Universitat de Girona

A STUDY ON THE PHYLOGENY AND THE ECOLOGY OF AMMONIA-OXIDIZING BACTERIA USING A NEW MOLECULAR MARKER BASED ON THE GENE AMOB

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Vist-i-plau
El Director de la Tesi

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Els antics costums i les faules de les velles són coses vulgars, però no s'han de sepultar en l'oblit. Encara es pot trobar en elles certa veritat i alguna cosa útil. A més, és un autèntic plaer comprovar que les generacions anteriors cometien tants errors...com la d'ara.

John Aubrey

Para llegar a ser sabio, es preciso querer experimentar ciertas vivencias, es decir, meterse en sus fauces. Eso es, ciertamente, muy peligroso; más de un sabio ha sido devorado al hacerlo.

Friedrich Nietzsche

Els problemes són una guia per aconseguir objectius

Anònim

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Abbreviations

AMO	Ammonia monooxygenase
amoA	Gene encoding the subunit A of AMO
amoB	Gene encoding the subunit B of AMO
amoC	Gene encoding the subunit C of AMO
AmoA	Subunit A of AMO
AmoB	Subunit B of AMO
AmoC	Subunit C of AMO
AOB	Ammonia Oxidizing Bacteria
CI	Chlorophorm Isoamyl alcohol
CY3	Fluorescent Indocarbocyanine
DAPI	4',6-diamiine-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
EDTA	Etilenediamine-sodium tetraacetate
FISH	Fluorescent *in situ* hybridization
MOB	Methane Oxidizing Bacteria
Nc	*Nitrosococcus*
Nm	*Nitrosomonas*
NOB	Nitrite Oxidizing Bacteria
Ns	*Nitrosospira*

pb	base pair
PBS	Posphate buffered saline
PCR	Polymerase Chain Reaction
pmoA	Gene encoding the subunit A of AMO
pmoB	Gene encoding the subunit B of AMO
pmoC	Gene encoding the subunit C of AMO
r.p.m.	revolutions per minute
RDP	Ribosomal Database Project
SBR	Sequencing Batch Reactor
TBE	Tris-Borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylendiamide
Tris	Tris-hydroxymethyl-aminomethane
WWTP	Wastewater Treatment Plant

Summary

Human activities such as farming and industrialization have produced a significant increase in the number of ammonium-rich environments. The presence of nitrogenated compounds reduces water quality causing toxicity problems, deteriorating the environment and even affecting human health. Consequently, nitrification has recently become a widespread process involving the cycling of nitrogen in the biosphere, which is mainly due to microbial activities. Ammonia oxidizing bacteria (AOB) are an essential component of the global cycling of nitrogen, being responsible for the aerobic oxidation of ammonium to nitrite.

Although the first ammonia oxidizing bacteria were isolated by the end of the XIX century, the slowness of their growth and the difficulties in culturing impeded achieving a full knowledge of this bacterial group until the 80s, when the first studies based on the gene 16S rDNA where performed. Nowadays, the databases contain huge numbers of entries of 16S rDNA sequences belonging to ammonia oxidizing bacteria.

The aim of this work was to find, develop, and evaluate useful and reliable tools for the study of ammonia oxidizers in environmental samples.

In this work we describe the use of Fluorescence *In Situ* Hybridization (FISH), based on the use of DNA probes specifically targeting the ammonia-oxidizers 16S rRNA molecule. Ammonia oxidizing bacteria were detected and enumerated by using this technique. However, unknown sequences are hardly detectable by using this method, and therefore, new tools were needed.

For this purpose we tried applying the sequence of the probe Nso1225 together with a universal oligonucleotide for eubacteria in a PCR reaction. The possibility of specifically amplifying a 16S rDNA gene fragment resulted in a new fingerprinting tool to assess the presence and diversity of ammonia-oxidizers in natural environments. Even so, some β–Proteobacterial non–ammonia oxidizer sequences were also retrieved by using this technique. Moreover, one of the main disadvantages of using 16S rDNA as a molecular marker is the impossibility of simultaneously detecting both the β and the γ–Proteobacterial ammonia oxidizing bacteria.

The gene *amoA*, which encodes for the subunit A of the enzyme ammonia monooxygenase, was then being extensively used as a marker for the detection of AOB in environmental samples. We describe the use of this marker for the identification of several ammonia oxidizing sequences in sludge samples from a sequencing batch reactor (SBR). Although useful, the use of *amoA* as a marker requires cloning, which is a tedious and time-consuming technique when dealing with large number of samples in microbial ecology studies. Besides, detection of non–AOB sequences has been reported by other authors when using *amoA* in a PCR-DGGE approach.

Aiming at obtaining a fast and rigorous analytical tool allowing AOB detection and identification, we developed a new set of primers targeting the gene *amoB*, which encodes for the transmembrane domain of the enzyme ammonia monooxygenase. This gene has been shown to be a good molecular marker for AOB, since it can be used for easy detection and identification of ammonia oxidizers, providing high specificity, sensitivity and reliability regardless of phylogenetic affiliations.

A real-time PCR assay for the detection and quantification of the γ–proteobacterial genus *Nitrosococcus* based on the *amoB* gene sequence is also presented. This newly designed primer set allows a highly sensitive and specific enumeration of all known Nitrosococci.

We finally performed a comparison and evaluation of the markers *amoA*, *amoB* and 16S rDNA, and built a polygenic based tree.

As a result we conclude that *amoB* is a suitable molecular tool for detecting and identifying AOB in environmental samples, yielding consistent grouping when performing phylogenetic inferences. In turn, the whole sequence of the gene 16S rDNA is indicated for taxonomical and phylogenetic purposes when working with ammonia oxidizing isolates.

Resum

L'agricultura i la industrialització han causat un augment significatiu del nombre d'ambients rics en amoni. La presència de compostos nitrogenats redueix la qualitat de l'aigua, causant problemes de toxicitat, deteriorant el medi ambient i fins i tot afectant la salut humana. En conseqüència, la nitrificació s'ha convertit en un procés global que afecta al cicle del nitrogen a la biosfera. Aquest cicle és principalment degut a diverses activitats microbianes. Els bacteris oxidadors d'amoni (AOB) són els responsables de l'oxidació de l'amoni a nitrit, i juguen un paper essencial en el cicle del nitrogen.

Malgrat que els primers oxidadors d'amoni van ser aïllats a finals del segle XIX, la lentitud del seu creixement i les dificultats per cultivar-los feren que fins a finals dels anys 80, quan es van realitzar els primers estudis amb el gen 16S rDNA, no s'assolís un coneixement complert d'aquest grup bacterià. Actualment les bases de dades contenen multitud d'entrades amb seqüències corresponents a bacteris oxidadors d'amoni.

L'objectiu d'aquest treball era el de trobar, desenvolupar i avaluar eines útils i fiables per a l'estudi dels bacteris oxidadors d'amoni en mostres ambientals.

En aquest treball primer descrivim la utilització de la hibridació *in situ* amb fluorescència (FISH), mitjançant l'aplicació de sondes amb diana específicament en el 16S rRNA dels bacteris oxidadors d'amoni. La FISH ens va permetre detectar i recomptar aquest grup bacterià; no obstant, aquest mètode no permetia la detecció de noves seqüències, pel que es necessitava una nova eina.

Amb aquesta intenció vam intentar aplicar la seqüència de la sonda Nso1225 juntament amb un oligonucleòtid universal per eubacteris en una PCR. El fet de poder amplificar específicament un fragment del 16S rDNA dels AOB va suposar el desenvolupament d'una nova eina molecular que permetia detectar la presència i la diversitat d'aquests bacteris en ambients naturals. Malgrat tot, algunes seqüències pertanyents a bacteris no oxidadors d'amoni del subgrup β dels proteobacteris, eren també obtingudes amb aquesta tècnica. Així mateix, un dels inconvenients de l'ús del 16S rDNA com a marcador molecular és la impossibilitat de detectar simultàniament els oxidadors d'amoni que pertanyen als subgrups β i γ dels proteobacteris.

El gen *amoA*, que codifica per la subunitat A de l'enzim amoni monooxigenasa (AMO), era aleshores àmpliament utilitzat com a marcador per a la detecció dels AOB en mostres ambientals. En aquest treball també descrivim la utilització d'aquest marcador en mostres de fangs procedents d'un reactor SBR (Sequencing Batch Reactor). L'ús d'aquest marcador ens va permetre identificar seqüències de bacteris oxidadors d'amoni en la mostra, però la necessitat de detectar *amoA* mitjançant clonatge fa que l'ús d'aquest marcador requereixi massa temps per a la seva utilització com a eina en estudis d'ecologia microbiana amb moltes mostres. Per altra banda, alguns autors han assenyalat l'obtenció de seqüències de bacteris no oxidadors d'amoni en utilitzar *amoA* en un protocol de PCR-DGGE.

Amb la finalitat d'obtenir una eina ràpida i rigorosa per detectar i identificar els AOB, vam desenvolupar un joc nou d'oligonucleòtids amb diana en el gen *amoB*, que codifica per a la subunitat transmembrana de l'enzim AMO. Aquest gen ha demostrat ser un bon marcador molecular pels AOB, oferint, sense tenir en compte afiliacions filogenètiques, una elevada especificitat, sensibilitat i fiabilitat.

En aquest treball també presentem una anàlisi de PCR a temps real basada en la detecció del gen *amoB* per a la quantificació del gènere *Nitrosococcus* (dels γ–proteobacteris). El nou joc d'oligonucleòtids dissenyat permet una enumeració altament específica i sensible de tots els γ–*Nitrosococcus* coneguts.

Finalment, vam realitzar un estudi poligènic, tot comparant i avaluant els marcadors *amoA*, *amoB* i 16S rDNA, i vàrem construir un arbre filogenètic combinat.

Com a resultat concloem que *amoB* és un marcador adequat per a la detecció i identificació dels AOB en mostres ambientals, proporcionant alhora

agrupacions consistents en fer inferències filogenètiques. Per altra banda, la seqüència sencera del gen 16S rDNA és indicada com a marcador en estudis amb finalitats taxonòmiques i filogenètiques en treballar amb cultius purs de bacteris oxidadors d'amoni.

Resumen

La agricultura y la industrialización han causado un aumento significativo del número de ambientes ricos en amonio. La presencia de compuestos nitrogenados reduce la calidad del agua, causando problemas de toxicidad, deteriorando el medio ambiente e incluso afectando la salud humana. En consecuencia, la nitrificación se ha convertido en un proceso global que afecta al ciclo del nitrógeno en la biosfera. Este ciclo está principalmente mediado por la actividad de varios microorganismos, entre ellos las bacterias oxidadoras de amonio (AOB), que son las responsables de la oxidación del amonio a nitrito.

Los primeros oxidadores de amonio fueron aislados a finales del siglo XIX, pero la lentitud de su crecimiento y las dificultades por cultivarlos hicieron que hasta finales de los años 80, cuando se realizaron los primeros estudios con el gen 16S rDNA, no se lograra un conocimiento completo de este grupo bacteriano. Actualmente las bases de datos contienen multitud de entradas con secuencias correspondientes a bacterias oxidadoras de amonio.

El objetivo de este trabajo era el de encontrar, desarrollar y evaluar herramientas útiles y fiables para el estudio de las bacterias oxidadoras de amonio en muestras ambientales.

En este trabajo primero describimos la utilización de la hibridación *in situ* con fluorescencia (FISH). Mediante la aplicación de sondas con diana específica en el 16S rRNA de las bacterias oxidadoras de amonio, la FISH nos permitió detectar y contar este grupo bacteriano. No obstante, este método no permitía la detección de nuevas secuencias, por lo que se necesitaba una nueva herramienta.

Con esta intención intentamos aplicar la secuencia de la sonda Nso1225 junto con un oligonucleótido universal para eubacterias en una PCR. El hecho de poder amplificar específicamente un fragmento del 16S rDNA de las AOB supuso el desarrollo d'una nueva herramienta molecular que permitía detectar la presencia y la diversidad de las bacterias oxidadoras de amonio en ambientes naturales. No obstante, con esta técnica se obtenían también algunas secuencias pertenecientes a bacterias no oxidadoras de amonio del subgrupo β de las proteobacterias. Asimismo, uno de los inconvenientes del uso del 16S rDNA como marcador molecular es la imposibilidad de detectar simultáneamente las oxidadoras de amonio pertenecientes a los subgrupos β y γ de las proteobacterias.

El gen *amoA*, que codifica por la subunidad A de la enzima amonio monooxigenasa (AMO), era entonces ampliamente utilizado como marcador para la detección de las AOB en muestras ambientales. En este trabajo también describimos la utilización de este marcador en muestras de fangos procedentes de un reactor SBR (Sequencing Batch Reactor). El uso de este marcador nos permitió identificar secuencias de bacterias oxidadoras de amonio en la muestra, pero la necesidad de detectar *amoA* mediante clonación requiere demasiado tiempo para la utilización de este marcador como herramienta en estudios de ecología microbiana con multitud de muestras. Por otra parte, algunos autores han señalado la obtención de secuencias de bacterias no oxidadoras de amonio al utilizar *amoA* en un protocolo de PCR-DGGE.

Con la finalidad de obtener una herramienta rápida y rigurosa para detectar e identificar las AOB, desarrollamos un nuevo juego de oligonucleótidos con diana en el gen *amoB*, que codifica para la subunidad transmembrana de la enzima AMO. Este gen ha demostrado ser un buen marcador molecular para las AOB, ofreciendo, sin tener en cuenta afiliaciones filogenéticas, una elevada especificidad, sensibilidad y fiabilidad.

En este trabajo también presentamos un análisis de PCR a tiempo real basada en la detección del gen *amoB* para la cuantificación del género *Nitrosococcus* (de las γ–proteobacterias). El nuevo juego de iniciadores diseñado permite la enumeración altamente específica y sensible de todos los γ–*Nitrosococcus* conocidos.

Finalmente realizamos un estudio poligénico comparando y evaluando los marcadores *amoA*, *amoB* y 16S rDNA individualmente y entre sí, y construimos un árbol filogenético combinado.

Como resultado concluímos que *amoB* es un marcador adecuado para la detección e identificación de las AOB en muestras ambientales, proporcionando a la vez agrupaciones consistentes al hacer inferencias filogenéticas. Finalmente, la secuencia entera del gen 16S rDNA es indicada como marcador en estudios con finalidades taxonómicas y filogenéticas al trabajar con aislados de AOB.

ÍNDEX

1 INTRODUCTION

1.1 BIOLOGICAL REMOVAL OF NITROGEN

In the last decades farming and industrial activities have almost doubled the concentration of nitrogen fixed per year in the biosphere (Vitousek *et al.*, 1997). Nowadays, since a great part of this nitrogen gets to the water systems, it is one of the most important polluting agents to eliminate from wastewater. The decrease in the water quality hinders its recycling, deteriorating the environment, causing toxicity problems to aquatic organisms and even affecting human health. For example, the presence of nitrate in the water (nitric acid salts) may cause some diseases in infants.

Stemmed water systems, lakes, lagoons, dams and marshes, are extremely vulnerable to eutrophication. Eutrophication is a process resulting from an excessive nutrient incorporation to these ecosystems. As a consequence, vegetation grows disproportionately, which causes not only aesthetic problems –such as stink, seaweed and mold accumulations– but also oxygen extinction in the water. Moreover, it promotes the accumulation of sediments and precipitates at the bottom of the lakes. It is therefore essential to find processes to maintain the nitrogenated compounds in acceptable amounts.

First sewer systems date from the Assyrian era. Piping systems pouring the residues to the rivers were used until the XIX century, and it was not until the beginning of the XX century that some cities and industries built wastewater treatment installations for the first time.

At first, physical and chemical methods consisting in moving the polluting agent from one environment to another were used. In contrast, the biological

methods used today, eliminate the pollutants and, often, end up with N_2 and CO_2 as final products.

Life depends on the element nitrogen (Madigan *et al.*, 2003), which can be found in nature in the following oxidation states:

–III: NH_3: ammonia
0: N_2: dinitrogen, the most frequently constituent of the atmosphere
+I: N_2O: dinitrogen oxide
+II: NO: nitrogen monoxide
+III: NO_2^-: nitrite
+IV: NO_2: nitrogen dioxide
+V: NO_3^-: nitrate

In addition, chemical instability and nitrogen transformations performed by prokaryotes, release other forms of nitrogenated compounds that have a transient existence.

Nitrogen cycling in the biosphere is complex, and is mainly due to microbial activities. Microbes reduce the dinitrogen from the atmosphere to ammonium, which is further incorporated into cell material (amino acids). Organic nitrogen compounds are mineralized to ammonia by a microbial process known as ammonification or mineralization. Ammonia (NH_3) /ammonium (NH_4^+) is the most common form of nitrogen in the biosphere. In contrast, nitrite is usually only found in traces, although it may accumulate at low oxygen partial pressures. Nitrification is the biological oxidation of reduced forms of nitrogen to nitrite and nitrate, and is mediated by two physiological groups of microorganisms: the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB). In aerobic habitats, the nitrite produced by the AOB is immediately oxidized by the NOB. Therefore, nitrite concentration is extremely low in habitats such as soil, sea, and freshwater (Schmidt, 1982). Moreover, when organic matter is available, nitrate is used as electron acceptor for anaerobic respiration under anoxic or oxygen limited conditions. Then, nitrate is converted to ammonia (respiratory ammonification) or dinitrogen (denitrification).

1.1.1 Nitrification

Nitrification is the main aerobic process of the nitrogen cycle, and consists in the oxidation of ammonium to nitrate. It takes place in two steps catalyzed by two phylogenetically distinct bacterial groups. Ammonium oxidizing bacteria

(AOB) first oxidize ammonium to nitrite. Then, nitrite oxidizing bacteria (NOB), oxidize the nitrite to nitrate:

$NH_4^+ + 1\,½\,O_2 \rightarrow NO_2^- + 2H^+ + H_2O$ $\Delta G = -66$ Kcal
$NO_2^- + ½\,O_2 \rightarrow NO_3^-$ $\Delta G = -17$ Kcal

All AOB species studied to date perform ammonium oxidation to nitrite in two successive metabolic steps (Gottschalk, 1986). First, the enzyme ammonium monooxygenase (AMO) oxidizes ammonium to hydroxylamine, which is further oxidized to nitrite by the enzyme hydroxilamine oxidoreductase (HAO). These two processes are detailed in the section 1.2.1.

1.1.2 Denitrification and DRNA

1.1.2.1 Denitrification

Denitrification consists in the reduction of nitrate NO_3^- to nitrogen gas; which is finally released to the atmosphere (Knowles, 1982). This anaerobic respiration process is performed by heterotrophic microorganisms from very distinct genera such as *Pseudomonas*, *Thiobacillus*, *Paracoccus* and *Alcaligenes*, and some of them can also grow autotrophically (Tiedje, 1988). Denitrification is performed in four steps (Thomsen *et al.*, 1994), and it starts with the reduction of nitrate to nitrite. This step is catalyzed by a three–domain transmembrane enzyme named nitrate reductase. Then the nitrite reductase transforms the nitrite to nitrogen monoxide (NO), which is further transformed to dinitrogen oxide (N_2O) by the nitric oxide reductase. N_2O is the final product of some denitrifying genera that do not possess the enzyme nitrous oxide reductase (Stouthamer, 1988), which is the enzyme responsible for the last step of denitrification, the release of the produced N_2 to the atmosphere.

1.1.2.2 Dissimilatory reduction of nitrate to ammonium (DRNA)

Another way of reducing nitrogen is the dissimilatory reduction of nitrate to ammonium (RDNA), which is performed by a nitrate reductase not linked to the membrane that uses $NADH_2$ and formate as the electron source (Jackson *et al.*, 1981). Since this process hinders the final reduction of nitrate (NO_3^-) to nitrogen gas (N_2), this way is considered to be a short–circuit into the nitrogen cycle (Cole and Brown, 1980). One of the environmental factors determining which will be the reductive way of nitrate is C/N proportion. In general, RDNA is favored when C/N proportions are high (Cervantes–Carrillo *et al.*, 2000).

1.1.3 Anaerobic ammonium oxidation (Anammox)

Anammox (anaerobic ammonia oxidation) bases on the oxidation of ammonium to N_2 by using nitrite as electron acceptor in anaerobic conditions (Mulder *et al.*, 1995; Van de Graaf *et al.*, 1996; Van de Graaf *et al.*, 1997). To date, only five microorganisms supposed to be responsible for anaerobic ammonium oxidation have been identified: Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensis*, Candidatus *Scalindua brodae*, Candidatus *Scalindua wagneri* and Candidatus *Scalindua sorokinii* (Egli *et al.*, 2001; Schmid *et al.*, 2000; Schmid *et al.*, 2003; Kuypers *et al.*, 2003), and they have never been isolated in pure culture yet. All these microorganisms are classified into the Planctomycetes phylum, planctomycetacia class, order planctomycetales.

One of the most promising proposals for wastewater treatment is the combination of nitrification and anammox. This process, known as CANON (Completely Autotrophic Nitrogen removal Over Nitrite) is based on a partial oxidation of ammonia to nitrite, followed by an anaerobic oxidation of ammonium to dinitrogen (N_2) using nitrite as electron acceptor. The combination of these two processes reduces both operational costs and CO_2 emissions (Jetten *et al.*, 1998; Jetten *et al.*, 2001; Strous *et al.*, 1997b).

1.2 AMMONIUM OXIDIZING BACTERIA

1.2.1 Characteristics and metabolism

Ammonia oxidizing bacteria (AOB) are gram negative chemolithotrophic bacteria. They obtain energy and reducing power from ammonium, and fix carbon from CO_2 in the atmosphere through the Calvin cycle. Table 1.1 shows AOB main characteristics.

Table 1.1 Characteristics of the main genus of ammonia oxidizing bacteria

Characteristics	*Nitrosococcus*	*Nitrosolobus*[2]	*Nitrosomonas*	*Nitrosospira*	*Nitrosovibrio*[2]
Phylogenetic affiliation	Class γ–Proteobacteria[1]; Order Chromatiales; Family Chromatiaceae	Class β–Proteobacteria; Order Nitrosomonadales; Family Nitrosomonadaceae	Class β–Proteobacteria; Order Nitrosomonadales; Family Nitrosomonadaceae	Class β–Proteobacteria; Order Nitrosomonadales; Family Nitrosomonadaceae	Class β–Proteobacteria; Order Nitrosomonadales; Family Nitrosomonadaceae
Cell form	Spherical Ellipsoids	Pleomorphic lobulated	Straight rods	Spiral	Slightly curved rods
Cell size	1,5–1,8 × 1,7–2,5	1,0–1,5 × 1,0–2,5	0,7–1,5 × 1,0–2,4	0,3–0,8 × 1,0–8,0	0,3–0,4 × 1,1–3,0
Flagellation of mobile cells	Grouped flagella	Peritrichal	Polar Subpolar	Peritrichal	Polar Subpolar
Intracytoplasmatic rearrangement of membranes	Grouped as flat vesicles. Central position	Forming an internal pseudo–compartmentalization	Peripheric and flat	External membrane invaginations	External membrane invaginations
Described species	*Nc mobilis*[1] *Nc halophilus* *Ns oceani*	*Nl multiformis*	*Nm europaea* *Nm eutropha* *Nm halophila* *Nm communis* *Nm marina* *Nm aestuarii* *Nm nitrosa* *Nm ureae* *Nm oligotropha* *Nm cryotolerans*	*Ns briensis*	*Nv tenuis*

1 except *Nc mobilis*, which is included into the β–Proteobacteria; order Nitrosomonadales.
2 nowadays *Nitrosovibrio* and Nitrosolobus are species included into the genera *Nitrosospira*. In this table they have been treated by separate due to their distinctive morphology.

Chemically, the transformation occurs in two consecutive steps: ammonium is first oxidized to hydroxylamine by the enzyme ammonia monooxygenase (AMO), and then, hydroxylamine is oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO). Equations 1 and 2 describe the two half-reactions of the oxidation of ammonia to hydroxylamine. The overall equation is depicted in equation 3. No oxygen is required for hydroxylamine oxidation (equation 4). Two electrons are transferred back to equation 2, and the remaining electrons pass to the respiratory chain (equation 5). Oxidation of hydroxylamine is given in equation 6. The global reaction, illustrated in equation 7, shows that nitric acid is produced during the biological oxidation of ammonia.

