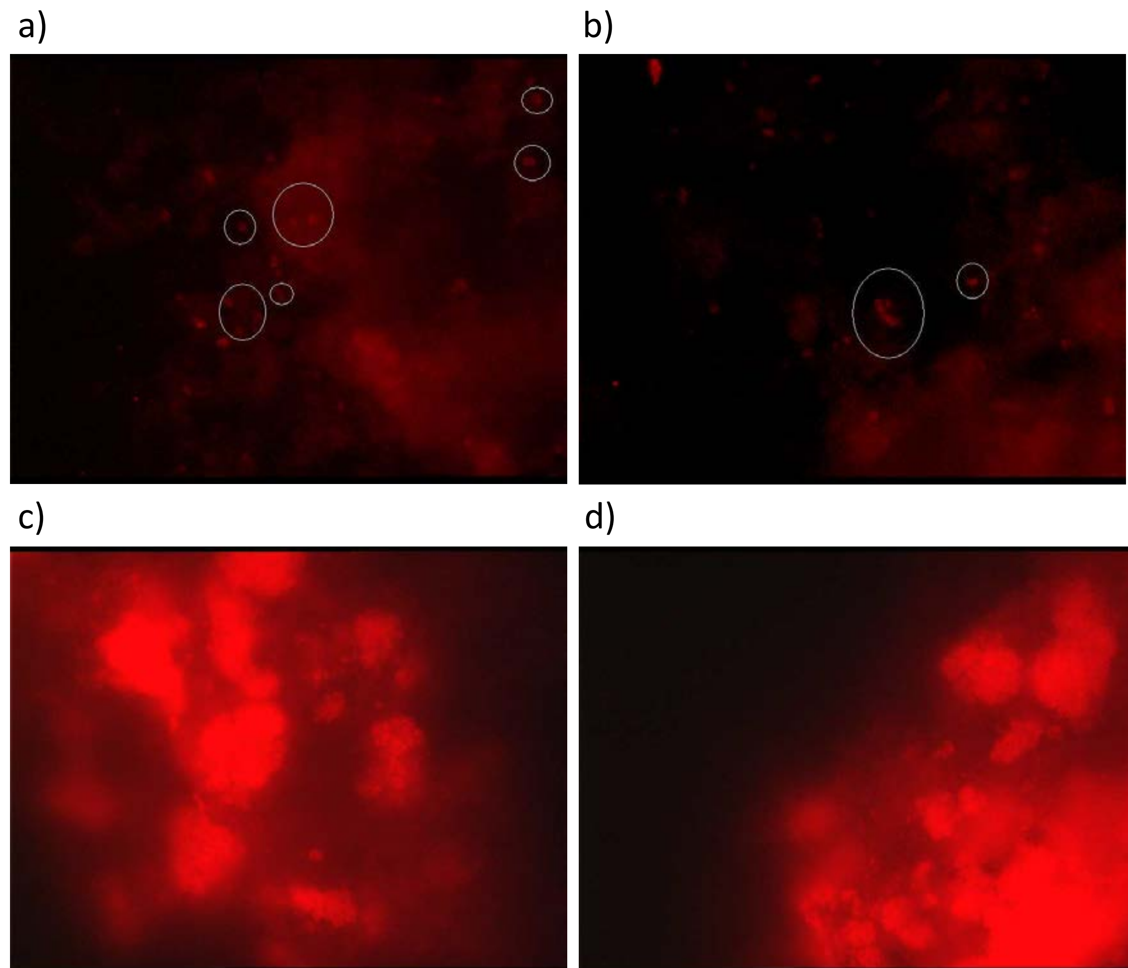


Figure 3.1.2: FISH image gallery of the E3 captured in an epifluorescence microscope after different enrichment periods. White circles point out anammox cells. (a) day 305th (1000x); (b) day 519th (1000x); (c) (d) day 823th (1000x). Anammox cells were hybridized with the Cy3-labelled probe Amx820, depicting them red.

The structures formed by growing anammox populations from early (fig. 3.1.3a) and advanced stages of the E3 (fig. 3.1.3b,c,d,e) were captured by CLSM. The latter pictures showed anammox cells (hybridized by both DAPI and the probe) clustering in microcolonies inside complex aggregates and surrounded by other bacterial cells that were only stained with DAPI. Although these FISH analyses were not used for anammox bacteria quantification, an increase in the aggregate size together with an increase of their complexity throughout the enrichment period was confirmed.

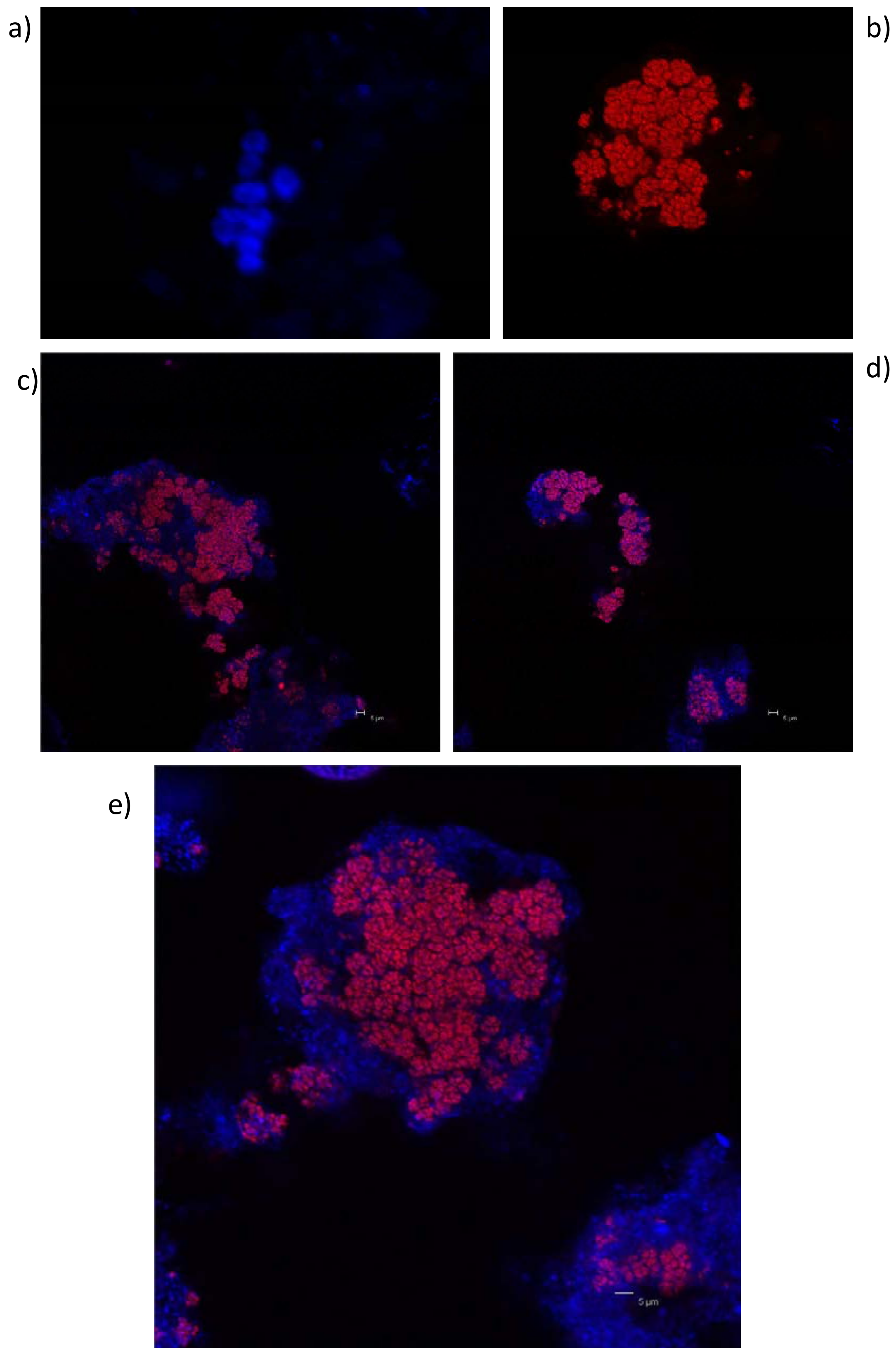


Figure 3.1.3: FISH image gallery of the E3 captured with a CLSM in different enrichment periods. (a) day 670th (b), (c), (d), (e) day 884th; (a) (b) were only hybridized with Cy3-labelled Amx820 probe (c) (d) and (e) were hybridized with Cy3-labelled Amx820 probe for anammox cells and also stained with DAPI.

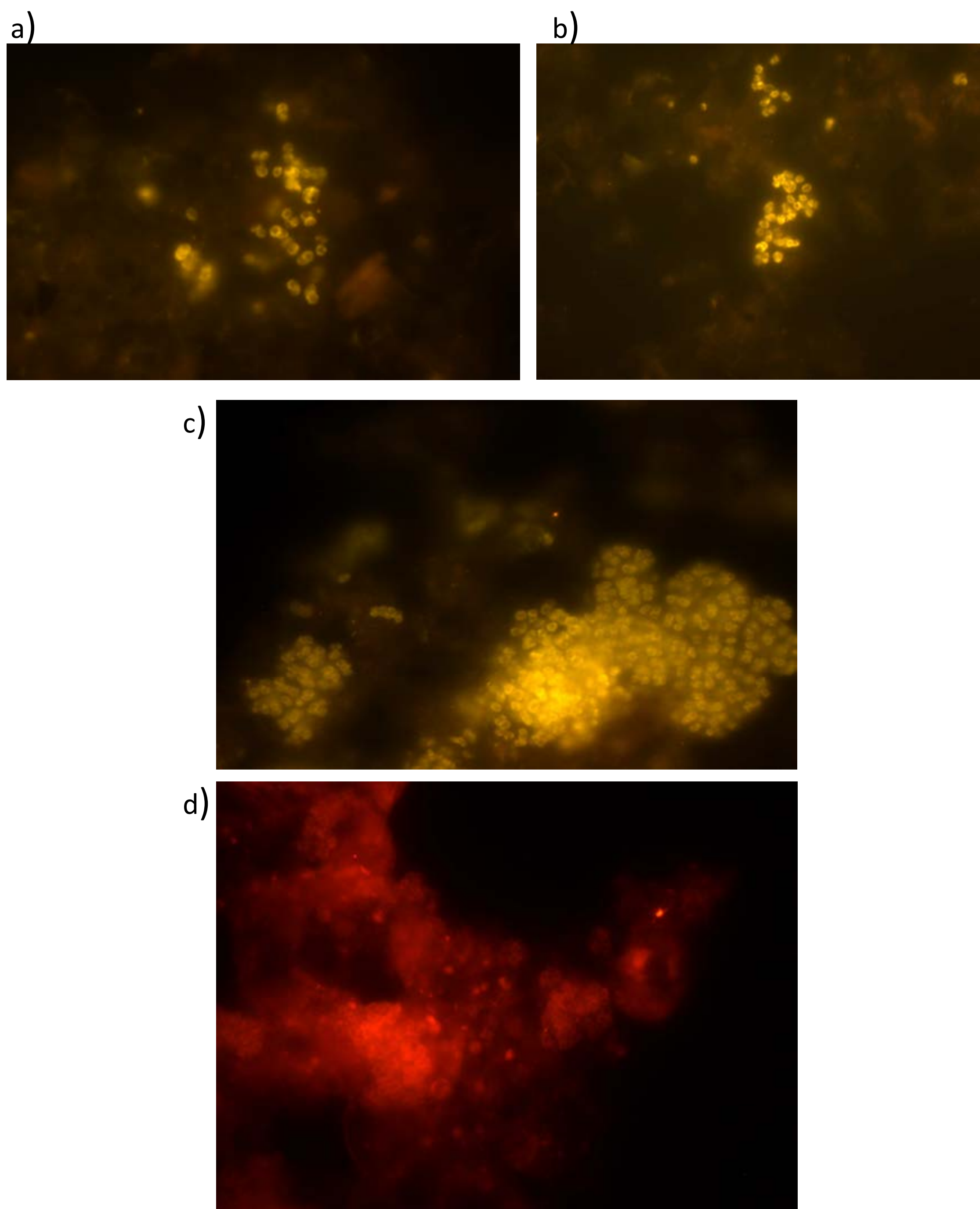


Figure 3.1.4: FISH image gallery captured with an epifluorescence microscope. Initial (a, b) and advanced (c) stages of the E5 (1000x) (day 521th and 850th, respectively); cells were hybridized with Cy3-labelled probe Amx820 and they were depicted in yellow. E11, day 250th (d); cells were hybridized with Cy3-labelled probe Amx820 and they were depicted in red (1000x).

FISH analyses were also performed for the rest of the batch cultures containing *Ca.* Brocadia anammoxidans populations, confirming the presence of cells only in E5 and

E11. The observation showed anammox cells with the same shape and similar aggregate structures with a lower complexity (fig 3.1.4) than the aggregates observed in the E3. It was not possible to obtain any anammox bacteria image from E6 and enrichment 8 (E8) during all their lifespan, although the anammox activity was clearly evident and *Ca.* Brocadia anammoxidans was identified in both cultures.

3.1.3 DISCUSSION

BATCH CULTURE as an ANAMMOX ENRICHMENT TOOL

Nowadays, two procedures have proved to be successful to enrich anammox bacteria. Strous and collaborators (1998) described the biological reactors (working in SBR configuration) as the best way to enrich the anammox slow-growing bacteria. From there on, different reactor types were used for the anammox enrichment (Egli et al., 2001; van Dongen et al., 2001; Furukawa, 2003; Vlaeminck et al., 2009b) with successful results. On the other hand, the achievement of potentially useful anammox bacterial populations can also be based on their enrichment in closed batch cultures (Toh and Ashbolt, 2002; Toh et al., 2002; Suneethi and Joseph, 2011) as it was done in the present work.

The strategy based on biological reactor was not chosen for several reasons. One of the major aims of the work was to embrace the maximum of anammox potentially sites to develop anammox species to be used as inocula for an anammox reactor. The more the sites screened, the more chances to find anammox populations. Thus, a high number of biological reactors should have to be simultaneously used to screen the large amount of seeds, involving large investments, which was not possible. Otherwise, if only one or few reactors were used, significant time would be required for the different attempts to grow anammox (unless the right inocula had been chosen by chance at the first trial). By using batch cultures, an array of different seeds can be tested economically, increasing the probability to achieve a successful

enrichment and even to obtain different anammox species by screening different environments.

Although the large enrichment period required to obtain fully grown anammox populations in a closed system, such as the batch cultures used in the present work, the aforementioned facts favoured its election. The main goal was the obtaining of enrichments that could be useful as inocula for bioreactors, not attaining a considerable amount of cells. The anammox bioreactor (linked to the PN reactor) was inoculated with 50 mL of homogenized sludge from E3 and E10 (López et al., 2008).

A few problematic aspects of the batch cultures operation required special attention. It had been reported that the first tested batch cultures decreased their activity within a period of 30 days, even after repetitive additions of ammonium and nitrate (Mulder et al., 1995). N-compounds were added, but there was not a restoration of C-sources, which were consumed by anammox bacteria for biosynthesis. Thus, C-limitation might have been responsible for growth reduction. In the batch cultures under study, this problem had been overcome by periodically refreshing the medium. Every two months, after an overnight sedimentation, most of the medium was removed and subsequently 250 mL of fresh medium were added. Nitrite concentrations were accurately checked and controlled after the refreshing, avoiding to reach inhibition levels and thus impairing the growth of anammox bacterial populations.

Although a strict surveillance of all the parameters was performed, some cultures developed unexpected processes. Some nitrate was added in all the enrichments during the start-up period to prevent the generation of H_2S by sulfate-reducing bacteria (Wang et al., 2009). Nevertheless, a single batch culture had to be replaced due to the presence of high sulfide concentrations, which are considered critical for anammox surviving (Jin et al., 2013; Russ et al., 2014). On the other hand, the nitritation and nitrification activities detected in two enrichments were probably due to an oxygen leakage through the butyl rubber stoppers.

The dominance of denitrification in some enrichments prior to anammox unveiling metabolism was also reported by Dapena-Mora et al. (2004), Third et al. (2005) and Suneethi and Joseph (2011). The addition of low concentration of nitrate in the initial steps of the enrichment would be essential in the dominance of denitrification in most of the enrichments. It probably favoured the activity of the denitrifiers to remove the organic matter present in the seed, thus avoiding the activity of sulfate-reducing bacteria and finally remaining only non-degradable organic matter and inorganic nutrients.

DETECTION of ANAMMOX BACTERIA

Ca. Brocadia anammoxidans was detected and identified in five enrichments (3,5,6,8 and 11) by chemical and molecular approaches. Anammox activity was detected in the first moment by the chemical techniques, and subsequently, PCR and FISH analyses were performed to confirm and identify the bacteria responsible of the process.

Although anammox bacteria were successfully enriched by using batch cultures, some mentions have to be done about the differences in the way the populations developed. Not every anammox enrichment disclosed their populations at the same rate. Anammox populations had detectable specific activity in the E3, E8 and E11 after 180, 280 and 250 days, respectively, whereas anammox populations from E5 and E6 took more than 450 days to clearly show it (table 3.1.2). Since all the enrichments were treated the same way, the origin of the seeds coupled with their intrinsic structure should be responsible of these differences through their development. The enrichments that derived from activated sludge quickly developed anammox populations, rather than those belonging to sediments from natural and modified environments. It is possible that anammox cells were in higher concentrations in the former. Moreover, they could have an easier acclimatization to the media and better growth conditions within the enrichments, because they were already treating wastewater. Thus, these enrichments could became into a swiftly detection.

The combination of the chemical and molecular techniques confirmed the presence of active anammox populations in the five enrichments. Anammox activity detection was always the first hint about their presence, but some differences were observed regarding to the periods of anammox detection by activity and molecular methods (fig. 3.1.5).

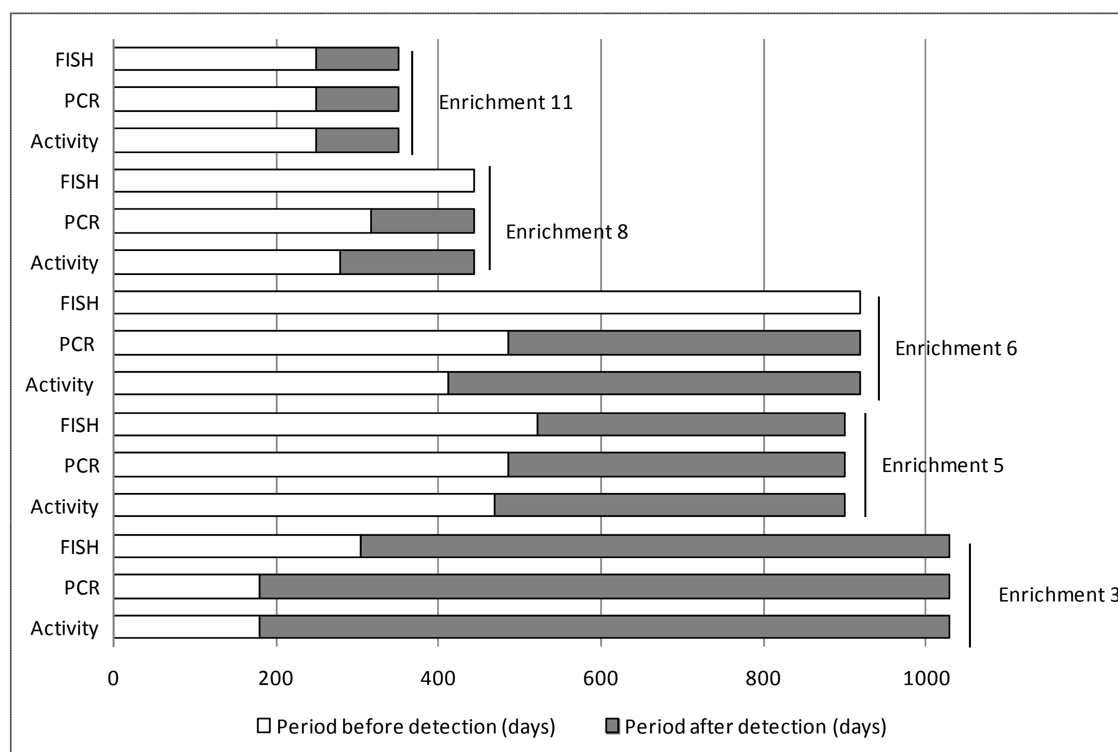


Figure 3.1.5: Lifespan of each successful anammox enrichment highlighting the period before and after the detection through chemical and molecular approaches. FISH detection values in E6 and E8 were not obtained.

It was expected that once the activity were showed, the anammox populations were enough concentrated to be detected by PCR and FISH procedures. Nonetheless, only E11 agreed in the three detection methodologies. Conversely, E3 only agreed in the PCR and activity detection period (FISH detection was not able since more than 100 days later) and the rest of the enrichments have a sequential detection. Clearly, chemical procedures have the highest sensibility whereas FISH analyses, in most cases, showed positive results only after a longer enrichment period. The late visualization of the anammox cells by FISH could be due to the inherent observer limitations together with high levels of background autofluorescence from the

aggregates. Concerning FISH analyses, there were two enrichments without any FISH positive image. In the case of the E6, it is possible that the sediment nature of the inoculum contained some sort of matter that made impossible the visualization of the anammox cells (Fujii et al., 2002). However, there is no explanation for the lack of FISH images from E8 since samples from activated sludge usually reported good analyses (even better than sediments). Again, the observer limitations and an important background fluorescence could be a reason.

Ca. Brocadia anammoxidans was detected and identified in all of the successful anammox enrichments. However, the inocula were obtained from different origins as well as diverse environmental conditions, such as the concentrations of measured nitrite, ammonium, salinity, and hydrogen sulfide. The man-made systems were the major, although different environments were screened in a vain effort to obtain some anammox populations diversity. The seeds of successful enrichments were mainly collected from sludge digesters with high ammonium concentrations and low nitrite levels, which were good candidates to harbour anammox populations.

Nowadays anammox bacteria can be detected in many different environments, most of them natural (Penton et al., 2006; Schubert et al., 2006; Humbert et al., 2009; Hu et al., 2013). As it was previously stated, phylotypes of *Ca.* Brocadia anammoxidans are commonly related to WWTPs and a few populations are known to occur in natural environments, such as freshwater sediments or lakes (Amano et al., 2007; Zhang et al., 2007; Dale et al., 2009; Yoshinaga et al., 2011). Interestingly, E5 was the first case of *Ca.* Brocadia anammoxidans to be retrieved from saline samples, in particular from the sediments of a brackish coastal environment, the Massona lagoon (fig. 3.1.6). However, it is possible that their active populations were not developed close to the sediment, but at water depths with low salinities. To fully understand this argument, it must be considered that this lagoon receives different water inputs: continuous freshwater from the "Corredor" irrigation channel, which is rich in nutrients due to the surrounding wetlands, marine seepage from the Mediterranean sea and surface intermittent marine water inputs during strong eastern sea storms. Since the former source provides water inputs with a lower density than marine water, salinity

conditions in the lagoon are different through depth and time. *Ca.* Brocadia anammoxidans phylotypes were retrieved from sediments of the lagoon (fig. 3.1.6, S), but their conditions were probably too limited for anammox development due to the high salinity values (hypolimnetic salinity was approximately 5%), very low redox potentials (-350 mV) and high sulfide concentrations (up to 7 mM) coupled with high salt concentration. These values are higher than sulfide IC50, reported to range between 10 µM and 3mM (Jin et al., 2013; Russ et al., 2014). It is possible, therefore, that the active populations developed upper in the water column, closer to the oxic/anoxic interface (Fig 3.1.6, W), which had lower salinities (0.1%), lower sulfide concentrations (3.2-32 µM) and lower redox potentials (-150 / -200 mV), rather than the bottom of the lagoon.

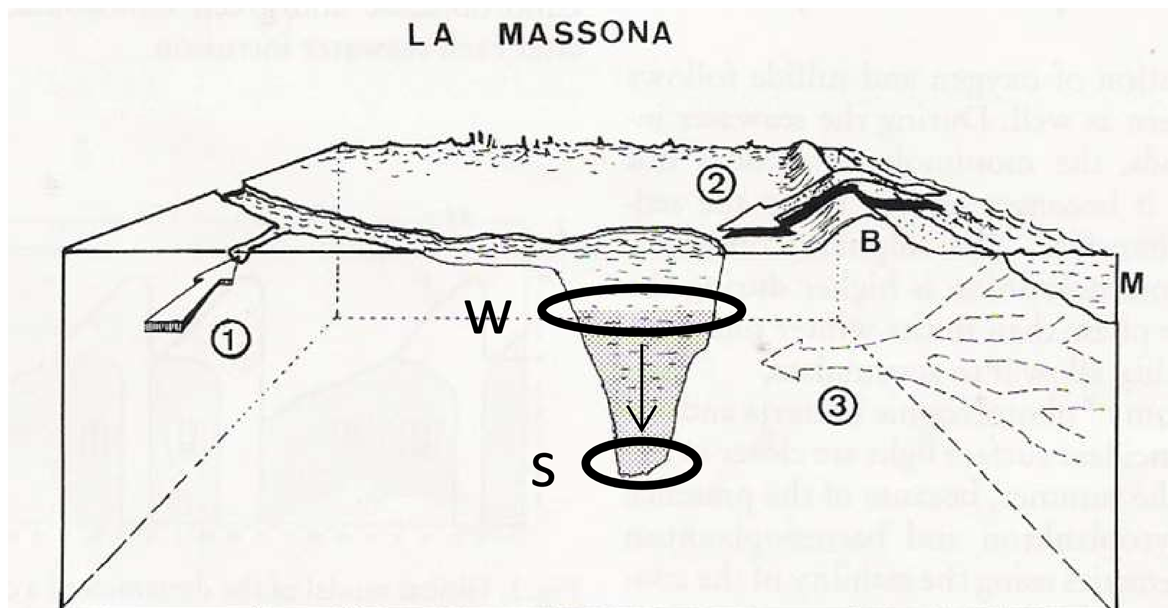
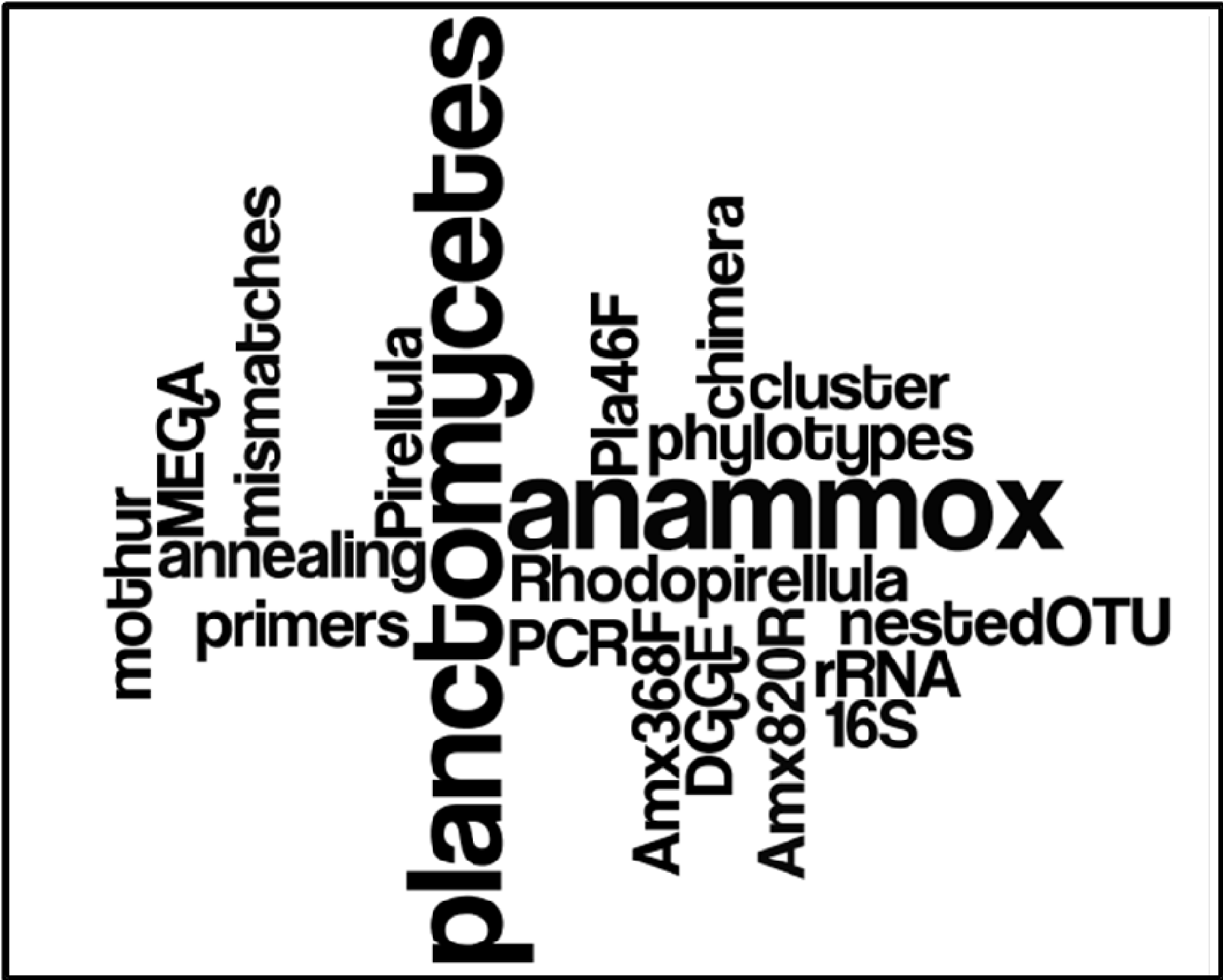


Figure 3.1.6: Schematic view of the Massona brackish coastal lagoon, showing their limnological features and hydrological behaviour (modified from Domínguez-Planella et al. (1987)). W: oxic/anoxic interface in the water column, at moderate salinity values; S: anoxic sediment with high salinity and extremely low Eh values; M: Mediterranean sea; B: sand bar; 1: Continuous freshwater inputs from the "Corredor" irrigation channel 2: Intermittent marine water inputs by wave transport over the sand bar during strong east storms; 3: Continuous seepage inputs throughout the sand bar.



Chapter II

Optimization of molecular techniques for the detection of anammox bacteria

3.2.1 BACKGROUND

After 15 years of anammox bacteria research, they were discovered all around the world. The uncovering of anammox ubiquity was mainly based on the application of culture-independent methods in environments where their presence was suspected or even previously unexpected. These detection methods have become essential to reveal the wide environmental distribution of their natural populations.

The application of rRNA and non-rRNA-based methods was essential for the anammox molecular detection. The information achieved by both methods can be considered complementary. Non-rRNA-based methods primarily include detection of single-ladderane lipids (Sinninghe Damste et al., 2002; van Niftrik et al., 2004) and also encompass tracer experiments with ^{15}N-labelled ammonium and ^{14}N-labelled nitrite (Kuypers et al., 2003; 2005). Both methods have been useful to confirm anammox presence and assess their contribution to nitrogen conversions in natural environments, respectively, where anammox cell concentrations are usually low (Schmid et al., 2005). The use of rRNA-based methods is mainly focused on targeting 16S rRNA gene (Schmid et al., 2001). Several primers and probes have been described (Schmid et al., 2005) to amplify and detect anammox 16S rDNA by performing PCR-based approaches or FISH analyses.

By applying the aforementioned methods, anammox presence and activity have been reported in diverse ecosystems such as WWTPs (Date et al., 2009; Bae et al., 2010a), freshwaters (Penton et al., 2006; Schubert et al., 2006; Hamersley et al., 2009), marine sediments (2002; Kuypers et al., 2003; 2005; Meyer et al., 2005) and even in terrestrial ecosystems (Humbert et al., 2009; Hu et al., 2011).

As reported in the previous chapter, batch cultures were used to screen a wide array of sludge and sediments from different origins to search for anammox bacteria. Their use shorten the long time period typically required for trial-and-error experiments in biological reactors or the need for a higher number of available reactors. After the enrichment period, *Ca.* Brocadia anammoxidans populations were detected in some

batch cultures by carrying out PCR analyses using *Planctomycetales*-anammox primer set (Pla46F-Amx368R). However, this approach was unable to detect these anammox populations before their activity became evident. The aim of this work is to assess a molecular method based on PCR for early detection and identification of active anammox organisms, even if they were at low concentrations and their activity was still undetectable. This method, combined with information on environmental parameters, could facilitate the choice of possible biomass sources to be used as potentially useful inocula for laboratory, semitechnical, or full-scale anammox reactor.

DNA isolations from the initial stages of the successful batch cultures, which contained low concentrations of anammox bacteria, were used as templates. They were chosen because their concentrations were clearly below the previously tested limit of detection of the *Planctomycetales*-anammox primer set (see Chapter I). On the other hand, initial-stage templates of the non-successful enrichments were used to confirm whether anammox populations were never present or they not further developed. A nested-PCR approach was used since the DNA isolations did not show any amplification with conventional PCR, as it was shown in the previous chapter. The nested-PCR first round was performed with a wide-range primer set, 27F-1492R. It was considered to be a suitable option to increase the sensitivity of the detection.

Different approaches were tested to evaluate to improve the detection lower the detection limits by the use of the nested-PCR. The first test (A) was focused on applying the most commonly used primer set, Pla46F-Amx368R (Schmid et al., 2003), at the recommended annealing temperature (56°C). In the second test (B), the same primer set was used but the annealing temperature was increased until its maximum (64°C). Finally, in the third test (C), a more specific primer set (Amx368F-Amx820) was chosen to try to target only anammox 16S rDNA.

The PCR products derived from the different tests were loaded in DGGE gels and the most prominent bands were excised and sequenced. Sequences retrieved from each test were aligned and later assigned to OTUs (defined at a 97% cutoff) with MOTHUR using the latest SILVA bacterial database as reference alignment. Representative

sequences for each OTU were also identified using the implemented tool in MOTHUR. For each test, tree topology and phylogenetic distances were computed using the maximum-likelihood method in the MEGA V5 package.

3.2.2 RESULTS

TEST A (PLA46F-AMX368R at 56ºC)

In the first test, after all DNA isolations were amplified with the wide-range primer set, 93% of the samples showed positive amplifications in the second round. The high amplification rate was considered unexpected since some of the enrichments did not develop any anammox populations.

From DGGE results (fig. 3.2.1), a change in the dominant microbial populations was observed throughout the enrichment period. However, any of them showed a band pattern similar to E3/d245 sample, where *Ca.* Brocadia anammoxidans presence was previously detected by conventional PCR.

The comparison of DGGE band patterns must be taken into account (fig. 3.2.1). E3/d1 and E4/d1 showed the same band pattern although they came from different origins, but only the former developed *Ca.* Brocadia anammoxidans. Concerning the rest of the samples, E5 culture increased the complexity of their *Planctomycetes*-anammox populations during the enrichment period whereas E6 lost most of them. In both enrichments, anammox populations were further developed. E1 showed an increasing appearance of *Planctomycetes*-anammox bands. They were maintained rather stable in E7, showing no significant changes.

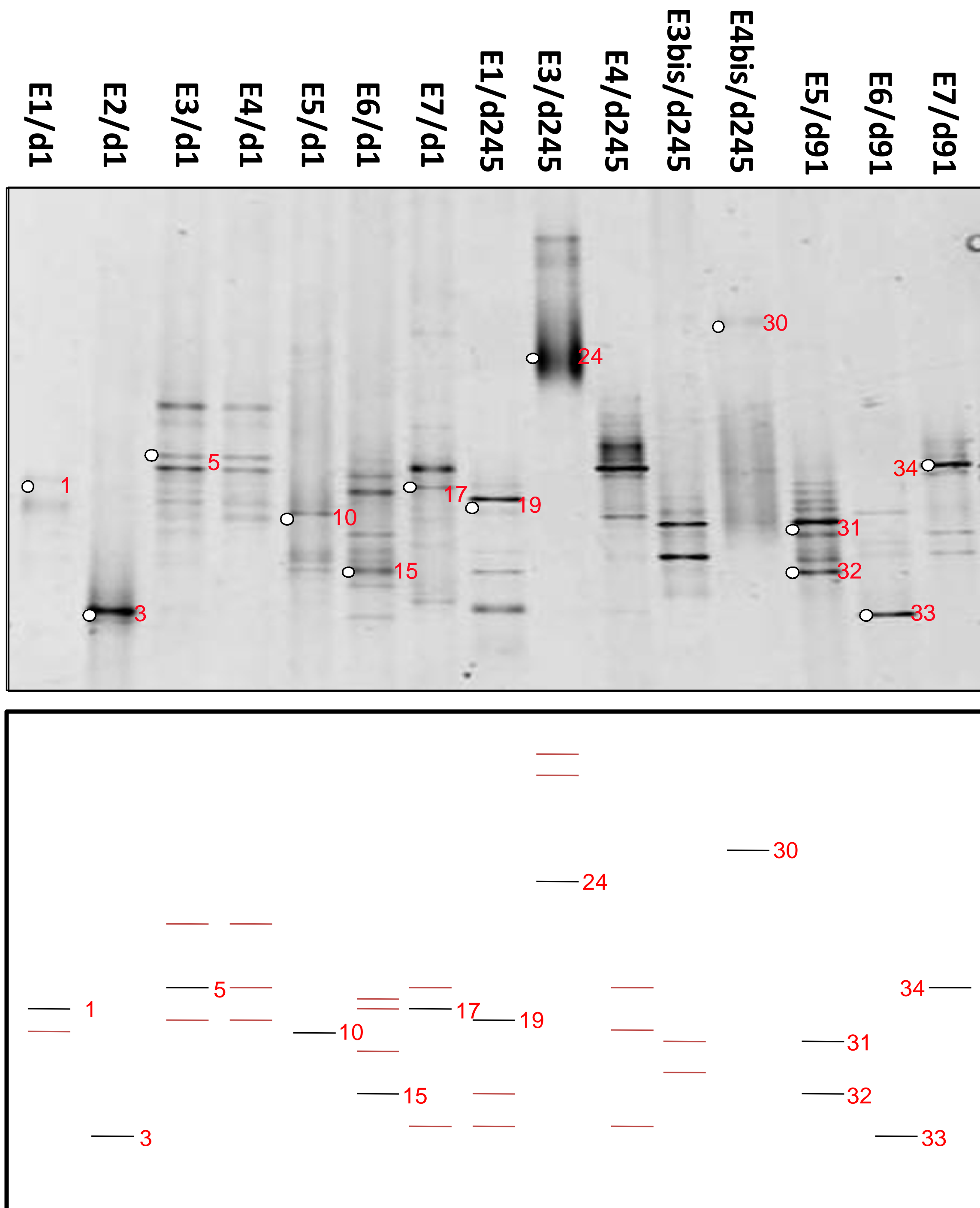


Figure 3.2.1: Gel image (up) and schematic representation (down) of the DGGE performed with a denaturing gradient from 30-70% using Pla46F-Amx368R at 56°C annealing temperature. White dots and red numbers in the gel image show only the sequences that were properly added in the phylogenetic tree whereas discarded bands were depicted in red in the schematic representation.

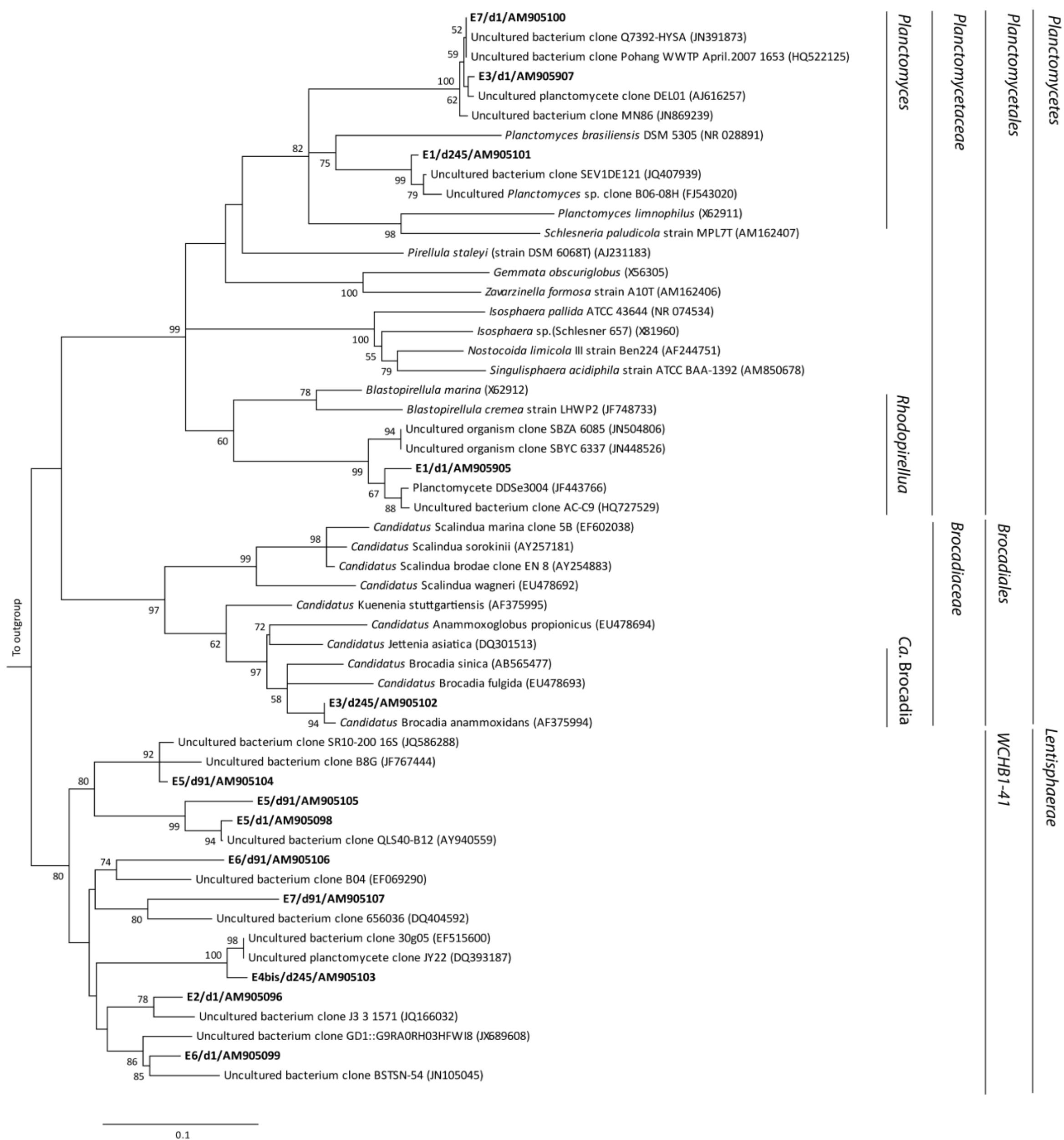


Figure 3.2.2: Maximum-likelihood phylogenetic tree based on the 16S rRNA gene retrieved from DGGE bands obtained by nested PCR using Pla46F-Amx368R at 56ºC annealing temperature. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. Accession numbers of external 16S rDNA sequences are given within brackets. The bar represents 10% estimated sequence divergence. Codes for sequences indicate: enrichment number / days of enrichment / accession number.

Most of the bands were excised and sequenced, but 55% of the sequences were considered PCR malfunctions (table 3.2.1) such as double sequences or chimeras. MOTHUR and MEGA software were used to discern the taxonomy of the sequences and to calculate a maximum-likelihood phylogenetic tree (fig. 3.2.2). The clustering of

the 16S rDNA sequences calculated by MOTHUR did not show any OTU by applying a 0.03 cutoff, meaning that the homology among sequences was less than 97%.

The taxonomic analyses and the tree topology showed five sequences clustering within *Planctomycetes*, four of them not belonging to *Brocadiaceae*. Three sequences clustered into *Planctomyces* (E3/d1/AM905097, E7/d1/AM905100, E1/d245/ AM905101) and one into *Rhodopirellula* (E1/d1/AM905095). Only the phylotype retrieved from E3 after 245 days of enrichment (E3/d245/AM905102) was clearly identified as *Ca.* Brocadia anammoxidans. The rest of the phylotypes were clustered inside *Lentisphaerae* phylum and phylogenetically close to uncultured bacteria. Thus, the application of a nested PCR with the commonly used Pla46F-Amx368R primer set at the recommended PCR conditions was unable to retrieve any anammox sequence from the DNA isolations at the first stages of the enrichments.

TEST B (PLA46F-AMX368R at 64ºC)

Since most of the samples showed positive reactions in test A but only a few sequences clustered into *Planctomycetes*, the annealing temperature of the Pla46F-Amx368R primer set was increased not only to enhance the sensitivity of the process but also to improve the specificity. The highest temperature where the primer set amplified (without losing yield) was determined by applying *Ca.* Brocadia anammoxidans DNA isolations in a gradient thermal cycler, from 56°C to 68°C (data not shown), and it was found to be 64°C. All the tested DNA isolations were amplified in the PCR first round but after the performance of the second round only 50% positive reactions were obtained (instead of the 93% obtained at 56°C) (table 3.2.1). This low amplification ratio was closer to the initial expectations.

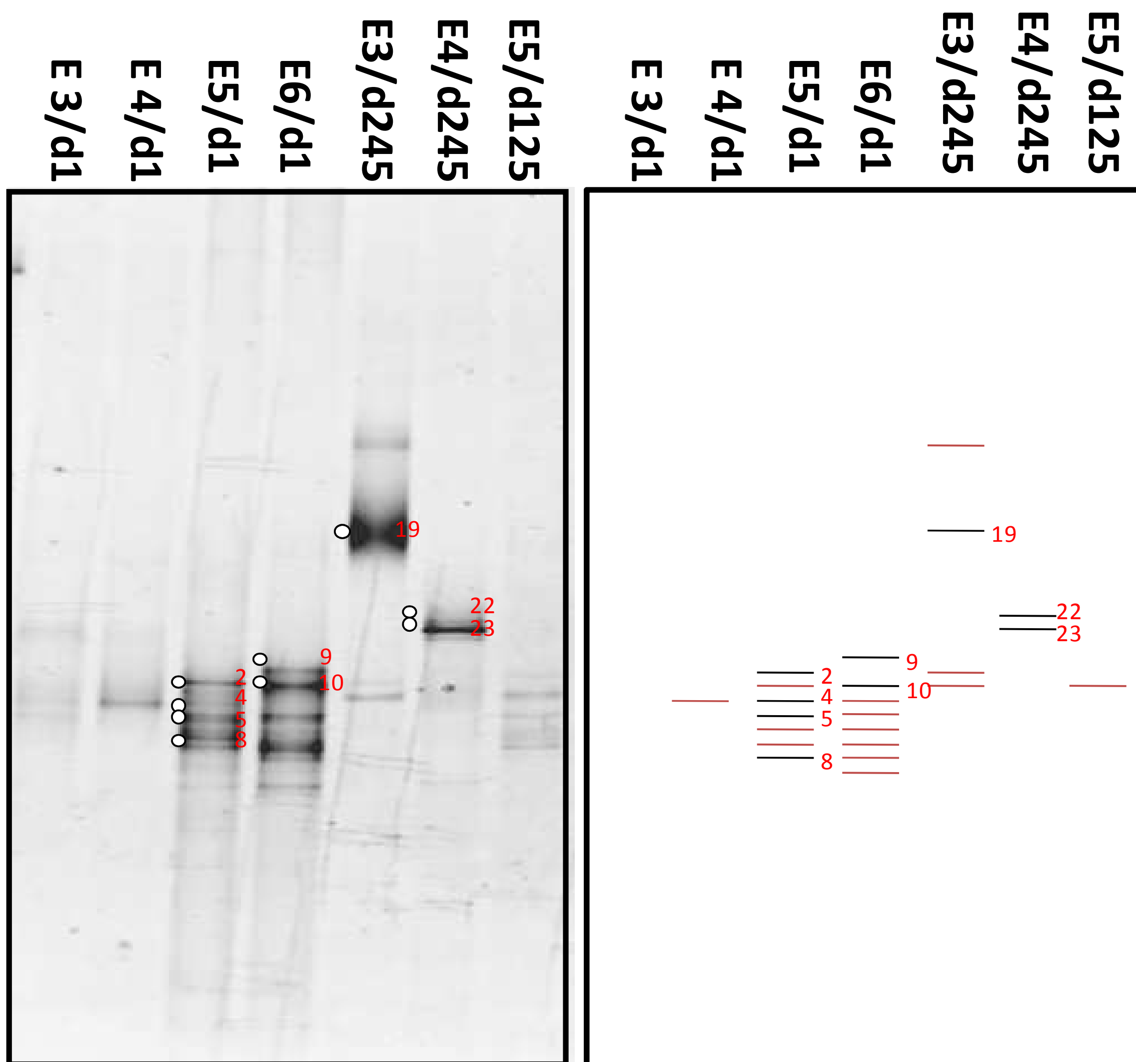


Figure 3.2.3: Gel image (left) and schematic representation (right) of the DGGE with a denaturing gradient of 30-70% using Pla46F-Amx368R at 64°C annealing temperature. White dots and red numbers in the gel image show only the sequences that were properly added in the phylogenetic tree whereas discarded bands were depicted in red in the schematic representation.

Again, after DGGE analyses (fig. 3.2.3), no sample showed a similar pattern to E3/d245. The band patterns found in samples E5/d1, E6/d1 and E3/d245 were rather similar to the ones obtained with an annealing temperature of 56°C (fig. 3.2.1). Concerning the rest of the samples, a reduction of the complexity was observed in comparison to their respective band patterns at 56°C, especially in E3/d1 and E4/d1.