(1) $NH_3 + 0.5\ O_2 \rightarrow NH_2OH$ $\Delta G_0' = +17\ kJ{\cdot}mol^{-1}$

(2) $0.5\ O_2 + 2H^+ + 2e^- \rightarrow H_2O$ $\Delta G_0' = -137\ kJ{\cdot}mol^{-1}$

(3) $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$ $\Delta G_0' = -120\ kJ{\cdot}mol^{-1}$

(4) $NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-$ $\Delta G_0' = +23\ kJ{\cdot}mol^{-1}$

(5) $0.5\ O_2 + 2H^+ + 2e^- \rightarrow H_2O$ $\Delta G_0' = -137\ kJ{\cdot}mol^{-1}$

(6) $NH_2OH + 0.5\ O_2 \rightarrow HNO_2 + 2e^- + 2H^+$ $\Delta G_0' = -114\ kJ{\cdot}mol^{-1}$

(7) $NH3 + 1.5\ O_2 \rightarrow HNO_2 + H_2O$ $\Delta G_0' = -235\ kJ{\cdot}mol^{-1}$

Since the redox values of ammonium/hydroxylamine and hydroxylamine/nitrite pairs are too low to reduce the NAD^+, the electron flow generated at the electronic transport chain is only capable of producing ATP. Therefore, an inverse electronic transport chain is required in most chemolithotrophic bacteria to obtain reducing power, which is needed in great amounts to fix CO_2 through the Calvin cycle. All these requirements cause the extremely low yield of these organisms.

Ammonium oxidation to nitrite produces a proton release to the media, which results in a decrease in the pH, which has to be periodically adjusted in culture media when no buffers are added. Optimal pH for their growth ranges from 7.5 to 7.8 units, and temperature must be set between 25 and 30º C.

1.2.2 AOB taxonomy and phylogeny

The first ammonia oxidizers were isolated at the end of the XIX century (Frankland and Frankland, 1890; Winogradsky, 1890). Although S. Winogradsky postulated the existence of a great diversity of species capable of growing upon ammonium oxidation, new species of AOB were not isolated until the late 60s (Watson 1965; Watson *et al.*, 1971a,b,c; Harms *et al.*, 1976; Jones *et al.*, 1988; Koops *et al.*, 1990, 1991).

Nowadays, studies dealing with 16S rDNA sequences have classified the AOB into two main monophyletic groups. The first, corresponding to the γ-subgroup of the Proteobacteria include strains of *Nitrosococcus oceani* and *Nitrosococcus halophilus*. The second is composed by the genera *Nitrosomonas* and *Nitrosospira*, both included into the β-subgroup of the Proteobacteria (Woese *et al.*, 1984, 1985; Head *et al.*, 1993; Teske *et al.*, 1994; Utåker *et al.*, 1995; Purkhold *et al.*, 2000). The genera *Nitrosolobus* and *Nitrosovibrio* are enclosed within the *Nitrosospira* group (Head *et al.*, 1993; Teske *et al.*, 1994). Besides, *Nitrosococcus mobilis* is included within the *Nitrosomonas* (Figure 1.1).

Recently, the *amoA* gene has been used as an additional molecular marker molecule for ammonia oxidizers (Mc Tavish *et al.*, 1993; Klotz and Norton, 1995; Rotthauwe *et al.*, 1995; Hommes *et al.*, 1998; Purkhold *et al.*, 2000, 2003). PCR primers flanking a 453 pb fragment are used in these studies (Rotthauwe *et al.*, 1997 modified by Stephen *et al.*, 1999). Results obtained with deduced amino acid sequences of the *amoA* gene fragments are in general consistent with the 16S rRNA phylogeny of AOB. Members of the *Nitrosospira* lineage form a tight monophyletic group with no evident substructure. Within the Nitrosomonads, *N. europaea /N. eutropha* are no longer monophyletic. The same occurs with *N. oligotropha / N. ureae*, and within the members of the *N. communis* cluster (Figure 1.2).

In this work we present *amoB* as a new molecular marker for AOB. If *amoB* nucleic sequences are used for phylogenetic analysis, basically, the same picture obtained with 16S rRNA and *amoA* gene sequences emerges (see chapters 6 and 7).

The diverse lineages associated to the *Nitrosomonas–Nitrosospira* group described by Purkhold *et al.* (2003) on the basis of 16S rDNA and *amoA* sequences are presented in Table 1.2. Only a few of the described lineages take account of cultured species. The rest owe their existence to DNA sequences recuperated from environmental samples.

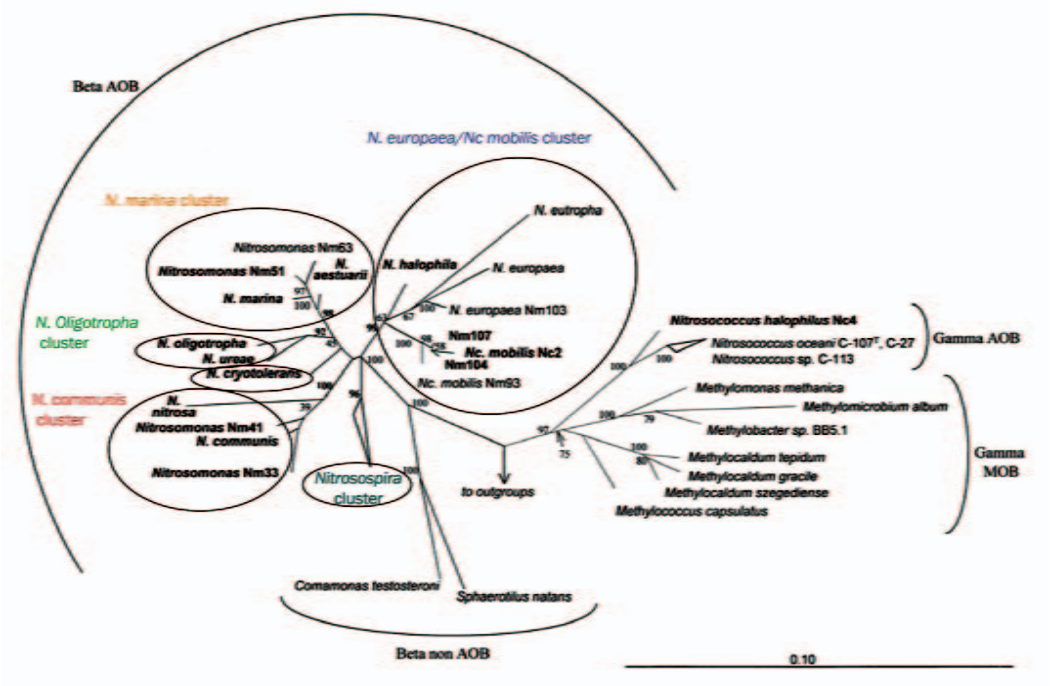


Figure 1.1 Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms. The tree is based on results of neighbor-joining analysis using a 50% conservation filter for the *Bacteria*. An encompassing collection of organisms representing all major lineages of the *Archaea* and *Bacteria* were used as outgroups for treeing. The multifurcation connects branches for which a relative order could not be unambiguously determined by applying different treeing methods. Parsimony bootstrap values (100 replicates) for branches are reported. Scale indicates 10% estimated sequence divergence. MOB, methane-oxidizing bacteria (figure adapted from Purkhold *et al.*, 2000).

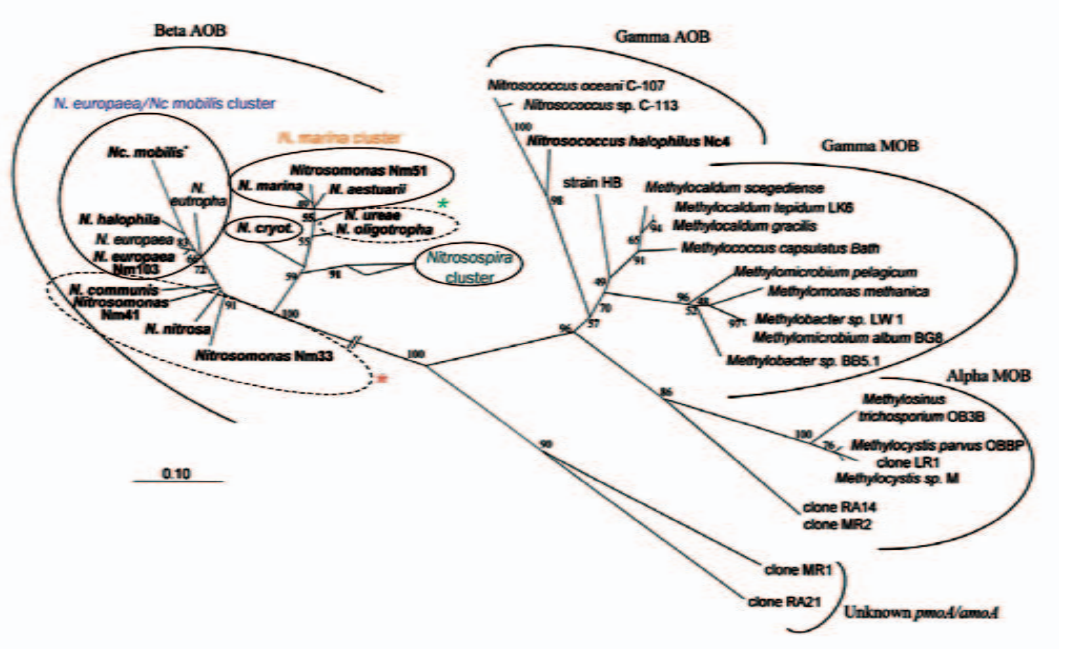


Figure 1.2. Phylogenetic Fitch-Margoliash tree (using global rearrangement and randomized input order [7 jumbles]) reflecting the relationships of AOB and methane-oxidizing bacteria (MOB) based on deduced AmoA and PmoA sequences. Parsimony bootstrap values (100 replicates) for branches are reported. Scale indicates 10% estimated sequence divergence. (*: to enhance clarity, AmoA sequences of *Nitrosococcus mobilis* Nm93 and of the isolates Nm104 and Nm107, which are identical in sequence to the AmoA sequence of *Nitrosococcus mobilis* Nc2, are not shown in the tree). *, * show *N communis* and *N oligotropha* clusters no longer monophyletic (figure adapted from Purkhold *et al.*, 2000).

Table 1.2. The diverse lineages associated to the Nitrosomonas–Nitrosospira group described by Purkhold *et al.* (2003) on the basis of 16S rDNA and *amoA* gene sequences.

	Lineage	Main characteristicss	Cultured species	Characteristics of cultured species
Nitrosomonas (β Proteobacteria)	*Nitrosomonas oligotropha*	Two cultured species and several genospecies described. Most of them can be found in oligotrophic waters and occasionally in moderately acidic soils (pH 6.00). Low affinity constants for ammonia (few μM). Almost all of them urease +, and sensitive to salt presence.	*Nitrosomonas oligotropha*	Spherical or rod shaped, with blunt ends. Excretion of polysaccharides causes the formation of cell aggregates. Mobility or carboxysomes not observed. G+C: 49.4–50.0
	Nitrosomonas marina	Two described species and one genospecie. Isolated from marine environments. Salt requirements. Most of them urease +.	*Nitrosomonas ureae*	Phenotype very similar to *Nm oligotropha*. Lower G+C (45.6–46.00)
	Nitrosomonas europaea/ Nitrosococcus mobilis	Relatively high salt tolerance. *Nm halophila* and *Nc mobilis* require moderate salt presence. Affinity constant for ammonia: 50 a 100 μM. Urease –. *Nm europaea* and *Nm eutropha* present 15–16% DNA–DNA similarity. Similarity with *Nm halophila* is minimal and inexistent with *Nc mobilis*.	*Nitrosomonas eutropha*	Pleomorphic cells, free or in short chains. Mobile and with carboxysomes. Rod shaped, with one or both ends sharp. Tolerates up to NaCl 400 mM. G+C: 47.9–48.5
			Nitrosomonas europaea	Short rods with sharp ends. Immobile and without carboxysomes. Eutrophic environments. Tolerates up to NaCl 400 mM. G+C: 50.6–51.4
			Nitrosomonas halophila	Very short rods or coccoids. Mobile cells, with flagella aggregates and carboxysomes. Salt requirements, tolerate up to NaCl 900 mM. Some tolerate media basification up to pH 10.00. Detected in North Sea samples and in salty lakes in Mongolia. G+C: 53.8
			Nitrosococcus mobilis	Cocci or very short rods. No carboxysomes. Mobile cells present flagella aggregates. Salt requirements. Isolated from the North Sea and a wastewater treatment plant. G+C: 49.3.
	Nitrosomonas communis	DNA similarity and phenotype causes a division in two sub–lineages: –*Nm comunis* + 2 cultured genospecies: urease – and can be found in farming soils with neutral pH.	*Nitrosomonas communis*	Long blunt–ended rods. Neither carboxysomes nor mobility. Found in soils moderately eutrophic and neutral pH. G+C: 45.6–46.0

Table 1.2. (Cont.)

		–*Nm nitrosa*: urease + and can be found in aquatic environments.	*Nitrosomonas nitrosa*	Spherical or short rods with blunt ends. Immobile. Prefer freshwater habitats although they have also been found in seawater and wastewater. G+C: 47:9
	Nitrosomonas sp. Nm143	There are several marine isolates, not described, and corresponding to this lineage.		
	Nitrosomonas cryotolerans	Only one species described, isolated from Kasitsna Bay (Alaska), salt requirements.	*Nitrosomonas cryotolerans*	No carboxysomes. Urease +. Immobile. Intracitoplasmatic membranes often surrounding the nucleoplasma. Tolerate low temperatures (down to 5°C). G+C: 45.5–46.1.
Nitrosospira (β Proteobacteria)	*Nitrosospira*	Three genera described: *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*. Only one species cultured and described for each genera. Several genospecies described. Although 16S rDNA sequences do not indicate a subestructuration, some authors defined several phylogenetic clusters.	*Nitrosospira briensis*	Mobile, with 1 to 6 peritrichal flagella. Some strains isolated from soil simples. Type strain urease –, but other isolates are urease +. G+C: 54.0
			Nitrosolobus multiformis	Pleomorphic cells, lobulated and mobile, with 1 to 20 peritrichal flagella. Cell division by constriction. Internal compartmentalization by invaginations of the membrane. Isolated from agricultural soils with neutral pH and from wastewater. G+C: 53.5
			Nitrosovibrio tenuis	Vibrio shaped. The three strains of N. tenuis are urease +, and have been isolated from soils. Mobile, with 1 to 4 subpolar flagella. G+C: 53.9
Nitrosococcus (γ–Proteobacteria)	*Nitrosococcus*	AOB gamma–proteobacteria are only represented by two Nitrosococcus species: *Nc oceani* and *Nc halophilus*. There was a third specie described (*Nc. Nitrosus),* but the culture was lost.	*Nitrosococcus oceani*	Spherical or ellipsoidal. Free–living or diplococci. Mobile with groups of flagella. Flat aggregation of intracitoplasmatic membranes. Urease +. Isolated from seawater. G+C: 50.0–51.0
			Nitrosococcus halophilus	Morphologically not distinguishable from *Nc oceani*. Differs in salt requirements and in ammonium tolerance. *Nc halophilus* is urease –. G+C: 50.0–51.0

1.2.3 AOB in the environment

AOB are ubiquitous in soils, freshwater, and marine aerobic environments where ammonia is available. AOB have been isolated from systems with anthropogenic nitrogen sources such as waste and fertilizers, or were there is strong mineralization of organic matter (Koops and Möller, 1992). Although AOB are assumed to be aerobic, they have also been isolated or enriched from low-oxygen environments such as salty water systems (Smorzewski and Schmidt, 1991), anoxic sediments layers and soils, and in building materials (Bock and Sand, 1993). Besides, most of the isolated species tend to be restricted to specific ecosystems (Belser, 1979).

AOB are well-known for their slow growth and low yield, which makes their isolation difficult and time-consuming (Schmidt and Belser, 1982). Also, the culture media may not really represent the most abundant populations present. In addition, the portion of viable cells in a given environmental sample that is actually cultivable may be quite small (Amann *et al.*, 1995; Wagner *et al.*, 1993). Consequently, the physiological properties of isolated strains often cannot explain the nitrification activities found in the environments from which they were recovered; nevertheless, the study of pure cultures has obviously increased our understanding of AOB physiology. Although 16S rRNA sequences of most of the AOB have been detected in any environment studied, in this section, only information provided by AOB isolation and culturing is mentioned.

1.2.3.1 Aquatic environments

– Marine environments

  As expected the AOB inhabiting marine environments present salt tolerance or requirement. Therefore, the only AOB regularly isolated from these ecosystems are the members of *Nitrosomonas marina* lineage and the γ-proteobacterial *Nc. oceani*. In addition, one strain of *Nm cryotolerans* has also been detected in marine samples. Occasionally, members of the lineage *Nitrosomonas europaea/Nitrosococcus mobilis*, presenting noticeable salt tolerance, have also been isolated from marine environments (Koops *et al.*, 1976; Koops and Pömmerening–Röser, 2001).

– Salt lakes

Little is known about the AOB populations inhabiting ecosystems where pH and salt composition and concentration vary a lot. A strain of *Nitrosococcus halophilus* presenting extremely high salt tolerance was isolated from a salt lake in Saudi Arabia (Koops *et al.*, 1990). Besides, alkali tolerant representatives of *Nitrosomonas halophila* were isolated in Mongolian soda lakes (Sorokin *et al.*, 2001).

– Freshwater environments

The dominant representatives in natural freshwater habitats are the members of the *Nitrosomonas oligotropha* lineage (Koops and Harms, 1985). 16S rRNA sequences of *Nitrosospira* lineage members were detected by molecular and immunological approaches (Kowalchuck *et al.*, 1998; Whitby *et al.*, 1999; Burrell *et al.*, 2001; Smorzewski and Schmidt, 1991), but these results are uncertain since quantitative data are lacking and the PCR primers used in these studies fail in covering the *Nitrosomonas oligotropha* cluster (Purkhold *et al.*, 2000).

– Artificial aquatic environments

In wastewater, AOB oxidize ammonia to nitrite, which is subsequently converted to nitrate by the nitrite-oxidizing bacteria. The dominant AOB isolated from municipal wastewater are *Nitrosomonas eutropha*, *Nitrosomonas europaea* and *Nitrosococcus mobilis* (Watson and Mandel, 1971b; Koops and Harms, 1985; Juretschko *et al.*, 1998; Koops and Pömmerening-Röser, 2001). In contrast, in industrial wastewater treatment plants, the AOB most frequently isolated are representatives of the *Nitrosomonas oligotropha* lineage, with high tolerance to the presence of heavy metals.

1.2.3.2 Soils

Only species of the genera *Nitrosospira* and *Nitrosovibrio* have been isolated from acidic soils (Stephen *et al.*, 1996; Smith *et al.*, 2001). In neutral soils, members of the *Nitrosomonas communis* lineage, and strains of *Nitrosolobus multiformis* prevail (Mac Donald, 1986; Koops and Pömmerening-Röser, 2001). In oligotrophic, moderately acidic, soils, strains of the *Nitrosomonas oligotropha* lineage, *Nitrosovibrio*, and members of the genera *Nitrosospira* (Koops and Pömmerening-Röser, 2001) have been isolated.

1.3 METHODS USED FOR AOB DETECTION AND STUDY

1.3.1 Classical techniques

The study of diversity and ecology of most of the bacterial groups has been traditionally based on culture–dependent techniques. Most of the culture media and conditions do not appropriately reproduce the natural nitrifying habitats. Therefore, quantification and recuperation of ammonia oxidizers has always underestimated the real numbers. Moreover, nitrifying organisms form cell aggregates, which contributes to this pitfall. Thus, when using for example the Most Probable Number (MPN) method, only 0.05% of the AOB present in a sample are detected (Hall 1986).

Besides, the characterization of natural populations of ammonia oxidizing bacteria is particularly time–consuming. Due to their extremely low yields, it takes months to isolate the dominant species. Even though, nowadays this is the only approach available for studying the physiological properties of the principal species, and it could be useful to determine why some species dominate in a given environment and not in another one.

To date, phenotypic identification of new AOB isolates (including the Ks value of ammonia oxidation, salt requirements or tolerance, urease possession, intracitoplasmatic membranes rearrangement, cell size and shape, carboxysome presence, and GC content) is only possible if they belong to species previously described. In the cases of ambiguous identification DNA–DNA hybridization with the type strain is required.

1.3.2 Immunological techniques

During the 70s, studies based on culture–dependent methods were complemented with techniques applying fluorescent antibodies. These techniques allowed the *in situ* identification of the nitrifying bacteria for the first time. However, the use of fluorescent antibodies requires a previous culture of the AOB, and the potential presence of false negatives and positives must also be taken into consideration.

1.3.3 Molecular techniques

Emile Zuckerkandl and Linus Pauling (1965) were the first to state that “molecules can be documents of evolutionary history” as well as “molecular chronometers”. Some years later, Woese and Fox (1977), recognized the 16S rRNA

as a useful and powerful molecule for phylogenetic purposes, and built the first 16S rRNA–based trees. The 16S rRNA molecule has been shown to be a valuable phylogenetic tool due to the following properties:

- It is a universal molecule, which can be used both in eukaryotes and in prokaryotes
- It turns up from a direct transcription of the DNA sequences
- It accomplishes an important function in the cells, and therefore, its secondary structure has conserved along evolution
- Its size is adequate to the present molecular methods.

Nowadays, thanks to the development of techniques such as sequencing and, most notably, PCR–based DNA amplification methods, the databases contain large numbers of 16S rDNA sequences. Moreover, the current bacterial classification is based on the phylogenetical relationships among microorganisms described by 16S rRNA trees. Up to 36 divisions have been described within the domain Bacteria, and ammonia oxidizers are included within the division named Proteobacteria (Figure 1.3).

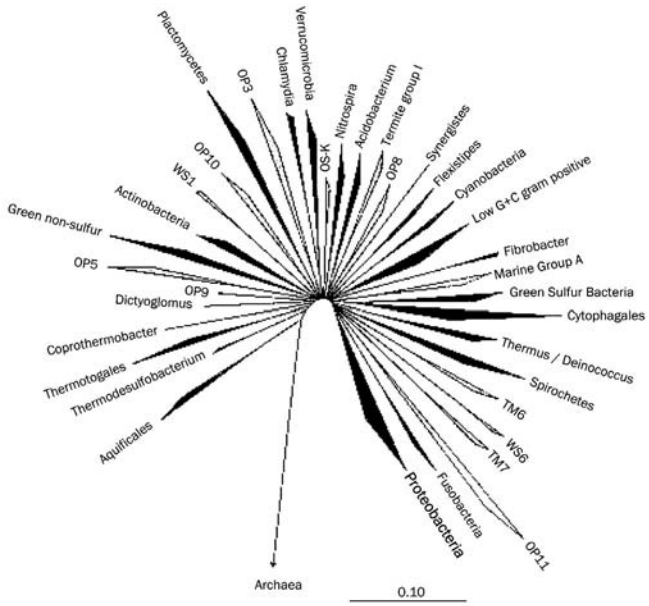


Figure 1.3 Evolutionary distance tree of the bacterial domain showing both the current recognized and putative divisions. Divisions enclosing cultured representatives are depicted in black, whereas the white ones are divisions represented only by environmental sequences (from Hugenholtz *et al.*, 1998).

Using 16S rDNA sequences provides an exhaustive view of the taxons present in a given environment, but overlooks the functional role of each phylogenetic group in the habitat. Besides, many new microbial genome projects provide new perspectives for bacterial phylogeny and taxonomy, and therefore, rRNA–based phylogeny is again under critical scrutiny. Intradomain relationships described by rRNA need to be corroborated by alternative markers fulfilling several prerequisites such as wide distribution, functional constancy, genetic stability, and a reasonable number of independently evolving positions or regions.

The study of *functional* genes, that is, those encoding key functions or enzymes specific of a given group, has often been used as a taxonomical tool to compare between distinct isolates. Nevertheless, to date, functional genes have rarely been used to obtain information about the diversity of a particular environment. The use of such markers is complicated due to several reasons:

- The degeneracy of the genetic code: the same protein can be encoded by several DNA sequences.
- Internal variability within natural populations is unknown for this type of genes.
- Functional genes do not possess phylogenetic value: lateral gene transfer is accepted to happen more frequently than previously assumed.
- There are no universal primers for a concrete functional gene, and the design relies on the previous existence of sequences.