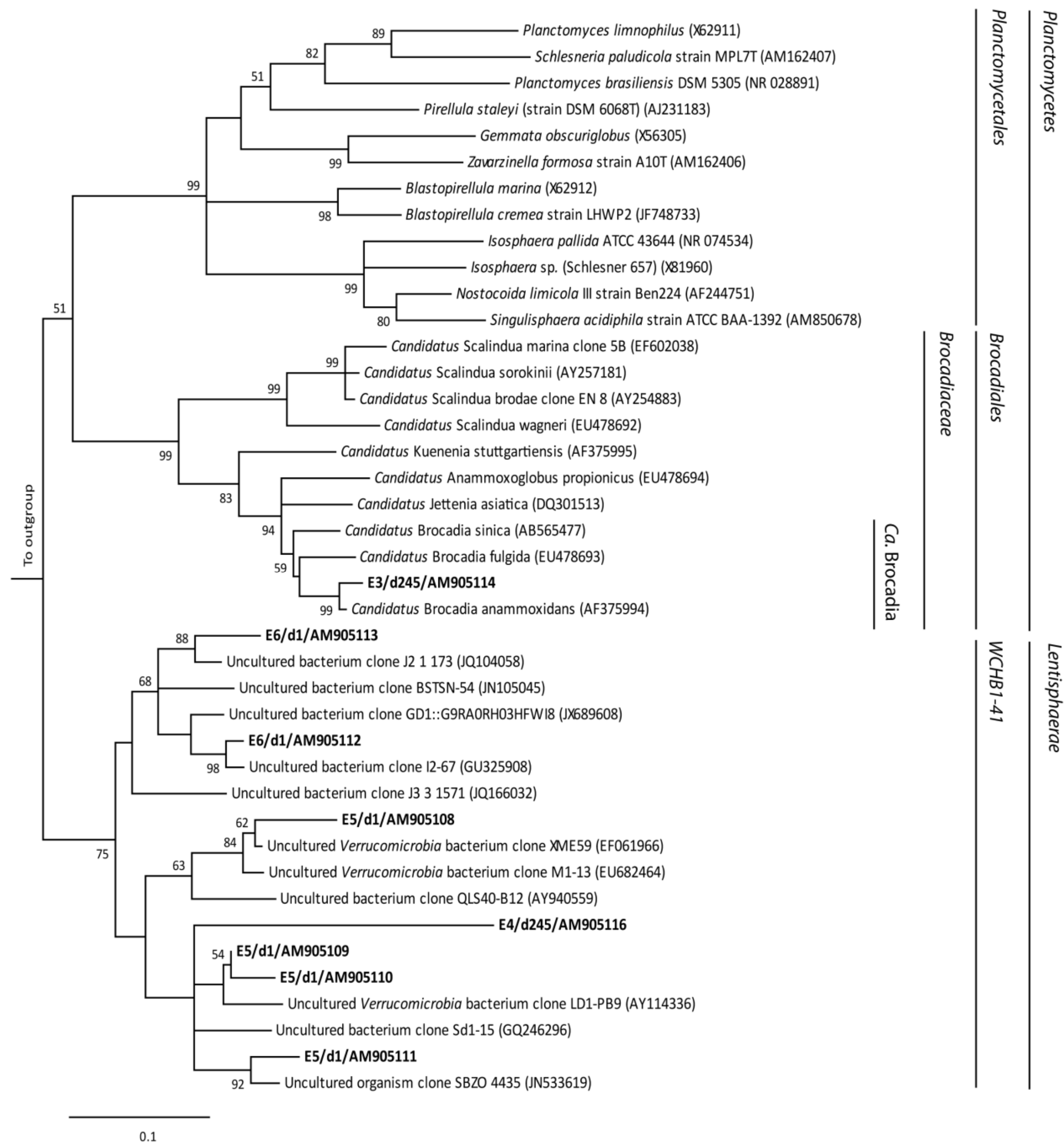


Figure 3.2.4: Maximum-likelihood phylogenetic tree based on the 16S rRNA gene retrieved from DGGE bands obtained by nested PCR using Pla46F-Amx368R at 64ºC annealing temperature. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. Accession numbers of external 16S rDNA sequences are given within brackets. The bar represents 10% estimated sequence divergence. Codes for sequences indicate: enrichment number / days of enrichment / accession number.

After sequencing, 69% of the retrieved sequences were considered PCR malfunctions (table 3.2.1). Clustering analysis performed by MOTHUR (applying a 0.03 cutoff for all 16S rRNA gene sequences) was unable to calculate any OTU since the homology among sequences was less than 97%. Regarding the maximum-likelihood phylogenetic tree obtained after MOTHUR and MEGA calculations (fig. 3.2.4), a single phylotype was

affiliated to *Planctomycetes*, which corresponded to an E3 sequence (E3/d245/ AM905114) closely related to *Ca.* Brocadia anammoxidans. The remaining sequences were located into *Lentisphaerae* phylum, only clustering with uncultured bacteria. Therefore, the increase of the annealing temperature not only did not result into obtaining anammox sequences but even reduced the number and quality of the retrieved 16S rDNA sequences.

TEST C (AMX368R-AMX820R)

In the third test, the primer set for the nested-PCR second round was changed to Amx368F-Amx820, in another attempt to increase the sensitivity without losing specificity. Amx368F is specific for all anammox bacteria whereas Amx820R was designed to hybridize only for *Ca.* Brocadia and *Ca.* Kuenenia genera (Schmid et al., 2003). PCR conditions were tested and optimized because there was only one experience previously reported with this primer combination, performed at the unique annealing temperature of 56°C (Amano et al., 2007). Thus, the first step was to look for the optimum annealing temperature with *Ca.* Brocadia anammoxidans DNA isolations in a gradient thermal cycler from 56°C to 68°C (data not shown). It was experimentally determined to be 62°C.

After all DNA isolations amplified in the first round, 88% of the nested-PCR reactions resulted in positive amplifications. This high amplification ratio was unexpected, as in test A. After DGGE performance (fig. 3.2.5), no band patterns similar to E3/d245 were observed. Interestingly, although their respective band patterns from day 1 were practically identical, E3 and E4 cultures suffered a different evolution until day 245th. Moreover, the E3 and E3bis (replicate enrichments) band patterns at day 245th also differed, not showing any trace of the band corresponding to *Ca.* Brocadia anammoxidans. Most of the samples kept a similar band pattern than in test A (E3/d1, E4/d1, E5/d1, E6/d1, E3/d245, E4/d245, E6/d91) and the rest (E3bis/d245, E4bis/d245, E5/d125, E7/d91) showed significant changes, mostly involving an increase of the richness.

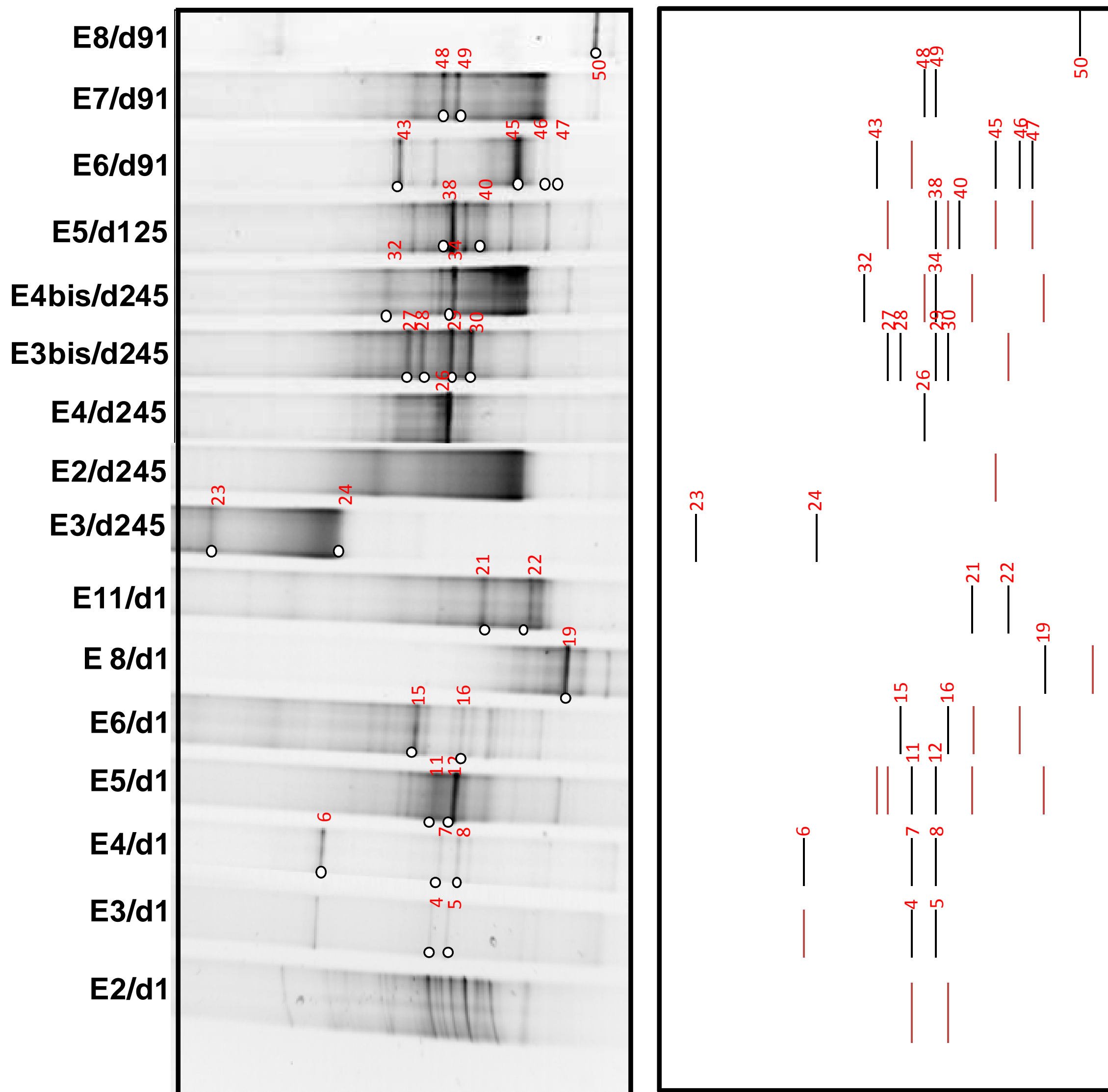


Figure 3.2.5: Gel image (left) and schematic representation (right) of the DGGE with a denaturing gradient of 30-70% using Amx368F-Amx820R. White dots and red numbers in the gel image show only the sequences that were properly added in the phylogenetic tree whereas discarded bands were depicted in red in the schematic representation.

Only 20% of the sequences retrieved from the DGGE were found to correspond to PCR malfunctions (table 3.2.1). MOTHUR calculations delivered 6 different OTU at a 0.03 cutoff value (table 3.2.2), whose representative sequences were added to the singletons to construct a maximum-likelihood phylogenetic tree (fig. 3.2.6). Up to 96.5% of phylotypes were found to belong to the *Planctomycetes* phylum and remarkably 9 sequences (20%) were affiliated within *Brocadiaceae* family. However, only the two sequences retrieved from E3/d245 (OTU 5) were clustered close to *Ca.* Brocadia anammoxidans. The rest of the phylotypes belonging to *Brocadiaceae* were

not closely related to any known anammox bacteria, although they clustered near uncultured bacteria that were mainly retrieved from anammox studies (Terada et al., 2009; Kalyuzhnyi et al., 2010; Hou et al., 2013; Wang et al., 2013). These potential anammox phylotypes were mostly retrieved from enrichments that further developed anammox populations (E5, 6, 8 and 11). The rest of the phylotypes in the *Planctomycetes* phylum were mainly located within *Pirellula*, except for 3 sequences that clustered into WS3 phylum.

Table 3.2.1: Summarized results from the nested PCR amplifications performed with different primer sets and annealing temperatures.

	Pla46F-Amx368R (56°C)	Pla46F-Amx368R (64°C)	Amx368F-Amx820
n (number of samples)	15	15	17
% positive amplifications	93	50	88
% methodological malfunctions	23 (duplicate sequence) 32 (chimera)	31 (duplicate sequence) 38 (chimera)	5 (duplicate sequence) 15 (chimera)
% sequences within *Planctomycetes*	36	12.5	96.5
% sequences within *Brocadiales*	7	12.5	34

Table 3.2.2: Summary of the OTUs calculated from the sequences derived from the use of Amx368F-Amx820R, the representative sequence and the sequences included in each OTU.

OTU	Representative sequence	Sample sequence
1	E6/d91/AM905139	E4/d1/AM905117, E6/d91/AM905139, E6/d91/AM905138, E6/d91/AM905140
2	E3bis/d245/AM905131	E4/d245/AM905129, E3bis/d245/AM905132, E3bis/d245/AM905133, E3bis/d245/AM905130, E3bis/d245/AM905131, E4bis/d245/AM905134
3	E4/d1/AM905119	E4/d1/AM905119, E4/d1/AM905118, E3/d1/AM905146, E3/d1/AM905147
4	E6/d91/AM905141	E6/d91/AM905141, E7/d91/AM905142
5	E3/d245/AM905128	E3/d245/AM905128, E3/d245/AM905127
6	E5/d1/AM905119	E5/d1/AM905121, E5/d1/AM905122

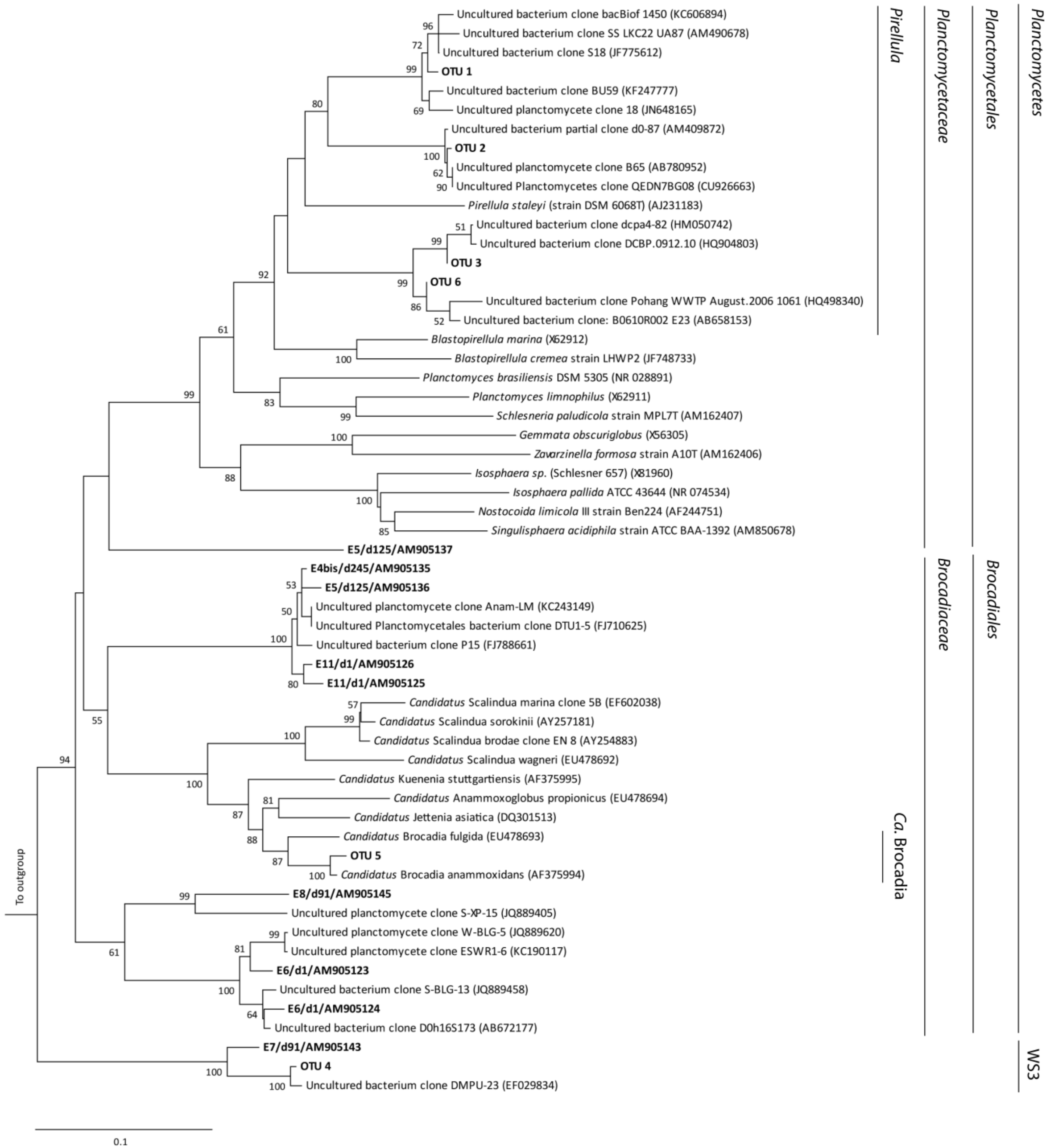


Figure 3.2.6: Maximum-likelihood phylogenetic tree based on the 16S rRNA gene retrieved from DGGE bands obtained by nested PCR using Amx368F-Amx820R. OTU identification numbers are indicated in table 3.2.2. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. Accession numbers of external 16S rDNA sequences are given within brackets. The bar represents 10% estimated sequence divergence. Codes for sequences indicate: enrichment number/days of enrichment/accession number.

3.2.3 DISCUSSION

As it was aforementioned in Chapter I, most of the studies related with the achievement of anammox enrichments are based on pilot plants and bioreactors, because these configurations favour the accumulation of the slow-growing bacteria (Strous et al., 1998; Dapena-Mora et al., 2004; Gilbert et al., 2013). In the previously mentioned studies, inocula usually were collected from other existing anammox reactors or from suitable WWTPs seeds, assuring potentially dense and active anammox populations, that will easily grow in the reactor under study. Nonetheless in Chapter I it has been suggested that batch culture enrichment could be appropriate as a previous step to test a large array of seeds. Therefore, anammox bacteria could be found without great investments (especially for those studies without initial enrichment or for new species research), although it has to be still considered as a time-consuming step. However, to be used as a suitable approach, the batch culture enrichment needs to shorten the required time to detect anammox bacteria in their early stages.

METHODOLOGICAL ISSUES

Currently, molecular methods based on 16S rRNA gene detection by PCR are the most common strategies for quick and easy anammox detection (Schmid et al., 2003) although some problems can be found when anammox bacteria are not sufficiently concentrated, underestimating their populations (mainly occurring in environmental samples and inocula). In these cases, rRNA-based-methods should be improved (for example, by using a nested-PCR approach) to enhance their detection prior to finding anammox activity evidences and thus shorten the batch culture step. However, nested-PCR also has some issues, mainly involving inherent PCR biases.

Initially, because of the few successful anammox enrichments and the high specificity of the primers and PCR conditions tested, a small number of positive amplifications was expected from the earlier stages of the study. After PCR, test B (high annealing

temperature) provided a low amplification ratio. Before the DGGE and the sequencing analyses, this lack of amplification was considered as an improvement, related to an increase in PCR specificity. Unfortunately, these expectations were overshadowed after PCR results of test C and specially after the sequencing of the overall DGGE partial sequences.

Firstly, high amplification ratios (similar to test A) were obtained after nested PCR step with a more specific primer set (test C). Secondly, different bacterial sequences were recovered in different tests. Finally, both primer sets retrieved unspecific phylotypes (whatever PCR conditions used), not always belonging to anammox bacterial group. Therefore, these results suggest that in these cases a high amplification ratio was not always related to a lower specificity of the primers.

The retrieved sequences from the tests based on the use of Pla46F-Amx368R primer set (A and B) were spread through *Planctomycetes* and *Lentisphaerae*, thus decreasing the chance to have amplified a high ratio of anammox DNA and, in consequence, the usefulness of this primer set. An extreme situation was found in test B, where all the phylotypes (except the one corresponding to *Ca.* Brocadia anammoxidans) were clustered into *Lentisphaerae* phylum, close to several uncultured bacteria. After analyzing the phylogenetic trees of tests A and B, the sequence clustering into this phylum was thought as a failure of the test, related to the nested-PCR biases. However, the late literature gave some hints about the present results. It has been recently described that *Lentisphaerae* is a novel phylum highly related to the PVC (*Planctomycetes*, *Verrucomicrobia* and *Chlamydia*) superphylum (Fuerst, 2013). Members of this phylum were detected in contaminated sludge and groundwater (Imfeld et al., 2010; Das and Kazy, 2014), in an anaerobic sludge digester (Chouari et al., 2005) and in landfill leachate (Limam et al., 2010). Indeed, a high recovery of *Lentisphaerae* clones was obtained in the anaerobic digester and the landfill leachate (98% and 85.9% respectively) by using Pla46F together with 1390R (universal primer). According to this, the clustering of the DGGE partial sequences into *Lentisphaerae* probably could not be longer considered as a bias caused by the chosen PCR approach, but from the election of the forward primer set.

Pla46F was initially described as specific for the *Planctomycetes* phylum but it was later recognized as being applicable to a broader phylogenetic range, as it targeted some members of the phylum *Lentisphaerae* (http://www.microbial-ecology.net/ probebase/search.asp) (Limam et al., 2010). Moreover, some studies revealed that Pla46F underestimates anammox bacterial abundance due to the lack of specificity of the oligonucleotides (Schmid et al., 2000; Egli et al., 2001; Schmid et al., 2003). Thus, it is possible that Pla46F primer tend to bind with *Lentisphaerae* in samples with low ratios of *Planctomycetes*. The decrease of the positive reactions in test B was probably because the primer set was unable to detect *Planctomycetes* at high annealing temperature and it was only attached to *Lentisphaerae*.

On the other side, the test based on the use of Amx368F-Amx820R primer set (test C) tended to retrieve unspecific phylotypes that clustered into *Pirellula* (which are also included in the *Planctomycetes* phylum) and WS3 division, but not *Lentisphaerae*. Moreover, other phylotypes obtained with these primers that were not specifically identified as known anammox bacteria belonged to the *Brocadiaceae*. According to these statements, the choice of Amx368F-Amx820R seem to allow a more specific detection of anammox-like bacteria, although it is still unable to detect anammox bacteria in the initial stages of the enrichments. This primer set offered a better counterbalance between specificity and sensitivity.

"PUTATIVE" ANAMMOX BACTERIA

Anammox bacteria, which were the target of these tests, are a novel group with a low number of known candidatus species and, even more, any pure cultured strain. In this context, what is the meaning of an unespecific sequence? To which extent do we consider that a sequence affiliated into *Brocadiaceae*, but not closely related to any sequence of the described anammox bacteria, is a failure in the detection of anammox phylotypes? The high amount of unspecificities retrieved using these primers must be accurately analyzed from this critical point of view.

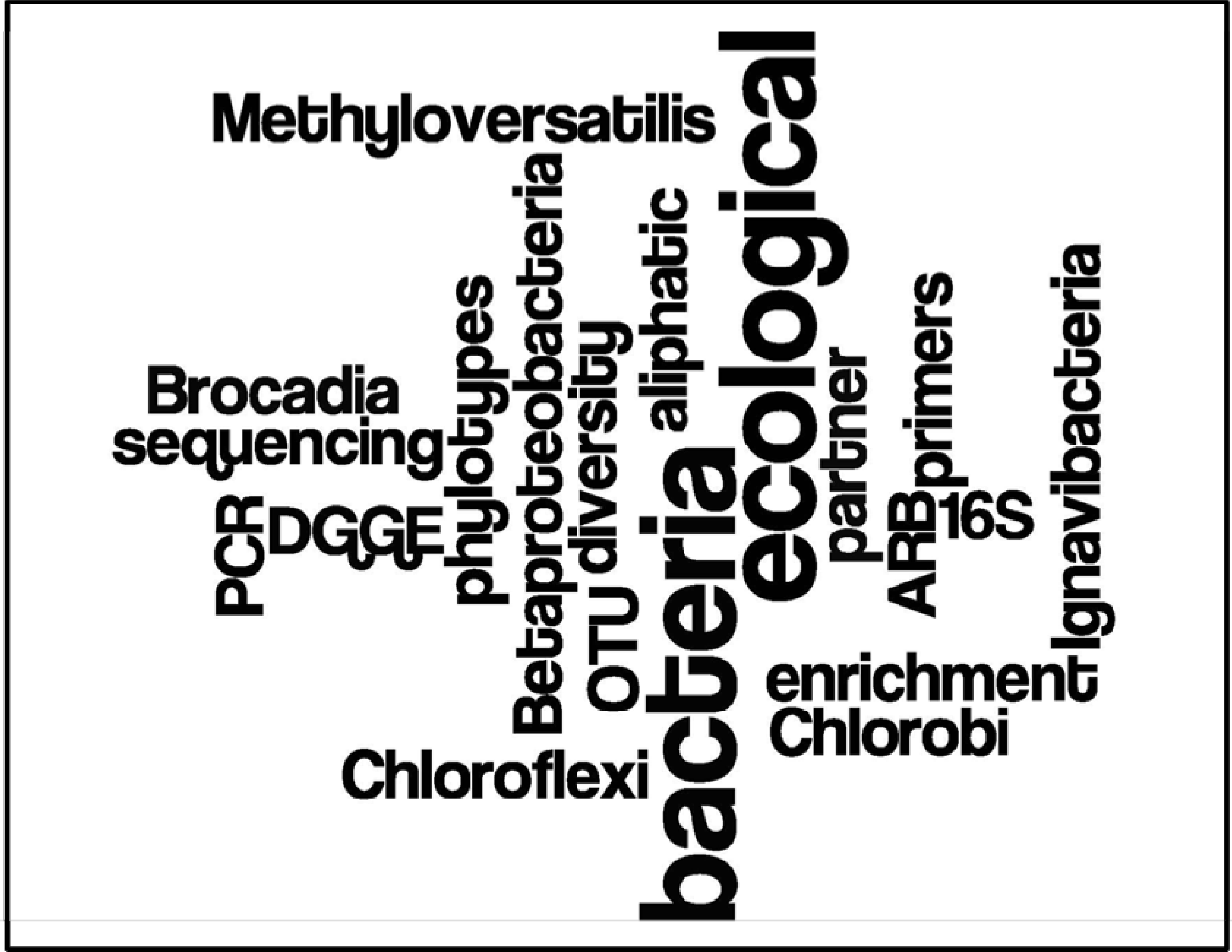
The highest ratio of sequences inside *Planctomycetes*, but even more importantly, inside *Brocadiaceae*, was delivered by test C. These sequences were clustered close to known anammox bacteria, but located in a different subgroup. There are two possible explanations about their presence. Firstly, it is feasible that too many PCR cycles delivered some "low quality" sequences because nested-PCR forced DNA template to be processed by a high number of PCR cycles. However, this hypothesis was ruled out since the DGGE partial sequences clustered together with database-retrieved sequences that were described in anammox-related studies. Secondly, it is possible that the unspecific sequences affiliated into this subgroup could actually belong to anammox bacteria that are still unknown. Probably in the upcoming years, when more anammox-related sequences will be loaded to databases, most of these *brocadiaceae* sequences will be identified as novel anammox bacteria. Indeed, Bae and collaborators (2010b) suggested, after the identification of a similar kind of sequences, to refer to them as "putative" anammox bacteria.

Assuming also these sequences as "putative" anammox bacteria, test C allowed to detect them in enrichments that developed *Ca.* Brocadia anammoxidans populations (E5, E6, E8 and E11). Again, more questions arise. What did happen to these "*putative*" anammox bacteria populations throughout the enrichment period? Were they removed due to *Ca.* Brocadia anammoxidans growth? Some studies (Kartal et al., 2006) reported that one growing anammox species (*Ca.* Anammoxoglobus propionicus) could outcompete other well-established anammox species (*Ca.* Brocadia anammoxidans) when operation conditions changed. In our case, hypothetically, environmental conditions in the inocula or during the first period of enrichment could benefit these "putative" anammox bacteria, but after a certain time *Ca.* Brocadia anammoxidans may have outcompeted them due to its more favourable growing conditions in the batch cultures.

The importance of environmental conditions during the enrichment is also exemplified by the differences in the evolution of the populations between E3 and E3bis. Both enrichments shared the same inoculum and most of the treatment process but only E3 developed *Ca.* Brocadia anammoxidans. However, E3bis had a previous washing step,

which could have changed the environmental conditions. Thus, performing replicate batch cultures to have more diversity of growth conditions could be a good choice to increase the chance of achieving anammox-enriched cultures.

Therefore, although the major aim of the work (the early detection and identification of active anammox organisms in environmental and inocula) was not completely accomplished, the detection of these “putative” anammox bacteria seem to be related to the potential for developing anammox populations if the environmental conditions in the batch enrichment cultures are appropriate for their growth.



Chapter III

Bacterial diversity in anammox enrichments

3.3.1 BACKGROUND

Nowadays, finding anammox bacteria and growing them in enrichments for wastewater treatment processes has derived in a large number of published studies. They differ in the anammox species present, the reactor configuration, the inocula used for seeding and the influent concentrations of ammonium and nitrite, but all of them have one thing in common: an anammox pure culture has never been obtained.

Strous and collaborators (1999a) achieved a 99.6% pure culture by developing a Percoll density gradient centrifugation procedure to separate cells of *Ca.* Brocadia anammoxidans from other members of the community. They considered that the residual presence of contaminant species did not contribute significantly to the anammox activity of the purified preparations (Kuenen and Jetten, 2001). However, Strous and collaborators (1999a) already suggested the necessary presence of some ecological partners for successful anammox activity. In addition, other studies also reached high anammox enrichment values (70-90%) in different kinds of reactors: (Egli et al., 2001; Fujii et al., 2002; Tsushima et al., 2007b; van de Vossenberg et al., 2008), among others.

The presence of ecological partners in communities with active anammox bacteria has never become a main research topic even though it could be a peculiar feature of the anammox bacterial enrichments. Several studies sought out the characterization of the anammox bacterial populations in various environments, but most of them avoid the whole microbial community description. Besides, some studies that tried to describe it relied on FISH analyses using only eubacterial, anammox and/or AOB probes, leaving out the rest of the community (Sumino et al., 2006; Chamchoi and Nitisoravut, 2007; Saricheewin et al., 2010; Vázquez-Padín et al., 2010).

The objective of this chapter is to analyze the microbial populations going with anammox bacteria. With this purpose, bacterial 16S rDNA sequences were retrieved from the fully developed anammox enrichments and compared by phylogenetic analysis to themselves and to a cluster of highly related sequences obtained from

online database. The relationships of the retrieved sequences with the already described bacteria provide a better approach to the ecophysiology of the anammox ecological partners. This knowledge can be useful for future optimization and increasing efficiency in the anammox process design and development.

DNA isolations from the successful anammox enrichments (E3,5,6,8 and 10, see Chapter I) at different incubation times were processed by PCR-DGGE approach using a bacterial primer set, 357F-GC and 907R. Although it was previously mentioned that E11 also developed *Ca.* Brocadia anammoxidans populations, it was not used for the microbial characterization analysis because it started up after the completion of the present study.

A DNA isolation from a 6-months lab-scale anammox bioreactor (named as AmxReac) was also included in the analysis. This bioreactor was inoculated with E3 and E10 (50 mL from each one). The presence of well-established anammox bacterial populations in the anammox bioreactor was previously checked by FISH analyses (data not shown). PCR products were processed by DGGE, using a 40-80% vertical gradient of denaturing chemicals (urea and formamide). After the electrophoresis, the most prominent DGGE bands were excised and further sequenced. Representatives sequences for each OTU (defined at a 0.03 cutoff) and initial phylogenetic relationships among the DGGE's sequences were obtained by using MOTHUR (using the latest SILVA bacterial database as reference alignment) and MEGA V5 package, respectively. Afterwards, ARB package was used to compare the own sequences with the ones obtained from databases.

3.3.2 RESULTS

CHARACTERIZATION of the MICROBIAL COMMUNITIES in ANAMMOX SOURCES

DGGE gel showed a high number of bands (fig. 3.3.1), suggesting the existence of a relevant specific richness in the microbial communities. Changes in the microbial

populations were detected throughout the enrichment period. As a general trend, day one samples (d1) showed lower richness in comparison with further enrichment periods. The batch cultures that developed *Ca.* Brocadia anammoxidans populations showed different patterns but they shared several bands located at the same position, suggesting that they could harbour similar species, even when they came from different inocula. Besides, AmxReac sample, which was inoculated with E3 and E10 (originally coming from the same seed), shared some bands with both enrichments, although some differences were still detected between them in the DGGE gel.

The microbial communities containing fully-developed anammox populations were the main target to search for the anammox ecological partners. It was considered that the last stages of the enrichments were the best option to fulfil this situation. Thus, only the DGGE bands from these final samples of the respective enrichment periods were excised and sequenced (fig. 3.3.1). Some chimera sequences were removed by applying MOTHUR specific tool and they were not included in the phylogenetic analysis. Several partial sequences (from 4 to 7) were obtained from every sample except for enrichment 8, from which only one sequence could be retrieved. Finally, 28 proper partial sequences were obtained. MEGA and MOTHUR were used to discern the taxonomy of the partial sequences and to calculate the maximum-likelihood phylogenetic tree (fig. 3.3.2).

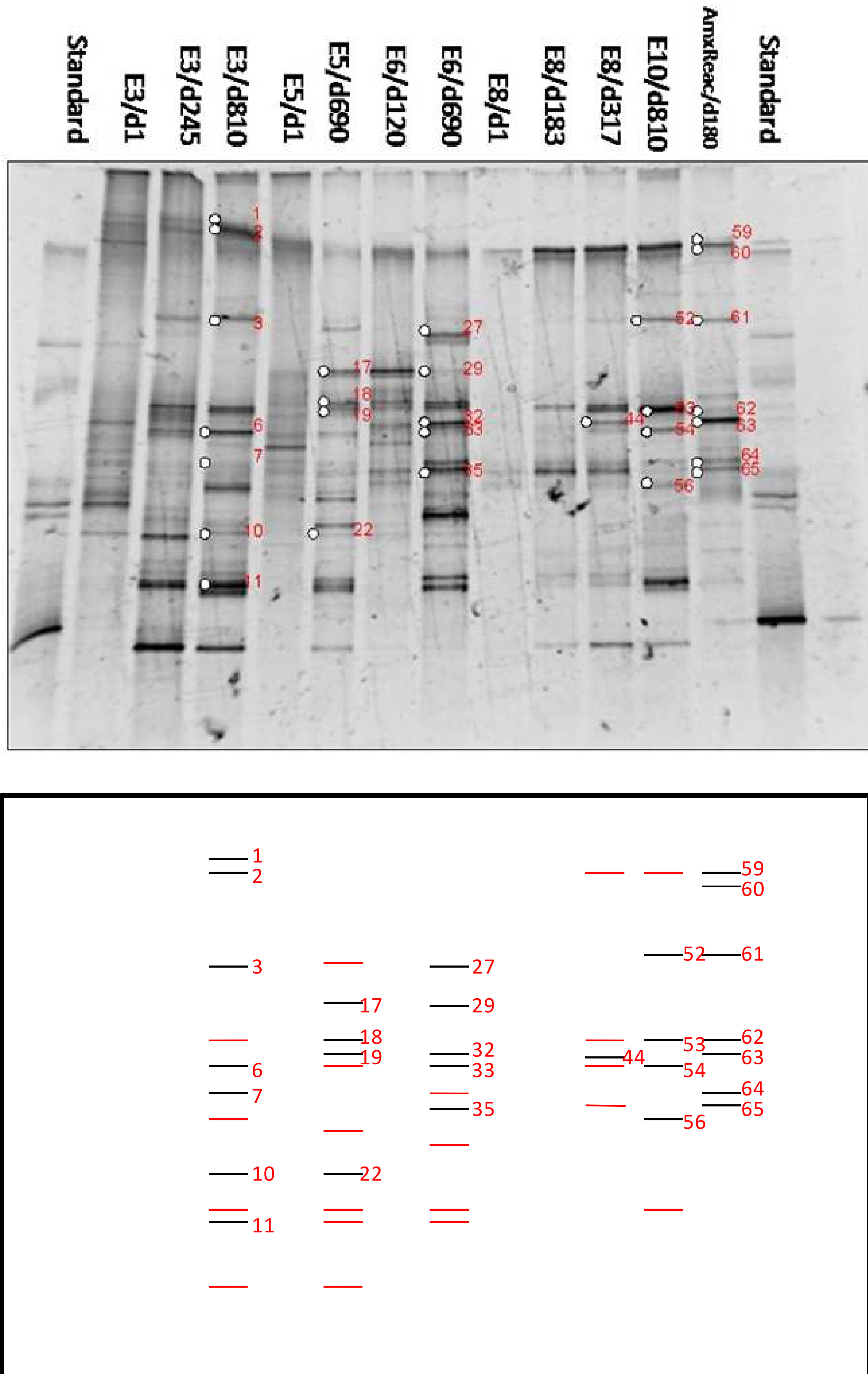


Figure 3.3.1: Gel image (up) and schematic representation (down) of the DGGE with a denaturing gradient from 40 to 80% using 357F-907R primer set. White dots and red numbers in the gel image show only the sequences that were properly added in the phylogenetic tree whereas discarded bands were depicted in red in the schematic representation.

Most of the retrieved phylotypes (table 3.3.1; fig. 3.3.2) belonged to the *Proteobacteria* phylum (43%) and spread over two subdivisions: *Betaproteobacteria* (29%), *Alphaproteobacteria* (14%). *Chlorobi* phylum was the second most important group, with 28.5% of the retrieved phylotypes. More distantly, the 11% of the sequences were affiliated into *Chloroflexi* class. The rest of the phylotypes (17.5%) were clustered into *Acidobacteria*, *Actinobacteria*, *Gemmatimonadales*, *Planctomycetales* and SBR1093 phyla (3.5% each). However, anammox bacteria related sequences were not detected. Only one phylotype, which was obtained from the anammox bioreactor (AmxReac/d180/AM900587), was affiliated within *Planctomycetales*, but its phylogenetic distance to the *Brocadiales* group was not closer enough to be clearly considered as an anammox phylotype.

The identification of these bands did not disclose any common species, although the similarity of their positions in the respective DGGE patterns. The analysis with MOTHUR revealed 5 different OTU, with a similarity of 97%, including 43% of the analyzed sequences (table 3.3.1). OTU1 is specially relevant, containing sequences retrieved from E6, E8 and the anammox reactor. It was affiliated to the *Methyloversatilis* genera, from *Betaproteobacteria* subdivision. Interestingly, the OTU1 sequences have different origins, whereas the phylotypes grouped into OTU2, OTU3 and OTU5 mainly have the same origin: E3. OTU4 follows the same trend than OTU1 but only involving sequences from E5 and E6. Perhaps the addition of more DGGE partial sequences could had been increased the number of common species found among samples. Focusing onto the singletons, several phylotypes, belonging to E3 and anammox reactor samples, were affiliated into *Ignavibacteriaceae* (*Chlorobi*). Another group of singletons were found into the *Chloroflexi*, where phylotypes from E3, E5 and E10 were clustered. Lastly, two phylotypes retrieved from anammox reactor and E6 samples, were branched together into *Sinobacteraceae*.

Table 3.3.1: Summary of the OTUs calculated from the sequences derived from DGGE bands, the representative sequence and the sequences included in each OTU.

OTU	Representative sequence	Sample sequences
1	E6/d690/AM900574	E6/d690/AM900574,AmxReac/d180/AM900586,E8/d317/AM90577
2	E10/d810/AM900578	E3/d810/AM900563,E10/d810/AM900578,AmxReac/d180/AM900584
3	E3/d810/AM900564	E3/d810/AM900564, E10/d810/AM900580
4	E6/d690/AM900573	E6/d690/AM900573, E5/d690/AM900568
5	E10/d810/AM900581	E10/d810/AM900581, AmxReac/d180/AM900588

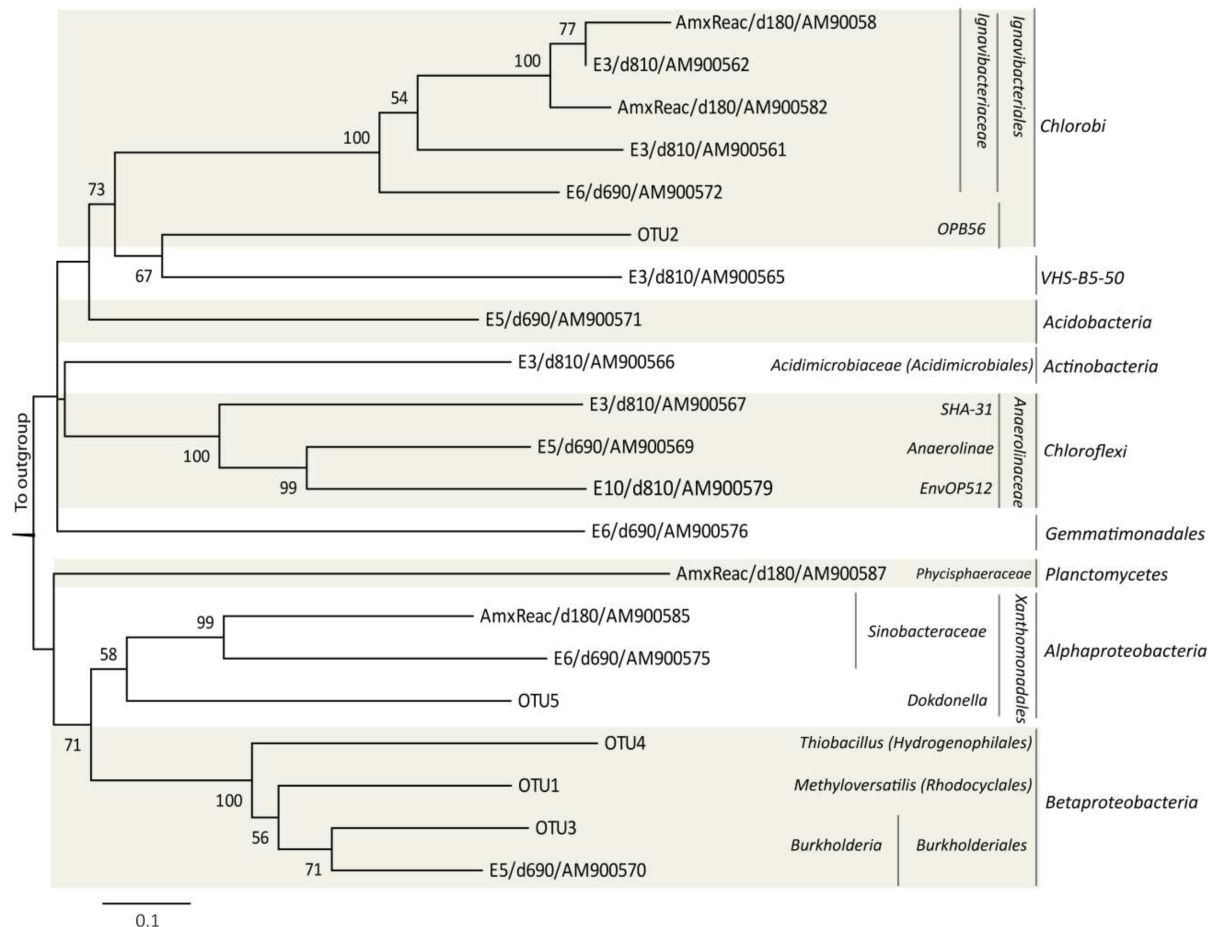


Figure 3.3.2: Maximum-likelihood phylogenetic tree based on the 16S rRNA gene from DGGE bands. OTU identification numbers are indicated in table 3.3.1. The alphanumeric code of each sequence from the DGGE bands indicate: enrichment number/days of enrichment/accession number. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. The affiliations of the sequences are shown in the right edge from genera to phylum, when feasible. The bar represents 10% estimated sequence divergence.

After the completion of the phylogenetic tree (fig 3.3.2), it was considered that more information was required to have a better resolution for analyzing the relationships among the bacterial phylotypes commonly found together with anammox bacteria. Thus, a high number of sequences similar to the DGGE partial sequences (minimum homology of 98-99%) were retrieved from the NCBI database (Satoh et al., 2007; Chuang et al., 2008; Liou et al., 2008; Park et al., 2008; Date et al., 2009; Riviere et al., 2009; Sueoka et al., 2009; Bae et al., 2010a; Cho et al., 2010; Li et al., 2010; Matsumoto et al., 2010; Militon et al., 2010; Yamamoto et al., 2011; van der Zaan et al., 2012). All the sequences were aligned by MOTHUR software and, finally, a phylogenetic tree was constructed by adding the DGGE partial sequences and the ones obtained from NCBI to the ARB backbone tree (fig 3.3.3). This phylogenetic tree was performed with ARB instead of MEGA to increase the number of similar sequences available for the analysis by adding those included in ARB database. After the completion of the ARB phylogenetic tree, the similarity between the affiliations obtained for DGGE partial sequences by ARB and MOTHUR was checked.

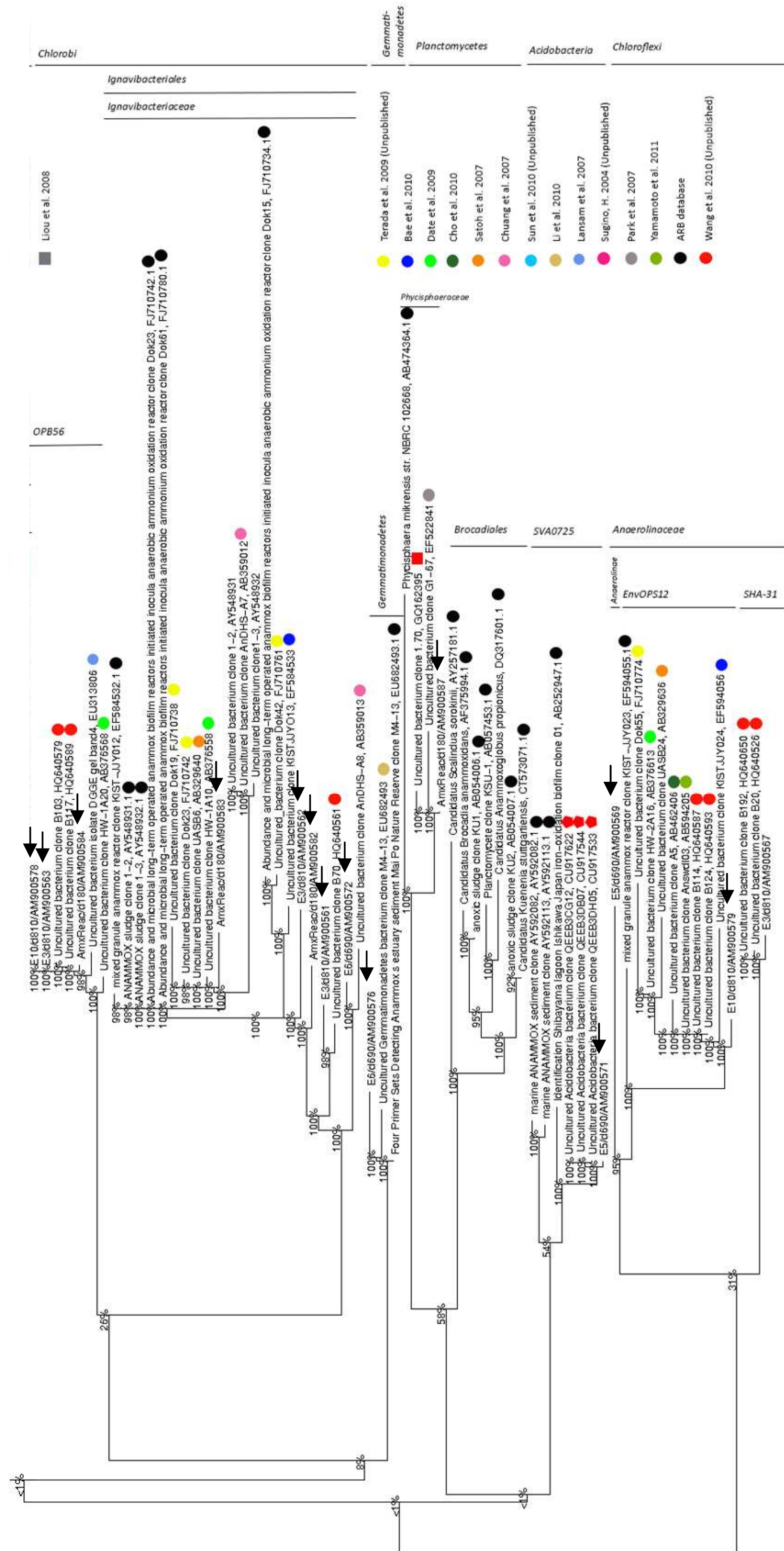
The external sequences introduced in the phylogenetic tree could be split into three main categories: (i) sequences obtained from studies based on the characterization of the microbial diversity of anammox reactors (as well as some unpublished related studies), (ii) sequences from the description of the microbial populations found on activated sludge and (iii) sequences from aromatic and aliphatic compound degradation studies.

Figure 3.3.3 (next pages): Phylogenetic tree showing the affiliations of the phylotypes retrieved in this work, as well as those obtained from other studies. The alphanumeric code of each sequence from the DGGE bands indicate: enrichment number/days of enrichment/accession number. Own sequences are highlighted with black arrows. External sequences are classified by a symbol in the right side, pointing out whether they were obtained from studies related to anammox bacteria (circles), degradation of aliphatic compounds (squares) or activated sludge (hexagons); each symbol has a different colour depending on the reference where these sequences were published. The bar represents 10% estimated sequence divergence.