The latest methodological tendencies in the study of microbial ecology rely on the study of whole genomes, and are based on two basic strategies: metagenomes, and BACs (bacterial artificial chromosomes). The basis in both cases is the comparison of the global genomic structure. Nowadays, approximations using BACs are used to obtain a global view of the genic composition of natural samples (Rondon et al 2000). Another new method consists in amplifying genic boxes by PCR, profiting the repetitive sequences (integrons) existing in numerous bacteria (Stokes *et al.*, 2001).

The design of DNA chips and arrays containing oligonucleotide sequences complementary to mRNA, or to genes encoding key enzymes, is nowadays much easier due to the increasing number of published genomes available. Recent studies have used this technique to analyze functional aspects of genes such as the genes encoding the nitrate reductase (Wu *et al.*, 2001), the ammonia monooxygenase (Daims *et al.*, 2001; Wagner *et al.*, 2002), and the methane monooxygenase (Murrell *et al.*, 2000).

1.3.3.1 The use of 16S rRNA in the study of AOB

Woese and co-workers, in the 80s, were the first to use 16S rDNA sequences in the study of the phylogeny of ammonia oxidizing bacteria. They divided this group in two major monophyletic subgroups, affiliated to the β- and the γ-proteobacteria respectively (see section 1.2.2). Today, almost entire 16S rRNA gene sequences from the 14 described species of β-proteobacterial AOB are available in the databases forming a well-supported evolutionary group (Teske *et al.*, 1994).

One of the main problems in the study of ammonia oxidizing bacteria through 16S rDNA is the impossibility of using a single AOB–specific set of primers. Since ammonia oxidizers can be found in both the subgroup β and γ– of the Proteobacteria, at least two different sets of primers would be required. Moreover, identifying new sequences often requires the application of time–consuming cloning technologies. Nevertheless, several studies have assessed the diversity and distribution of the β–AOB in natural environments (McCaig *et al.*, 1994; Hiorns *et al.*, 1995; Stephen *et al.*, 1996; Hovanec and Delong, 1996), and new sequences have been discovered and identified through phylogenetic analysis.

Either directly extracted rRNA, or 16S rDNA fragments recovered by PCR, can be detected by hybridization with oligonucleotide probes. This technique is quicker than the formerly mentioned cloning–assisted methods, but the incapability of the probes to detect both new sequences, and variations outside of the probe target area, confer a serious deficiency in microbial ecology studies (Hiorns *et al.*, 1995; Voytek and Ward, 1995; Hovanec and Delong, 1996). Nonetheless, whole–cell hybridization with fluorescently labeled oligonucleotide probes supplies accurate spatial information (Wagner *et al.*, 1995; Mobarry *et al.*, 1996), but reliable quantification of cell numbers can only be obtained by real–time PCR (Hermansson and Lindgren, 2001).

Some of the 16S rDNA targeted primers published to date (NITA and NITB, CTO189f and CTO654r, CTO178f, and CTO637r or β–AOBf and β––AOBr) have been used for diversity fingerprinting of AOB communities (McCaig *et al.*, 1994; Voytek and Ward, 1995; Kowalchuck *et al.*, 1997), but nested PCR is required in most of the cases. Besides, the use of degenerate primers yields heterogeneous PCR products, which is reflected in multiple banding from single templates in denaturing gradient gel electrophoreses (Kowalchuck *et al.*, 1998; McCaig *et al.*, 1999; Ward *et al.*, 2000; Regan *et al.*, 2002).

1.3.3.2 Amonia monooxygenase

– Characteristics, properties and function

The enzyme ammonia monooxygenase (AMO) catalyzes the oxidation of ammonium to hydroxylamine, which is the first step in nitrification. It has never been isolated in its active form, although indirect evidences indicate that it is a copper–containing monooxygenase (Hooper and Terry, 1973). Therefore, most of the knowledge about this enzyme has been obtained from studies performed with whole cells.

Hyman and Wood (1985) identified a membrane–associated protein when incubated *Nitrosomonas europaea* with ^{14}C labeled acetylene. The N–terminal amino acidic sequence determination of this protein, allowed the derivation of an oligonucleotide, which was further used to identify and clone the gene *amoA*. This gene encodes one of the subunits of the enzyme ammonia monooxygenase (AmoA). The protein AmoA contains the active site of the enzyme AMO (Hyman and Arp, 1992). It has a molecular weight of 31.8 KDa (McTavish *et al.*, 1993), and encloses 5 transmembrane sequences and a periplasmic loop. The gene *amoB* can be found adjacent to the gene *amoA,* and encodes the AmoB subunit, of 43 KDa, of the enzyme AMO (Bergmann and Hooper, 1994). This subunit is formed by two transmembrane domains, and two periplasmic loops (Vanelli *et al.*, 1996). Upstream of these two genes, a third gene, *amoC*, of the same operon is detected. It is believed that AmoC encodes a chaperone protein that helps the subunits AmoA and AmoB to properly integrate in the membrane (Klotz *et al.*, 1997).

A 6% of the total proteic content of *Nitrosomonas eutropha* is AmoA and AmoB when cells grow in standard conditions (Pinck *et al.*, 2001). The specific AMO cell content is regulated by ammonium concentration, thus, the amount of AMO enzyme in the cell is higher at ammonium limiting conditions than at high ammonium concentrations (Pinck *et al.*, 2001). Besides, AMO has been detected in great amounts in one year starving cells, which indicates that the quantity of ammonia monooxygenase cannot be straightly correlated to ammonium oxidation activity.

Ammonia monooxygenase can also oxidize other compounds such as some hydrocarbons and alcohols, and therefore, special care must be taken in microbial ecology studies (Hall 1986). Although cells cannot use these compounds for growing, this oxidation capacity has been widely studied, focusing on bioremediation purposes (Vanelli *et al.*, 1990). Even though, due to the wide range of substrates for ammonia monooxygenase, there is great quantity

of inhibiting substances such as: methyl fluorides, dimethyl ethers, acetylene, alkanes, alkenes, aromatic compounds, carbon monoxide, chelating agents such as the diethyldithiocarbamate, etc.

– The CAB operon genes, characteristics and applications in phylogeny
The genes encoding the three subunits of the enzyme ammonia moooxygenase (*amoA*, *amoB* and *amoC*) are arranged in an operon named *CAB operon* (Figure 1.4).

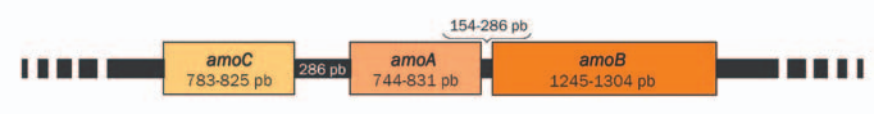


Figure 1.4 CAB operon in ammonia oxidizing bacteria.

Multiple *amo* operons have been identified in several ammonia oxidizers (Table 1.3). For example, *Nitrosomonas europaea* encloses a duplicated *amo* operon. A third copy of the gene *amoC*, neither associated with *amoA* nor *amoB*, was detected in 1998 (McTavish *et al.*, 1993; Sayavedra-Soto *et al.*, 1998).

Hommes *et al.* (1998) disrupted each of the two *amoA* copies in *Nitrosomonas europaea* demonstrating that both copies were fuctional in the cell. However, the knockout of one of the *amoA* copies, but not the other, resulted in a decrease in the growth rate of the culture, suggesting an independent regulation of each copy.

With the exception of the isolated *amoC* gene, the sequences of the duplicated genes are very similar, even identical, within a single ammonia oxidizer species. Lower similarities are found among different species of AOB, which indicates that recent duplication happenings, and not lateral gene transfer, might be the key to the presence of multiple gene copies (Klotz and Norton, 1998).

The presence of multiple genes seems to confer several advantages to the AOB:

– Maintain an appropriate proportion of the CAB operon gene products (Bergmann and Hooper, 1994).
– Allow a faster production of messenger RNA when ammonia concentration rises in the environment (Hommes *et al.*, 1998).

Table 1.3 Number of amo operons and *amoC* copy numbers in different ammonia oxidizers.

Organism	*amo* operon number	*amoC* copy number
Nitrosomonas europaea ATCC 19178	2	3
Nitrosomonas eutropha C-91	2	3
Nitrosospira briensis C-128	3	4
Nitrosospira sp NpAV	3	4
Nitrosolobus multiformis ATCC25196	3	4
Nitrosospira sp. 39-19	3	4
Nitrosovibrio tenuis NV-12	2	3
Nitrosococcus oceani	1	1
Nitrosococcus sp. C-113	1	1

1.4 OBJECTIVES

The main goal of this project was to find, develop, and evaluate useful and reliable tools for the study of ammonia oxidizing bacteria. At the time this work was started, the first phylogenetic analyses performed in the 80s were confirmed and extended via comparative 16S rRNA gene sequence analysis (Head *et al.*, 1993; Teske *et al.*, 1994; Utaker *et al.*, 1995; Purkhold *et al.*, 2000). Likewise, the *amoA* gene was presented as an additional phylogenetic molecular marker (Rotthauwe *et al.*, 1997; Stephen *et al.*, 1999), and was (and still is) extensively used for the detection and study of ammonia oxidizers, particularly in natural environments (Aakra *et al.*, 2001; Jiang 1999). Phylogenetic and taxonomical AOB information was being compared to that obtained with other markers such as the ISR 16SrDNA–23SrDNA fragment (Aakra *et al.*, 1999). Purkhold *et al.*, (2003) recently demonstrated a higher resolution of 16S rDNA versus *amoA* within the traditional classification. Nowadays, the databases contain a huge number of entries of both 16S rDNA and *amoA* partial sequences. In addition, the whole genome (approx 2.2Mb) of *Nitrosomonas europaea*, the first AOB ever isolated has already been sequenced and annotated (Chain *et al.*, 2003).

Our first attempt (chapter 3) was to use the 16S rRNA-targeted probe Nso190 for *in situ* whole cell hybridization. Although this technique allowed quantification and an *in situ* detection of the AOB present in a sample, several disadvantages, such as the impossibility of recognizing new sequences, lead us to the second attempt (chapter 4): developing a new set of 16S rRNA-targeted primers for fingerprinting analysis of AOB communities. The main disadvanta-

ge of this new approach was the impossibility of simultaneously detecting the whole group of β- and γ-proteobacterial ammonia oxidizers. Thus, to cover all AOB lineages a new molecular tool, other than the 16S rRNA, was needed.

Therefore, we developed a new set of primers for the use of partial *amoB* sequences as a new molecular marker molecule for studying the ammonia oxidizing bacteria (chapter 6). In addition, to facilitate comparative *amoB* sequence analyses in diversity or distribution studies, the construction of a database of *amoB* sequences was performed.

Besides, nowadays, one of the main issues in microbial ecology studies is the quantification of the members of any population. Few molecular tools based on quantitative PCR methods have been recently published (Harms *et al.*, 2003; Okano *et al.*, 2004; Aoi *et al.*, 2004), but none of them targets the AOB belonging to the γ–subgroup of the Proteobacteria. Thus, we developed an *amoB*–based RTi-PCR method for the enumeration of this subgroup of the ammonia-oxidizing bacteria.

Our final goals consisted in evaluating the 16S rRNA, *amoA* and *amoB* sequences as molecular markers in order to situate our newly developed *amoB* marker within the existing AOB molecular markers, as well as to build a polygenic tree containing the information enclosed in all the markers separately (chapter 7). Additionally, regarding our results, we proposed which of the available markers provides faster and more reliable information depending on the type of study we intend to perform.

2 MATERIAL AND METHODS

2.1 SAMPLING

Samples from three different wastewater treatment environments and from two controlled laboratory scale pilot plants were collected and used in this work: nitrifying biofilms growing on rotating biodiscs, sediments from tertiary treatment wetlands, activated sludge from a wastewater treatment plant, and two sludge samples coming from a nitrifying pilot plant and an anoxic pilot plant, respectively.

2.1.1 Sampling locations and methods

Environmental samples used in this study were collected in Campdorà and Empuriabrava (N.E. Spain). The pilot plants sampled in this project were located at the University of Girona, N.E. Spain (Figure 2.1).

Figure 2.1 Geographical locations of the sampling sites.

2.1.1.1 Nitrifying Biofilms from Campdorà

The nitrifying biodiscs in Campdorà (NE, Spain) were implemented to treat the secondary effluent of Girona's wastewater treatment plant, which processes c.a. 35,000 m^3 wastewater day^{-1}. The biodiscs were installed into a shed, protected from the light, and consisted of a submerged biological contactor with four compartments in series (Figure 2.2). The total volume of the tank was 561 liters, with rotating polyethylene wheels of 0.5 m of diameter, and the total area of the biodiscs was 25.26 m^2, with a percentage of immersion of 70% operating with air diffusion from the bottom of the tank. At 70% of immersion the volume was 422 liters, and the specific area was 60.6m^2 m^{-3}. The influent entered the biodiscs at the top of the first stage, and consecutively passed through the rest of the stages by lateral flux at a 40% of immersion. Ammonium concentrations in the influent were 25.1 mg l^{-1}, and there was less than 1 mg l^{-1} of organic nitrogen. A solution of ammonium chloride was added when needed. Dissolved organic carbon (DOC) never surpassed 55 mg l^{-1}, with an average value of 36 mg l^{-1}. Total Suspended Solids concentration presented a maximum value of 37 mg l^{-1}, with an average of 12 mg l^{-1}. Nitrifying microorganisms present in the influent colonized the biodiscs system producing the biofilms studied in this work. Construction, start up, and maintenance of the pilot plant was performed by members of the LEQUIA laboratory of the University of Girona (Amer, 1998).

Figure 2.2 Pilot plant consisting of four biodiscs connected in series in Girona's wastewater treatment plant (Campdorà, NE Spain).

According to ammonia oxidation rates calculated by Amer (1998), sampling was performed in each stage of the pilot plant the following dates: 30.01.1998, 23.02.1998, 13.03.1998, 19.05.1998, 06.08.1998.

2.1.1.2 Empuriabrava's wastewater treatment plant

The WWTP situated in Empuriabrava consists of two lines of sewage treatment disposed in parallel with a pre–treatment, a biological reactor, one settling tank, and three tertiary treatment lagoons. Finally, a mechanic centrifugation step dries the sludge (Figure 2.3). The inflow of this WWTP is typically domestic, with the peculiarity of presenting big seasonal oscillations ranging from 800 $m^3\,day^{-1}$ in the winter, to 6,000 $m^3\,day^{-1}$ in the summer.

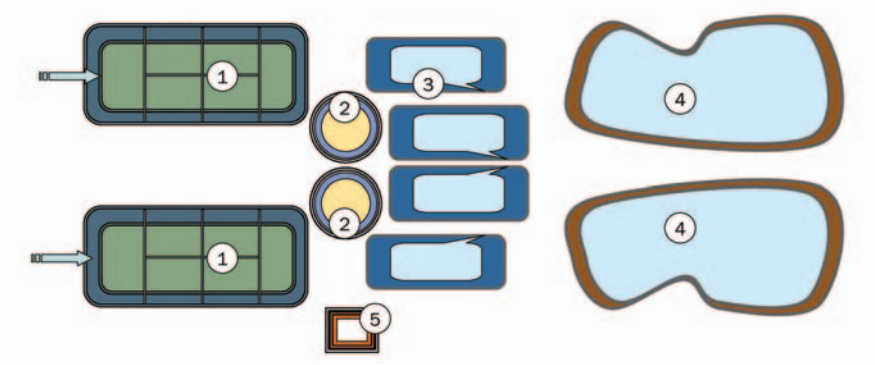


Figure 2.3 Scheme of the WWTP in Empuriabrava. 1 Carrousel biological system; 2 Settling tank; 3 and 4 Tertiary treatment lagoons; 5. Sludge centrifuge.

The biological reactor consists of a carrousel, were the water moves around in an elliptical stream. Along this circuit there are points where air is supplied, and where nitrification and BOD decrease are detected, and areas were the water becomes anaerobic and denitrification occurs.

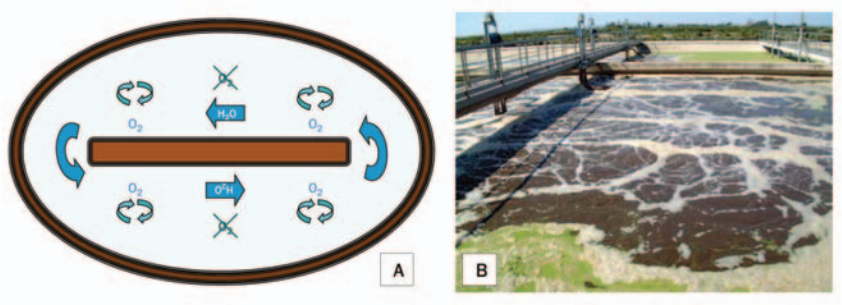


Figure 2.4 Biological reactor in Empuriabrava's wastewater treatment plant. A. Scheme of the carrousel; B. Aeration area of the carrousel.

The main technical characteristics of this WWTP are shown in Table 2.1

Table 2.1 Main technical characteristics of the wastewater treatment plant of Empuriabrava (Sala *et al.*, 2001).

Concept	Value
Maximum inflow	8,750 m^3 day^{-1}
Equivalent habitants	35,000
Theoretical incoming BOD	240 mg l^{-1}
Primary settling	No
Biological reactors volume	14,000 m^3
Air diffusers	400
Secondary settling	2 units
Settling tank diameter	15 m
Settling tanks volume	1,160 m^3
Tertiary treatment tanks volume	12,000 m^3
WWTP total volume	35,600 m^3

Activated sludge samples from this WWTP were collected from the most aerated areas of the carrousel. This WWTP is part of as project oriented to fulfill with clean water some lagoons belonging to the nearby Parc Natural els Aiguamolls de l'Empordà, during the dry season. Although the final purified water had an excellent quality in terms of BOD_5 and TSS, the amount of nutrients in the water was still too high to be delivered to El Cortalet (see 2.1.1.3).

2.1.1.3 Artificial tertiary treatment wetlands in Empuriabrava

Artificial wetlands were constructed next to the WWTP in Empuriabrava (Figure 2.5) with the following objectives:

- To deliver water to El Cortalet to avoid its desiccation during the summer. This would prevent damages to aquatic flora and fauna.
- To reduce the discharges to the river La Muga.
- To avoid the eutrophication that would cause the use of the water coming from the WWTP of Empuriabrava, which is purified, but still contains too many nutrients.
- To maximize the biodiversity, both in the wetlands, and in El Cortalet.

Figure 2.5 Panoramic view of Empuriabrava wastewater treatment plant and the associated tertiary treatment wetlands.

This tertiary treatment system includes three lagoons arranged in parallel (8,000 m^2 each, 40 cm depth), and a fourth lagoon named Europa (44,000 m^2, 15–20 cm maximum depth). The water flow equally fills the three lagoons, and moves following a piston flow. At the end of the ponds the water is diverted to the Europe lagoon (Figure 2.6). In order to protect the aquifer from a possible contamination, all the wetlands were impermeabilized with a clay layer of approx. 25 cm at the bottom. Since vegetation is an essential element for the functioning of the system, the growth of rush and bulrush species (*Phragmites communis*, *Sparganium* spp.) were favored. These plants absorb nutrients and provide a structural support for the microbial communities that complete the nutrient removal process.

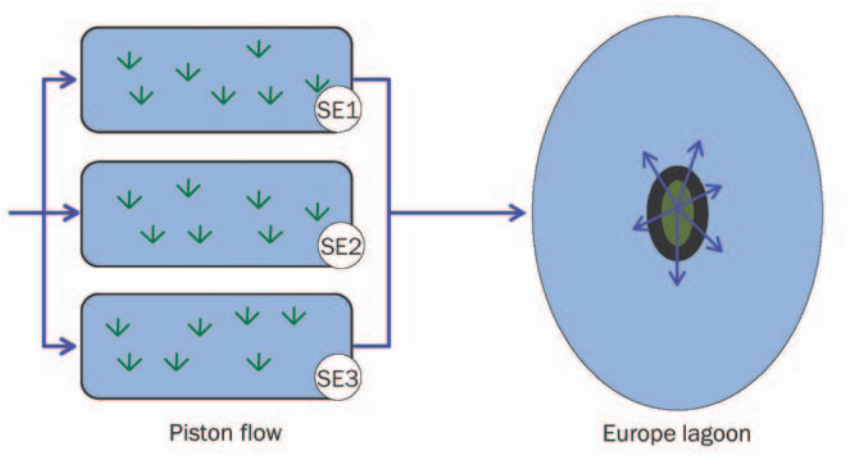


Figure 2.6. Scheme of the tertiary system of artificial wetlands in Empuriabrava. All samples were obtained from the SE3 lagoon.

The effluent of the Europe lagoon is finally collected and conducted to a bombing station from where the water is pushed along 2.4 km to El Cortalet.

Soil samples were collected by using metacrylate cores (Figure 2.7). The upper water fraction was carefully removed, and the sediment samples were conserved in a portable icebox until arriving to the laboratory. All the samples used in this work were obtained from the third lagoon SE3 (Figure 2.6).

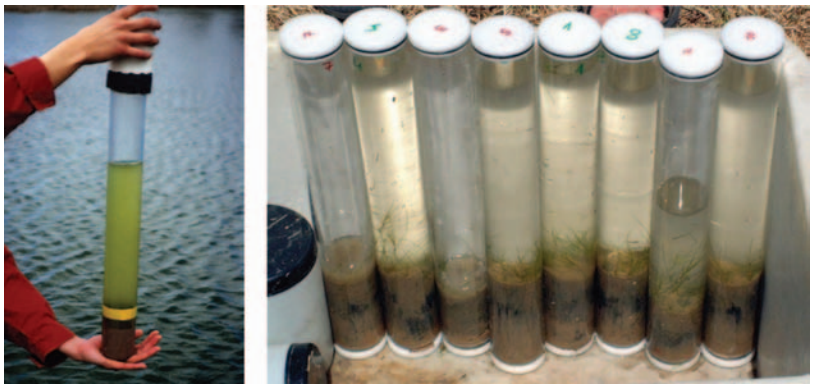


Figure 2.7 Cores obtained from SE3 sampling site.

2.1.1.4 The nitrifying pilot plants at the University of Girona

The nitrifying pilot plant at the university of Girona consisted of an aerated bioreactor treating wastewater from the WWTP of Campdorà. Ammonium was added when needed. Activated sludge samples from this reactor were used in this study.

Besides, SBR (sequencing batch reactor) samples were obtained from a 30 litres maximum volume biological reactor (Vives *et al.*, 2003) treating 30 l day^{-1} of urban landfill leachate at 20ºC. Leachate was stored at 4ºC and renewed once a week from real landfill. Aeration conditions in this pilot plant were as follows: 50% of the reaction time under anaerobic conditions, and with 8 h/cycle. The SBR (Sequencing Batch Reactor) technology, based on a fill–reaction–draw strategy has been applied to different treatment options top cope with high ammonia contents in wastewater. In order to achieve high nitrification and denitrification efficiencies different operational conditions (such as cell residence time, applied ammonia load or aerobic–anoxic–anaerobic conditions) can be set by programming a reaction phase with different DO set–points, raw wastewater loads, or mixing conditions.

2.2 ISOLATION AND CULTIVATION OF AOB AND MOB

2.2.1 Strains, origin and characteristics

2.2.1.1 Cultured strains and media used

Methylobacterium extorquens DSM1337 and *Methylomonas methanica* NCIMB11130 were purchased at the Deutsche Sammlung von Mikroorganismen und ZellKulturen (DSMZ), and at the National Collections of Industrial food and Marine Bacteria (NCIMB), respectively. They were grown at the laboratory by using the following medium, at pH 7.00 and 30ºC:

Peptone	5.00 g
Meat extract	3.00 g
Agar–agar	15.00 g
Distilled water	1,000 ml
Methanol	10.00 ml

Nitrosococcus oceani ATCC 19707 was obtained from the American Type Culture Collection (ATCC) and grown in the recommended medium ATCC 928:

Ammonium sulfate	1.32 g
$MgSO_4 \cdot 7H_2O$	380.00 mg
$CaCl_2 \cdot 2H2O$	20.00 mg
Chelated iron (Sequestrene)	1.00 mg
$Na_2MoO_4 \cdot 2H_2O$	100.00 µg
$MnCl_2 \cdot 4H_2O$	200.00 µg
$CoCl_2 \cdot 6H_2O$	2.00 µg
$ZnSO_4 \cdot H_2O$	100.00 µg
K_2HPO_4	8.70 mg
Phenol Red 0.04%	3.25 ml
Seawater filtered (Ø=0.22µm)	1,000 ml

Originally the medium is red to pink, but becomes transparent–yellow as a consequence of bacterial growth (Figure 2.8). pH was maintained within the recommended margins (7.5 to 7.8) with a sterile solution of 10% K_2CO_3. Every 4 to 6 weeks the culture was transferred to fresh medium as recommended by the ATCC.

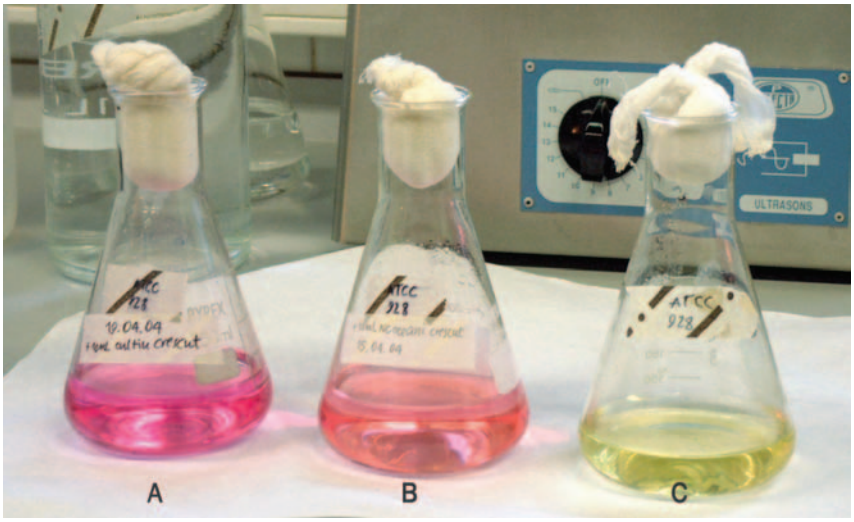


Figure 2.8 *Nitrosococcus oceani* ATCC19707 cultures. A: ATCC928 medium freshly inoculated with *Nitrosococcus oceani* ATCC19707. B: three weeks old culture. The acidification of the medium has started, and the phenol red becomes orange. C: five weeks old culture. The acidification of the medium has turned phenol red into yellow.

2.2.1.2 Other strains used

The rest of the AOB strains used in this work (Table 2.2) were kindly provided as cell lysates or genomic DNA by Dr. J Beate Utåker (Lab. of Microbial Gene Technology, Dept. of Chemistry and Biotechnology. Agricultural University of Norway), and Dr. Annette Bollmann (Netherlands Institute of Ecology, Center for Limnology. Maarsen).

Table 2.2 Strains used in this work, source and references.

Strain	Source	Reference
Nm europaea Nm50	Soil (USA)	NCIMB 11850
Nm europaea	ATCC 19781	ATCC 19781
Nm eutropha Nm57	Municipal sewage (Chicago)	Koops and Harms, 1985
Nm sp. Nm33(*)	Soil (Japan)	Koops *et al.*, 1991
Nm aestuarii Nm36	Sea water (North Sea)	Koops *et al.*, 1991
Ns briensis	ATCC 25971	ATCC 25971
Ns sp. 40KI	Loam (Norway)	Utåker *et al.*, 1995
Ns sp. Ka4	Lead contaminated soil (Norway)	Aakra *et al.*, 1999 a,b
Ns sp. B6	Sewage treatment column (Norway)	Utåker *et al.*, 1995
Ns sp. Nv6	Soil, Goroko (Danlo Pas)	Koops and Harms, 1985
Ns sp. Nsp1	Soil (Sardinia)	Koops and Harms, 1985
Ns multiformis Nl13	Soil (Surinam) NCIMB 11849	Koops and Harms, 1985
Ns sp. Nsp17	Soil (Iceland)	Koops and Harms, 1985
Ns sp. Nsp2	Soil (Hamburg)	Koops and Harms, 1985
Ns sp. AF	Sandy Loam (Zambia)	Utåker *et al.*, 1995
Nc halophilus. Nc4	γ–AOB	Purkhold *et al.*, 2000

(*) The strain *Nitrosomonas* sp. Nm33 has been used along the whole work considering it to be a *Nitrosomonas*. We were recently informed (J.B. Utåker, personal communication) that this genomic DNA appeared to be contaminated with DNA from a *Nitrosospira*, and that the real Nm33 strain has already been sequenced by Dr. M. Wagner and collaborators. The name of the strain hereby used is now "uncultured ammonia–oxidizing bacteria" and its new accession number is AJ298706.

2.3 MOLECULAR MICROBIOLOGY METHODS

2.3.1 Genomic DNA purification methods

Genomic DNA from environmental samples was obtained using the phenol/chloroform method described by Moore (1996), whereas the Wizard™ Genomic DNA purification kit was used to extract genomic DNA from cultures.

2.3.1.1 Solutions and reactives

- Tris–Cl 1M (pH 8.0) buffer
  Dissolve 121.1 g of Tris in 800 ml of Milli–Q water. Adjust pH to 8.0 with concentrated HCl (≈42 ml), and set the final volume to 1 l. Autoclave at 121ºC for 15 minutes.

- EDTA 0.5M (pH 8.0)
  Dissolve 186.1 g of sodium–EDTA·2 H_2O in 800 ml of Milli–Q water. Adjust pH to 8.0 with NaOH (≈ 20 g), and autoclave.

- NaCl 5M
  Dissolve 292.2 g of sodium chloride in 800 ml of Milli–Q water. Adjust the final volume to 1 l and autoclave.

- Sodium acetate 3M (pH 5.2)
  Dissolve 408.1 g of sodium acetate·3 H_2O in 800 ml of Milli–Q water. Adjust pH with glacial acetic acid (≈12.75 ml); set the volume to 1 l and autoclave.

- 20% SDS
  Dissolve 20 g of SDS in 90 ml of Milli–Q water pre–heated to 65 ºC. Adjust pH to 7.2 with concentrated HCl. Set the final volume to 100 ml, and autoclave.

- Chloroform:Isoamyl alcohol (C.I.)
  Mix 480 ml of chloroform and 20 ml of isoamyl alcohol in a sterilized bottle. Store at 4ºC.

- S Buffer (pH 8.0)
  Composition: Tris–HCl (pH 8.0), 100 mM EDTA (pH 8.0), 50 mM NaCl, 1.5 M CTAB, 1 %. For a final volume of 500 ml, mix into a sterile bottle: 50 ml of Tris–HCl 1M, 50 ml of EDTA 0.5M, 50 ml of CTAB 10%, 150 ml of NaCl 5M, and 200 ml of milli–Q sterile water.

– Proteinase K (10 µg µl^{-1})
  Dissolve 25 mg of proteinase K (Boehringer Mannheim) in 2.5 ml of Milli–Q sterile water. Store at –20ºC in aliquots of 100 µl.

2.3.1.2 Cell lysis

Collected samples were always centrifuged at 12,000 r.p.m. for 10 minutes and supernatant was discarded. The pellet was either stored at –20ºC or subjected to the following cell lysis protocol.

Cells were lysed with S buffer before proceeding to the DNA extraction through phenol/chloroform method:

1. Transfer the sample to a 50 ml sterile tube (NALGENE) and ressuspend the pellet in 20 ml of S buffer
2. Add 100 µl of proteinase K (10 µg µl^{-1}) and vortex.
3. Add 2 ml of SDS and gently mix by inversion.
4. Incubate in a water–bath at 65 ºC for 3–4 hours (5 hours maximum) and invert the tube every 15 to 20 minutes. Cool to room temperature.

2.3.1.3 Phenol/Chloroform extraction

This method is based on the denaturing and solubilization capability of the phenol (Kirby 1957). As detailed in the protocol described hereby, the DNA is maintained in the aqueous phase, apart from the organic one (Marmur, 1961), and finally isopropanol is used to precipitate the obtained DNA (Wilson *et al.*, 1990).

5. Add 1V of phenol:chloroform:isoamyl alcohol 25:24:1 (v/v/v) and mix by inversion for 10 minutes. For a volume of 20 ml, 10 ml of BIOPHENOL (phenol saturated with Tris–HCl pH 8.0 and 0.1% 8–hydroxiquinoleine LABGEN) and 10 ml of chlorofom–isoamyl alcohol (CI) must be added.
6. Centrifuge at 12,000 r.p.m. for 10 minutes with a SS–34 rotor (SORVALL RC–5B). Transfer the aqueous phase into a clean 50 ml tube.
7. Repeat steps 5 and 6 to ensure a complete protein removal.

8. Add 0.6 V of isopropanol and mix by inversion. In this step sometimes DNA can be seen as a white filamentous precipitate.
9. Store the tube at –20ºC overnight.
10. Centrifuge the sample at 12,000 r.p.m. for 20 minutes.
11. Discard the supernatant and wash the pellet with 70% ethanol.
12. Ressuspend the pellet with 400 µl of sterile Milli–Q water.
13. Add 500 µl of Cl solution and mix by inversion for 5 minutes. Centrifuge at 12,000 r.p.m. for 10 minutes and transfer the aqueous solution to a clean 1.5 ml tube.
14. Add 1 V of sodium acetate 3 M (pH 5.2) and 2 V of ethanol 100%. Mix gently by inversion.
15. Centrifuge at 12,000 r.p.m. for 15–20 minutes and discard the supernatant.
16. Wash the pellet twice with 70% ethanol and allow to air–dry.
17. Ressuspend the purified DNA with buffer Tris–HCl 100mM and quantify.

2.3.1.4 Wizard™ Genomic DNA purification Kit

This kit distributed by Promega allowed the purification of genomic DNA from 5 ml of AOB and MOB cultures. Since this method was faster than the phenol–chloroform protocol (see 2.3.1.3), this kit was used to purify DNA from all the AOB and MOB cultures. Nevertheless, it was never used with environmental samples. In those cases, phenol/chloroform method was used to ensure both the recuperation of DNA belonging to the whole community, and the purity of the obtained DNA. This kit was used as specified by the manufacturer, and detailed below:

1. Centrifuge 5–10 ml of liquid culture at 12,000 r.p.m. for 10 minutes. Discard the supernatant.
2. Ressuspend the pellet in 600 µl of Nuclei Lysis Solution and transfer to a new microcentrifuge tube
3. Incubate at 80ºC for 5 minutes and cool to room temperature.
4. Add 3µl of RNAse solution and mix by inversion. Incubate 30 minutes at 37ºC.
5. Add 200 µl of Protein Precipitation Solution. Vortex for 20 seconds and let the sample sit on ice for 5 minutes.
6. Centrifuge at 12,000 r.p.m. for 3 minutes. Transfer the supernatant into a clean tube containing 600 µl of isopropanol. Mix by inversion.
7. Centrifuge at 12,000 r.p.m. for 2 minutes and carefully discard the supernatant.
8. Wash the pellet with 70% ethanol and centrifuge again at 12,000 r.p.m. for 2 minutes. Pour off the ethanol and allow to air dry at room temperature.
9. Ressuspend the pellet with Elution Buffer or with Tris–HCl 100mM.

2.3.2 DNA cleaning methods

Different protocols were used depending on the DNA size and grade of purity needed:

2.3.2.1 DNA precipitation with ethanol and sodium acetate

Precipitation with sodium acetate and ethanol (Sambrook *et al.*, 1989) is the most used method to concentrate DNA. The washing step with 70% ethanol removes completely any salt excess caused by the use of sodium acetate. The method hereby described was always used after DNA extraction, as well as to concentrate diluted DNA samples.

1. Add 0.1 V sodium acetate 3M (pH 5,2) and 2 V cold ethanol (–20ºC) to 1V of the aqueous DNA solution. Incubate 10 minutes at room temperature.
2. Centrifuge at 12,000 r.p.m. for 10 minutes to precipitate the DNA and discard the ethanol.
3. Add 700 µl 70% etanol and gently mix by inverting the tube two or three times.
4. Centrifuge at 12,000 r.p.m. for 10 minutes and discard the 70% ethanol.
5. Allow the pellet to air–dry and resuspend the pellet with Tris–HCl 100 mM.

2.3.2.2 DNA precipitation with ethanol and magnesium chloride

This protocol was exclusively used to precipitate the sequencing reaction products:

1. For 10 µl of sequencing reaction, add 9,5 µl of MgCl2 2mM and 33 µl of cold 100% ethanol (–20ºC).
2. Mix and incubate at room temperature for 15 minutes.
3. Centrifuge at 12,000 r.p.m. for 15 minutes and discard very carefully the supernatant by using a micropipette or a sterile pasteur pipette.
4. Incubate the pellet at 37ºC until it dries.
5. The dry pellet can be either stored at –20ºC or resuspended and analyzed through the ABI PRISM 377 device (section 2.3.7).

2.3.2.3 QIAquick® columns

The QIAquick® columns (QIAgen) were used to readily purify PCR products. Those columns provide a quick and efficient purification of the DNA, removing primers, salts and superfluous dNTPs. The basis of this system is the sylica–gel membranes, which retain the DNA in presence of high concentrations of caotropic salts (Vogelstein and Gillespie, 1979).

1. Add 5 V PB buffer (QIAgen) to 1V PCR product and mix.
2. Transfer the sample into a QIAquick column previously placed into a 2 ml collecting tube.
3. Centrifuge the QIAquick® column at 14,000 r.p.m. for 1 minute.
4. Discard the supernatant and maintain the column into the same collecting tube.
5. To wash the DNA, add 750 µl of PE buffer (QIAgen) into the column and centrifuge 1 minute at 14,000 r.p.m.. Discard the effluent.
6. Centrifuge the column again 1 minute at 14,000 r.p.m. to completely eliminate the PE buffer.
7. Insert the QIAquick® column to a clean 2 ml microcentrifuge tube.
8. To elute the DNA, add 30 µl of eluting buffer (EB – QIAgen) onto the center of the membrane. Incubate 1 minute and centrifuge 1 more minute at 14,000 r.p.m.
9. Store at –20 ºC.

2.3.2.4 QIAquick® gel extraction kit

PCR products presenting unspecific bands or undesirable DNA smears were selected by using the QIAquick® gel extraction kit (QIAGEN). DNA bands with different molecular weight can be easily differentiated by running them in agarose gels (See section 2.3.5.2). The expected band can be distinguished and recovered by cutting the appropriate agarose portion. Agarose can be easily removed and DNA efficiently recuperated by applying the QIAquick® gel extraction kit, which is designed for treating DNA fragments ranging from 70pb to 10Kb.

1. Run the samples in a 1% low melting point agarose gel (Ecogen), at 80–100V. A molecular marker (1Kb or 100 bp DNA ladder – GIBCO BRL) should also be run in order to have a size reference to recognize the DNA band to recover.
2. Cut the agarose portion containing the desired DNA fragment with a sterile scalpel and drop it into a clean sterile tube previously weighted.
3. Add 3 V of buffer QG (QIAgen) to 1V of gel (consider 100 mg of gel as 100 µl). The maximum capacity of a QIAquick® column is 400 mg.
4. Incubate at 50ºC for 10 minutes or until the agarose has melted. Vortex every 2–3 minutes.
5. Once the agarose fragment has completely melted check the colour to be yellow. If the colour is orange add 10 µl of sodium acetate 3 M (pH 5.0). Buffer QG incorporates a pH indicator that turns to orange if pH ≥ 7.5.
6. Add 1 V of isopropanol and mix.
7. Transfer the sample into a QIAquick® column previously inserted into a 2 ml collecting tube.
8. Centrifuge the column at 14,000 r.p.m. for 1 minute. Now the DNA is retained on the membrane of the column. Discard the effluent.
9. Keeping the collecting tube in place, add 0.5 ml of QG buffer to remove any residual agarose and centrifuge at 14,000 for 1 minute.
10. Add 0.75 ml of PE buffer (QIAgen) and incubate for 2–5 minutes at room temperature. Centrifuge at 14,000 r.p.m. for one minute. Discard the effluent.
11. Centrifuge the column again at 14,000 for 1 minute to remove any residual PE buffer.
12. Transfer the column to a 1.5 ml clean eppendorf.
13. To recuperate the DNA, add 30 µl of EB buffer (QIAgen) onto the membrane of the column. Incubate 15 minutes at 4 ºC, and centrifuge at 14,000 r.p.m. for 15 minutes.
14. Store at –20 ºC.

2.3.3 DNA quantification methods

2.3.3.1 Spectrophotometric determination

Purity and concentration of extracted genomic DNA was determined spectrophotometrically. An absorbance spectrum from 200 to 400 nm was performed and absorbance was measured at 230, 260 and 280 nm (A230, A260 and A280 respectively). The coefficient A260/A280 provided an analytical measure of the genomic DNA purity (Sambrook *et al.*, 1989). Thus, a DNA sample is considered to be pure when A260/A280 value ranges from 1.8 to 1.9 (Gallagher, 1989). Besides, a coefficient A260/A230 > 2.2 denotes a protein–free DNA sample.

Genomic DNA concentration was calculated according A260 absorbance value of 1, corresponds approximately to 50 µg ml^{-1} dsDNA (Sambrook *et al.*, 1989).

2.3.3.2 Picogreen® determination

Picogreen® reactive is a fluorochrome that spontaneously attaches to dsDNA. Fluorescent quantification allowed the detection of little amounts of DNA impossible to quantify spectrophotometrically. Measured fluorescence is mainly provided by dsDNA, which ensures that ssDNA nor RNA in the sample are quantified (Sambrook *et al.*, 1989). Samples were excited at 460 nm, and fluorescence was measured at 540 nm. The procedure is described below.

1. Dispense 1–5 µl of the sample into a 1.5 ml microcentrifuge tube. A standard solution of salmon sperm DNA (100 µg ml^{-1}) is used for internal calibration.
2. Add TE buffer to set the final volume to 900 µl and mix.
3. Add 100 µl of Picogreen© solution, previously diluted 40 times. Mix.
4. Incubate 10 minutes at room temperature in the dark (the Picogreen© fluorochrome is light–sensitive).
5. Measure fluorescence emission at 540 nm.
6. DNA concentration is obtained on the basis of a standard curve built with known concentrations of salmon sperm DNA.

2.3.4 Polymerase chain reaction (PCR)

Polymerase chain reaction is a quick procedure for *in vitro* amplification of specific DNA fragments. Kleppe (1971) exposed the theoretical basis of the PCR, which was first performed by Mullis and Faloona (1987) and Saiki (1988) to obtain great amounts of single–copy genes. Efficiency of amplification can be altered by any parameter or reactive used in the reaction, such as:

- Purity of the reactives.
- Distance, complementariety, length, and sequence of the nucleotide primers.
- Quantity and purity of the DNA template.
- Type of enzyme used.
- Salt and triphosphate deoxyribonucleotides (dNTPs) concentration.
- Temperatures and length of denaturing, annealing and extension steps.

All these factors must be carefully evaluated and programmed before proceeding to any amplification (Innis and Gelfand, 1990), particularly when dealing with degenerate primers, i.e. in our case, when amplifying *amoA* and *amoB* genes.

In this work, the polymerase chain reaction has been used to both isolate and amplify 16S rDNA, *amoA* and *amoB* genes from cultures or environmental samples, as well as to recuperate inserts from pGEM®–T Easy Vector (Promega) (section 2.3.6).

2.3.4.1 PCR conditions

PCR mixes used in the reactions were prepared in a laminar flow cabinet. All the material was sterilized and used to perform polymerase chain reactions only.

2.3.4.2 16 rDNA targeted primers

In this study several sets of primers were used (Table 2.4). The previously described primers with specificity for 16S rDNA gene are detailed in this section altogether with our newly designed Nso1225 reverse oligonucleotide. The degenerated primer pairs targeting the *amo* genes and pGEM ®–T Easy Vector are further described in sections 2.3.4.3 and 2.3.4.4, respectively. All the primers used in this work were synthesized and purified by reverse phase chromatography by Roche Diagnostics© GmbH. The used concentration was 50 pmol μl^{-1} (µM) and all of them were stored at –20 ºC.

– Eub787f and Nso1225r

This set of primers was used to semi–specifically amplify a fragment of β–AOB's 16S rDNA gene. Eub787f (Lane, 1991) is an oligonucleotide specific for all Eubacteria, complementary to the positions 772 to 787 of 16S rDNA according *E. coli* numbering. Nso1225r is a primer specifically targeting the 16S rDNA of ammonia oxidizing bacteria. The sequence of this oligonucleotide was obtained from the in–situ hybridization probe Nso1225, designed by Mobarry *et al.* (1996) to study wastewater environmental AOB communities.

– Eub27f and Nsv443r

This set of primers was used to semi–specifically amplify a fragment of some *Nitrosospira*'s 16S rDNA gene. Eub27f (Lane, 1991) is an oligonucleotide specific for all Eubacteria, complementary to the positions 8 to 27 of 16S

rDNA according *E. coli* numbering (Brossius *et al.*, 1978). Nsv443r is a primer complementary to the 16S rDNA of some *Nitrosospiras*. The sequence of this oligonucleotide was obtained from the in–situ hybridization probe Nsv443, designed by Mobarry *et al.* (1996) to study wastewater environmental AOB communities.

– NmVf and Eub907r

This set of primers was used to semi–specifically amplify a fragment of *Nitrosococcus mobilis* 16S rDNA gene. Eub907r (Lane, 1991) is an oligonucleotide specific for all Eubacteria, complementary to the positions 887 to 907 of 16S rDNA according *E. coli* numbering (Brossius *et al.*, 1978). The sequence of NmVf was obtained from the in–situ hybridization probe NmV, designed by Juretschko *et al.* (1998).

– Eub984f and Eub1492r

This universal set of primers was used to amplify a fragment of the 16S rDNA gene of all the Eubacteria. Eub984f and Eub1492 (Lane, 1991) are complementary to the positions 968 to 984, and 1492 to 1513 of 16S rDNA according *E. coli* numbering, respectively.

– Amx820 and Pla46r

Those primers were designed by Strous (2000) and Neef *et al.* (1998) respectively, and are specific for the 16S rDNA gene of the Planctomycetale bacterial order. All the anaerobic ammonia oxidizers (anammox bacteria) described to date belong to this group.

2.3.4.3 Degenerate primers targeting functional genes

Oligonucleotide primers used in PCR commonly present a sequence exactly matching the beginning or the end of the DNA fragment to amplify. However, a mix of primers sharing the length but not the base composition is often used (Compton 1990). These types of oligonucleotide are known as degenerate primers, and have recently been used in the search for new genes, or genes not yet well characterized (Mack and Sninsky, 1988; Pedersen *et al.*, 1996). In our case degenerate primers have been used to partially amplify the genes *amoA* and *amoB* of ammonia oxidizing bacteria.

– *amoA*–1F and *amoA*–2R

This set of primers designed by Rotthauwe *et al.* (1997) and modified by Stephen *et al.* (1999) specifically amplifies a 500bp fragment of the *amoA* gene belonging to AOB. The *amoA* gene encodes for the active site of the ammonia monooxygenase, the key enzyme in ammonia oxidation (Mc Tavish *et al.*, 1993). The sequences of these primers are presented in Table 2.4.

– amoBMf and amoBMr

In this work a new set of primers was designed from all the available *amoB* gene sequences in the databases DDBJ/EMBL/GenBank (Table 2.3). The *amoB* gene belongs to the CAB operon, and encodes for the AmoB subunit of the ammonia monooxygenase enzyme in AOB. *amoB* gene sequences were aligned and consensus areas were used to design several sets of primers. The oligonucleotides presenting less degeneration, similar annealing temperatures, and yielding products longer than 300bp were selected.

Table 2.3 Source *amoB* sequences used for the design of the degenerate primers amoBMf and amoBMr

Organism (Accession number)	Reference
Nitrosococcus sp. C–113 (AF153344)	Alzerreca *et al.*, 1999
Nitrosospira sp. NpAV (AF071774)	Sayavedra–Soto *et al.*, 1998
Nitrosococcus oceanus (AF047705)	Norton *et al.*, 2002
Nitrosospira sp. NpAV (AF016003)	Klotz and Norton, 1995
Nitrosospira sp. NpAV (AF032438)	Klotz and Norton, 1995
Nitrosospira sp. NpAV (U92432)	Klotz and Norton, 1995
Nitrosomonas sp. TK794 (AB031869)	Yokoyama *et al.*, Unpublished
Nitrosospira sp. (X90821)	Rotthauwe *et al.*, 1995
Nitrosolobus multiformis (X90822)	Rotthauwe *et al.*, 1995
Nitrosomonas europaea (L08050)	Mc Tavish *et al.*, 1993

All the selected oligonucleotide primer sets were then tested with the NetPrimer software (PREMIER Biosoft International, Palo Alto, California). This software calculates annealing temperatures as well as the probability of hairpin, dimer and crossdimer formations. NetPrimer program also looks for palindromes, calculates the G+C content, molecular weight, and the 5' and 3' end stability for each primer. As a result, the set of primers selected were named amoBMf and amoBMr, and their sequence is presented in Table (2.4). Degenerate positions are indicated according to IUB notation: Y=C o T; K=G o T; W=A o T; R=A o G and S=G o C.