Alphaproteobacteria
Xanthomonadales
Sinobacteraceae
Dokdonella
Betaproteobacteria
Rhodocyclales
Rhodocyclacea
Methyloversatilis
Hydrogenophilales
Hydrogenophilaceae
Thiobacillus
Burkholderiales
Burkholderiaceae
VHS-B5-50
SBR1093
EB1017
Acidomicrobiales
Actino-bacteria

Uncultured bacterium clone Field7, EU499701
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 496, AB252883.1
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 478, AB252882.1
AmxReac/d180/AM900585
Uncultured bacterium clone HW-1A09, AB376557
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 486, AB252884.1
Uncultured bacterium clone mbl-b7, AB426189
Uncultured bacterium clone AMGF11, AM935415
Uncultured bacterium clone CM38C4, AM936447
Uncultured bacterium cloneB129, HQ640596
E6/d690/AM900575
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 31, AB252886.1
Uncultured bacterium clone HW-2A03, AB376600
Uncultured bacterium clone NG-270micro-01, AB518138
E10/d810/AM900581
AmxReac/d180/AM900588
Pseudoxanthomonas taiwanensis, AB210278
Dokdonella ginsengisoli str. Gsoil 191, AB245362.1
mixed granule anammox reactor clone KIST-JJY035, EF654704.1
situ activity and spatial organization anaerobic ammonium-oxidizing (anammox) biofilms clone: clone HU4, AB290147.1
Abundance and microbial long-term operated anammox biofilm reactors initiated inocula anaerobic ammonium oxidation reactor clone Dok59, FJ710778.1
ANAMMOX sludge clone 1-7, AY548934.1
ANAMMOX sludge clone 2-7, AY548943.1
mixed granule anammox reactor clone KIST-JJY037, EF654706.1
Uncultured bacterium clone KISTJJY037, EF654706
anammox sludge clone, AB194898.1
Nitrogen removal performance and microbial anaerobic up-f granular bed reactor anammox enriched clone A2, AB462403.1
mixed granule anammox reactor clone KIST-JJY030, EF654699.1
Uncultured bacterium clone:A, AB194898
Uncultured bacterium clone KISTJJY036, EF654705
Uncultured bacterium clone:A2, AB462403
mixed granule anammox reactor clone KIST-JJY032, EF654701.1
E8/d317/AM900577
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 196, AB252923.1
Identification Shibayama lagoon Ishikawa Japan iron-oxidation biofilm clone 333, AB252924.1
Abundance and microbial long-term operated anammox biofilm reactors initiated inocula oxygen-limited autotrophic nitrification-denitrification reactor clone Ge45, FJ710695.1
Abundance and microbial long-term operated anammox biofilm reactors initiated inocula oxygen-limited autotrophic nitrification-denitrification reactor clone Ge24, FJ710674.1
Bacterium enrichment culture clone_1.73, GQ162398
Uncultured bacterium clone Ge63, FJ710713
Uncultured bacterium clone KISTJJY038, EF654707
Bacterium enrichment culture clone 1.86, GQ162411
Uncultured bacterium clone B90, HQ640568
Nitrosospira sp. clone P96, GQ255608
E6/d690/AM900574
AmxReac/d180/AM900586
Thiobacillus thioparus strain THI_115, HM535225
Uncultured bacterium clone B10, HQ640517
E6/d690/AM900573
E5/d690/AM900568
Thiobacillus sp. str. ST22, FJ982929.1
Thiobacillus sp. K6.2, EF079668.1
Burkholderiales bacterium enrichment culture clone ANA-RAS-6, HM769659
mixed granule anammox reactor clone KIST-JJY028, EF654697.1
E10/d810/AM900580
E3/d810/AM900564
Uncultured bacterium clone B16, HQ640523
Uncultured bacterium clone B169, HQ640627
Uncultured bacterium clone B40, HQ640539
E5/d690/AM900570
ANAMMOX sludge clone 1-5, AY548933.1
ANAMMOX sludge clone 2-12, AY548944.1
suspended sludge anammox reactor clone KIST-JJY041, EF654710.1
Uncultured bacterium clone AMEF1, AM935326
Uncultured bacterium clone Kaz2, AB491166
Uncultured bacterium clone BACDER07-3H7, FN597995
E3/d810/AM900565
E3/d810/AM900566
Uncultured bacterium clone QEDR3CG08, CU922479

Riviére et al. 2009
Sanchez et al. 2009
Matsumoto et al. 2010
Sueoka et al. 2009
Boden et al. 2012
Dong et al. 2009 (Unpublished)
Sakai et al. 2008 (Unpublished)
van der Zaan et al. 2012
Militon et al. 2010


Chlorobi
Ignavibacteriales
Ignavibacteriaceae
OPB56
Gemmatimonadetes
Planctomycetes
Acidobacteria
Chloroflexi
Phycisphaeraceae
Brocadiales
SVA0725
Anaerolinaceae
Anaerolinae
EnvOPS12
SHA-31
Liou et al. 2008
Terada et al. 2009 (Unpublished)
Bae et al. 2010
Date et al. 2009
Cho et al. 2010
Satoh et al. 2007
Chuang et al. 2007
Sun et al. 2010 (Unpublished)
Li et al. 2010
Lansam et al. 2007
Sugino, H. 2004 (Unpublished)
Park et al. 2007
Yamamoto et al. 2011
ARB database
Wang et al. 2010 (Unpublished)
0.10

The first group was the dominant and their sequences had a widespread distribution along the phylogenetic tree, clustering close to sequences of each enrichment. Three phylogenetic groups harboured a great number of sequences from different studies: *Methyloversatilis* (*Betaproteobacteria*), *Ignovibacteriaceae* (*Chlorobi*) and *Anaerolinaceae* (*Chloroflexi*). *Methyloversatilis* sequences were related to the sequences belonging to OTU1, *Ignovibacteriaceae* contained sequences related to E3, E6 and AmxReac, and *Anaerolinaceae* had a mixture of phylotypes clustering close to E3, E5 and E10. The distribution of the sequences involved in the aliphatic compounds degradation was quite homogenous, exhibiting high homology with E3, E6 and anammox bioreactor sequences. They clustered into *Alphaproteobacteria*, *Betaproteobacteria*, SBR1093 and *Gemmatimonadetes* phyla. The sequences retrieved from activated sludge were mainly related with E3, E5 and E6. They distributed into *Chloroflexi*, *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*. In both categories, there was no clear differentiation due to the origin of the enrichment.

3.3.3 DISCUSSION

DO ECOLOGICAL PARTNERS PLAY an IMPORTANT ROLE in the DEVELOPMENT of COMMUNITIES with ANAMMOX ACTIVITY?

Any unique ecological partner for anammox bacteria has been described up to now. However, some specific ecological relationships can be established since most of the studies involved in the characterization of the microbial composition of anammox bioreactors exhibited a similar microbial pattern. Interestingly, the composition of the microbial populations obtained in this work after the performance of the fingerprinting techniques and the further sequencing showed a similar ecological and phylogenetic composition, agreeing with other studies (table 3.3.2). Some new groups came out (*Acidobacteria*, *Actinobacteria*), but the main phyla (*Proteobacteria*, *Chlorobi*, *Chloroflexi*) were present in most of the studies. Not only at phyla level, but even at

family/genus depth, the concordance with other studies is similar (fig. 3.3.3). This microbial pattern was obtained even though the present work used several anammox sources from different origins whereas the microbial populations described in the previous studies mostly used one source.

Proteobacteria phylotypes are found to be the dominant populations in most of the studies, not only in anammox-related ones but also in those studies characterizing microbial communities from WWTPs sludge without evident anammox activity (Wagner et al., 2002). *Proteobacteria* encompass enormous morphological, physiological and metabolic diversity, and are of great importance to global carbon, sulfur and nitrogen cycles (Kersters et al., 2006). Unfortunately, in the published studies, the retrieved phylotypes belonging to *Proteobacteria* were not usually classified into the different classes, and they grouped only as *Proteobacteria*. In the present work, this subdivision could be achieved, and the dominance of *Beta*- class over *Alpha*- class was clearly evident. Fujii and collaborators (2002) already suggested the dominance of *Betaproteobacteria* over other groups through the use of confocal images. Although the functionality description of each group is hard to define, in some cases it can be hypothesized. *Methyloversatilis* genera, belonging to *Rhodocyclaceae* family, is a recurrent group present in several anammox-related studies (fig. 3.3.3), although their role is not clear. *Zoogloea*, a *Rhodocyclaceae*-related bacteria, was found to form a thin layer around the anammox agglomerated spheres (Fujii et al., 2002). It was postulated that *Zoogloea* presence in the external sections of the aggregates could protect the living anammox present in the internal regions from O_2. Although *Zoogloea*-related sequence was not detected (fig. 3.3.3), the main presence of *Methyloversatilis* sequences in anammox environments suggests that they might be have an important role in the anammox aggregate structure and viability.

The coexistence of *Chlorobi* (green sulfur bacteria) and *Chloroflexi* (green non sulfur bacteria) with anammox bacteria must be taken into account since their presence was also reported in several studies (Strous et al., 2006; Gong et al., 2007; Liu et al., 2007; Li et al., 2009; Xiao et al., 2009). The dominance of *Chlorobi* over *Chloroflexi* populations was usually detected (table 3.3.2).

Table 3.3.2: Bacterial community composition in different types of anammox reactor systems. Modified from Li et al. (2009).

Reactor type	NH_4^+-N and NO_2^--N in influent water	Influent water type	Phylotypes abundance[a]	Reference	Primer set
Non-woven biomass carrier	250 mg L^{-1}	Mixed in groundwater of low total	Anammox 38% *Chlorobi* 7% *Proteobacteria* 55%	Fujii et al. (2002)	16S-1//16S-2 (designed in the study)
Rotating biological contactor	100-500 mg L^{-1}	Wastewater	*Planctomycetes* *Proteobacteria* *Firmicutes*	Egli et al. (2003)	6F-1510r (mod. from Weisburg et (1991)
Anoxic gas-lift reactor[b]	NM[c]	Synthetic wastewater	Anammox 2% *Chlorobi* 36% *Proteobacteria* 31% *Bacteroidetes* 15% *Chloroflexi* 13%	Strous et al. (2006)	
Rotating disk reactor	1.4-4.6 mg L^{-1} [d]	NM	*Proteobacteria* 27% Others not mentioned	Tsushima et al. (2007b)	Bac11F-1387R (Weisburg et al., 1991)
Cylindrical up-flow anaerobic reactor	50-1000 mg L^{-1}	Artificial wastewater	*Chlorobi* 54% *Proteobacteria* 13% *Bacteriodetes* 11% *Chloroflexi* 9% Anammox 9%	Li et al. (2009)	27F-1390R (Lane, 1991)
Up-flow granular bed anammox reactor	15 kg N m^{-3} d^{-1}	Synthetic nutrient medium	Anammox 33% *Proteobacteria* 23% *Nitrosomonas* 4% *Chloroflexi* 35% *Bacteroidetes* 2% *Acidobacteria* 2%	Cho et al. 2010 (2010)	27F-1492R (Lane, 1991)
Up-flow anaerobic sludge bioreactor	NM	NM	*Proteobacteria* 42% *Chloroflexi* 22% *Anammox* 20% *Chlorobi* 7% *Bacteriodetes* 5% *Acidobacteria* 2% *Actinobacteria* 2%	Bae et al. 2010	27F-1492R (Lane, 1991)
Batch anammox cultures and anammox bioreactor	10-100 mg L^{-1}	Synthetic nutrient medium	*Betaproteobacteria* 29% *Chlorobi* 28.5% *Gammaproteobacteria* 14% *Chloroflexi* 11% *Planctomycetales* 3.5% *Actinobacteria* 3.5% *Acidobacteria* 3.5% *Gemmatimonadales* 3.5% *Deltaproteobacteria* 3.5%	This work	357F-907R

[a] % of main taxonomic categories derived from the total of sequences retrieved in each study.
[b] This result was a comparative analysis of all 16S rRNA gene sequences retrieved from metagenome sequencing.
[c] Not mentioned.
[d] These concentrations were used in batch tests.

Relevant information about the relationship between anammox bacteria and *Chlorobi* species was described in other studies. Chuang and collaborators (2008) suggested that uncultured *Chlorobi* could use organic matter derived from the microbial catabolism of anammox bacteria, while Park and collaborators (2010) found a relationship between the presence of bacteria from the *Bacteroidetes/Chlorobi* phyla and the development of the sludge granulation process.

Today is quite clear that *Chlorobi* have an ubiquous presence in most of the anammox sludge, although most of the members of the phylum *Chlorobi* are obligatory photoautrotophic bacteria (Frigaard and Bryant, 2004). So, the continuous presence of obligate phototrophic bacteria in batch cultures that always remained in the dark (not only the enrichments from the present work, but most of the bioreactors and pilot plants are commonly protected from the light) could be surprising. Focusing only in the phylogenetic relationships discerned in this work (including DGGE and database sequences), it was observed that all the *Chlorobi* sequences belonged to *Ignavibacteraceae*. Recently, Liu et al. (2012) described one species from *Ignavibacteria* genera (*I. album*) as the only non-phototrophic member of the phylum *Chlorobi* that has been isolated and grew anoxically in the laboratory. Moreover, it was suggested that this species can live under both oxic and anoxic conditions by using a variety of electron donors and acceptors. It is feasible that other members from the *Ignavibacteria*ceae family have similar features to *I. album*. Their theoretical features might convert them in suitable ecological partners for the anammox bacteria, and could explain the reason why *Chlorobi* is usually related with anammox bacteria in activated sludge with non-phototrophic growth conditions.

On the other hand, the presence of *Chloroflexi*-like could be related to the aggregate structures. A high detection frequency of *Chloroflexi*-like bacteria suggested that this group may be important for granulation (Bae et al., 2010a). FISH analyses showed that *Chloroflexi*-like filamentous bacteria were mainly present at the surface of the granules and around bacterial clusters, suggesting that they could probably be responsible for the construction of web-like structures (Li et al., 2008; Li et al., 2009). Their co-

existence with anammox bacteria might suggest hypothetically similar capabilities to grow in ammonium- and nitrite-rich, oligotrophic, and oxygen-deficient environments.

Interestingly, most of the microorganisms hitherto described are responsible of community organization in aggregates, floccules or similar structures. All the evidences point out that anammox bacteria do not depend of a single ecological partner but the presence of surrounding species may be essential. It could be speculated that not only the presence of members of *Planctomycetales* but also *Chlorobi*, *Chloroflexi* or *Betaproteobacteria* encompassing families such as *Rhodocyclaceae,* would confer structural integrity, playing a major role in the consecution of environmental conditions for a successful anammox reaction.

The presence of phylotypes related with bioremediation studies (Liou et al., 2008; Militon et al., 2010; van der Zaan et al., 2012) in the sequences obtained from the databases was an unexpected result (fig. 3.3.3). These sequences were recovered from all the seed range (constructed wetlands, WWTPs, and anammox bioreactor) and clustered into *Planctomycetales*, *Beta-*, *Gamma-* and to *Deltaproteobacteria*. Thus, the presence of bacteria able to degrade recalcitrant compounds (such as benzene derivatives) as anammox ecological partners in activated sludge not only increases the global species richness in anammox environments (involving bacteria with very different metabolisms) but also points out the singular characteristics of some of these anammox bacteria partners.

IS the DETECTION of ANAMMOX POPULATION in the WHOLE BACTERIAL COMMUNITY BIASED by the ELECTION of the PRIMER SET ?

Fingerprinting techniques were used to identify the main bacterial populations that coexisted with the anammox bacteria after a long enrichment period. The bacterial primer set (357F-907R) was selected to screen the overall 16S rRNA gene diversity in anammox batch cultures and bioreactor samples. As explained before, the bacterial

population composition described in the present work mostly agrees with the rest of the studies (table 3.3.2) but surprisingly no anammox phylotypes were retrieved.

There are two possible hypotheses for this lack of amplification with the bacterial primers: first, the inherent PCR template-to-product ratio bias (Polz and Cavanaugh, 1998) and second, possible mismatches between the primer sequence and the anammox 16S rRNA gene (Sipos et al., 2007). The phylogenetic analyses were only based on the last stages of the enrichments, when anammox bacteria were quite well-developed to initially overcome the first hypothesis. Even a sample from a 6-months enriched anammox bioreactor was used. On the other hand, Kuenen & Jetten (2001) and Penton and collaborators (2006) already described the difficulties of retrieving 16S rRNA anammox sequences from environmental samples by the use of “universal” primers.

During a PCR, the polymerase could have more problems to recognize the primer and to extend the duplex when the number of mismatches increases (Kwok et al., 1990), producing a decrease in the efficiency of the reaction. Thus, as higher is the number of mismatches between the bacterial primer and the anammox 16S rRNA gene as lower is the anammox phylotypes appearance in the characterization of the community. The comparison of anammox 16S rRNA gene sequences with the primer sequences used in the studies included in the table 3.3.3 could be useful to analyze the efficiency of these primers, since the relative abundance of detected anammox bacteria seemed to be rather diverse in these studies.

Table 3.3.3: The number of mismatches between the commonly primers used in the identification of bacterial community and the 16S rRNA gene sequence of the known anammox.

	27F	1492R	16S-1	16S-2	6F	1510R	Bac11F	1387R	27F	1390R	357F	907R
***Ca.* B. anammoxidans**	X	2	1	0	X	9	X	0	X	0	1	2
***Ca.* B. fulgida**	X	2	1	0	X	9	X	0	X	0	1	2
***Ca.* K. stuttgartiensis**	X	2	0	0	X	9	X	0	X	0	1	2
***Ca.* A. propionicus**	X	2	2	2	X	9	X	0	X	0	1	2
***Ca.* S. wagnerii**	X	2	0	0	X	9	X	0	X	0	1	2
***Ca.* S. brodae**	X	2	1	0	X	9	X	0	X	0	3	2
***Ca.* S. sorokinii**	X	X	1	0	X	9	X	0	X	0	3	2
***Ca.* J. asiatica**	X	2	1	0	X	8	X	0	X	0	1	2

X= evaluation cannot be done

This analysis (table 5.3) shows that 357F-907R primer set contains only 1 mismatch in the forward primer, close to the 3' region, and 2 mismatches in the reverse primer (in the middle region of the primer sequence) with most of the known anammox bacteria (excepting *Ca.* Scalindua brodae and sorokinii). Therefore, since the results from Chapter I showed that only *Ca.* Brocadia anammoxidans populations were present in the batch cultures used in the present work, the potential efficiency problems should be quite inexistent. According to the complementary correspondence between primers and targets, the 357-907R primer set should be suitable enough to detect anammox populations in the samples under study without any critical biases. It can not be considered as a bad primer set choice for the detection of *Ca.* Brocadia anammoxidans, although other primer sets (such as 16S-1/16S-2) might provide better characterization of the anammox populations in the whole community. On the other hand, the 6S/1510R primer set seems to be the worst combination for a proper anammox retrieval.

Similar conclusions can be achieved by comparing the studies shown in the table 3.3.2. The studies that mostly have contributed to the characterization of the bacterial populations in anammox environments were performed by Li et al. (2009), Cho et al. (2010) and Bae et al. (2010a). In all three studies, 27F was used as the forward primer but in the former it was coupled with 1390R while the others combined with 1492R. As it was shown in table 3.3.2, 1390R has no mismatches with any species whereas 1492R has two mismatches with the 16S rRNA gene of all the anammox species. It was not possible to evaluate the 27F suitability since it is commonly used for the whole 16S rRNA gene sequencing and this region is rather available in the online database: the first 20 bases, which correspond to the primer sequence, are commonly poor resolved with the Applied Biosystems capillary DNA sequencers (ABI sequencers), according to the DNA Sequencing Troubleshooting Guide (Korch, 2010). If the primer set election, according to the number of mismatches, should have effects on the retrieval of a high amount of anammox phylotypes in a study of bacterial community, it should be Li et al. (2009) who showed the highest anammox rate. Unlikely, it was Cho and collaborators (2010) who presented the highest anammox clone retrieval (33%). Since the three reactors seemed to operate at high rates (table 3.3.2), a feasible explanation could be

that the number of anammox 16S rRNA gene copies present in the DNA isolations was rather different, explaining this significant variation of the clone recovery.

Hence, in the present work and despite the presence of *Ca.* Brocadia anammoxidans in all the samples, it seems more suitable the explanation of PCR biases due to a low copy number of 16S rRNA gene (below the limit of detection of the eubacterial primers), rather than the presence of bacterial primer mismatches. These detection difficulties when "universal" primer sets were used, in relation to the low copy numbers of anammox PCR targets, could be the reason of underrating their populations during long time, although anammox activities and ecological consequences could be more significant than the scientific community assumed. Nowadays and in the future, pyrosequencing analyses should help to correct the underestimation of anammox presence and role because of the overwhelming average of retrieved sequences comparing with DGGE and cloning procedures.

Chapter IV

AOB and NOB dynamics in a high ammonium concentrated bioreactor

3.4.1 BACKGROUND

Autotrophic nitrification followed by heterotrophic denitrification is currently the most widely used method for nitrogen removal in wastewater treatment plants (WWTPs). Nonetheless, this treatment is disadvantageous when dealing with wastewater with a high nitrogen concentration and low biodegradable organic matter; high oxygen demand and the need for an external carbon supply are the main drawbacks of the process (van Dongen et al., 2001; Ganigué et al., 2009). Other processes have been developed on the basis of anaerobic ammonium oxidation instead of heterotrophic denitrification. Thus, to allow its activity, a partial nitritation (PN) step must be previously performed by ammonium-oxidizing bacteria (AOB) to oxidize only part of the ammonia to nitrite while avoiding its subsequent conversion to nitrate by nitrite-oxidizing bacteria (NOB). Consequently, it is necessary to limit AOB activity and to impair NOB in the PN reactor.

Nitrifying bacteria are key microorganisms in the PN process, and they are spread over the prokaryotic phylogenetic tree. *Nitrosomonas* and *Nitrosospira* genera are the most frequent AOB found in this environment, belonging to *Betaproteobacteria* subdivision (Bothe et al., 2000), whereas *Nitrobacter* (*Alphaproteobacteria* subdivision) and *Nitrospira genera*, split into several classes (Daims et al., 2001) are the most commonly detected NOB.

Both AOB and NOB are chemolithotrophs and relative slow growers, with low biomass yields, long incubation times and specific growth requirements, therefore limiting the use of traditional microbiological methods for their study (Bernhard et al., 2005; Mota et al., 2005). Currently, the most common techniques for the detection and identification of these bacteria are based on PCR amplifications of their DNA, targeting 16S ribosomal subunit operons or key genes coding for enzymes involved in N transformations, such as ammonia monooxygenase (*amo*A) (Bothe et al., 2000) or nitrite oxidoreductase (Nxr) (Yamanaka and Fukumori, 1988; Starkenburg et al., 2008) AOB and NOB quantification has been attempted using other molecular methods, such as competitive PCR (Dionisi et al., 2002; Cébron and Garnier, 2005b) and qPCR

(Hermansson and Lindgren, 2001; Tsushima et al., 2007a; Hu et al., 2010; Kim et al., 2011b; Yao et al., 2011).

The major aim was to study the population dynamics of AOB and the main NOB genera in response to ammonium-increasing conditions in a SBR designed to perform PN with a remarkably high ammonium leachate concentration (up to 2000 – 3000 mg N L^{-1} of both ammonium and nitrite in the reactor) during its start-up and subsequent stable operation. With this purpose, a previous goal had to be accomplished: the optimization for qPCR of several primer sets that were barely reported to have been used for quantification. Concerning AOB, it was also interesting to determine the species that were capable of growing at these concentrations of nitrite and ammonium, which should be fairly outstanding for most bacteria. Although the conditions within the reactor were intended to wash out NOB populations, they were still looked for, as small amounts of nitrate were produced during the working period of the reactor. The information derived from the present study should help to obtain a better understanding of the effects of the reactor's working features over the nitrifying bacteria populations.

Identification and quantification of AOB and NOB main genera were performed by PCR coupled with cloning procedures and qPCR analyses, respectively. Initial screening of both chemolithotrophic populations was also attempted by DGGE analyses. The identification of AOB populations was done by using CTO189F mix (working as a 2:1 mixture of A/B and C primers) coupled with CTO654R. *Nitrobacter* and *Nitrospira* populations characterization was carried out with the FGPS872F-FGPS1269R primer set for the former, and the NSR1137F-NSR1269R primers for the latter. Sequences retrieved from each cloning library were aligned and later assigned to OTUs (defined at a 97% cutoff) with MOTHUR using the latest SILVA bacterial database as reference alignment. Representative sequences for each OTU were also calculated using the implemented tool in MOTHUR. For each test, tree topology and phylogenetic distances were computed using the maximum-likelihood method in the MEGA V5 package. For quantification, qPCR analyses performance compelled to change AOB and *Nitrobacter* primer sets. Thus, RT1R was used for AOB and Nwi70F coupled with Nwi165R for NOB.

Standard curve for each primer set was constructed by 10-fold dilution of clones containing the specific target.