Table 2.4 Primers used in this study

Primer		Sequence		Reference
Eub787f	5'	TTA GAT ACC CTG GTA	3'	Lane 1991
Eub1492r	5'	ACG GTT ACC TTG TTA CGA CTT	3'	Lane 1991
Eub907r	5'	CCG TCA ATT CCT TTA AGT TT	3'	Lane 1991
Eub27f	5'	AGA GTT TGA TCC TGG CTC AG	3'	Lane 1991
Eub984f	5'	A A C G C G A A G A A C C T T A C	3'	Lane 1991
Nsv443	5'	CCG TGA CCG TTT CGT TCC G	3'	Mobarry *et al.*, 1996
NmVr	5'	T C C T C A G A G A C T A C G C G G	3'	Juretschko *et al.*, 1998
Nso1225r	5'	CGC CAT TGT ATT ACG TGT GA	3'	Mobarry *et al.*, 1996
Amx820	5'	AAA ACC CCT CTA CTT AGT GCC C	3'	Strous 2000
Pla46r	5'	GGA TTA GGC ATG CAA GTC	3'	Neef *et al.*, 1998
amoA-1F	5'	GGG GTT TCT ACT GGT GGT	3'	Rotthauwe *et al.*, 1997
amoA-2R	5'	CCC CTC KGS AAA GCC TTC TTC	3'	Rotthauwe *et al.*, 1997
amoBMf	5'	TGG TAY GAC ATK AWA TGG	3'	Calvó and Garcia-Gi,I 2004
amoBMr	5'	RCG SGG CAR GAA CAT SGG	3'	Calvó and Garcia-Gil, 2004
M13f	5'	G T A A A A C G A C G G C C A G T G	3'	Promega Ltd.
M13r	5'	G G A A A C A G C T A T G A C C A T G	3'	Promega Ltd.

2.3.4.4 M13 primers

The pair of primers M13f and M13r was used for PCR amplification of the inserts ligated into a cloning vector. The plasmid used in this work was the pGEM®-T Easy Vector from Promega (section 2.3.6), which presents the target for these two primers surrounding the area where the cloned fragment of DNA inserts (See 2.3.7.2). The sequence of these primers is shown in Table 2.4.

2.3.4.5 PCR programs

General conditions for PCR amplification were defined as described elsewhere (Innis *et al.*, 1990. Giovannoni, 1991). Polymerase chain reactions were carried out in total volumes of 50 µl, in 200 µl, thin wall polypropilene tubes (QSP). 50 to 150 ng template DNA was used in each reaction, and a non–template control was carried out every time.
Amplification Mix:

Amplification buffer (10×)	5 µl
dNTP (2mM)	5 µl
Primer (forward)	1 µl
Primer (reverse)	1 µl
DNA polymerase	1 U
Sterile Milli–Q water	to 50 µl

Although mix proportions were standardized, amplification programs varied according to the set of primers used. All reactions were performed in a thermal cycler (model 9600 P.E. Applied Biosystems, Foster City, CA, USA).

PCR products were checked by loading 4 µl of the reaction product on 1.5% agarose gels (see section 2.3.5.2). The rest of the reaction was then purified (see section 2.3.2), and stored at –20ºC.

Used temperature programs were based on the previously described protocols by Wilson *et al.* (1990) and Weisburg *et al.* (1991) to amplify 16S rDNA gene.

– Program for partial amplification of 16S rDNA gene with primers Eub787f and Nso1225

1. Denaturing	94ºC	5 min
2. 30 cycles:	94ºC	30 sec (denaturing)
	56ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for partial amplification of 16S rDNA gene with primers Eub27f and Nsv443

1. Denaturing	94ºC	5 min
2. 10 cycles	94ºC	30 sec (denaturing)
	57ºC	1 min (annealing)
	72ºC	1 min (extension)
3. 20 cycles:	94ºC	30 sec (denaturing)
	52ºC	1 min (annealing)
	72ºC	1 min (extension)
4. Extension	72ºC	7 min
5. Conservation	4ºC	Pause

– Program for partial amplification of 16S rDNA gene with primers NmVf and Eub907r

1. Denaturing	94ºC	5 min
2. 30 cycles:	94ºC	30 sec (denaturing)
	56ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for partial amplification of 16S rDNA gene with primers Eub984f and Eub1492r

1. Denaturing	94ºC	5 min
2. 30 cycles:	94ºC	30 sec (denaturing)
	52ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for partial amplification of 16S rDNA gene with primers Amx820 and Pla46r

1. Denaturing	94ºC	5 min
2. 35 cycles:	94ºC	45 sec (denaturing)
	56ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for partial amplification of *amoA* gene with primers *amoA*–1F and *amoA*–2R

1. Denaturing	94ºC	5 min
2. 42 cycles:	94ºC	45 sec (denaturing)
	58ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for partial amplification of *amoB* gene with primers amoBMf and amoBMr

1. Denaturing	94ºC	5 min
2. 42 cycles:	94ºC	45 sec (denaturing)
	58ºC	1 min (annealing)
	72ºC	1 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

– Program for amplification of an insert in pGEM®–T Easy Vectors with the primers M13f and M13r

1. Denaturing	94ºC	5 min
2. 30 cycles:	94ºC	30 sec (denaturing)
	48ºC	1 min (annealing)
	72ºC	2 min (extension)
3. Extension	72ºC	7 min
4. Conservation	4ºC	Pause

2.3.5 Real–Time PCR

Real–Time PCR is a technique that allows the quantification of initial amounts of template in a PCR reaction by directly monitoring the synthesis of new amplicons. This technique was first developed in the mid 1990s (Heid *et al.*, 1996), and supposed a revolution in nucleic acid quantification due to its high precision over a wide range of concentrations (Schmittgen *et al.*, 2000). Real–Time PCR instruments consist of specialized light sources and detectors to measure the emitted fluorescence of PCR products appropriately labelled. In this work the ABI Prism 7700 Sequence Detection System (SDS) RTi–PCR system (Applied Biosystems) was used.

In RTi–PCR, an increase in the fluorescent signal is directly proportional to the number of amplicons generated, and due to the different labelling methods available, DNA quantification is possible together with melting curve analysis and allelic discrimination. In general there are several Real–Time PCR methods according to the detection format used (Figures 2.9 and 2.10). There are unspecific detection strategies (independent of the target sequence and based on the use of dsDNA–binding dyes such as SYBR green), and specific detection strategies (based on the use of sequence–specific fluorescent oligonucleotide probes).

In this work the SYBR Green methodology has been set up for quantifying members of the genus *Nitrosococcus* by measuring the amount of *amoB* gene copies.

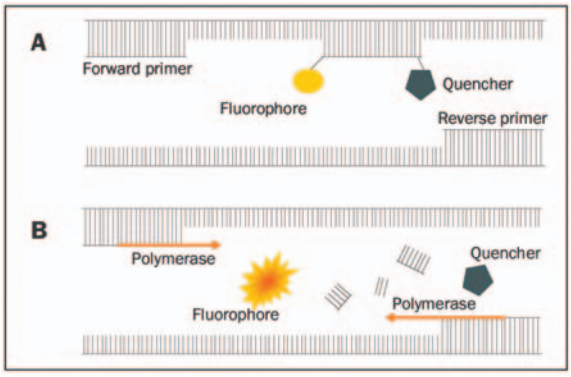


Figure 2.9 TaqMan system. A– Primers and probe anneal to the target. The proximity between the fluorophore and the quencher avoids fluorescence emission. B– The 5'→3' enzymatic activity of *Taq* polymerase cleaves the probe during the phase of extension. The quencher and fluorophore are separated and, therefore, fluorescence is emitted by the reporter dye. Adapted from Giulietti *et al.* (2001).

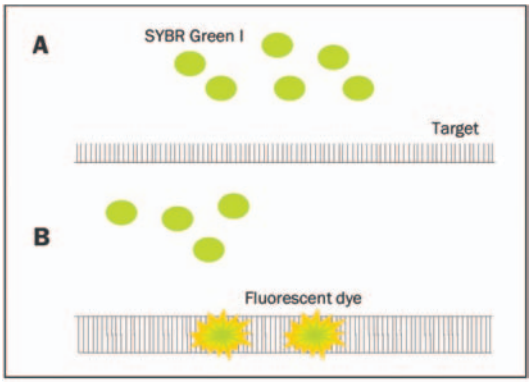


Figure 2.10 DNA–binding dyes (SYBR Green). A– The dyes are free in the solution and do not emit fluorescence. B– Fluorescence occurs as soon as the dye binds to the dsDNA. Adapted from Giulietti *et al.* (2001).

2.3.5.1 Experimental design guidelines

Sensitivity, efficiency and reproducibility are important criteria to consider when optimizing an assay. Therefore, optimization of the reagents used to perform PCR is critical to obtain reliable results.

– Target properties: The length of the target should not exceed 100–299bp when working with SYBR Green, and 60–120 bp with all probe–based methods.

– Primer properties: The length of the primers should be 18–30bp, and with a GC content of 20–80%. Both primers (forward and reverse) should present the same Tm (55–60ºC, above 60ºC for Taqman experiments). In addition, BLAST must be performed to ensure that the primers do not hybridize with unintended targets. For the design of the primers used in this study (Table 2.5) the software Primer Express v2.0 (Applied Biosystems) was used as detailed in chapter 6.

Table 2.5 Primers used for RTI–PCR in this study

Primer		Sequence		Reference
Nc133f	5'	AATGGTATGTTCGTCCCTCGTT	3'	This work
Nc216r	5'	CCCTTGCATCGTCATCTCAA	3'	This work

– Probe properties (if used): The length of the probe should be 20–30bp, and with a GC content of 20–80% (more C's than G's). The Tm should be 10ºC above the primers'. No 3 base runs, specially G's, and no 5' end G. The 5' end of the probe should be located within 3bp (10–12bp maximum) of the 3' end of the primer on the same strand.

– Dye parameters: It is important to avoid spectral overlap.

– PCR program used for RTi–PCR

1. Denaturing	95ºC	10 min
2. 50 cycles:	95ºC	15 sec (denaturing)
	60ºC	1 min (annealing and extension)

2.3.5.2 Quantification analysis

In both methods described above the fluorescent emission is proportional to the synthesized DNA, which can be represented as an amplification curve showing three different phases (Figure 2.11): during the first cycles of amplification the measured fluorescence does not surpass the baseline, which is known as the initiation phase. Subsequent PCR cycles are reflected in an exponential increase in the emitted fluorescence (exponential phase), which reaches a maximum value known as the plateau phase.

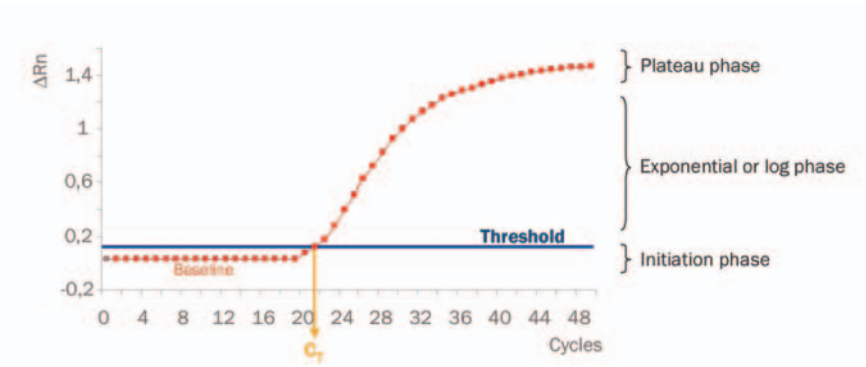


Figure 2.11 Model of a PCR amplification curve showing the three phases (Initiation, exponential, and plateau). The blue line corresponds to the threshold and the C_T is represented in orange.

The fluorescence values obtained during the initiation phase are used to calculate the baseline and to establish a threshold. The Threshold Cycle (C_T) corresponds to the cycle at which the fluorescence equals the defined threshold, and is included into the defined exponential phase. Quantification is performed in the exponential phase, where the increase in fluorescence is lineal and, therefore, proportional to the amount of DNA generated.

The amount of template DNA used in a PCR can then be quantified by interpolating the obtained C_T value in a linear standard curve of C_T values. This standard curve is previously built by performing the RTI–PCR with several dilutions of quantified, known standards, which results in a series of C_T values corresponding to different amounts of initial template DNA (Figure 2.12).

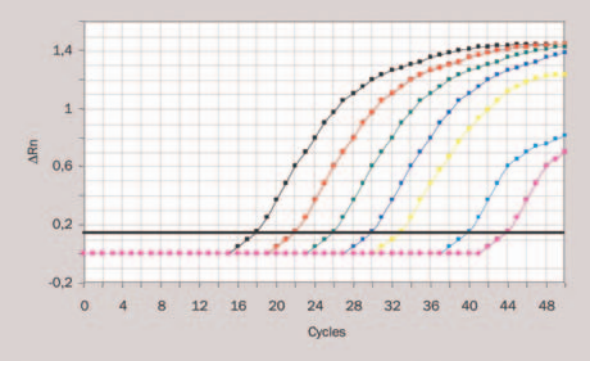


Figure 2.12 Linear representation of RTI–PCR amplification curves. ΔRn is the normalized fluorescence. The black horizontal line corresponds to the threshold. The amplification curves depicted in several colours correspond to serially diluted known–amount standards

2.3.6 Electrophoresis methods

2.3.6.1 Solutions and reactives

– TBE 0.5× buffer pH 8,0
  Prepare a TBE 5× buffer solution by dissolving 54 g Tris in 800 ml of Milli–Q water. Add 20 ml of EDTA 0.5M. Adjust pH with concentrated boric acid, set the volume to 1 l with Milli–Q water, and autoclave. TBE 0.5× buffer is then prepared by mixing 100 ml of TBE 5× with 900 ml of Milli–Q water.

– TAE buffer 50×
  Dissolve 242 g Tris in 800 ml of Milli–Q water and 100 ml of EDTA 0.5M. Adjust pH by adding glacial acetic acid (≈ 57 ml), fill up to a final volume to 1 l, and autoclave.

– Ethidium bromide (10 mg ml^{-1})
  Dissolve 0.1 g ethidium bromide in 10 ml of Milli–Q water and mix with a magnetic stirrer overnight. Store at 4ºC protected from the light. This compound is both mutagenic and toxic.

– Loading buffer
  The loading buffer was used for increasing the sample density in order to ease the loading of the samples in electrophoresis gels. Besides, the colorants of the loading buffer also migrate in the electrophoresis, which helps to locate the samples in the gel along the run. The loading proportion was 4 µl sample : 1µl loading buffer. Loading buffer composition in Milli–Q water was: 0.25% Bromophenol blue, 0.25% Cyanol Xylene FF, and 30% glycerol.

– Ammonium persulfate 10%
  Dissolve 1 g ammonium persulfate in 10 ml of sterile Milli–Q water. Store at –20ºC.

– Molecular weight ladders

  100 bp DNA ladder (Promega)
  The ladder consists of eleven fragments that range in size from 100–1,000bp in 100bp increments, plus an additional fragment at 1,500bp. The 500bp fragment is present at increased intensity to allow easy identification.

  1Kb DNA ladder (Promega)
  The ladder consists of thirteen blunt–ended fragments with sizes ranging from 250bp to 10,000bp. The 1,000bp and 3,000bp fragments are present at increased intensity for easy identification.

2.3.6.2 Agarose gels

Horizontal electrophoresis in agarose gels was performed for both the analysis of PCR or restriction products, and for purification of DNA preparations. The agarose gels were prepared by dissolving agarose powder (Pronadisa) in TBE 0.5× buffer. Electrophoresis device used was MINI–SUB® CELL GT (BioRad). The electric source was a Power–PAC300 (BioRad) Agarose concentration varied depending on the experimental needs, ranging from 0.8% to 1.5% agarose in TBE 0.5× buffer.

Gel casting
1. Weigh the amount of agarose needed according to the desired agarose percentageand mix with the indicated volume of TBE 0.5× buffer. 2. Melt the agarose in a microwave oven. Make sure the agarose is completely melted. 3. After cooling the solution to about 60ºC pour it into the casting tray containing a comb to form the wells. 4. Let it cool until the agarose is completely solid and the wells are formed. Carefully remove the comb by lifting it gently at one end.

Sample loading and gel running
5. Cover the gel with TBE 0.5× buffer. 6. Add ¼ (vol/vol) loading buffer to the samples and load them into the wells using a micropipette. Load the molecular weight ladder too. 7. Apply a voltage 2–10 V cm2, which corresponds to approximately. 100V in the MINI–SUB® CELL GT gel box. 8. Let the samples run until the first blue colorant of the loading buffer (bromophenol blue) reaches 3/4 of the length of the gel.

Gel staining
9. Submerge the gel into 150 ml of TBE 0.5× buffer with 12 µl ethidium bromide (10 mg ml^{-1}). The staining should be performed into a light–protected box. 10. DNA bands can be seen on a transiluminator providing UV light of 312 nm (TFX–20M). Images can be digitalized with a PC images capturing system (Scion Image, TDI).

2.3.6.3 Poliacrylamide gels

Acrilamyde gels were used to analyse restriction DNA patterns at a concentration 8%. Gels were run in a vertical system, DCode™ System (BioRad), in TBE 0.5× buffer, for 2 hours, and at 200 V.

Gel casting
1. Wash the glasses with water, detergent and ethanol. Wash the combs and spacers with water, soap and distilled water. Assemble the two glasses with the spacers and place the gel sandwich with the clamps in the DCode™ System (BioRad) casting stand. Prepare the acrylamide solution.
2. To obtain 50 ml of acrylamide solution, mix 10 ml of acrylamide/bisacrylamide 40% solution, 1 ml of TAE buffer 50×, and 39 ml of sterile Milli–Q water.
3. Add 400µl ammonium persulfate (APS) 10%, and 10 µl TEMED. Both APS and TEMED accelerate the polymerization of the acrylamide.
4. Pour or pipette the gel solution into the sandwich until it covers the wells of the comb. Allow the gel to polymerize for about 1–2 hours.
Sample loading and gel running
5. Put the polymerized gel sandwich inside the electrophoresis tank and check the upper chamber is filled up with TAE buffer 1×.
6. Prepare the samples with ¼ (vol/vol) loading buffer and load them with a micropipette. Add a molecular weight ladder.
7. Run the gel at 200V for about 2 hours.

Gel staining
8. Submerge the gel in 500 ml of TAE buffer 1× with 12 µl ethidium bromide (10 mg ml^{-1}) for 15 minutes.
9. DNA bands can be seen on a transiluminator providing UV light of 312 nm (TFX–20M). Images can be digitalized with a PC images capturing system (Scion Image, TDI).

2.3.6.4 DGGE (Denaturing gradient gel electrophoresis)

Denaturing gradient gel electrophoresis (DGGE) is an electrophoretic method to distinguish among DNA sequences having the same length but differing in base composition (Muyzer *et al.*, 1993). This method allows the electrophoretic separation and screening of heterogeneous PCR product mixtures. In the last decade DGGE has been used to assess the diversity and composition of environmental communities (Muyzer *et al.*, 1993; Ferris and Ward, 1997; Overmann *et al.*, 1999; Casamayor *et al.*, 2000).

Poliacrylamide gels containing a linear denaturing gradient decrease the electrophoretic mobility of double strand, partially denatured DNA. The behavior of DNA molecules with different sequence varies in front of a denaturing agent. Therefore, every sample stops migrating at a specific position along the gel (Fisher and Lerman, 1983). DGGE performance requires

one of the two primers to contain a GC clamp at 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 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 is a variable parameter which basically depends on the size and sequence of the fragments to be run. Thus, wide denaturing ranges allows discriminating among fragments containing very different sequences, whereas narrow ranges are used to distinguish fragments whose sequences differ only in a few nitrogenated bases.

In this work, to determine the appropriate denaturing range in every case a previous DGGE was always prepared at 20–80% denaturing gradient. Once the samples were located in the gel, narrower gradients were selected to sharpen detection of different DNA sequences. The denaturing range of DGGE is set by the amounts of urea and formamide added.
DNA samples in the gel can be recuperated by cutting the appropriate acrylamide band (Ferris *et al.*, 1996). The system used in this work was DCode™ System (BioRad).

Sample preparation
Samples used in DGGE must be obtained by PCR with a primer containing a GC clamp at the 5' end. The PCR program to be used is the same described for use with regular primers (see section 2.3.4).

Gel casting
1. Wash the glasses with water, detergent and ethanol. Wash the combs and spacers with water, detergent and distilled water. Assemble the two glasses with the spacers and place the gel sandwich with the clamps in the DCode™ System (BioRad) casting stand. Prepare the acrylamide solution.

2. Two solutions must be prepared to cast a 6% polyacrilamide, 20–80% denaturing gradient gel:

	A Solution (20%)	B Solution (80%)
Acrylamide/Bisacrylamide 40%	3,75 ml	3,75 ml
TAE 50×	0,50 ml	0,50 ml
Formamide	2,00 ml	8,00 ml
Urea	2,10 g	8,40 g
Ammonium persulfate 10%	100 µl	100 µl
TEMED	5,00 µl	5,00 µl
Milli–Q water	To 25 ml	To 25 ml

A narrower denaturing gradient can be easily prepared by changing the amounts of formamide and urea used.

3. Since ammonium persulfate and TEMED cause a quick polymerization of the acrylamide, they must be added in the last minute.

4. With the help of a gradient former and a peristaltic bomb, the two solutions are introduced into the gel sandwich.

5. Place the comb, and allow the gel to polymerize for about 1–2 hours at room temperature or overnight at 4ºC.

Sample loading and gel running

5. Put the polymerized gel sandwich inside the electrophoresis tank with the TAE buffer 1× preheated to 60ºC, and check the upper chamber is filled up with TAE buffer 1×

6. Prepare the samples with ¼ (vol/vol) loading buffer. Wash every well with the help of a syringe and load the samples with a micropipette.

7. Run the gel at 200V for about 5 hours or at 120V for 16 hours.

Gel staining

8. Submerge the gel in 500 ml of TAE buffer 1× with 12 µl ethidium bromide (10 mg ml^{-1}) for 15 minutes.

9. DNA bands can be seen on a transiluminator providing UV light of 312 nm (TFX–20M). Images can be digitalized with a PC images capturing system (Scion Image, TDI).

The DNA containing bands seen in the gel can be recuperated for further characterization. The method hereby described was used in this work to separate the DNA molecules from the poliacrylamide.

1. Cut the desired band with a sterile scalpel. This step must be quick in order to avoid timine dimer formation caused by overexposure to UV radiations.
2. Put the acrylamide band in a 1.5 ml microcentrifuge tube and add 100 µl of Milli–Q sterile water or sterile Tris–HCl buffer 100mM pH 7.4. Add some 0.5 mm diameter glass beads (BioSpec Products Inc.).
3. Vigorously shake the sample in a Bead–beater, at maximum speed for 1 minute. Repeat this step twice.
4. Incubate overnight at 4ºC.
5. Reamplificate the samples by PCR, using the same primers without the GC clamp. Use 1–15 µl template DNA in 50 µl PCR reactions.l

2.3.7 Cloning and restriction analysis

The amplification of any gene (see section 2.3.4) from environmental samples generates a mixture of fragments from all the members of the community holding this gene. To identify the different genes, they must be previously isolated. In this work, cloning with *Escherichia coli* was used to separate a mixture of *amoA* gene fragments to further sequence and compare with the databases.

Cloning is based on inserting the mixture of amplicons in a vector. Every vector containing one single gene or gene fragment (insert) is then transformed into an *E. coli* strain. Spread plating in Petri dishes yields colonies that carry one single insert. By selecting the colonies and growing them independently every insert is obtained individually.

2.3.7.1 Strains, origin and characteristics

The strain used for cloning was *Escherichia coli* strain DH5α (Invitrogen), which presents the genotype modified in the following way:

*end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 Δ(*lac*ZYA-*arg*F) U169 φ80Δ(*lac*Z)M15 F-

The φ80Δ(*lac*Z)M15 marker provides α–complementation of the β–galactosidase gene from the vector and, therefore, can be used for the blue/white

screening of colonies on bacterial plates containing X–Gal. Besides, mutations in *end*A1 and recA1 enhance plasmid stability and quality.