3.4.2 RESULTS

PARTIAL NITRITATION (PN) REACTOR PERFORMANCE

The system under study was successfully operating for 450 days (fig. 3.4.1), treating leachate with high ammonium concentrations. The influent ammonium concentrations varied significantly over the course of the study (Ganigué et al., 2009). The initial concentration was 5000 mg NH_4^+-N L^{-1}, but during certain periods (days 109-194 and 279-355), the supplied leachate presented lower concentrations, with values around 2500 mg NH_4^+-N L^{-1}. Effluent concentrations of ammonium and nitrite changed according to the influent ammonium concentration and the available bicarbonate. Over the majority of the study, their concentrations were kept in the range of 500-3400 mg NH_4^+-N L^{-1} and 700-3200 mg NO_2^--N L^{-1}, respectively. These high concentrations, coupled with the elevated temperature and pH, resulted in free ammonia and free nitrous acid concentrations of up to 346 mg NH_3 L^{-1} and 0.34 mg HNO_2 L^{-1}, respectively. The ammonium oxidation rate (AOR), calculated as the amount of ammonium oxidized per unit volume per day, was around 0.5 kg N m^{-3} d^{-1} for the majority of the study, with peak values up to 0.8 kg N m^{-3} d^{-1}. In addition, some traces of nitrate production (irrelevant from an operational outlook) were detected over the course of the study, with concentrations seldom higher than 25 mg NO_3^--N L^{-1}.

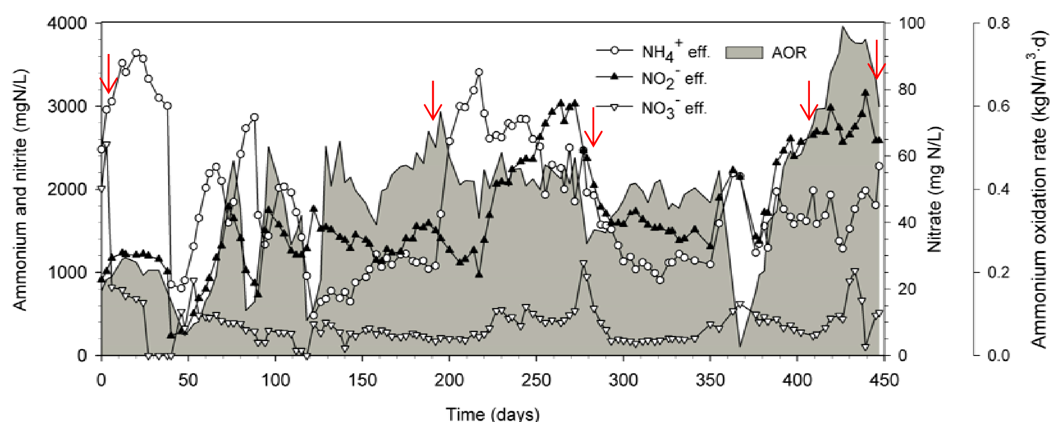


Figure 3.4.1: Evolution of the main nitrogen compounds and the ammonium oxidation rate (AOR) at the effluent of the reactor. Red arrows indicate the period throughout the reactor performance where samples for DNA isolation were collected.

MOLECULAR DETECTION and IDENTIFICATION of AOB

Since changes in the AOB community were expected throughout the reactor performance, several samples were collected and analyzed throughout the process (fig. 3.4.1). DNA isolations from day 0 (R0), day 192th (R192), day 288th (R288), day 415th (R415) and day 450th (R450) were amplified with CTO189F mix (with GC clamp) together with CTO654R, and the PCR products were subsequently loaded into a DGGE with a denaturing gradient from 30-70% (data not shown). Fingerprints from the time of inoculation (R0) showed a characteristic band pattern, without any DNA bands shared with the rest of the samples, whereas the samples from day 192th (R192) to 450th (R450) presented the same fingerprint pattern. According to these results, cloning of samples R0 and R450 were carried out to screen the AOB diversity in more detail.

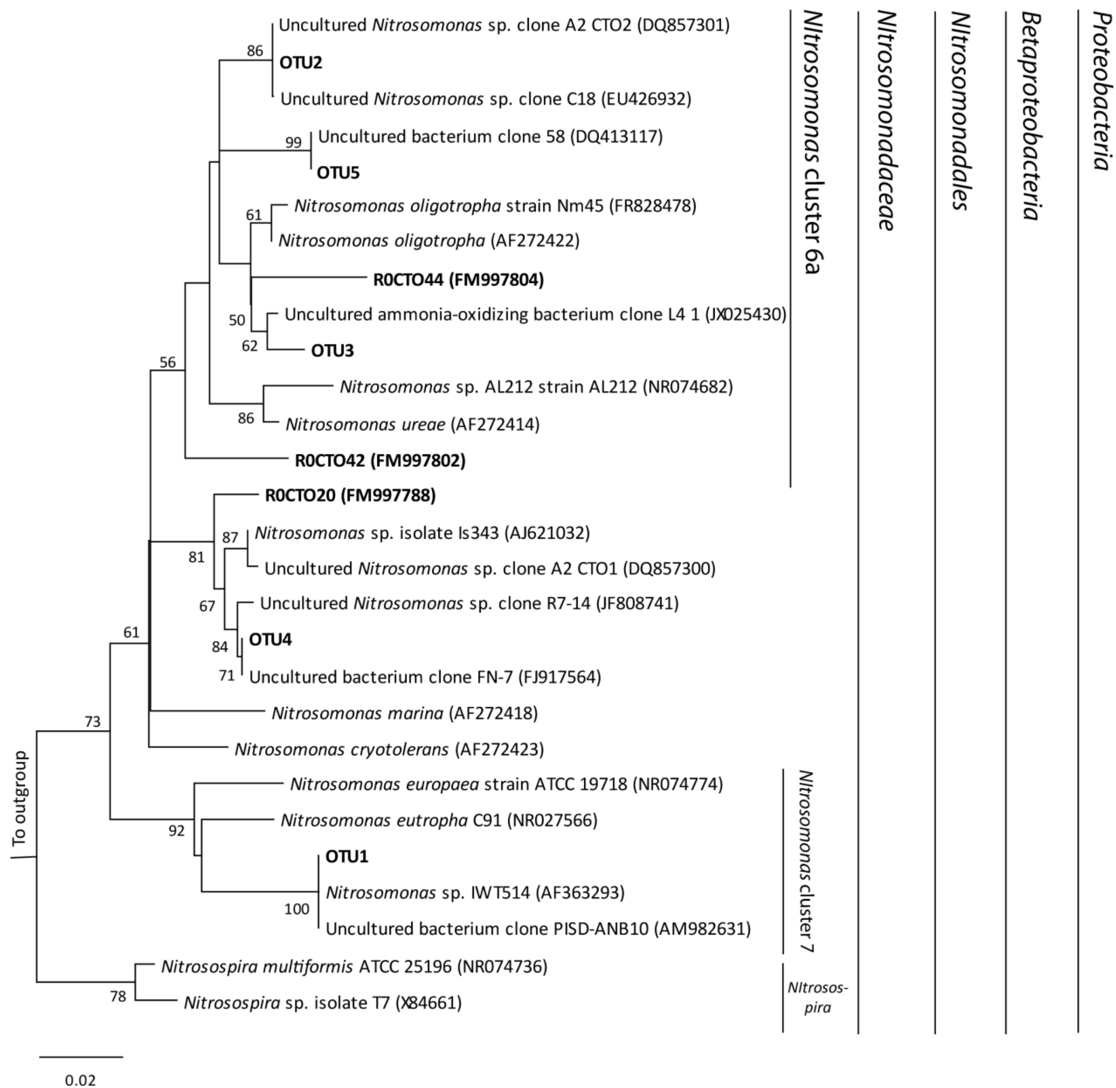


Figure 3.4.2: Maximum-likelihood phylogenetic tree based on 16S rRNA gene retrieved from cloning library after PCR with CTO mix, from R0 to R450 samples. OTU identification numbers are indicated in table 3.4.1. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. Accession numbers of external 16S rDNA sequences are given within brackets. The bar represents 2% estimated sequence divergence.

Up to 67 proper sequences (Ca. 465 bp) were finally retrieved from both samples after removing chimera and double sequences (<5%). MOTHUR calculations by applying a 0.03 cutoff allowed to group the sequences of both samples in 5 different OTUs (table 3.4.1). The representative sequences from each OTU together with the singletons and several external 16S rDNA sequences, from both cultured and uncultured related bacteria, were loaded into MEGA to obtain a maximum-likelihood phylogenetic tree (fig 3.4.2). Besides, the taxonomic affiliation of each sequence was also obtained by the tool included in the MOTHUR software.

All clone sequences belonged to *Nitrosomonadaceae*, within *Betaproteobacteria* subdivision (fig 3.4.2). Interestingly, all sequences arisen from R450 were grouped exclusively into OTU1, clustering inside *Nitrosomonas europaea/eutropha* lineage (also called *Nitrosomonas* cluster 7) and they were highly related to *Nitrosomonas* sp. IWT514 (98-99% homology) (accession number: AF363293). In contrast, sequences belonging to all 5 OTUs were retrieved from R0. Interestingly, sequences related to *Nitrosomonas* sp. IWT514 (OTU1) represented only the 11.5% of the total amount of sequences recovered from R0. OTU2, OTU3 and OTU5 clustered close to *Nitrosomonas oligotropha/ureae* lineages (71.1%) (fig. 3.4.3), also called *Nitrosomonas* cluster 6a. OTU4 was more related to *Nitrosomonas* sp. Is343 (accession number: AJ621032) and uncultured *Nitrosomonas* (17.4%).

Table 3.4.1: Summary of the OTUs calculated from the sequences derived from the clone library using the CTO primer set, the representative sequence and the sequences included in each OTU. Accession numbers of each sequence are given within brackets.

OTU	Representative sequence	Sample sequences
1	R450CTO13 (FM997772)	R0CTO6 (FM997782), R0CTO26 (FM997793), R0CTO36 (FM997798), R0CTO74 (FM997819), R0CTO83 (FM997825), R0CTO89 (FM997829), R450CTO2 (FM997763), R450CTO3 (FM997762), R450CTO5 (FM997764), R450CTO6 (FM997765), R450CTO8 (FM997767), R450CTO9 (FM997768), R450CTO10 (FM997769), R450CTO11 (FM997770), R450CTO12 (FM997771), R450CTO13 (FM997772), R450CTO14 (FM997773), R450CTO15 (FM997774), R450CTO16 (FM997775), R450CTO17 (FM997776), R450CTO20 (FM997777)
2	R0CTO10 (FM997783)	R0CTO10 (FM997783), R0CTO15 (FM997786), R0CTO22 (FM997790), R0CTO23 (FM997791), R0CTO24 (FM997792), R0CTO33 (FM997797), R0CTO40 (FM997800), R0CTO41 (FM997801), R0CTO49 (FM997808), R0CTO50 (FM997809), R0CTO51 (FM997810), R0CTO52 (FM997811), R0CTO59 (FM997814), ROCTO76 (FM997820), R0CTO82 (FM997824), R0CTO86 (FM997827), R0CTO93 (FM997833),
3	R0CTO48 (FM997807)	R0CT013 (FM997784), R0CTO31 (FM997795), R0CTO32 (FM997796), R0CTO48 (FM997807), R0CTO53 (FM997812), R0CTO58 (FM997813), R0CTO60 (FM997815), R0CTO67 (FM997817), R0CTO73 (FM997818), R0CTO91 (FM997831), R0CTO92 (FM997832), R0CTO94 (FM997834)
4	R0CTO47 (FM997806)	R0CTO5 (FM997781), R0CTO18 (FM997787), R0CTO43 (FM997803), R0CTO47 (FM997806), R0CTO80 (FM997822), R0CTO81 (FM997823), R0CTO84 (FM997826), R0CTO90 (FM997830)
5	R0CTO38 (FM997799)	R0CTO1 (FM997778), R0CTO2 (FM997779), R0CTO14 (FM997785), R0CTO21 (FM997789), R0CTO38 (FM997799), R0CTO61 (FM997816)

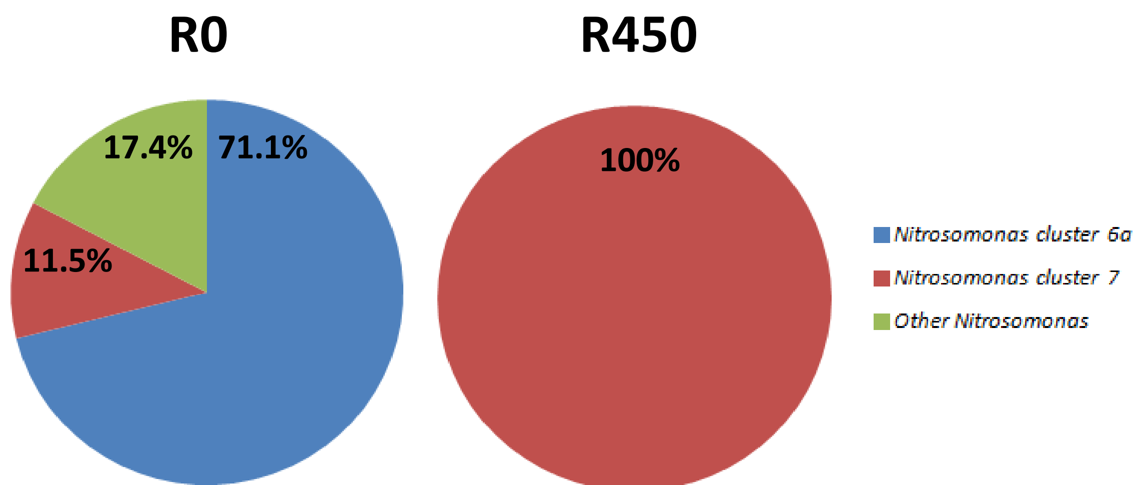


Figure 3.4.3: Percentages of the clone sequence affiliation obtained by using CTO mix primer set and related to *Nitrosomonas* spp. in samples R0 and R450.

MOLECULAR DETECTION and IDENTIFICATION of NOB

A universal NOB primer set targeting the 16S rRNA gene could not be used because NOB is a polyphyletic group. Thus, a different primer set was chosen for each of the main WWTPs NOB genera (*Nitrobacter* and *Nitrospira*). For *Nitrobacter* populations, PCR analyses using FGPS primer set (with GC-clamp) were performed in all DNA isolations with the purpose of comparing DGGE band patterns. Several PCR attempts were performed, but no positive results were obtained in any of the samples. It was thought that the addition of the GC-clamp probably decreased the yield of the PCR amplification because positive PCR results were easily achieved in all samples without it. Thus, in front of this lack of DGGE results, the same strategy used to determine the changes of *Nitrobacter* populations for AOB was followed, and R0 and R450 samples were cloned with PCR products derived from the use of FGPS872F-FGPS1269R.

A total of 52 partial sequences (Ca. 397 bp) were finally retrieved from the clones and MOTHUR calculations allowed to group most of the sequences into 6 OTUs by applying a 0.03 cutoff. No chimera or double sequences were detected. The representative

sequences of each OTU together with singletons and external 16S rDNA sequences, (from both cultured and uncultured bacteria) were uploaded into MEGA and a maximum-likelihood tree was constructed (fig. 3.4.4). The mainstream of the 16S rDNA partial fragments (88.5%) were affiliated within *Rhizobiales* order. Within it, the two main OTUs (OTU1 and OTU2) arose, clustering to *Bradyrhizobium* and *Nitrobacter* genera respectively. OTU1 was mainly formed by R450 sequences whereas OTU2 contained a high number of R0 sequences. The other OTUs found inside *Rhizobiales*, which were only retrieved from R450 (fig. 3.4.5 and table 3.4.2), were affiliated into *Xanthobacter* and *Mesorhizobium* genera.

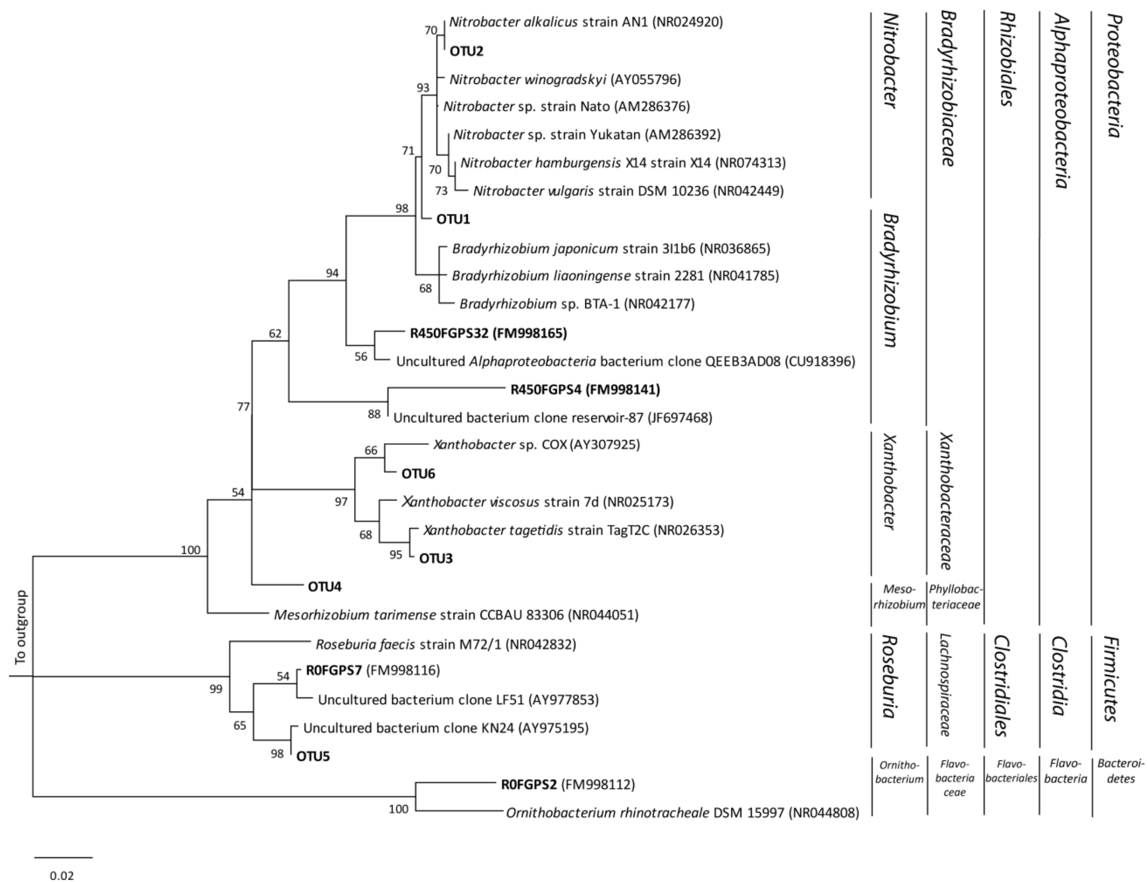


Figure 3.4.4: Maximum-likelihood phylogenetic tree based on 16S rRNA gene retrieved from cloning library after PCR with FGPS primer set, from R0 to R450 samples. OTU identification numbers are indicated in table 3.4.2. The resulting bootstrap values higher than 50% are displayed in the nodes of the tree. Accession numbers of external 16S rDNA sequences are given within brackets. The bar represents 2% estimated sequence divergence.

There were also sequences that not belonged to *Alphaproteobacteria*, all of them coming from R0. The sequences included in OTU5 and R0FGPS7 (20.9%) (fig. 3.4.5)

were related with *Roseburia* genera (*Firmicutes* phylum) and the singleton R0FGPS2 (8.2%) grouped inside *Ornithobacterium* genus (*Bacteroidetes* phylum).

For *Nitrospira* spp. characterization, positive PCR results applying the NSR1113F-NSR1264R primer set indicated their presence in all samples. In this case, direct sequencing of the PCR products was performed and results indicated that only one species was present in each sample. Therefore, neither DGGE nor cloning approaches were carried out. The same sequence was retrieved in all the samples, with 100% of homology with *Candidatus* Nitrospira defluvii (accession number: DQ059545).

Table 3.4.2: Summary of the OTUs calculated from the sequences derived from the clone library using the FGPS primer set, the representative sequence and the sequences included in each OTU. Accession numbers of each sequence are given within brackets.

OTU	Representative sequence	Sample sequences
1	R450FGPS31 (FM998164)	R0FGPS11 (FM998118), R0FGPS12 (FM998119), R0FGPS13 (FM998120), R0FGPS21 (FM998125), R0FGPS26 (FM998128), R0FGPS27 (FM998129), R450FGPS1 (FM998138), R450FGPS8 (FM998144), R450FGPS11 (FM998145), R450FGPS12 (FM998146), R450FGPS13 (FM998147), R450FGPS20 (FM998154), R450FGPS21 (FM998155), R450FGPS29 (FM998162), R450FGPS31 (FM998164), R450FGPS34 (FM998167)
2	R450FGPS28 (FM998161)	R0FGPS5 (FM998114), R0FGPS6 (FM998115), R0FGPS8 (FM998117), R0FGPS16 (FM998121), R0FGPS19 (FM998123), R0FGPS20 (FM998124), R0FGPS22 (FM998126), R0FGPS25 (FM998127), R0FGPS28 (FM998130), R0FGPS29 (FM998131), R0FGPS31 (FM998133), R450FGPS3 (FM998140), R450FGPS28 (FM998161), R450FPGS36 (FM998169)
3	R450FGPS17 (FM998151)	R450FGPS2 (FM998139), R450FGPS7 (FM998143), R450FGPS17 (FM998151), R450FGPS19 (FM998153), R450FGPS26 (FM998159), R450FGPS30 (FM998163), R450FGPS35 (FM998168)
4	R450FGPS25 (FM998158)	R450FGPS15 (FM998149), R450FGPS18 (FM998152), R450FGPS22 (FM998156), R450FGPS25 (FM998158), R450FGPS27 (FM998160),
5	R0FGPS18 (FM998122)	R0FGPS18 (FM998122), R0FGPS30 (FM998132), R0FGPS32 (FM998134), R0FGPS35 (FM998137)
6	R450FGPS14 (FM998148)	R450FGPS14 (FM998148), R450FGPS24 (FM998157)

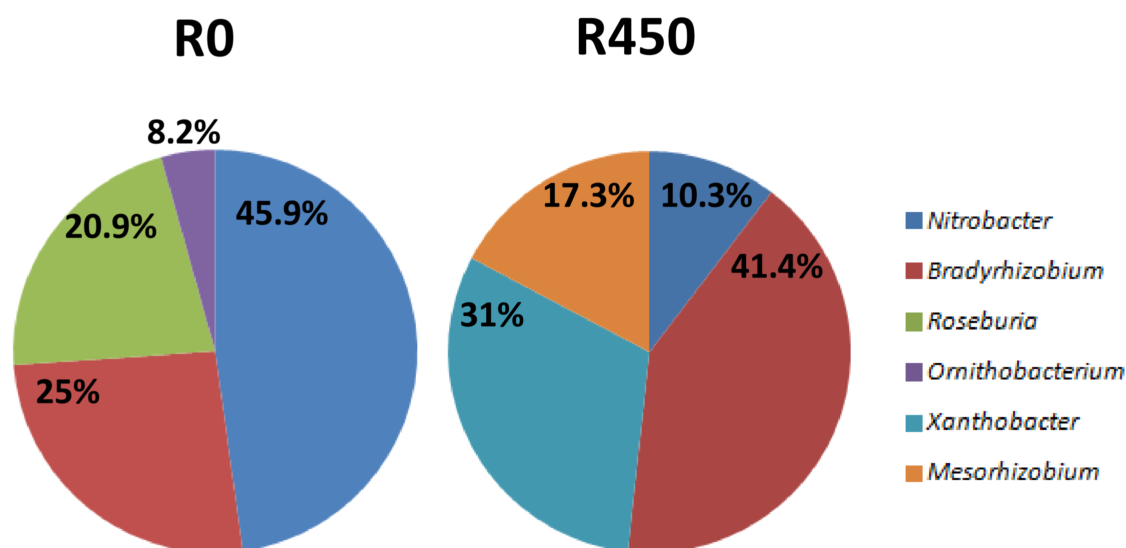


Figure 3.4.5: Percentages of the clone sequence affiliation obtained by using FPGS primer set in samples R0 and R450.

OPTIMIZATION of qPCR ASSAYS for AOB and NOB QUANTIFICATION

Three qPCR assays were optimized for the quantification of AOB, *Nitrobacter* and *Nitrospira* populations, respectively, using standard curves obtained by cloning specific PCR products into plasmids. DNA concentration values were used to calculate the number of plasmids containing the inserts. The CTO189F-RT1R vector presented a DNA concentration of 100.90 ng μL^{-1}, and the plasmid concentration was gauged to be 2.26 x 10^{10} molecules μL^{-1}. For the NSR1113F-NSR1264R vector, the DNA concentration was 22.13 ng μL^{-1}, and the plasmid concentration was 4.90 x 10^9 molecules μL^{-1}. For the Nwi70F-Nwi165R vector, the DNA concentration was 27.37 ng μL^{-1}, corresponding to 6.26 x 10^9 plasmids μL^{-1}.