2.3.7.2 Plasmids

The plasmid pGEM®–T Easy Vector (Promega) was used as a cloning vector for an *amoA* gene fragment (Figure 2.13). This plasmid contains the lacZα portion of the Lac operon in *E. coli*, which encodes a 146 aa fragment of the β–galactosidase N–terminal. The β–galactosidase enzyme consists of this portion together with the Ω portion encoded by the host cell. Therefore, cells containing the vector form blue colonies in X–Gal (5–bromo–4–chloro–3–indolyl–β–D–galactose) supplemented medium.

The insertion of DNA between the l*ac* gene and its promoter in the vector causes the inactivation of the gene expression. Thus, cells containing a plasmid with an insert form white colonies. The pGEM®–T Easy Vector (Promega) also confers resistance to ampicillin. The plating on growth medium containing this antibiotic will only permit the growth of cells containing the vector. Of those, the white ones also contain the insert.

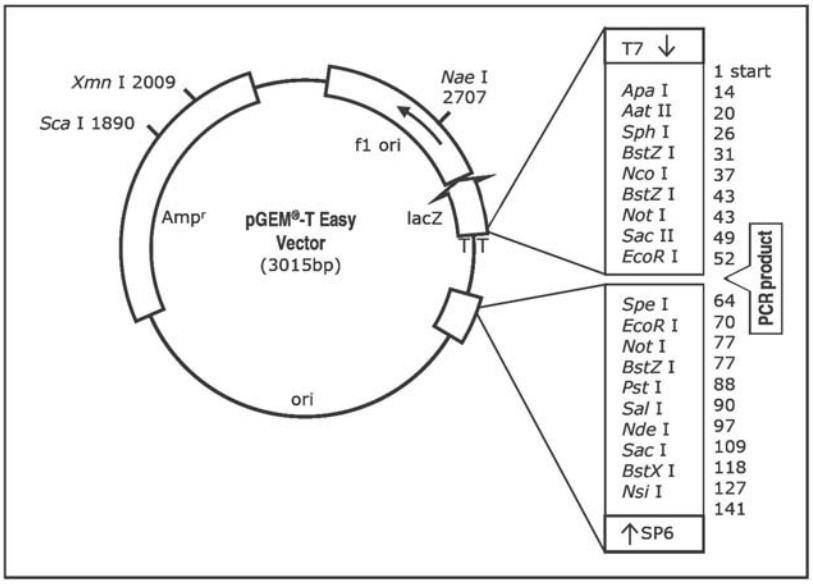


Figure 2.13 pGEM®–T Easy Vector (Promega) scheme showing the insert location and several targets for enzymatic restriction.

One of the advantages of pGEM®–T Easy Vector is that, flanking the insert area, it contains the target sequences for the pUC/M13 forward and pUC/M13 reverse primers. The presence of those targets was used in this work to reamplify by PCR the inserts with those primers.

2.3.7.3 Growth Media

The media used to grow the previously described *E. coli* strain (see section 2.3.6.1) were the following:

– Luria Bertani (LB)

Formulation to prepare 1 liter of LB medium:

Bactotryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	To 1 l

Adjust pH to 7.2 with NaOH, and autoclave at 120ºC for 20 minutes. To obtain agar plates, add 15 g agar–agar (1.5%) before adjusting pH and then autoclave and dispense in Petri dishes. After autoclaving, cool until 55ºC and add, if necessary, 100 µg ml^{-1} ampicillin.

– LB plates with X–Gal and IPTG

To prepare LB plates with IPTG and X–Gal incubate the plates with ampicillin at 37ºC for 10 minutes. Add 40 µl IPTG 100 mM (238 mg in 10mg Milli–Q water) to the agar surface and spread it with a Digralsky spreader. Then repeat the procedure with 100 µl d'X–Gal (10 mg ml^{-1} dissolved in dimethylformamide).

– SOC medium

SOC is a growth medium similar to LB but enhances bacterial growth further because of the presence of glucose. To prepare 1 liter of SOC medium:

Bactotryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
Distilled water	To 1 l

Add 10 ml of KCl 250 mM (1.86 g KCl in 100 ml of water), adjust pH to 7.0 with NaOH, and autoclave. Add 5 ml of a sterile $MgCl_2$ solution 2M (19 g Mg-

Cl2 in 100 ml of water). Also add 20 ml of glucose 1M sterile (18 g glucose in 100 ml of water, and filtered through a 0.22 µm of diameter filter).

2.3.7.4 Ligation

Ligation of DNA fragments permits the insertion of outsider genes in the sequence of the vectors. Amplified PCR products were ligated to the vector pGEM®–T Easy Vector (see section 2.3.6.2) taking advantage of the adenosine (A) added by the Taq Polymerase at the 3' end of the PCR products. The pGEM®–T Easy Vector (Promega) was intended to have blunt ends, and contains a timine (T) in the insertion area.

Both the concentration of the vector and the insert must be taken into consideration to obtain a good ligation reaction. The recommended ratio of vector:insert, (1:1) was used in all the ligation reactions throughout this work. To calculate the amount of PCR product (insert) to use to perform a ligation with the 1:1 proportion the following equation was used:

$$\frac{\text{ng (vector)} \times \text{Kb (insert size)}}{\text{Kb (vector size)}} \times \text{Insert:vector (molar proportion)} = \text{insert ng}$$

Ligation reactions were performed as specified by the pGEM®–T Easy Vector manufacturer (Promega):

2x Buffer rapid ligation	5 µl
pGEM®–T Easy Vector (50 ng)	1 µl
PCR product	Volume
T4 DNA Ligase	1 µl
Sterile Milli–Q water	To 10 µl

Reaction should be carefully mixed and incubated 1 hour at room temperature or 12 hours at 4ºC.

2.3.7.5 Transformation

Inserting a vector containing the sequence of interest into a host cell is known as transformation. In our case the plasmid pGEM®–T Easy Vector (See section 2.3.6.2) ligated with the insert was transferred into the strain DH5α (See section 2.3.6.1) as follows:

1. Thaw the SOC medium (see section 2.3.6.3) at room temperature.
2. Prepare LB plates with X–Gal, IPTG and ampicillin (see section 2.3.6.3) and incubate at 37ºC.
3. Thaw 50 µl of competent cells and add 2 µl of the ligation reaction (vector + insert). Mix gently.
4. Incubate on ice for 20 minutes.
5. Incubate the tubes in a bath at 42ºC for 45–50 seconds exactly, and return them to the ice for two more minutes.
6. Add 950 µl of SOC medium and incubate for 1.5 hours at 37ºC with gentle shaking (≈150 r.p.m.).
7. Spread off 50–200 µl of transformation product onto the LB plates with X–Gal, IPTG and ampicillin.
8. Incubate 16–24 hours at 37ºC. Afterwards they may be incubated at 4ºC for 2 more hours to ease the galactose positive cells to grow blue.

2.3.7.6 Insert recovering

All growing colonies carry the plasmid and are therefore resistant to the antibiotic. The blue colonies carry the plasmid, but do not carry the insert. In turn, the white colonies are the ones carrying both the plasmid and the insert. In this case, each recovered plasmid leads to the recuperation of a single *amoA* fragment from the original environmental sample.

The insert was directly amplified from the transformant colonies. The white colonies were directly used as a template in a PCR with the primers M13F and M13R (see section 2.3.4.4). The amplified fragments were further purified (see section 2.3.2).

2.3.7.7 Restriction analysis

Type II restriction enzymes recognize a specific sequence in a ds DNA chain as a target, and cut it in a particular site. As a result, two sequences with defined length and sequence are produced.

amoA fragments recovered from the plasmids should have been sequenced in order to identify all the AOB present in the initial environmental sample. Due to the huge number of inserts recuperated, restriction analysis was used previously as a screening method to distinguish the several types of *amoA* fragments. Thus, it was only necessary to sequence one fragment from every

variety. Two sites of the sequence were cut by either restriction enzyme. As a result, every insert provided a specific number of fragments with variable lengths. The number and length of the fragments were detected in an agarose gel (see section 2.3.5.2) which allowed the grouping of the inserts into restriction patterns.

The enzymes used in this work are presented in Table 2.6. All restriction enzymes and buffers were stored at –20ºC. In our case, the two enzymes used were compatible with the same buffer.

Table 2.6 Enzymes used and main characteristics

Enzyme	Target	Manufacturer	Buffer
Afa I (Rsa I)	5'–GT|AC–3'	Amersham Pharmacia	330 mM Tris–acetat pH 7,9 100 mM Mg–acetat
Alu I	5'–AG|CT–3'	Promega	5 mM Dithiothreitol 660 mM K–acetat

The volume of restriction analyses was 20 µl. The volume of enzyme added per reaction was always lower than 2µl. Restriction enzymes are provided in 50% glycerol solutions. In many cases a concentration of glycerol higher than a 5% may cause a specificity loss. This unspecific activity can also be caused by an excess of DNA compared to the amount of enzyme used (> 25 U μg^{-1}).
Restriction analysis reactions for 20 µl with the enzymes Alu I and Afa I were prepared as described below:

T buffer 10X	2 µl
BSA 0,1%	0,2 µl
Alu I (8U μl^{-1})	0,5 µl
Afa I (8U μl^{-1})	0,5 µl
Milli–Q water	to 15 µl

5 µl of the DNA (200 ng μl^{-1}) to analyse were added. The reaction was then incubated at 37ºC for at least 4 hours.

The restricted product was then precipitated with ethanol (see section 2.3.2.1) and loaded into a poliacrylamide gel (see section 2.3.5.3).

2.3.8 Sequencing

The dRhodamine Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used to sequence 16S rDNA, *amoA* and *amoB* genes as specified by the manufacturer. The primers used for sequencing were the same used in the polymerase chain reaction (Table 2.4).

Prior to sequencing, the PCR product was purified (see section 2.3.2) and DNA concentration was quantified (see section 2.3.3). The sequencing reaction was prepared as specified below in a 200 µl tube:

DNA template.....	15–45 ng
Terminator Ready Mix.....	4 µl
Primer.....	2 pmol
Milli–Q water.....	fins a 10 µl

The composition of the Terminator Ready Mix is: dNTP, dyed terminal ddATP, dyed terminal ddCTP, dyed terminal ddGTP, dyed terminal ddTTP, Tris (pH 9.0), $MgCl_2$, pyrophosphatase and AmpliTaq DNA polimerase, FS.

The program used in the thermocycler was the one specified by the manufacturer and described below:

1. Denaturing	94ºC	5 min
2. 30 cycles:	94ºC	30 sec (denaturing)
	50ºC	15 sec (annealing)
	72ºC	1 min (extension)
3. Extension	60ºC	4 min
4. Conservation	4ºC	pause

Later, the samples were precipitated with ethanol–sodium acetate method (see section 2.3.2.1) and resuspended as follows.

1. Add 25 µl of TCR buffer to each tube. Avoid bubbling.
2. Incubate at room temperature for 10 minutes.
3. Mix vigorously by vortexing 10 seconds and centrifuge for 5 seconds.
4. Incubate at 94ºC for 3 minutes.
5. Let sit on ice until it is cold.
6. Mix vigorously by vortexing 10 seconds and centrifuge for 5 seconds.
7. Transfer to a new sequencing tube.
8. Inject to ABI PRISM™ 310 Genetic Analyzer (Applied BioSystems), which provides the sequences as superposed chromatograms belonging to the four dyes of the different terminal ddNTP.

2.3.9 Analysis of DNA sequences

2.3.9.1 Alignment

Multiple sequence alignment of the sequences was carried out with ClustalW (Thompson *et al.*, 1994) and was further refined manually by using the software BioEdit Sequence Alignment Editor (Hall, 1999).

2.3.9.2 Comparison of sequences against the databases

Obtained sequences were compared to the databases EMBL (European Molecular Biology Laboratory) and NCBI (National Center for Biotechnology Information). Sequence similarities were checked using the BLAST (Basic Local Alignment Search Tool) software, which is available at the web pages: http://us.expasy.org/tools/blast/ and http://www.ncbi.nlm.nih.gov/BLAST/.

2.3.9.3 Phylogenetic reconstruction

Phylogenetic trees were constructed by using distance, maximum likelihood and maximum parsimony methods. Bootstrap was used to confer reliability to the obtained results.

– Distance Method

MEGA v 2.1. (Kumar *et al.*, 2001) was used to calculate distance matrixes. The distance matrix method uses estimated distances in a matrix form between all pairs of species (or genes) in a data set to reconstruct a phylogenetic tree. In all cases the required nucleotide substitution model was used for each genetic marker. The Neighbor–Joining (NJ) clustering method (Saitou and Nei, 1987) involves clustering of neighbor species that are joined by one node. It does not evaluate all the possible tree topologies, but at each stage of clustering the Minimum Evolution (ME) method is used. The ME method estimates the total branch length of each topology. After it evaluates all possible topologies, it chooses the topology with the least total branch length. The tree does not assume an evolutionary clock, so that it is actually an unrooted tree. This method is computationally intensive and therefore slow, and with a small number of species to compare, the NJ method usually gives the same result as the ME method in less time. Thus, the NJ method is considered a simplified version of the ME method.

– Maximum Parsimony (MP) Method

Maximum Parsimony (MP) method was first developed for morphological characters, and exists in several versions (Nei and Kumar, 2000). Maximum parsimony is a character–based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, i.e. by minimizing the total tree length. In MP inference a site is considered to be informative only when there are at least two different kinds of nucleotides at the site, each of which is represented in at least two of the sequences under study. Maximum parsimony searches for the optimal (minimal) tree. In this process more than one minimal tree may be found. In order to find the best possible tree an exhaustive evaluation of all possible tree topologies has to be carried out. However, this becomes impossible when there are more than 12 OTUs in a dataset.

Branch and Bound: is a variation on maximum parsimony that guarantees to find the minimal tree without having to evaluate all possible trees. By this method a larger number of taxa can be evaluated but the method is still limited.

Heuristic searches is a method with step–wise addition and rearrangement (branch swapping) of OTUs, and it does not guarantee to find the best tree. MEGA v 2.1 software includes both the methods for amino acid and for nucleotide sequences analyis (Eck and Dayhoff, 1966; Fitch 1971). Phylogenetic trees inferred by MP are unrooted. Nevertheless, they are often presented with a root to ease interpretation of results. Since the Maximum Parsimony method may result in more than one equally parsimonious tree, a consensus tree should be created. For the creation of a consensus tree see next section (Bootstraping).

– Maximum Likelihood (ML) method

Maximum Likelihood inference method was designed by Felsenstein in 1981 and can be applied to either amino acid or nucleotide sequences. Maximum Likelihood evaluates a hypothesis about evolutionary history in terms of the probability that the proposed model and the hypothesised history would give rise to the observed data set. The supposition is that a history with a higher probability of reaching the observed state is preferred to a history with a lower probability. For a given tree topology, and a given probability model, the method computes the probability for a nucleotide (or an amino acid) to be at a specific site of the sequence. The sum of the probabilities of all the sites is maximized to estimate the length of the tree branches. This procedure is repeated for all the branches of the tree, and the tree with highest global probability (or likelihood) is chosen as the final tree. The model of evolution that attributes to each possible nucleotide or amino–acid substitution a certain probability is es-

sential to obtain the correct tree. In this work ML analysis were performed with the program Treepuzzle (v. 5.0, Heiko *et al.*, 2000). An appropriate nucleotide substitution model was used for every marker.

Bootstrapping

The bootstrap test (Felsenstein 1985) is a way of testing the reliability of the dataset. A bootstrap of the Distance and Maximum Parsimony data was performed with MEGA v 2.1 software. Bootstrapping is based on the creation of pseudoreplicate datasets by randomly resampling the original matrix. This method creates new matrices of the same size as the original, and the frequency with which a given branch is found, is recorded as the bootstrap proportion. These proportions can be used as a measure of the reliability of individual branches in the original tree. Finally, a consensus tree is built, which incorporates the branches with higher bootstrap values. The bootstrap value indicates how many times, regarding the total number of replicates, a branch appears at a given position.

2.3.10 Deduced amino acid sequences

The study of peptidic sequences allows distinguishing among nucleotide differences that may affect the protein function, and differences that do not cause a functional variation. The same peptidic sequence can be encoded by different DNA sequences due to the genetic code degeneracy.

In this work, *amoA* and *amoB* nucleotidic sequences were translated to peptidic sequences to discriminate the mutations causing amino acid variations from the mutations having no effect. Subsequently, the peptidic changes that might cause functional changes in the enzyme ammonia monooxygenase were analysed.

The software TRANSLATE (which can be found at the web page of the proteomic server SIB (Swiss Institute of Bioinformatics: http://us.expasy.org/tools/dna.html) was used to deduce peptides from the DNA sequences. This program supplies the deduced peptidic sequences for the three reading frames.

2.3.11 Peptidic sequences analysis methods

Deduced peptidic sequences were aligned as previously described (see section 2.3.8.1) prior to similarity analysis.

2.3.11.1 PAM–DAYHOFF method

The term PAM–Unit (Point Accepted Mutation Unit) was introduced by Dayhoff *et al.* (1978) and is defined as the amount of evolution necessary to change an average of 1% of the amino acids of a given sequence. There are many inference methods based on PAM–distances. PAM–Dayhoff inferred trees are based on a PAM–Dayhoff distance matrix, which is constructed by computing the logarithm of the probability that two amino acids evolved from the same ancestor and are not two random sequences. Mathematically the Dayhoff matrix is:

$$DAB = 10 \log 10 \frac{P(A \text{ and } B \text{ from common ancestor } X)}{P(A \text{ and } B \text{ are independent})}$$

The tree is built using the distances of the Dayhoff Matrix as an estimation of the branch length.

2.3.12 Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) was first developed by Amann *et al.* (1990) and is based on the use of labeled probes with target in 16S rRNA and 23S rRNA sequences. This technique allows the detection of numerous bacterial groups at different phylogenetic levels in environmental samples.

Approaches depending on rRNA hybridization are excellent for the study of nitrifying bacteria (Wagner *et al.*, 1995). However, there are some inconvenients associated to this technique (i.e. low rRNA contents or low permeability of target cells) that must be defeated by using the appropriate hybridization protocols (Manz *et al.*, 1992).

Target molecules

Nowadays the study of the composition of microbial communities is focused on the analysis of molecules with phylogenetic value. For instance, ribosomical RNA is almost a perfect target. It is homogeneously distributed in the cell, the encoding DNAs are homologues, and lateral gene transfer has never been reported (Olsen *et al.*, 1986). Phylogenetic characterization has been

performed by comparing the three rRNA molecules (5S, 16S and 23S) (Woese *et al.*, 1983. Woese, 1987). Moreover, only 16S and 23S rRNA present the appropriate size to perform valid phylogenetic inferences.

The presence of conserved and variable regions in rRNA sequences allows finding signature sequences for microbial groups (Stahl and Amann, 1991). Most of the differences between the several bacterial groups are found in the variable regions of the rRNA. Therefore it is not always necessary to sequence the whole DNA sequence to obtain good signature sequences. This last property turns rRNA to be an appropriate target for in situ hybridization.

Besides, the quality of any hybridization depends directly on the availability of target sequences. Thus, using rRNA as a target is better than using DNA, since the relative abundance of the former is bigger (Olsen *et al.*, 1986; Ward *et al.*, 1992; Waters and McCutchan, 1990).

2.3.12.1 Solutions and reactives

– PBS 10x buffer (pH 7.2)

Prepare a solution 1.3 M NaCl, 70 mM Na2HP04, 30 mM NaH2P04 in Milli–Q water.

– Fixation buffer (pH 7.2)

Heat 40 ml of distilled water until 55–65 ºC. Add 2 g of paraformaldehyde and 150 µl of NaOH (1M). Maintain the solution at 65 ºC. Mix vigorously until the paraformaldehyde completely dissolves. Add 5 ml of buffer PBS 10x, and adjust pH tp 7.2 with concentrated HCl. Set the volume to 50 ml. Solution must be filtered through a nitrocellulose filter with 0.45 µm of diameter.

– Slide preparation

The slides used for fluorescent *in situ* hybridization present a teflon layer (Knittel Gläser, Germany) with 6 wells of 8 mm in diameter each. Prepare a 0.1% gelatine solution with 0.05 g of gelatine in 50 ml of water at 60ºC and 50 µl of 10% $KCr(SO_4)_2$. Bath the slides in the solution and allow it to air–dry.

– Hybridization buffer (pH 7.0)

Prepare in distilled sterile water: 20 mM Tris–HCl (pH 7.0); 0.9 M NaCl; 0.01% SDS; % Formamide variable depending on the probe to use.

– Washing buffer (pH 7.4)

Prepare in distilled sterile water: 20mM Tris–HCl (pH 7.0); 112 mM NaCl; 0.01% SDS; 5 mM EDTA. Depending on the percentage of formamide (%F) used for the probe, the concentration of NaCl varies as specified by Hahn and Zarda (1996) equation:

$$[NaCl] = 89.72\ e^{(-0.07(\%F))}$$

– Probes

The probes used in this work were Nso190 (5'–CGATCCCCTGCTTTTCTCC–3'), with specificity for AOB belonging to the β–subgroup of Proteobacteria (Mobarry *et al.*, 1996), and Nit3 (5'–CCTGTGCTCCATGCTCCG–3') with specificity for the nitrite oxidizer genus *Nitrobacter* (Wagner *et al.*, 1996. These probes were synthesized, labeled with fluorescent indocarbocyanine (Cy3) and purified through reverse phase chromatography by Boehringer Mannheim. When excited with light at 552 nm, the fluorochrome Cy3 presents, in PBS buffer, an emission peak at 568nm.

The oligonucleotides used in this work were considered to be the best probes to detect nitrifying bacteria due to their high specificity. Besides, both Nso190 and Nit3 enclosed most of the nitrifying bacteria known to date (Mobarry *et al.*, 1996; Juretschko *et al.*, 1998). However, it is not possible to establish a correlation between the number of hybridized cells with each probes and the real number of nitrifiers in the sample. Thus, for example the number of cells hybridized with the probe Nso190 cannot be considered as the total number of AOB, because in this case, the probe Nso190 doesn't include the members of the genus *Nitrosococcus* (Figures 2.14 and 2.15).

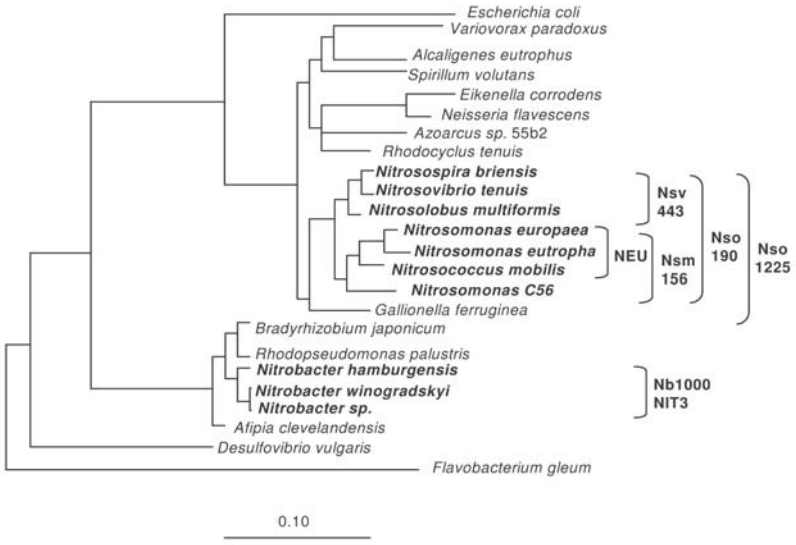


Figure 2.14 Phylogenetic tree constructed by comparison of 16S rRNA sequences. The target microorganisms for some of the published probes are indicated in boldface (Modified from Mobarry *et al*, 1996).