The consistency of the qPCR assay with the three primer sets was demonstrated from the strong linear inverse relationship between the threshold cycle numbers and the copy numbers of the 16S rRNA genes of each target, along with the appropriate amplification efficiency. It is considered that an acceptable qPCR run must have an

amplification efficiency ranging from 90-100% and a R^2 value higher than 0.98. The CTO189F-RT1R standard curve generated an equation slope of -3.598 and a R^2 value of 0.989, with an amplification efficiency (*E*) of 0.90; the Nwi70F-Nwi165R standard curve generated an equation slope of -3.284, and a R^2 value of 0.990, resulting in *E*=1.01; and the NSR1113F-NSR1264R standard curve generated a lower equation slope of -2.915, with a R^2 value of 0.9988 and a higher efficiency (*E*=1.20).

The dissociation stage (melting curve) included at the end of the run allowed the verification of the specificities of the primer sets. The melting curves from the three assays showed one peak at T_m= 82.5°C at different template concentrations. However, a second peak was detected at T_m= 72.5°C using the NSR primer set at low template concentrations, theoretically related to primer-dimer artefacts signal. Because they usually consist of short sequences, the primer-dimer melt at lower temperature than the targeted sequence and hence can be distinguished by their melting-curve characteristics (Kubista et al., 2006). To confirm the results of the dissociation stage, the qPCR products were separated by electrophoresis on a 2% (w/v) agarose gel. A primer-dimer band was observed only in the PCR products derived from the use of the NSR primer set at low concentrations.

AOB and NOB QUANTIFICATION by qPCR

Cell numbers for each target were determined in all DNA isolations from the number of copies of the 16S rRNA gene (fig. 3.4.6). 10-fold dilution of samples were used to avoid qPCR inhibition due to excess of DNA. Specific quantification of each target was performed using the appropriate standard vector. AOB and *Nitrobacter* concentrations (cells mL^{-1}) (±SD) were quite similar at the time of inoculation (4.24E+05 (±0.09) and 9.71E+05 (±2.42) cells mL^{-1}, respectively) whereas *Nitrospira* populations were found to be at slightly lower concentrations (1.32E+05 (±0.32) cells mL^{-1}). AOB populations grew throughout the reactor operation, reaching up to 4.65E+07 (±0.70) cells mL^{-1} (a 100-fold increase in 200 days). After the reactor process was completed (R450), the concentration was 2.18E+08 (±0.20) cells mL^{-1}. *Nitrobacter* populations, however, were

strongly washed out by the adverse reactor working conditions, and their concentrations decreased to 1% of the original values (5.90E+03 (±1.40) cells mL^{-1}). *Nitrospira* populations presented lower concentrations until day 288th (7.88E+03 (±1.48) cells mL^{-1}) but then increased from day 415th to the end of the study (from 1.25E+04 (±0.50) to 4.44E+04 (±0.50) cells mL^{-1}), surpassing *Nitrobacter* populations but also remaining very far from AOB cell numbers.

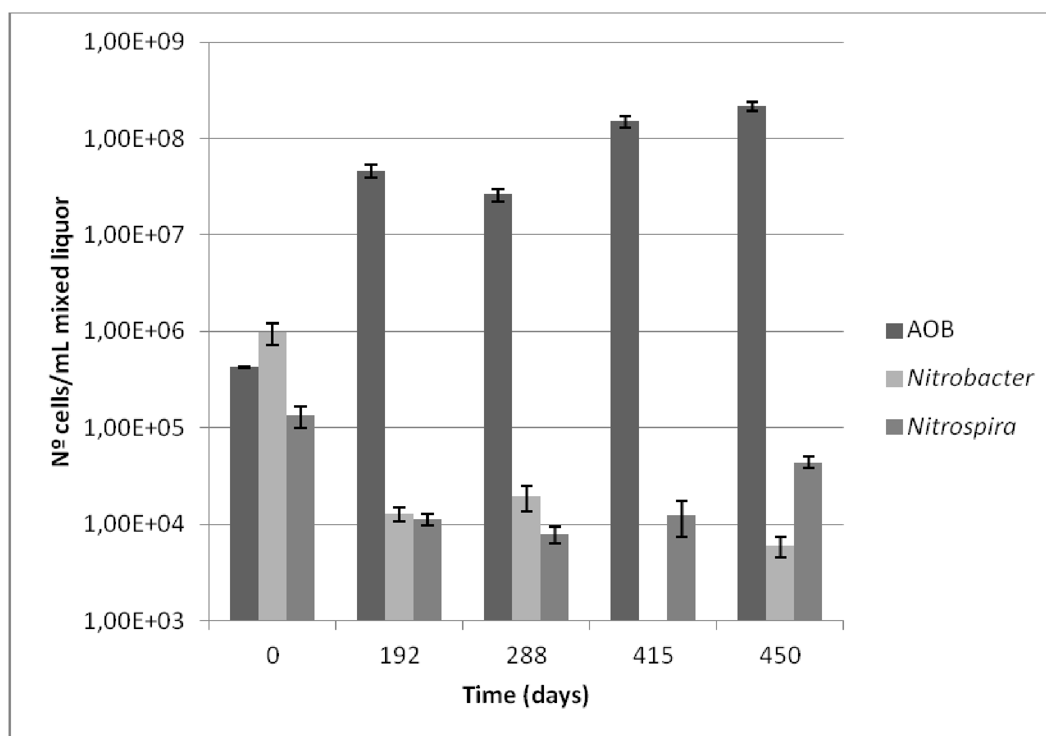


Figure 3.4.6: Concentrations of AOB, *Nitrobacter* and *Nitrospira* spp. over the course of the reactor's performance, obtained from qPCR quantifications. (*) *Nitrobacter* cell numbers were unable to quantify at day 415th.

Finally, an indicator value was calculated to evaluate the productivity of the reactor throughout its performance. Thus, specific oxidation rates of ammonium (AOR) per cell were gauged from cell concentrations and the ammonium removal of the PN reactor (table 3.4.3). Calculations indicated that the maximum value was reached when the reactor was seeded (138 pmol $cell^{-1}$ day^{-1}), a strong reduction was experimented until day 192 (5.91) and then the AOR was kept rather low until the end of the reactor performance (6.09, 1.39 and 1.45 pmol $cell^{-1}$ day^{-1} for days 288, 415 and 450, respecti-vely).

Table 3.4.3: Reactor parameters of the PN reactor for the calculation of the specific ammonium oxidation rates per cell.

Day	Ammonium removal (mol oxidized N m^{-3} $reactor^{-1}$ day^{-1})	AOB concentration (cell number ml^{-1} mixed liquor)	AOR (pmol $cell^{-1}$ day^{-1})
0	11.76	4.24E+05	138
192	35.79	4.65E+07	5.91
288	20.71	2.62E+07	6.09
415	42.14	1.51E+08	1.39
450	43.72	2.18E+08	1.45

3.4.3 DISCUSSION

NITRIFYING BACTERIA QUANTIFICATION USING qPCR ASSAYS

Quantitative PCR was considered to be more appropriate for measuring bacterial concentrations than other techniques such as FISH (Juretschko et al., 2002) or competitive PCR (Dionisi et al., 2002), according to the features of the samples to be analyzed (sludge samples from WWTPs or bioreactors, mainly composed of aggregates and biofilms). Moreover, qPCR is less time-consuming than FISH to obtain reliable results. Therefore, this assay allows for the screening of a large number of samples in a short time, with quick, reliable and objective results.

The 16S rRNA gene is considered useful as a reference for AOB quantification, because all species studied up to now are known to have only one rRNA gene copy per genome (Aakra et al., 1999; Liang et al., 2010). The *amoA* gene is also used for detection of AOB (Calvo and Garcia-Gil, 2004), but quantification could be challenging because the number of *amo*A gene copies per genome is generally unknown (Hermansson and Lindgren, 2001). In this sense, even when it is known, relative quantities of DNA fractions can hardly be converted into absolute cell counts in samples because the copy number of these genes per cell is variable (Koops and Pommerening-Röser,

2001). The 16S rRNA gene has also been chosen for use in the qPCR quantifications of *Nitrobacter* and *Nitrospira* populations instead of their respective functional genes because they are also known to have only one 16S rRNA gene copy (Navarro et al., 1992b; Dionisi et al., 2002), allowing a direct conversion from copy number to cell number.

To enhance the quantification precision of the bacteria specifically present in these samples, it was considered convenient to use specific DNA cloned into plasmids rather than bacterial strains in culture collections. The former strategy was found to be the most suitable because all the chemolithotrophic bacteria under study are considered uncultured bacteria. Furthermore, qPCR primer sets were checked for the absence of mismatches against both the vectors and the phylotypes identified in the reactor to avoid potential underestimations of the DNA quantity. With this procedure, the parameters obtained for the CTO189F-RT1R and Nwi70-Nwi165R standard curves (for AOB and *Nitrobacter* population quantifications, respectively) matched the values that were required to achieve acceptable qPCR results. Both assays had adequate amplification efficiencies that were close to 1 (E=0.90 and 1.01, respectively) and R^2 values near 0.990. However, *Nitrospira* spp. quantification was more challenging. The NSR1113F-NSR1264R standard curve showed some over-efficiency (E=1.20), a nuisance that also occurred when these primers were first described and used for *Nitrospira* quantification (Dionisi et al., 2002). This high value could be related to primer-dimer artefacts that were found to arise at low DNA concentrations for the vector. Unfortunately, the standard curve had to encompass this range of concentrations, because NOB were expected to be present at low densities in the processed samples. Thus, the over-estimation of *Nitrospira* populations must be assumed as a weakness of this assay, although it should be negligible when working with samples containing higher target DNA concentrations.

ECOLOGICAL SUCCESSION and QUANTIFICATION of the AOB ASSEMBLAGE

Phylogenetic analysis showed that *Nitrosomonas* spp. were the only AOB detected all over the duration of the reactor performance. This result agrees with recent studies that confirm that nitrosomonads are the most important bacteria for ammonium oxidation in WWTPs (Purkhold et al., 2000; Wagner et al., 2002). The *Nitrosospira* group (encompassing *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*) has low significance in the activity and composition of the nitrifying assemblage in this kind of environments. Concerning *Nitrosomonas* spp., different populations and temporal changes were found within the reactor. Several phylotypes were detected at the time of inoculation (clustered into OTU2 to OTU5), most of them belonging to the *N. ureae-oligotropha-marina* group, which is also named *Nitrosomonas* cluster 6a (Bollmann and Laanbroek, 2001). The bacteria affiliated in this cluster are known to have a high affinity for ammonium (low K_s values) and are dominant at low concentrations (Wagner et al., 1996; Purkhold et al., 2000; Bollmann and Laanbroek, 2001). These conditions are common in natural environments and some WWTPs, especially for one of the sludge samples collected for the reactor start-up.

However, the ecological succession inside the reactor derived to a single dominant phylotype (OTU1) with 100% homology with *Nitrosomonas* sp. IWT514, and phylogenetically close to *Nitrosomonas europaea-eutropha* group. This group is also named *Nitrosomonas* cluster 7 (Whitby et al., 1999; Otawa et al., 2006) and bacteria with low affinity to ammonium (high K_s values) are enclosed within. The selection of bacteria from the *N.europaea-eutropha* group has also been reported in SBR reactors and in activated sludge from WWTPs treating wastewater with similar high ammonium concentrations (Otawa et al., 2006).

The increase of AOB populations over the course of the reactor's working period, in combination with the fingerprinting results, suggests an active behaviour leading to the dominance of *Nitrosomonas* sp. IWT514 at these extreme conditions, which are

too hostile for most AOB. In fact, Bollmann and Laanbroek (2001) reported that *Nitrosomonas* cluster 6a populations could not withstand long-term ammonium concentrations above 10 mM and hypothesized that they were inhibited, as likely happened in this PN reactor. Moreover, the reactor operation parameters seemed to fit the requirements of *Nitrosomonas* sp. IWT514, which was found to be capable of oxidizing ammonium at high rates in a deodorizing plant (Satoh et al., 2004) at concentrations above 8000 mg NH_4^+-N L^{-1} (2- to 3-fold more concentrated than the leachate supplied to this reactor). The specific selection produced in the reactor during this successional change at high ammonium concentrations (Otawa et al., 2006) is uncommon in natural environments, where phylotypes of *Nitrosomonas* cluster 6a appeared to be dominant.

Some stages of the reactor operation seemed to have favoured AOB growth more than others, especially when their populations had lower densities or when the ammonium concentrations were higher. Nitrogen removal rates per cell, which were calculated from AOR and cell concentrations, could provide relevant information to better understand the AOB behaviour during the process and the PN reactor capabilities. Their decreasing values over time suggested that AOB could have higher metabolic activities during most of the reactor performance if the initial conditions were kept. From a microbiological standpoint, the reactor could potentially operate with higher AOR values; therefore, some environmental factors inside the reactor, such as free ammonia or alkalinity, should have been detrimental to the partial nitritation process. According to these statements, microbial composition and activity of the biomass are revealed as important factors to initiate and properly operate the PN reactor, and they should be continuously monitored (Calli et al., 2007). Moreover, qPCR coupled with chemical analyses can provide relevant information about the behaviour of the reactor in a short amount of time.

PN coupled with SBR configuration is a very suitable configuration prior to an anammox reactor (in the present work the two linked reactors are named PANAMMOX® process) to perform the N transformations necessary to feed the anammox reactor (Ganigué et al., 2009). Nonetheless, the high solid retention capacity

of the PN reactor, in comparison with other kinds of reactors, such as SHARON (also used in anammox pre-treatments), must be considered a negative factor for the fast development of community succession and the decrease of impaired bacteria, because outcompeted populations are not completely washed out from the reactor. Therefore, the inoculation of the PN reactor with appropriate seeds can be essential to prevent delays in the development of the AOB community by means of successional changes when the ammonium concentrations increase to their ecophysiological limits.

COMPOSITION and STATUS of the NOB ASSEMBLAGE

PN reactor was designed to operate at adverse conditions for NOB, such as high concentrations of free ammonia and free nitrous acid, which should theoretically result in their removal (Stüven et al., 1992). However, their presence and activity were suspected because traces of nitrate were detected over the course of the reactor's performance. Detection of NOB by molecular analyses based on the 16S rRNA gene is a more convoluted process than for AOB because NOB are split into different phylogenetic groups. As a result, it is not possible to generate a global primer targeting 16S rRNA gene for all NOB. Because of this, only the main NOB groups found in sewage disposals (*Nitrobacter* and *Nitrospira*) were search targets and all of them were detected. Other NOB (such as *Nitrococcus* and *Nitrospina*) were not tested because they were not expected to be present in WWTP (Otawa et al., 2006) or at any stage of the reactor's operation, since they are obligate halophilic bacteria (Koops and Pommerening-Röser, 2001). Therefore, negative results should not unequivocally indicate that NOB concentrations were too low to be detected.

Successional changes in the *Nitrobacter* populations were observed. *Nitrobacter alkalicus* was the only *Nitrobacter*-like sequence detected in R0 sample (45.1%), but it was nearly outcompeted (10.3%) in the last stages of the reactor by *Bradyrhizobium*-like members. *Nitrobacter* spp. belong to *Bradyrhizobiaceae* and it is closely related to *Bradyrhizobium* (97-98% 16S rDNA identity) (Starkenburg et al., 2008). FGPS primer

sequences (which targets 16S rRNA gene) were compared against most of the *Nitrobacter*, *Bradyrhizobium*, *Xanthobacter* and *Mesorhizobium* (all of them belonging to *Rhizobiales* order) 16S rDNA known member sequences and few mismatches were detected. FGPS primer set contained only one mismatch with all the known *Nitrobacter* spp. sequences, whereas 2 to 4 mismatches were found with *Bradyrhizobium*, *Xanthobacter* and *Mesorhizobium* sequences. Thus, it is understand-dable (because of their phylogenetic closeness) that FPGS primer set was not able to discriminate among the members inside *Rhizobiales* order.

According to these statements, the *Nitrobacter* spp. quantification obtained by qPCR became difficult to completely attribute to them. Nwi primer set composition was checked against the 16S rDNA sequences of *Nitrobacter, Bradyrhizobium*, *Xanthobacter* and *Mesorhizobium*. Nwi primer set also contained very few mismatches with both *Nitrobacter* and *Bradyrhizobium* sequences, as FGPS primers. Thus, it is possible that the quantitative decrease of *Nitrobacter*-like sequences all along the reactor performance could also be due to the reduction of *Bradyrhizobium* sequences. Anyway, adverse conditions in the reactor could have impaired *Nitrobacter* growth and favoured the dominance of *Bradyrhizobium*, who seemed to better withstand at the final reactor conditions. However, the ecological variation within the *Nitrobacter* group is still not clearly understood and ammonium or nitrite concentrations have not been reported to be a selective factor inside this group, as for AOB.

Besides the successional change of the *Nitrobacter* populations, several other bacterial populations not related to NOB were detected with the use of the FGPS primer set. Sequences clustered inside *Roseburia* and *Ornithobacterium* genus, from *Firmicutes* and *Bacteroidetes* phyla, respectively. Again, non-specificities in the primer set were thought to be responsible for the amplification of DNA fragments belonging to some of these groups, because all of the sequences contained the primer binding sites.

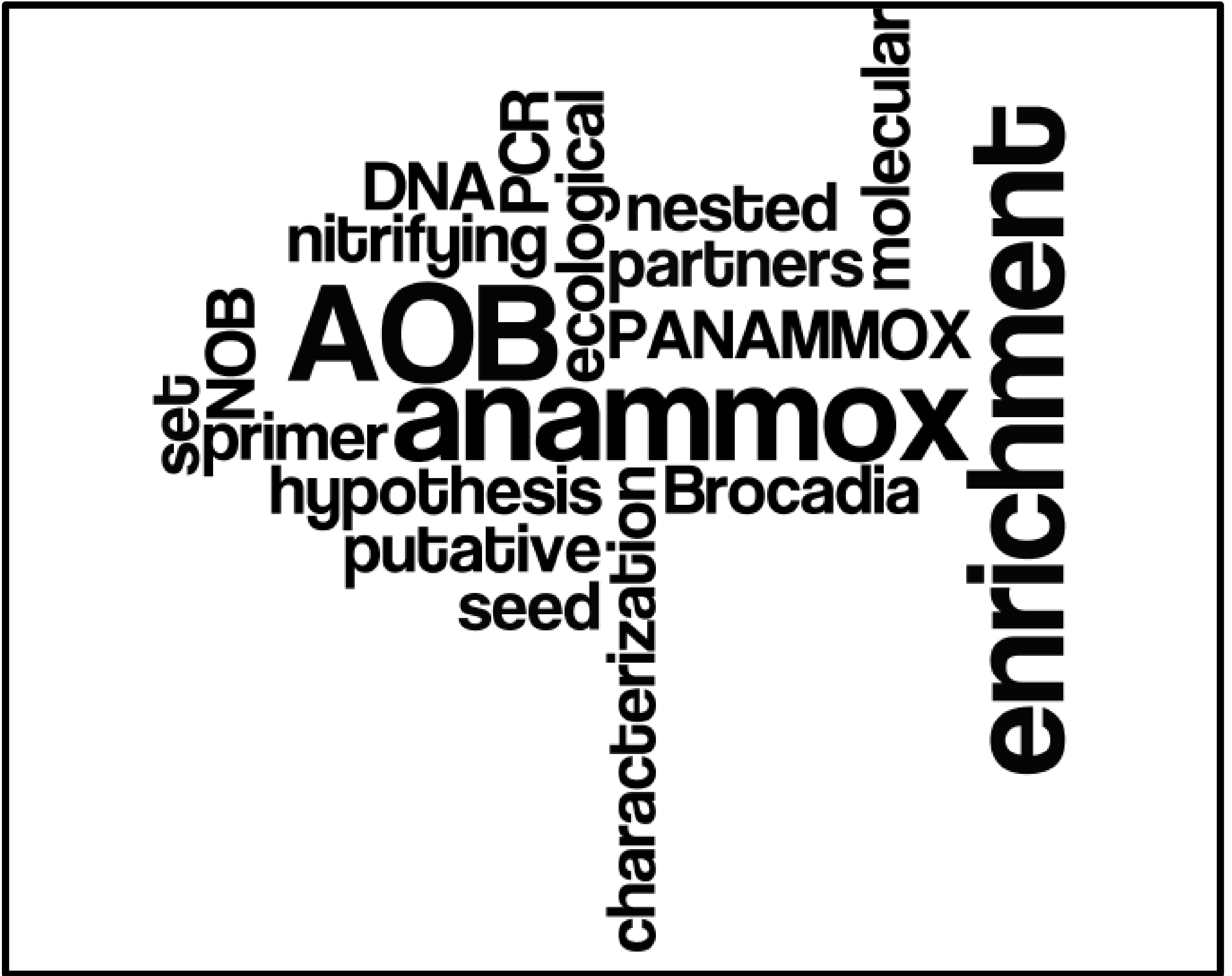
On the other hand, *Ca.* Nitrospira defluvii (Spieck et al., 2006b) was also detected over the course of the reactor's performance, demonstrating that *Nitrobacter* spp. were not the only NOB capable of resisting these conditions. Although the latter were considered to be the main bacteria responsible for nitrite oxidation in sewage

disposals (Bock and Koops, 1992), the overview of NOB species in WWTPs is changing. Currently, *Nitrospira* (instead of *Nitrobacter*) is considered to be the dominant nitrite oxidizer in most WWTP systems (Dionisi et al., 2002; Cébron and Garnier, 2005b) and in many natural habitats (Spieck et al., 2006b). Therefore, the coexistence of both *Nitrospira* and *Nitrobacter* in a single reactor where nitrite concentrations were temporally or spatially elevated (Wagner et al., 2002) is not yet an unexpected result, although they may require different ecological niches (Maixner et al., 2006). The presence of *Ca.* Nitrospira defluvii as the single *Nitrospira*-like bacteria in the PN reactor agreed with the results reported by Ren and collaborators (Ren et al., 2010), who only detected *Ca.* Nitrospira defluvii when the reactor was treating 100 mg N L^{-1}, 60 days after starting up. *Ca.* Nitrospira defluvii belongs to *Nitrospira* sublineage I, which is found to have competitive advantage at high nitrite concentrations over other *Nitrospira* sublineage (Maixner et al., 2006).

Results from qPCR showed the decrease of the dominant *Nitrobacter*-*Bradyrhizobium* populations over the course of the reactor's working period, while *Ca.* Nitrospira defluvii population increased when nitrite concentrations were at their highest. Indeed, *Nitrobacter* spp. are considered to be putative *r*-strategists with lower nitrite and oxygen affinities (Wagner et al., 2002). Nevertheless, any quantitative consideration about *Nitrospira* must be subject to reservations because their concentrations might be slightly overestimated by the qPCR assay, although this statement cannot be strictly considered a handicap for the comparison among different samples.

The identification and quantification of NOB, coupled to the detection of small amounts of nitrate, indicate that they likely still showed some degree of metabolic activity. The retention of some residual biomass, due to the configuration of the SBR, could also have contributed to the resiliance of NOB in the reactor. In some way, long-term performance allows some resistant NOB populations to thrive, to the extent that even *Nitrobacter* spp. and *Ca.* Nitrospira defluvii were capable of finding their own ecological niches in these restrictive conditions. The PN reactor operation was not restrictive enough to completely wash out these bacteria from the reactor, although

their activities are too low to significantly interfere in the global performance of the system as a previous step for anammox nitrogen removal.



Global remarks

4. GLOBAL REMARKS

Anammox bacteria are considered nowadays more than a promising N-cycle shortcut and their application in technological processes is growing day by day (Siegrist et al., 1997; Jetten et al., 1998; Furukawa et al., 2002; Robert Hamersley et al., 2009; Langone et al., 2014). However, although their unique and exceptional features, anammox bacteria are not able to perform N removal (converting ammonium and nitrite to N_2) directly from urban leachates or other effluents. One of the reasons is their need of an accurate ammonia:nitrite ratio (1:1) (Strous et al., 1999b), thus requiring part of the ammonia to be converted into nitrite. This critical drawback can be eluded throughout the use of the PANAMMOX® process, which includes a previous PN reactor, where each mol of ammonia is converted by AOB to 0.5 mol of ammonia and 0.5 mol of nitrite.

In this thesis, main chemolithotrophic bacterial communities and their ecological partners have been characterized by molecular techniques in anammox batch cultures and a PN reactor. Two main questions stand out after analyzing the results obtained from this work. The first one, a methodological topic, which lead up to wonder if the methodology applied is reliable and can contribute to a better anammox bacteria detection in environmental samples or reactors. A second issue is focused on the characterization of the bacterial assemblages in samples of batch cultures and PN reactor (both subjected to long enrichment periods in different conditions). Relevant differences in the community composition of the targeted chemolithotrophic bacteria and anammox ecological partners population were observed. Does this characterization give some hints to a better understanding of the enrichment processes and the reactors performance? Ultimately, could this knowledge help to obtain a better PANAMMOX® performance?