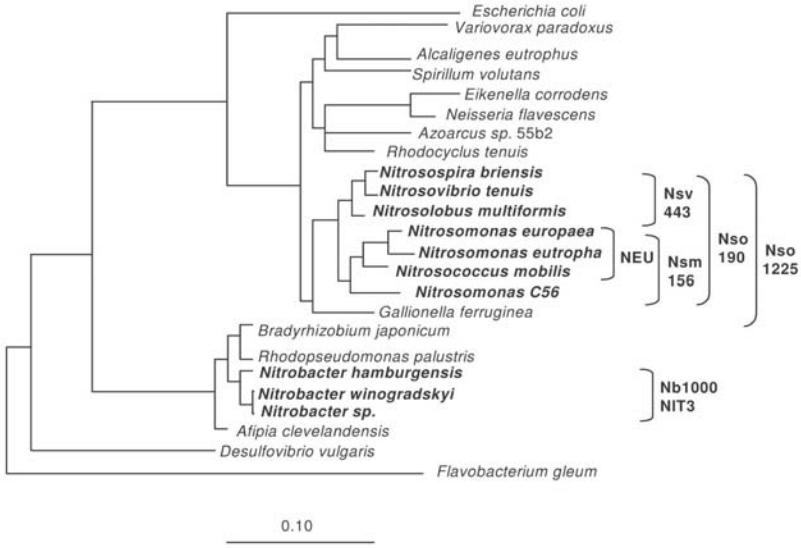


Figure 2.15 Phylogenetic tree based in maximum likelihood analysis of 16S rDNA sequences. The target microorganisms for Nit3 and S–*–Ntspa–1026–a–A–18 are indicated in boldface (Modified from Juretschko *et al.*, 1998).

2.3.12.2 Sample fixation

Cells are exposed to high temperatures, detergents and osmotic gradients along the hybridization protocol. Therefore, fixation is essential to ensure structural and morphological stabilization (Fry, 1988). Besides, fixating agents cause physical changes to cellular and extracellular components, usually increasing permeability of cell envelopes and cytoplasm.

Several recommended protocols can be found for sample fixation: 50% Ethanol–PBS (Phosphate buffered saline) (Spring *et al.*, 1992), 90% Ethanol/ 3.7% Formaldehyde (Braun–Howland *et al.*, 1992), although the most used method, and also in this study was 4% paraformaldehyde in PBS (Amann *et al.*, 1990).

1. Mix 200 µl of samples with 600 µl fixation buffer (1:3 v/v).
2. Incubate for 3–12 hours at 4 ºC. Avoid prolonging incubation more than 12 hours, since long fixations may produce sample autofluorescence.
3. Centrifuge for 5 minutes at 14.000 r.p.m. and discard supernatant. Mix vigorously.
4. Wash twice with PBS 1x buffer and centrifuge for 5 minutes at 14.000 r.p.m.
5. Discard supernatant and ressuspend the pellet in PBS 1x buffer:ethanol (1:1) solution (Amann *et al.*, 1990).
6. Store at –20ºC.

2.3.12.3 Slide preparation

6. Dispense 5 µl of sample in each slide well and let dry for 20 minutes at 45ºC.
7. Carefully wash with distilled water.
8. Dehydrate the sample by following three consecutive steps with a series of alcohols. (50%, 70% and 96% ethanol, 3 minutes each).

2.3.12.4 Hybridization and sample washing

In this work the method used was first developed by Amann *et al.* (1990) and subsequently modified by Hahn *et al.* el 1992.

9. Add 9 µl of hybridization buffer to each well, on the dehydrated sample.
10. Add 1 µl of Cy3 labelled probe (25 ng μl^{-1}). Fluorochromes are photodegradable, thus from this moment it is recommended to work in the dark.
11. Add 1 µl of DAPI 0.001% solution.
12. Put carefully the slide into a hybridization chamber and incubate for 2 hours at 45°C. To avoid evaporation of the hybridization buffer, a soaked paper is added into the hybridization chamber.
13. Transfer the slide into a 50 ml tube containing preheated washing buffer and incubate for 20 minutes at 45°C.
14. With the aid of a forceps remove the slide from the tube and wash it gently with distilled water. Allow it to air–dry.

2.3.12.5 Sample observation

15. Allocate a Citifluor AF1–glycerol (Citifluor products, University of Kent, UK) drop between the slide and the 20x60 mm cover. Citifluor solutions have been shown to reduce DAPI photofading.
16. Observations were performed in an epifluorescence microscope (Zeiss Axioskop) equipped with the appropriate filters for DAPI and the employed fluorochrome detection (DAPI: Bandpass F31–000; Cy3: HQ F41–007, both from AF Analysentechnick, Tübingen, Germany).
17. Preparations can be photographed with a 1600 ASA film (i.e. Kodak Ektachrome 1600 ASA).

3 STUDY OF AMMONIA OXIDIZERS IN A BIOFILM BY IN SITU HYBRIDIZATION

3.1 INTRODUCTION

One of the main difficulties in the study of microbial ecology using the current techniques is the impossibility of culturing more than a small portion (0.1-10%) of the bacteria that can be seen in a microscope (Head *et al.*, 1998). Therefore, studies based in environmental samples enriched in the laboratory are not representative of their initial microbial communities.

Although biases introduced by culture-based methods were already documented by Winogradsky (1949), it was not until the mid 1980s that molecular biology techniques allowed the acquisition and improvement of the knowledge about natural population's composition. Phylogeny studies presented by Zuckerkandl and Pauling (1965), and Woese (1987) set the basis for the identification of uncultivable bacteria. Nowadays, entire groups of microorganisms, quantitatively important, and from most of the natural communities, are known by their molecular sequences only.

The use of molecular biology techniques has increased the knowledge about environmental microbial communities, but their limitations have to be taken into consideration in order to minimize, eradicate or, at least, recognize them. Since total numbers of nucleic acids present in an environmental sample are usually unknown, spores won't be lysed as efficiently as vegetative cells, and gram-positive cells are more resistant to lysis than gram-negative cells, it is hard to assess the efficiency of DNA purification. Lysis efficiency can only be measured by counting the cells before and after the treatment (Head *et al.*, 1998). Lots of different methods for DNA purification have been developed, but efficiency of only a few has been exhaustively studied with samples from several origins (Head *et al.,* 1998; Frostegard *et al.,* 1999). Diversity measure-

ments can also be affected by the selectivity of enzymatic amplification. Thus, small differences in the sequences considered to be universally conserved may lead to a discriminatory amplification. Moreover, the number of ribosomal DNA gene copies in the genome of microorganisms varies in number from 1 to 14 (Cole and Girons, 1994). Therefore, it is not possible to associate a sequence frequency in a PCR product library to the relative abundance of this group of microorganisms in a microbial community. Rainey *et al.*, (1994) demonstrated that the same group of PCR products resulted in different gene libraries depending on the cloning vector used. Fidelity of PCR amplification is also influenced by the type of DNA polymerase used. Another source of problems is the formation of chimeric PCR products. They are produced when some DNA strands are not wholly copied during an extension step. Then, in following cycles, two of these partial fragments may fusion and generate a chimeric gene, which contains regions copied from several origins (Liesack *et al.*, 1991, Giovanonni, 1991). Besides, hybridization techniques also present inconveniients that can be divided into three main categories: cell permeability, target accessibility, and specificity and sensitivity of hybridizations (See section 2.3.11).

The study of nitrifying communities through methods depending on cultivation is particularly problematical due to the difficulty and slowness of their growth. Isolation methods usually consist of consecutive enrichments in mineral medium with ammonium or nitrite salts. The result is a collection of isolates that are not representative of the original environmental sample (Belser, 1979). To date, many culture-independent techniques have been developed to study the nitrifying communities: ELISA tests (Inamory *et al.*, 1997), *in situ* hybridization (Gall and Pardue, 1969; Wagner *et al.*, 1993; Wallner *et al.*, 1995; Amann *et al.,* 1995), polymerase chain reaction (PCR) (Rotthawe *et al.*, 1997; Teske *et al.*, 1996), dot-blot hybridization, denaturing gradient gel electrophoresis (DGGE) (Teske *et al.*, 1996), DNA hybridization analysis (Torsvik *et al.,* 1996), or restriction fragment length polymorphisms (RFLP) (Moyer *et al.,* 1994).

With the main goal of optimizing the protocol for the *in situ* detection of microbial populations with fluorescent probes, the present work focused on the characterization of the nitrifying microbial community inhabiting the rotating biodiscs of a pilot plant (see section 2.1.1.1). Small subunit ribosomal gene sequences from nitrifying organisms can be obtained from the databases (GeneBank and Ribosome Database Project), and oligonucleotide probes matching specific targets in this gene can be designed. The fluorescent labeling of these probes permits individual identification and enumeration of the cells by using an epifluorescence microscope. This technique eludes

the above described problems of culture-dependent methods, detecting both active and non-active (but not lysed) cells. Population dynamics and behavior when limiting the input of ammonium have been studied by employing several 16S rRNA targeted probes. Besides, the reliability of this technique has been corroborated, and its limitations established.

3.2 RESULTS

3.2.1 Determination of hybridization conditions

3.2.1.1 Probe Nso190

The percentage of formamide is one of the factors determining the specificity of a probe for its target. Mobarry *et al.*, (1996) recommended using 55% formamide with the probe Nso190, but hybridizations showed then unexpected results. Only few cells were labelled: the characteristic nitrifying cell aggregates did hybridize with the probe for eubacteria Eub338, and stained with DAPI, but could not be observed with Nso190 probe. To assess the best conditions for Nso190 hybridization, a series of hybridization buffers with different formamide percentages was tested. The percentages tested ranged from 30 to 55%, and the corresponding washing buffer with the appropriate NaCl concentration (Hahn and Zarda, 1996) was used in every case.

Epifluorescence microscopy observations indicated that for our samples the best percentage of formamide for hybridization with the probe Nso190 was 35% (Figure 3.1). The same results were obtained with several samples from diverse origins, and measuring fluorescence intensities by the group of S. Biesterfeld in Colorado (Biesterfeld 1998, personal communication).

In Figure 3.1 two of the performed tests are shown. Cells in the nitrifying aggregates could not be differentiated when using 50% formamide, and an orange background distorted the preparation (Figure3.1B). In contrast, when using 35% formamide (Figure 3.1D), cells belonging to the aggregates can be independently and clearly distinguished in a black background.

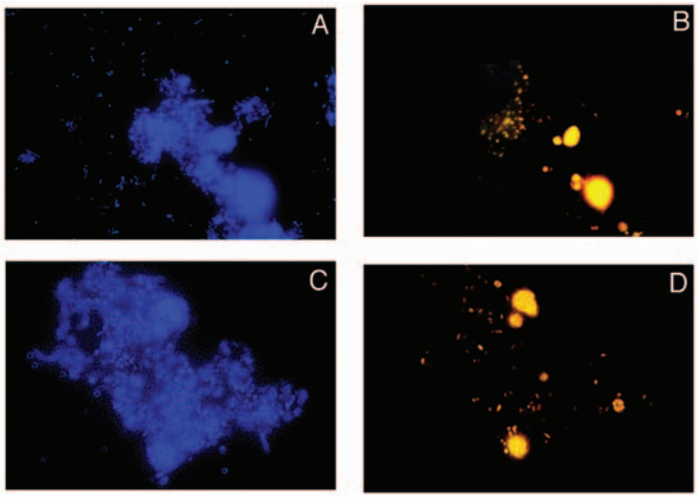


Figure 3.1 Hybridization with Nso190 probe labelled with fluorochrome CY3 using 50% (B) and 35% (D) formamide in the hybridization buffer. The same microscopic fields have been observed with DAPI (A and C respectively)

In order to check whether or not the new conditions altered the specificity of the probe Nso190, an online analysis was performed in the Ribosomal Database project (RDP). Results indicated that the probe Nso190 maintained the same specificity when applying either 55 or 35% formamide in the hybridization buffer.

3.2.1.2 Probe Nit3

Since hybridizations obtained with probe Nit3 using the recommended 40% formamide (Mobarry *et al.*, 1996, Wagner *et al.*, 1996) showed an orange background that even masked the fluorescence of hybridized cells, the same experiment described above was performed with this probe. In this case, formamide percentages ranged from 15 to 40%, and a 17.5% was obtained as optimal. The use of these new conditions gave as a result a clearer image, where the same number of CY3-labelled cells detected before could be easily distinguished in a black background (figure 3.2). Online analyses in the RDP indicated that using 17.5% formamide did not affect the specificity of the probe Nit3.

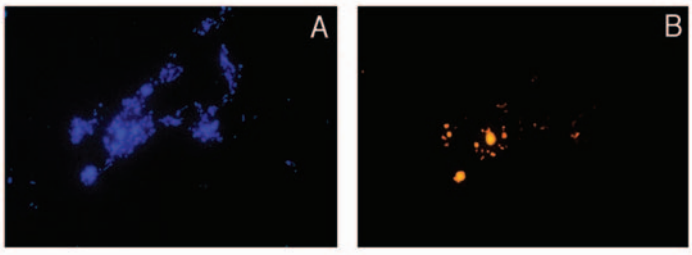


Figure 3.2 Microscopic field with DAPI staining (A). The same field showing cells hybridized with Nit3 probe using 17.5% formamide in hybridization buffer (B).

3.2.1.3 Probes Eub338 and Bet42a

Hybridizations using probes Eub338 and Bet42a, specific for eubacteria and β subgroup of the Proteobacteria respectively, were performed as specified (Amann *et al.,* 1990; Manz *et al.,* 1992) with no further refinements of the conditions (Figure 3.3).

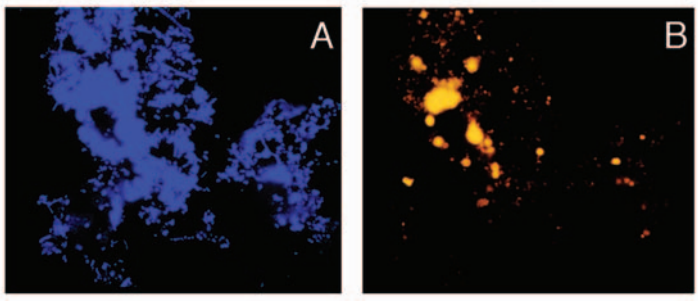


Figure 3.3. Microscopic field with DAPI staining (A). The same field showing cells hybridized with Bet42a probe using 35% formamide in hybridization buffer (B).

3.2.2 Sample permeability

Enumeration of Eub338 hybridized cells along the colonization process gave as a result percentages above 70% on total DAPI counts (Table 3.1). This measure can be considered as an indicator of the relative quantity of cells capable of being hybridized with a probe.

Table 3.1. Percentage of Eub338 hybridized cells (%) with respect to total DAPI counts.

	Stage 1	Stage 2	Stage 3	Stage 4
30.01.1998 (day 28)	88.5±1.8	84.6±1.9	70.7±8.8	83.9±5.3
13.03.1998 (day 50)	76.4±4.2	88.0±3.2	82.4±4.3	90.5±2.1
21.05.1998 (day 136)	71.1±4.0	78.1±1.7	77.8±3.3	78.3±3.8
06.08.1998 (day 216)	75.3±7.1	82.3±3.3	81.6±4.5	77.5±2.9

3.2.3 Biodiscs colonization

3.2.3.1 Nitrogen compounds evolution

Measures of several compounds revealed that nitrification started between the days 12 and 18, when oxidation of ammonium started in the four stages of the pilot plant (Figure 3.4). Ammonium oxidation rate presented a maximum at 2 g N m^2 day^{-1} by the day 60 (Amer 1998), and 120-130 days after the pilot plant was started, reached the steady state. Then, ammonium oxidation rate was stable at 1 g N m^2 day^{-1} (Figure 3.5).

As expected, nitrification caused a decrease in ammonium concentration (Figure 3.4). Nitrate concentration increased at the same rhythm that ammonium reduced, whereas nitrite remained stable, showing low concentrations along the whole process of colonization (Amer, 1998).

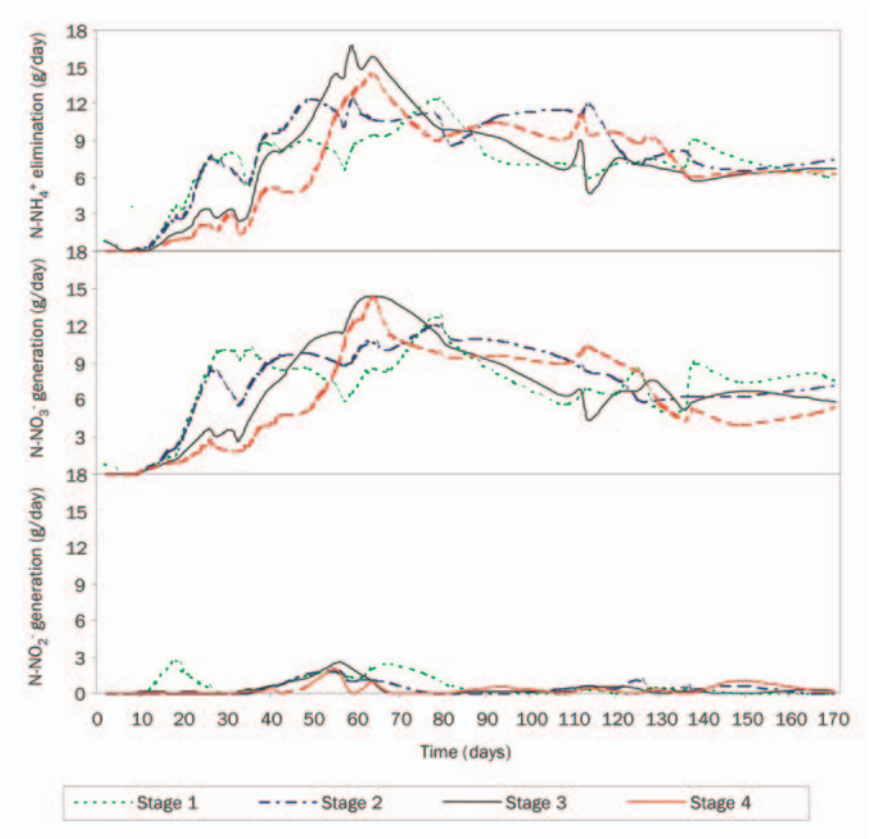


Figure 3.4 Evolution of ammonia, nitrite and nitrate along the colonization period in the four stages as measured by Amer 1998.

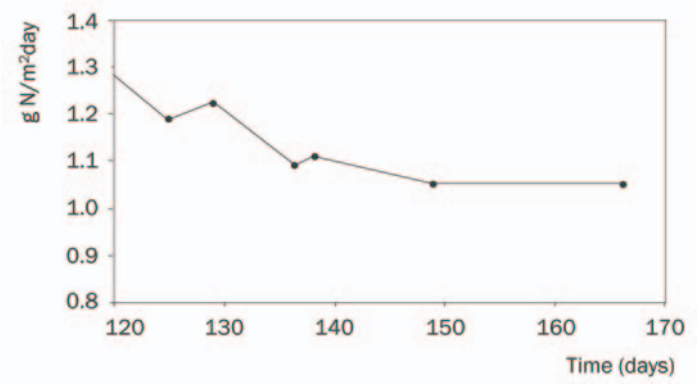


Figure 3.5 Evolution of ammonia oxidation rate in the nitrifying biofilm until achievement of the steady state (modified from Amer 1998).

3.2.3.2 Fish counts

The number of cells hybridized with Nso190 (AOB group) and Nit3 (NOB group) increased from the first day of sampling (day 28). Two months later, a maximum was achieved, but at the steady state the values obtained for Nso190 and Nit3 on total DAPI counts were 40% and 20% respectively (Figure 3.6).

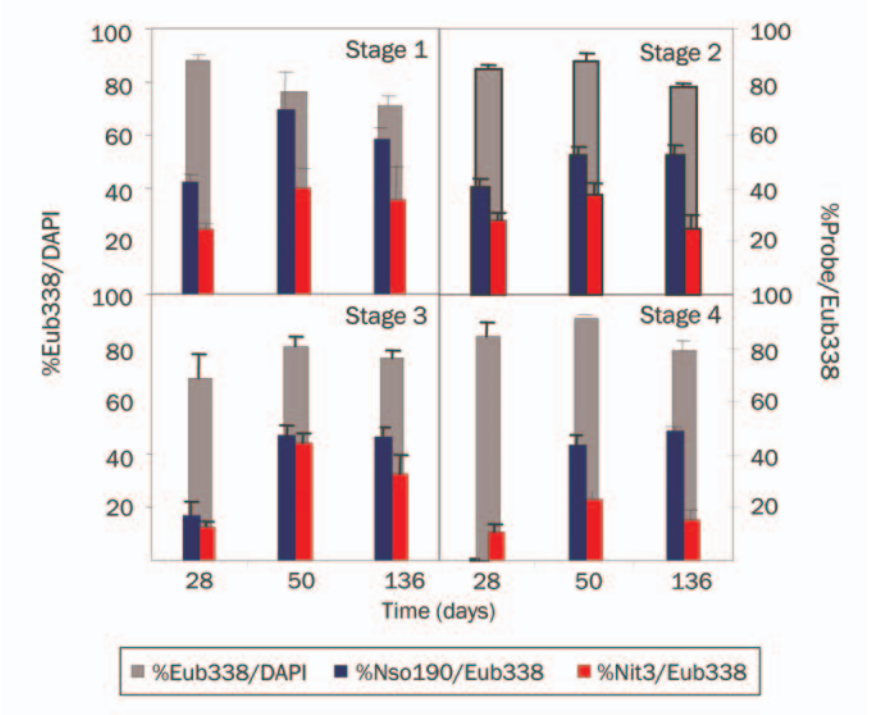


Figure 3.6 Evolution of the percentages of cells hybridized with probes Nso190, Nit3 (on Eub338 total counts) and Eub338 on total DAPI counts along the colonization period and in every stage of the bioreactor.

Compared to the first and second stages, colonization of the third and fourth was delayed, but once the steady state was reached, both ammonium oxidizers and nitrite oxidizers stabilized. The same values were then obtained in the four stages. Percentages of Eub338 hybridized cells on total DAPI counts were always around 80%.

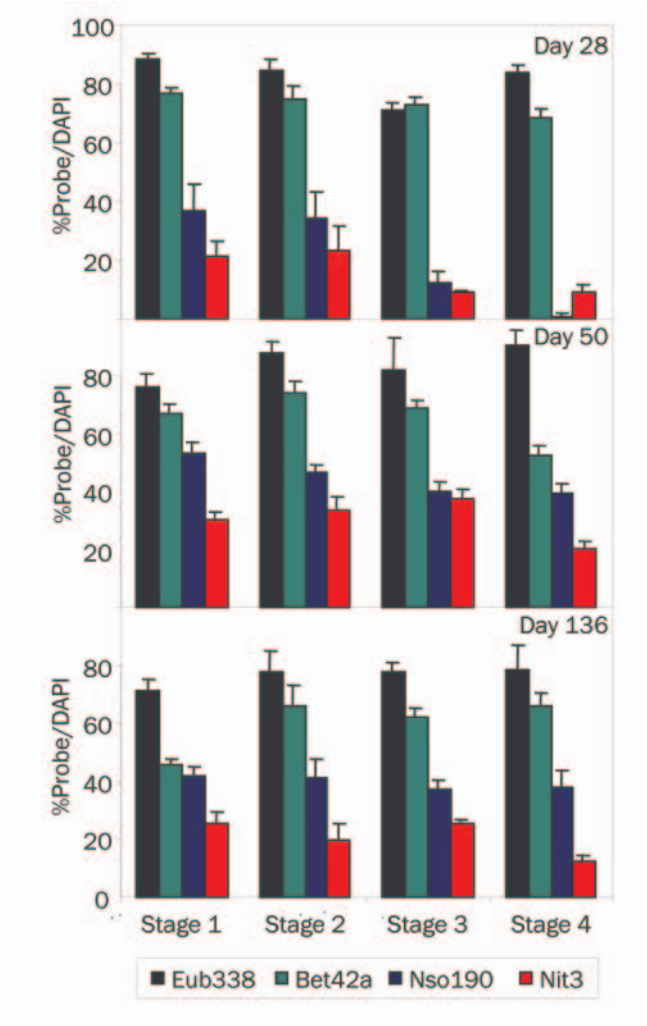


Figure 3.7 Evolution of the percentages of cells hybridized with the probes Nso190, Nit3, Eub338 and Bet42a as compared to total DAPI counts in every stage of the bioreactor along the colonization period of the nitrifying biodiscs.

3.2.3.3 Epifluorescence microscope observations

Hybridized cells labelled with Cy3 fluorochrome, could be observed by fluorescent microscopy. Cells appeared orange in a black background (figure 3.8A). The greatest part of hybridized cells was included in compact aggregates with

variable morphology. These aggregates were observed in both AOB (Nso190) and NOB (Nit3) hybridization preparations.

Nit3 hybridized cells were larger and forming denser aggregates than Nso190 hybridized cells. However, free hybridized cells were also observed in all fields, and with all probes.