PCR and ENRICHMENT CULTURES for an EARLY ANAMMOX BACTERIA DETECTION

PANAMMOX® process requires the presence of both AOB and anammox bacteria to fully operate. However, a well-developed anammox bacteria population is essential for a successful performance of N removal. From a microbiological outlook, the enrichment of AOB is not considered a major downside, since their populations are not uncommon in aerobic WWTP activated sludge (mainly *Nitrosomonas* populations) (Purkhold et al., 2000; Limpiyakorn et al., 2006; Ganigué et al., 2009). Finding anammox bacteria is something completely different. Although currently it seems that anammox bacteria are quite present anywhere the ammonia and nitrite disposition couple with anoxic conditions, only few years ago the situation was not so clear, because a methodological effort (specially focused on their detection and enrichment) is required to obtain successful results.

Different strategies can be followed to obtain a suitable inocula for an anammox bioreactor start-up. The main options were: (a) to sample small volumes of several seeds and enrich them in closed systems (batch cultures) (Toh and Ashbolt, 2002; Toh et al., 2002; Suneethi and Joseph, 2011) or (b) to collect activated sludge from WWTPs suspicious to harbor anammox populations, and enrich them using diverse technological processes (Sliekers et al., 2002; Kalyuzhnyi et al., 2006b; Vlaeminck et al., 2009b). Based on the goals of the present work, the former option fulfilled most of the requirements: several seeds could be tested without great investments, the chance of finding anammox bacteria increased because more samples were collected and, by sampling a wide-range typology seeds, the diversity of the anammox bacteria enriched could be higher. However, the use of batch cultures operating as closed-system (entailing limited and stepwise nutrient addition) give rise to some disadvantages. This configuration along with the slow growth rate and low cell yield of the anammox bacteria (Strous et al., 1999b; Oshiki et al., 2011; Ding et al., 2013) turns the achievement of anammox bacteria a remarkably time-consuming process. The slow-

growing disadvantage could be partially get over by the use of different technologies, but the need of screening a lot of seeds cannot be avoided.

Finally, after a long enrichment period (close to three years in some cases) the use of batch cultures lead to the detect *Ca.* Brocadia anammoxidans populations in five enrichments developed from several seeds (categorized as natural, modified and man-made environments). PCR and FISH performance with the Pla46F-Amx368R primer set and the Amx820 probe, respectively, pointed out the presence of anammox bacteria in most of the seeds. Interestingly, it was the first time that *Ca.* Brocadia anammoxidans was described in a brackish coastal lagoon with high salt content.

At this point, the first question has been partially answered. Active anammox populations, suitable to be used as inocula for a bioreactor, have been achieved by using batch cultures methodology. Enrichments 3 and 10 were successfully used as inocula for the start-up of the anammox bioreactor. Biomass from different origins, such as Sils-Vidreres and Orís WWTPs, and Ecoparc (a plant treating solid urban wastes) were also added. *Ca.* Brocadia anammoxidans was identified by PCR analysis when ammonium and nitrite simultaneously removal was detected after only 78 days (López, 2008).

Despite of their successful use as inocula, the time required to notice anammox activity and identify them by molecular methods in batch cultures was rather unacceptable from an engineering overview. Therefore, the PCR approach should be optimized to reduce the period of detection of anammox bacteria during the enrichment process. Nested-PCR assays with different primer sets (Pla46F-Amx368R/Amx368F-Amx820R) and different parameters (such as annealing temperature), coupled to DGGE, were carried out to attempt increasing the sensitivity of the process.

Pla46F-Amx368R was confirmed as a fine primer set when anammox bacteria populations are sufficiently dense (Chapter I), but the different PCR attempts carried out with these primers were unable to retrieve any anammox sequence from inocula or early stages of any enrichments (even from the ones that lately developed *Ca.*

Brocadia anammoxidans). Furthermore, most of the retrieved sequences from these analyses belonged to *Lentisphaerae* phylum, not *Planctomycetes*. Before going deeper into the conclusions derived from the use of the Amx368F-Amx820R, a brief preview is necessary, stating that most of the retrieved sequences from this test belonged to *Planctomycetes*, thus confirming that they were present in the samples. How can the lack of *Planctomycetes* and the uprising of *Lentisphareae* sequences in the Pla46F-Amx368R tests be explained? Some studies described that even though Pla46F is the most common forward primer used for anammox detection, it tends to underestimate anammox populations (Penton et al., 2006; Limam et al., 2010). Thus, it is possible that the detection of *Lentisphaerae* could happen because of primer underestimation when anammox bacteria are low concentrated, as well as *Lentisphaerae* phylogenetic closeness with the PVC superphylum (Limam et al., 2010; Fuerst, 2013). This last feature surely favored the primer to bind to *Lentisphaerae* rather than *Planctomycetes* when they are barely present. Although it seems to be a topic, it is actually true that the election of the primer set has great impact on the results of diversity and dynamics analyses using DNA fingerprinting, gene libraries or qPCR.

Fortunately, the election of a second primer set (Amx368F-Amx820R) retrieved more relevant information. The use of this primer set led to identify some highly-related anammox bacteria sequences in the early stages of the enrichments. According to MOTHUR calculations (based on SILVA bacterial database) these sequences belonged to *Brocadiaceae* but they were not directly branched into the known anammox bacteria group. These highly-related sequences were called as “putative” anammox bacteria, like Bae and collaborators (2010b) previously reported. The “putative” anammox bacteria were detected in the early samples in most of the successful enrichments, but they were lately removed for *Ca.* Brocadia anammoxidans populations. Probably, initial conditions inside batch cultures fit well with these populations, but the stepwise increase of ammonium and nitrite provided a suitable niche to residual *Ca.* Brocadia anammoxidans for growing and outcompeting these “putative” anammox bacteria species.

The presence of these highly related anammox sequences and the later achievement of a successful anammox bacteria enrichment does not seem to be a mere coincidence. Probably, the presence of “putative” anammox bacteria in seeds is a potential indicator that anammox bacteria are able to develop in this culture media. This hypothesis surely could not arose without screening several inocula from diverse environments. The use of a single DNA seed probably would not led to identify these highly-related sequences as “putative” anammox sequences. The knowledge obtained from the application of batch cultures and molecular assays to the early detection of anammox bacteria should help to reduce the time necessary for the further use of their enrichments as reliable inocula in anammox bioreactors.

BACTERIAL ASSEMBLAGES SHIFTS in the DIVERSE PANAMMOX® COMPONENTS

EVALUATION of the ECOLOGICAL SUCCESSION of NITRIFYING BACTERIAL ASSEMBLAGES in PN REACTOR

The PN reactor, as a previous step to feed the anammox reactor in the PANAMMOX® process, works at specific conditions that lead to partially repress AOB, whom oxidize only half of the ammonium to nitrite, and to impair NOB populations avoiding the subsequent nitrite oxidation step. Consequently, the effluent of the PN to the anammox biological reactor carries the 1:1 ratio of ammonium/nitrite suitable for anammox bacteria. Several studies investigated the presence of AOB (and even NOB) in different technologies designed to provide anammox bacteria with proper ammonium and nitrite concentrations, such as CANON, OLAND, DEAMOX (Sliekers et al., 2002; Kalyuzhnyi et al., 2006b; Vlaeminck et al., 2009b). Most of the novel technological processes treating and leachates from urban wastes must deal with high ammonium and nitrite concentrations. It was stated that ammonia oxidation could be inhibited at high ammonium concentration and *Nitrosomonas* spp. growth is reduced in presence of 1000-2000 mg L^{-1} of ammonium (Princic et al., 1998) but in natural

environments, mainly in soil and water containing waste from animal farms, this concentration can be risen to 5000 mg L^{-1} (Mahne et al., 1996).

A few number of investigations carried out AOB and NOB molecular characterization in PN reactor treating remarkably high ammonium leachate concentration (up to 2000-3000 mg L^{-1} of both ammonium and nitrite). Cloning and quantification by qPCR allowed to identify and quantify (respectively) AOB and even NOB (*Nitrobacter* and *Nitrospira*) thriving at these high N-compound concentrations.

The sequencing of a high number of random clones showed that *Nitrosomonas* was the only AOB genera identified all along the reactor performance, agreeing with those studies describing the predominance of *Nitrosomonas* over *Nitrosospira* in activated sludge from WWTPs and bioreactors (Wagner et al., 2002). However, the AOB population composition changed over time. Initial sample (consisting in activated sludge from Sils-Vidreres WWTP) harbored more specific richness, most of the sequences belonging to the *Nitrosomonas oligotropha*/*ureae* lineage (*Nitrosomonas* cluster 6a) (Bollmann and Laanbroek, 2001) and few others branching inside *Nitrosomonas europaea*/*eutropha* lineage (*Nitrosomonas* cluster 7) (Otawa et al., 2006) with perfect match with *Nitrosomonas* sp. IWT514 (Satoh et al., 2004). The community structure changed at the end of the reactor performance, where only *Nitrosomonas* cluster 7 sequences were identified. Quantification assays by qPCR showed a major increase of *Nitrosomonas* spp*.* cell numbers along the reactor performance, demonstrating that not only *Nitrosomonas* cluster 7 dominate, but even grew.

Similar species distribution was described in previous studies (Purkhold et al., 2000; Bollmann et al., 2002). Bodelier and collaborators (1998) described that AOB communities are highly specialized and a noteworthy example can be observed in this work. In oligotrophic and low-ammonium concentration habitats (similar conditions found in Sils-Vidreres WWTP), AOB have to compete with heterotrophic bacteria and plants for limiting amounts of ammonium, and it seems that species belonging to *Nitrosomonas* cluster 6a are more adapted at this conditions (Bollmann et al., 2002). *Nitrosomonas* cluster 6a members are considered *K*-strategists: they have higher

substrate affinity (lower K_S values), increased sensitivity to high ammonia/ammonium (Suwa et al., 2011) and lower ammonium threshold concentration for growth, which enable them to be better competitors in limiting ammonium conditions (Cerrone et al., 2013). On the other hand, the appearance and dominance of *Nitrosomonas* cluster 7 when high ammonium concentrations were treated also agrees with their described ecophysiological features (Whitby et al., 1999). Members of the cluster 7 nitrosomonads are assigned as *r*-strategist amongst the AOB, with a relatively high growth rate and predominance in habitats with high N input and turnover such as urban WWTPs.

One of the features that can change the desired nitrite/ammonium ratio of 1 in the PN effluent is the oxidation of nitrite to nitrate. There are some strategies on the PN operational process that can be helpful for the inhibition of NOB: low DO conditions, high reactor temperatures or pH values, unfavorable alkalinity/ammonium ratios and short sludge residence time, where NOB populations can be washed out. In the PN under study, pH was kept at low values by very strict control of the bicarbonate dosage, to limit the ammonium oxidation and to avoid the subsequent nitrite oxidation by free nitrous acid inhibition (Ganigué et al., 2009). Since residual nitrate formation was detected along the reactor performance and *Nitrobacter* and *Nitrospira* are the main NOB genera described in WWTPs and biological reactors, they were studied using a similar approach than it was performed for AOB. Populations of both *Nitrobacter* and *Nitrospira* spp. were detected, identified and quantified, giving some unexpected results. A single *Nitrospira* phylotype was detected in all the studied period, identified as *Ca.* Nitrospira defluvii. On the other hand, more specific richness was found with the *Nitrobacter* populations. Most of the sequences from the initial stage have high homology with *Nitrobacter alkalicus* (despite of several sequences not clustering inside *Nitrobacter* group, mostly due to the 16S rDNA closeness of *Rhizobiales* species) but they were nearly removed in the last stages where sequences belonging to *Bradyrhizobium* dominated.

Phylogenetic analyses were coupled with the molecular quantification of the 16S rDNA of *Nitrospira* and *Nitrobacter*. *Nitrobacter-Bradyrhizobium* populations considerably

reduced their cell number along the reactor but *Ca.* Nitrospira defluvii (despite a big reduction in the 200 initial days) found a way to grow in the disadvantage conditions even when nitrite concentrations were at their highest. These NOB populations also seemed to be highly specialized. *Nitrobacter* spp. are considered *r*-strategists, with lower oxygen and nitrite affinities (Wagner et al., 2002). Up to now few studies are based on the study of *Nitrospira* populations, so little is known about their ecophysiology. However, *Ca.* Nitrospira defluvii belongs to *Nitrospira* sublineage I, which is described to have competitive advantage over other *Nitrospira* sublineage (Maixner et al., 2006) and as far it has been observed in the present work, also over *Nitrobacter-Bradyrhizobium* populations. Probably due to the SBR configuration of the PN reactor, and its recently described ability to benefit from simple organic (Spieck et al., 2006a; Ushiki et al., 2013), *Ca.* Nitrospira defluvii was allowed to find an specific ecological niche to thrive in these adverse conditions.

ANAMMOX SYNERGISM in MICROBIAL COMMUNITIES

Although no anammox pure culture is already obtained (Kartal et al., 2012), anammox bacteria are undoubtedly the responsible of the anaerobic oxidation of the ammonium (using nitrite as the electron acceptor). Strous and collaborators (1999a) isolated anammox cells by Percoll density gradient centrifugation procedure and demonstrated that *Ca.* Brocadia anammoxidans were the unique responsible of their metabolic activity. Thus, if *Ca.* Brocadia anammoxidans isolated cells can carry out the described anammox metabolic activity, why it is not possible the achievement of an anammox pure culture? In natural and artificial ecosystems, anammox bacteria interact with other microorganisms, establishing synergism based on substrate, space and some key ecological factors (Ding et al., 2013). These ecological partners are also present in anammox biological reactors, as it was described in several studies (Li et al., 2009; Bae et al., 2010a; Cho et al., 2010). Could other bacterial species have an important task in the anammox development and activity? Little is known about this matter, since molecular characterization of the bacterial population structure linked with anammox bacteria has usually been more focused on the study of anammox ecophysiological

relationship with AOB or other N-cycle removal bacteria (Schmid et al., 2000; Third et al., 2001; Philips et al., 2002b) rather than the whole microbial community description (Li et al., 2009).

The identification of the bacterial populations in the later stages of the successful anammox enrichments was performed by PCR-DGGE approach, using 357F-907R primer set. Although DGGE technique usually underestimate the specific richness, this drawback was made up for by using several different samples, all of them containing well-developed *Ca.* Brocadia anammoxidans populations (commonly the studies only used a single DNA source). Not only AOB, NOB or similar chemolithotrophic species were intended to be identified, but the whole bacterial assemblage of each sample. Phylogenetic analyses from the partial 16S rDNA sequences retrieved from the excision of the DGGE bands showed phylotypes spread all along the *Bacteria* domain. None a single sequence was ubiquitously found in the all the enrichments although some clustering could be calculated by MOTHUR, integrating sequences from different origins. *Proteobacteria (Beta and Alpha-)*, *Chlorobi* and *Chloroflexi* are the phyla containing most of the clustered sequences, although single sequences (singletons) also branched inside *Acidobacteria*, *Actinobacteria*, *Gemmatimonadales* and *Planctomycetes*. This distribution of sequences is likely similar from other studies (Fujii et al., 2002; Egli et al., 2003; Strous et al., 2006; Tsushima et al., 2007b; Li et al., 2009; Bae et al., 2010a; Cho et al., 2010). Not only the sequences retrieved from aforementioned studies phylogenetically clustered in the same phyla that the ones obtained in this work, but a high number of sequences from NCBI database (mostly submitted by anammox-related studies) also showed high homology with the 16S rDNA partial sequences obtained from DGGE. Based on the origin of these external sequences, some ecological patterns can be observed, and therefore they can be classified into three categories: (a) from a anammox reactors, (b) from activated sludge or (c) from samples where aliphatic and aromatic compound degradation occurs.

Which role can these resilient bacterial populations that cohabit with anammox bacteria play in such different environments? It is possible that their importance falls on structural tasks. *Rhodocyclaceae* sequences (belonging to *Proteobacteria*) are found

to form a thin layer in the anammox granule, avoiding the excess amount of nitrite (Fujii et al., 2002). *Chlorobi* and *Chloroflexi* seemed to have an important role in the sludge granulation process (Gong et al., 2007; Bae et al., 2010a; Park et al., 2010). The synergism between these bacterial groups could guarantee the anammox aggregate structure and its viability and at the same time they can use some products from the anammox catabolism (Chuang et al., 2008). However, these ecological partners could be needless in reactors that not require the development of aggregates, such as the membrane bioreactors (MBR), where anammox bacteria were found in free-cell suspension. Recent studies (van der Star et al., 2008; Lotti et al., 2014) reported that anammox bacteria showed high metabolic activities after enrichment process in reactors with this kind of configuration, although they were completely dominant (>97% of cells). The role of the other groups remains unclear.

More ecophysiological studies are needed to clarify the microbial interaction between them and the anammox bacteria. A better understanding of the bacteria community structure, including the ecophysiology of this community, may lead to future optimization and efficient design of the anammox process.

Can the information obtained by molecular characterization of the different bacterial assemblages related to PANAMMOX® process help to better understand and improve its performance? For the anammox bacterial partners, several studies testing different technologies and inocula finally described a similar bacterial community. Bacterial populations cohabiting with anammox bacteria in aggregate structures seemed to be important in their growth and their activity. Surely, the granular process would not be possible without some of the most predominant bacterial populations (*Proteobacteria*, *Chlorobi* and *Chloroflexi*). For nitrifying bacteria, the molecular characterization showed that AOB and *Nitrobacter* ecological succession are strongly determined by the stringent conditions within the biological reactor. Probably the inoculation with appropriate seeds could prevent delays in the development of their activity. Moreover, the capability of the PN reactor to oxidize ammonium has revealed to be higher than it was actually indicated by its activity, as it was suggested by gauging the amount of

ammonia that can be processed by each single AOB cell. These remarks suggest that PN yield could be increased if operation conditions would better fit to the requirements of these bacterial populations.

CONCLUSIONS

5. CONCLUSIONS

1. Anammox bacteria can be enriched by using batch culture methodology. Chemical and molecular methods confirmed the presence of *Ca.* Brocadia anammoxidans populations in five of thirteen enrichments. Aliquots from two batch cultures were used as inocula for the start-up of a 50 L anammox bioreactor as the second stage of a PANAMMOX® system.

2. *Ca.* Brocadia anammoxidans populations were enriched from samples of natural, modified and man-made environments: an anoxic SBR, a brackish coastal lagoon, an artificial constructed wetland and several anoxic biological digesters. It was the first time they were detected in a brackish coastal lagoon with high salt content. Although they were identified in samples from the sediment of the lagoon, it is possible that the active populations developed close to the oxic/anoxic interface.

3. The use of the Pla46F-Amx368R primer set for PCR amplification, even using nested-PCR, did not allow to detect anammox bacteria prior to evidences of their activity from chemical analyses, usually the first sign of their presence. Most of the sequences retrieved with this primer set in low-concentrated anammox samples without anammox activity clustered into *Lentisphaerae* phylum, closely related to *Planctomycetes* in the PVC superphylum.

4. The use of the Amx368F-Amx820R primer set did not retrieve clear anammox sequences from inocula or batch cultures at their early stages of enrichment. However, it allowed to detect some *Brocadiaceae* sequences in the successful anammox enrichments. These bacteria were considered as “putative” anammox bacteria because they were not phylogenetically close enough to known anammox bacteria. Their identification could be considered as an indicator that a sample has the potential for developing anammox populations if the environmental conditions are appropriate for their growth.

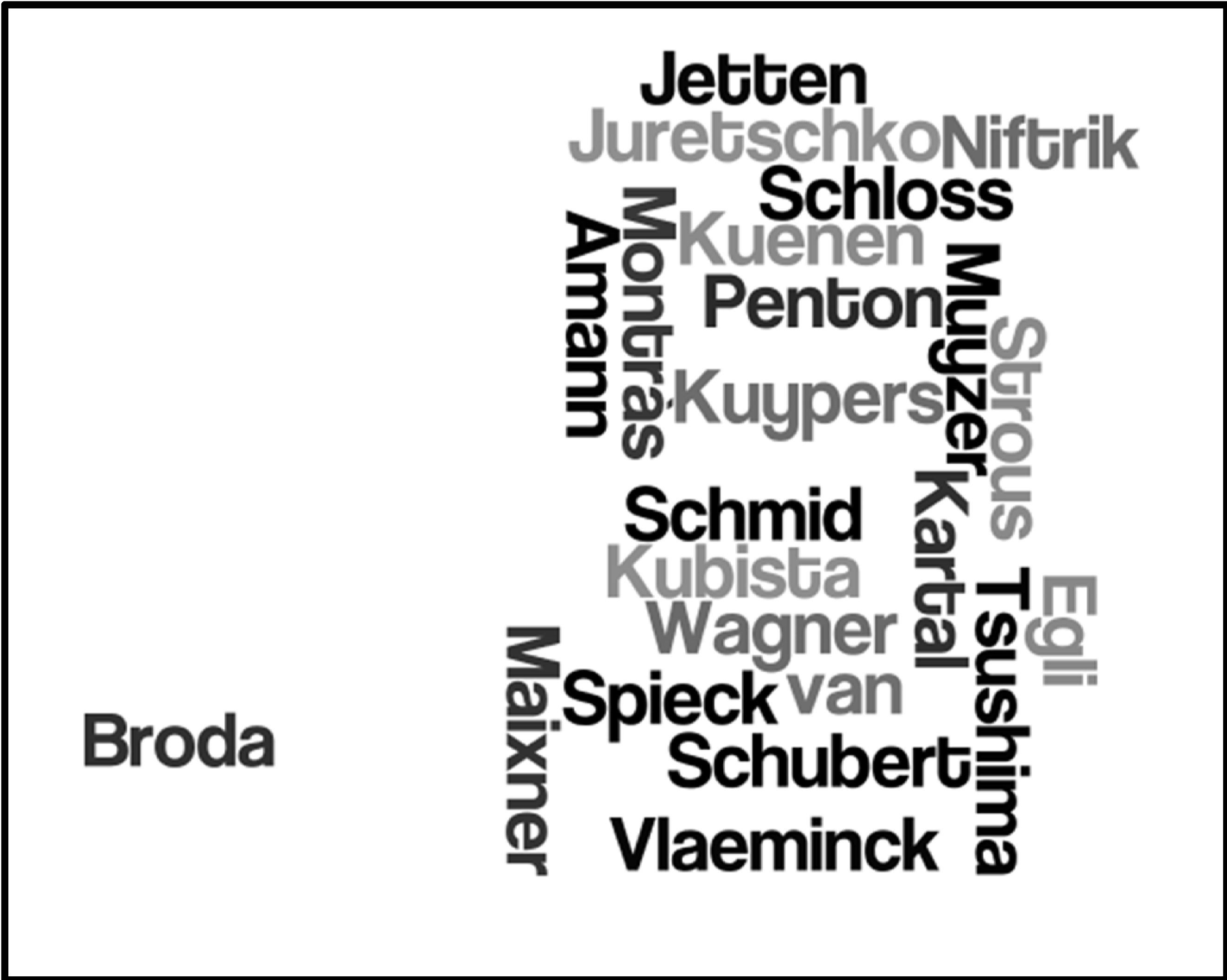
5. In the last stages of the successful anammox bacteria enrichments, bacterial primer sets were unable to detect any common sequence in all samples. A potential ubiquitous ecological partner of anammox, therefore, could not be identified. Furthermore, anammox bacteria phylotypes were not retrieved in any sample with these primers, which probably underrated them.

6. Although an ecological partner of anammox bacteria was not clearly identified, closely-related phylotypes were detected in different samples, and even in similar studies found on the literature. The whole community composition of the samples analyzed in the present work and these studies, at a phyla level, was quite similar. *Chlorobi*, *Rhodocyclaceae*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria* and *Actinobacteria* were the main bacterial groups identified. Probably their presence was related with the formation of the anammox aggregates, thus involving them in the anammox occurrence and development.

7. *Nitrosomonas* spp. were the dominant AOB all along the PN reactor performance. Phylogenetic analyses showed an ecological succession from initial *Nitrosomonas* cluster 6a species (*K*-strategists) to *Nitrosomonas* cluster 7 species (*r*-strategists). In the last stages of the reactor performance, all sequences were affiliated to *Nitrosomonas* sp. IWT514, a strain specialized in environments with high ammonium and nitrite concentrations.

8. *Nitrobacter* spp. were detected throughout the PN reactor performance. At the initial stages, high concentrations of *Nitrobacter alkalicus* where determined with specific primers for this genera. However, they decreased and other *Rhizobiales* populations arose (*Bradyrhizobium*, *Xanthobacter* and *Mesorhizobium)* at the final stages. The unexpected detection of these *Rhizobiales* sequences was probably due to unspecifities of the FGPS primer sequences.

9. *Ca.* Nitrospira defluvii was also detected all along the PN reactor performance, using specific primers for *Nitrospira* spp. This species belongs to the *Nitrospira* sublineage I, which is found to have competitive advantage at high nitrite concentrations over other *Nitrospira* sublineage. Quantification analyses suggested that this species found an ecological niche inside the reactor that enable it to be more competitive and overpass *Nitrobacter* spp.



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