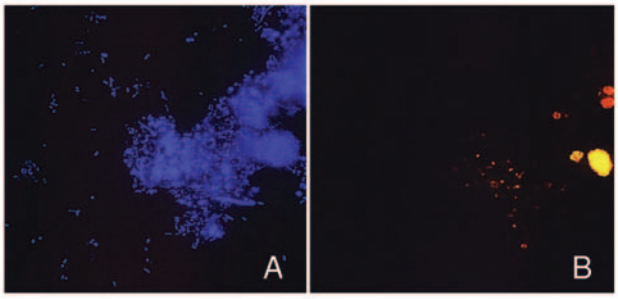


Figure 3.8 Micrography showing the same cells stained with DAPI (A) and hybridized with probe Nso190 labelled with the fluorochrome CY3 (B).

3.2.4 Effect of ammonium limitation

3.2.4.1 Nitrogen compounds evolution

After the steady state was reached, ammonium input was limited from the day 170 (Figure 3.9).

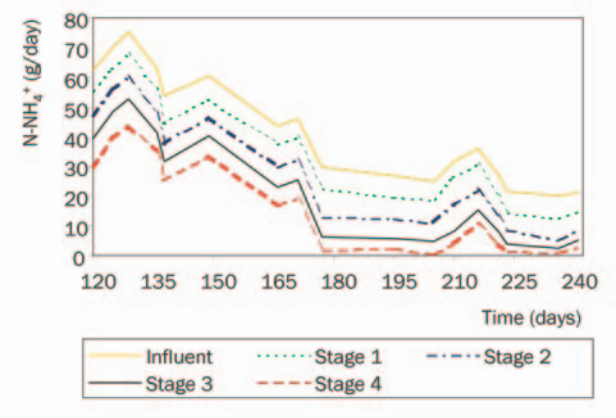


Figure 3.9 Time-course of ammonia (in g $N\text{-}NH_4^+$/day) in the inflow and in every stage of the bioreactor when limiting ammonium input. (Modified from Amer, 1998).

Although the input of ammonium was limited, nitrate was still being produced (Amer, 1998), whereas nitrite concentration remained low and constant. The first two stages were found to consume all the ammonium available. Therefore, and because of the connection in series of the stages, the 3rd and 4th were the ones presenting a real ammonium limitation. Ammonium oxidation rates in the two first stages during the period of ammonium limitation were the same as in the steady state (Table 3.2). The third stage remained stable until the rate decreased a 50% on the day 236. The rate in the fourth stage decreased when limiting ammonium input until showing the lowest value at 1.17 $gNm^{2}day^{-1}$.

Table 3.2. Ammonia oxidation velocity, Vn, (in $gNm^{-2}day^{-1}$) in every stage, on the steady state and when limiting the ammonium input (Modified from Amer, 1998).

	Vn Stage 1 ($gNm^{-2}day^{-1}$)	Vn Stage 2 ($gNm^{-2}day^{-1}$)	Vn Stage 3 ($gNm^{-2}day^{-1}$)	Vn Stage 4 ($gNm^{-2}day^{-1}$)
Day 125 Steady state	1,13	1,12	1,11	1,39
Day 149 NH_4^+ limitation	1,17	1,03	0,97	1,01
Day 171 NH_4^+ limitation	0,93	1,17	1,05	0,98
Day 205 NH_4^+ limitation	0,96	1,21	1,01	0,64
Day 236 NH_4^+ limitation	1,20	1,10	0,48	0,22

3.2.4.2 FISH counts

As expected, the ammonium limitation was reflected by a decrease in the percentages of cells hybridized with Nso190 and Nit3. Nevertheless, the cells hybridized with Eub338 maintained the same values (Figure 3.10).

The effect of ammonium limitation (day 216) was also evident when comparing the percentages calculated for probes targeting AOB with the same percentages calculated in the steady state (day 136). Cells hybridized with Nso190 decreased to similar values in the four stages, whereas the percentages of cells hybridized with the probe Nit3 (NOB group) decreased progressively from the first to the fourth stage.

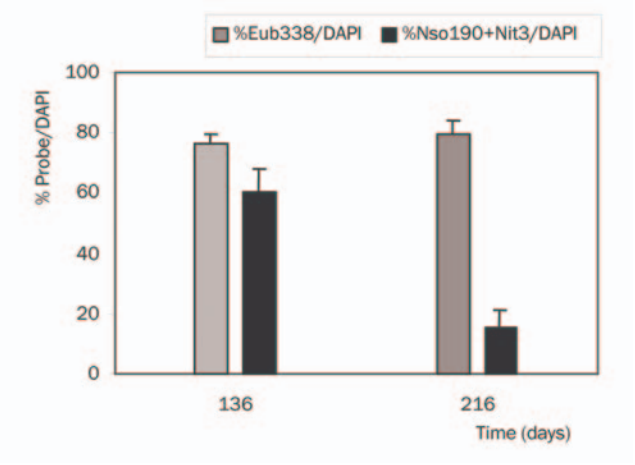


Figure 3.10 Percentage of cells hybridized with probe Eub338 and sum of the percentages of cells hybridized with probes Nso190 and Nit3 on total DAPI counts in the steady state and when limiting ammonium input in the whole bioreactor.

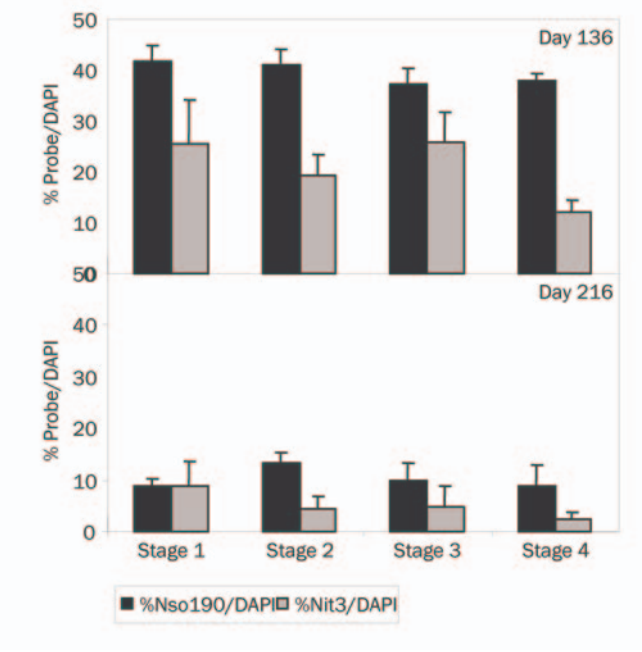


Figure 3.11 Percentages of bacteria hybridized with the two probes Nso190 and Nit3 on total DAPI counts in the steady state (day 136) and when limiting the ammonium input (day 216).

3.2.4.3 Epifluorescence microscope observations

Although cells hybridized with probes Nso190 and Nit3 still formed dense aggregates, free hybridized cells were also observed. Cells hybridized with Nit3 were bigger and formed denser aggregates than the cells hybridized with the probe Nso190, as previously observed.

Nevertheless, microscopic observation revealed in this case the existence of numerous filamentous cells. These cells stained with DAPI and hybridized with the probe Eub338, but did not with the probes specific for nitrifying bacteria or with Bet42a (Figure 3.12.).

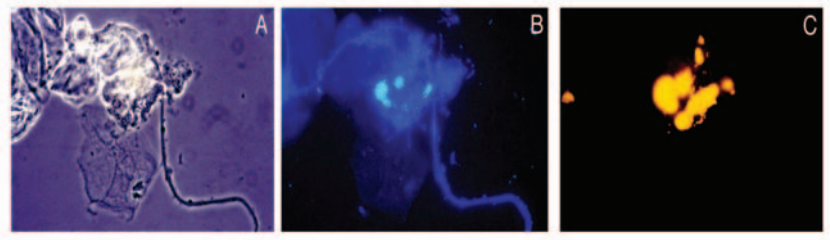


Figure 3.12 Micrography showing cells present in SBC first with light field microscopy (A), stained with DAPI (B) and hybridized with CY3 labelled Bet42a probe (C).

3.3 DISCUSION

3.3.1 Evaluation of cell percentages detected by FISH in the biofilms

The probes used in this work target different areas of the 16S rRNA sequence. The accessibility of the probes varies according to the 16S rRNA secondary structure. In this work, the percentage of cells hybridized with the probe Eub338, on total DAPI counts, was always above 70% (Table 3.1). Although this measure ignores the difficulties for any probe to find its target, it is an excellent indicator of the amount of cells taken into consideration in this study. Eub338 should hybridize with any eubacteria present in the sample, whereas DAPI stains all DNA. If we consider that most of the cells containing DNA were eubacteria, we can state that at least a 70% of these microorganisms were permeable to the probes, and therefore, susceptible of being hybridized. Thus, the results hereby presented would be valid at least for a 70% of the population in the biodiscs.

3.3.2 Colonization process

Proportions of ammonia- and nitrite-oxidizing bacteria (as detected by hybridization with probes Nso190 and Nit3, respectively) on total eubacteria counts (hybridized with probe Eub338), showed a good correlation with ammonium removal values (Figure 3.13). Both bacterial groups increased their presence among eubacteria from the start up of nitrification. Likewise, maximum AOB and NOB percentages were detected when the highest value for nitrogen elimination was obtained (day 60).

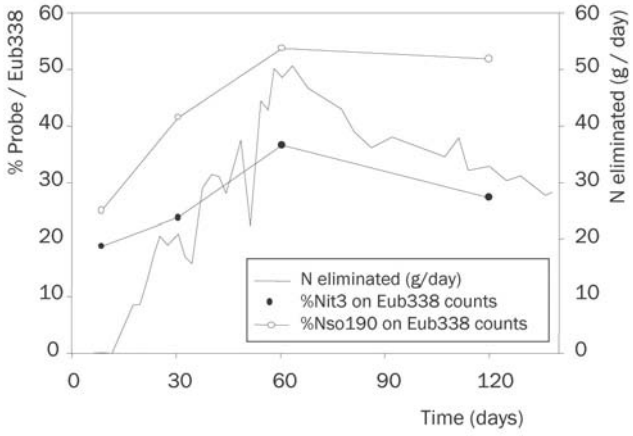


Figure 3.13 Relationship between the percentages of cells hybridized with probes Nso190 and Nit3 on total Eub338 hibridyzed cell counts and the nitrogen eliminated (in g of nitrogen per day) along the colonization period in the whole bioreactor.

Higher percentages of Nso190 were detected along the whole colonization process, in all stages and in all samples (Figure 3.13), which is probably due to a higher activity of nitrite oxidizing bacteria. Besides, since AOB produce the substrate NOB need to grow, their relationship is syntrophic. Moreover, nitrite is toxic to ammonia-oxidizers, who need the NOB to metabolize this compound and convert it to nitrate. Therefore, the proportion between both bacterial groups should remain constant when the population stabilizes. Calculated percentages of cells hybridized with Nso190 and Nit3 in the three first stages was quite constant from the first sampling date (Figure 3.14). Our results indicate that the proportion of ammonia- and nitrite- oxidizers stabilized quickly after the start-up of the pilot plant (in less than 28 days), and that when both percentages increased, the proportion of AOB and NOB remained constant. Nevertheless, the fourth stage needed 50 days to achieve the same percentages than the rest. This may be due to the low inputs of ammonium received

by this last stage, in which the colonization process was slower. AOB and NOB proportions also stabilized later in this stage.

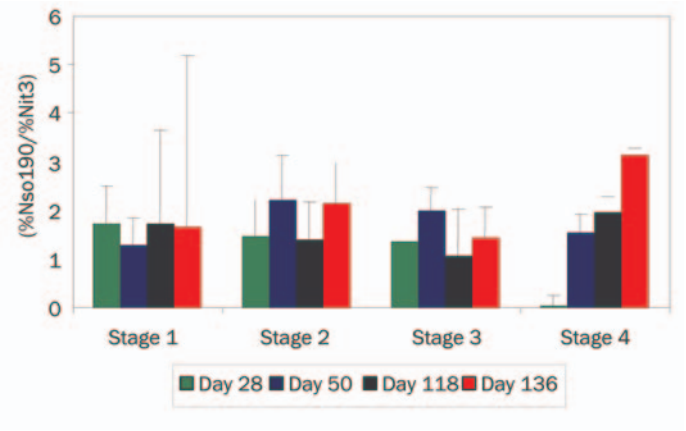


Figura 3.14 Ratio of the percentages of Nso190 (AOB group) and Nit3 (NOB group) hybridized cells during the colonization period.

3.3.2.1 Sequential colonization

The metabolic relationship between AOB and NOB leads to the formulation of a sequential colonization hypothesis. That is, a first colonization performed by ammonia oxidizing cells (Nso190 hybridization) which transform ammonium to nitrite. Then nitrite oxidizing cells start growing (Nit3 hybridization) and nitrite is finally converted to nitrate. Nitrogen compounds analysis (Amer, 1998) revealed a slight accumulation of nitrite in the first periods of colonization (Figure 3.4). Figure 3.15 illustrates the values obtained for the first days of colonization in the first stage, and shows a nitrite peak. It was measured between days 12 and 24, and corresponded to an increase of ammonium removal. Moreover, nitrate appears later, on the day 18, supporting the hypothesis of a sequential colonization. In Figure 3.16, especially in the fourth stage, the appearance of NOB days after the growth of AOB is also shown.

Colonization in the four stages is shown in Figure 3.16. Steady state was first reached by the two first stages. This indicates a fast colonization by both bacterial groups. In the two last stages colonization was slower, but after some days, the same values obtained by the two first were reached. This differential colonization process is a clear effect of the gradual decrease of ammonium

concentration from the first to the last stage, and is a consequence of the arrangement in series of the biodiscs.

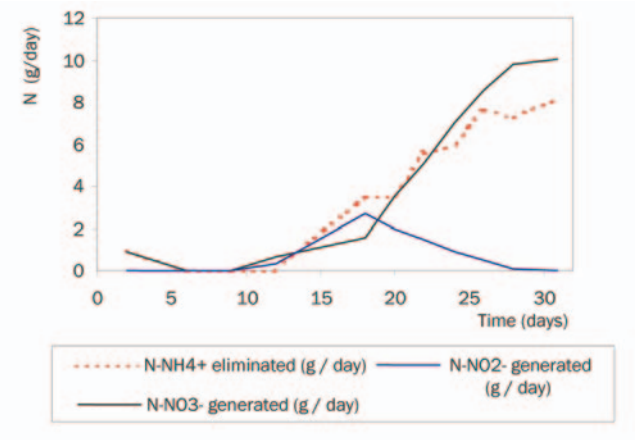


Figure 3.15 Evolution of ammonia eliminated and nitrite and nitrate generated during the first 30 days of the colonization of the first stage of the bioreactor.

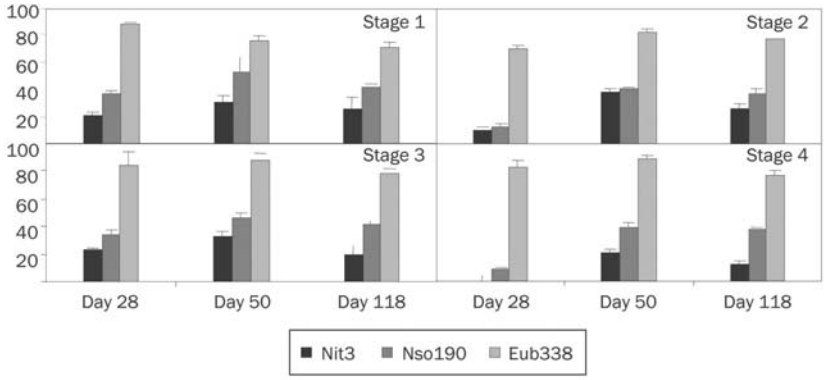


Figure 3.16 Evolution of the percentages of cells hibrydized with probes Eub338, Nit3 and Nso190 on total DAPI counts, for the four stages of the bioreactor along the colonization period of the biodiscs.

3.3.2.2 Spatial disposition of nitrifying bacteria

Both Nso190 and Nit3-hybridizing cells were observed after hybridization as free cells or forming aggregates. Mobarry *et al.* (1996) described *Nitro-*

somonas spp. and *Nitrobacter* spp. forming dense cell aggregates when hybridizing with probes NEU (specific for certain AOB) and Nit3 respectively. Both kinds of aggregates were found to be located near each other, which reflects the syntrophic relationship between ammonia and nitrite-oxidizing bacteria. Nitrite toxicity and the low yields obtained from its oxidation could be two factors promoting the association of ammonium- and nitrite-oxidizing aggregates. This competitive advantage would explain the highest proportion of aggregated cells in our samples.

3.3.3 Effects of ammonium limitation on the nitrifying bacteria

As reported in section 3.2.4.1, ammonium decrease mainly affected the two last stages of the pilot plant. Both AOB and NOB decreased drastically (Table 3.3), but the proportion of eubacteria (cells hybridizing with probe Eub338 on total DAPI counts) remained stable with the same values presented in the steady state. This indicates that the decrease of ammonium concentration caused a substitution of the nitrifying bacteria by other, probably heterotrophic, bacterial species.

Table 3.3 Percentage of cells hybridized with probe Eub338 and sum of the percentages of cells hybridized with probes Nso190 and Nit3 on total DAPI counts in the steady state and when limiting ammonium input in the whole bioreactor.

	%Nso190/DAPI + %Nit3/DAPI	%Eub338 / DAPI
Dia 136	60,12 ± 8,02	76,30 ± 3,20
Dia 216	15,32 ± 5,72	79,19 ± 4,47

Limitation of ammonium is also reflected along the four stages (Figure 3.17). The two first stages behave the same way, but percentages of cells hybridized with the probes specific for nitrifying bacteria, decreased in the third and especially in the fourth stage.

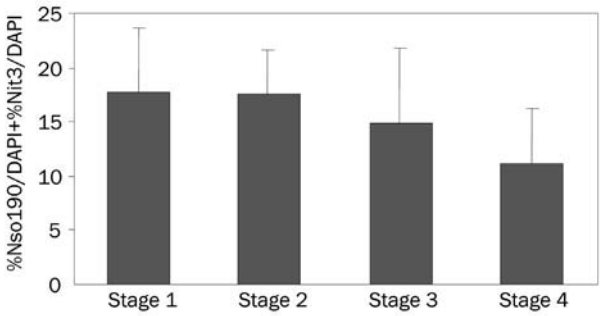


Figure 3.17 Evolution of the sum of the percentages of cells hibrydized with probesNit3 and Nso190 in the four stages of the bioreactor in limited ammonia input conditions (day 216).

As shown in table 3.4, ammonium oxidation rate maintained the same values calculated in the steady state in the first and second stages, even when ammonium input was limited. In contrast, ammonium oxidation was found to be slower in the third, and particularly in the fourth stage.

Table 3.4 Ammonia oxidation rates (in g/m^2day) in the four stages when ammonia concentration was reduced on the bioreactor.

	Stage 1	Stage 2	Stage 3	Stage 4
Ammonium oxidation rate	1.2047 g/m^2day	1.1004 g/m^2day	0.4814 g/m^2day	0.2206 g/m^2day

In the steady state, ammonium concentration was high, and was oxidized at maximum rate in the four stages (Fig 3.18). When reducing ammonium input a different behaviour of the four stages was noticed. Stages 1 and 2 continued oxidizing ammonium at maximum rate, but percentages of Nso190 and Nit3 hybridized cells decreased. Besides, in the two last stages, when both ammonium input and ammonium oxidation rate were lower, the number of Nso190 and Nit3 hybridized cells was lower than in the steady state.

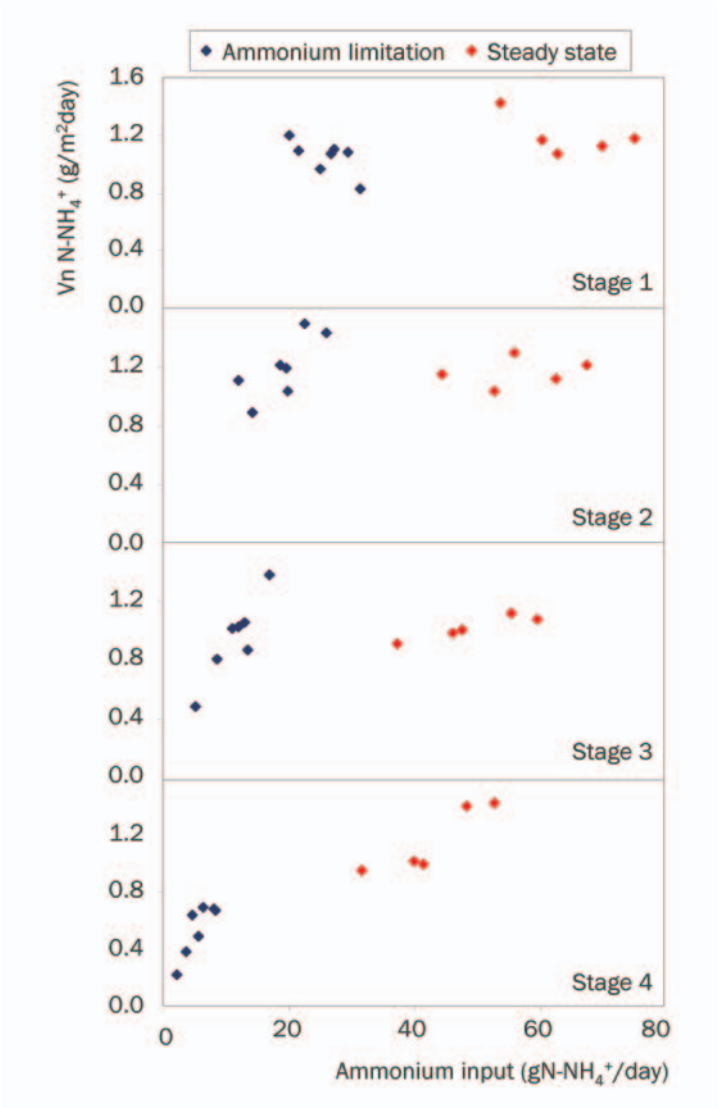


Figure 3.18 Behaviour of ammonia oxidation rate (in g/m2day) with respect to the quantity of ammonium added to every stage during the steady state (red dots) and while limiting the ammonium input (blue dots).

3.3.3.1 Presence of filamentous microorganisms when limiting ammonium input

Microscopic observations revealed the presence of filamentous cells when ammonium concentrations decreased. These cells hybridized with Eub338 probe but not with Bet42a, and had never been detected along colonization or in the steady state. Filamentous bulking is a situation that can affect activated sludge-based wastewater treatment plants and implies a fracture of biological equilibrium. This situation is characterized by a deficient solid-liquid separation in the secondary tank, and as a consequence, the effluent presents a reduced quality. Conditions favouring the growth of certain filamentous organisms are well known (low dissolved oxygen, pH variations, nutrient lack, sulphur presence, variable organic loads or low food/microorganisms (F/M) proportion (Balaguer *et al.*, 1998). When ammonium concentration decreased in the pilot plant, the percentage of nitrifying bacteria on total DAPI counts decreased too, whereas the percentage of eubacteria remained the same as in the steady state. Therefore, the number of heterotrophic microorganisms must have increased in the biodiscs of the pilot plant. Since the concentration of organic matter entering the plant did not vary, an increase in the number of heterotrophic microorganisms may have caused a low F/M proportion, which is one of the factors leading to a filamentous bulking situation (Balaguer *et al.*, 1998). Since low F/M proportions trigger bulking in activated sludge, it may explain the great number of filamentous cells detected in the biofilms hereby studied. A bigger area/volume proportion would represent a clear advantage for these cells when exchanging compounds with the medium. Besides, nitrification generates protons, causing an acidification of the medium. When limiting ammonium, pH increases (compared to that of the steady state), allowing the growth of certain microorganisms such as filamentous cells. In addition, pH variations might also determine bulking (Balaguer *et al.*, 1998).

3.4 CONCLUDING REMARKS

The colonization and effect of ammonium limitation in a submerged biological contactor has been studied using fluorescence in situ hybridization techniques (FISH).

Hybridization conditions for probes Nso190 and Nit3 have been established in 35 and 17.5% formamide in hybridization buffer, respectively. The calculated percentage of Eub338 hybridized cells indicated that along the whole study and in all the stages, more than 70% of the microorganisms inhabiting the

biodiscs were susceptible of being hybridized with oligonucleotide probes. Microorganisms from the secondary effluent of Girona's wastewater treatment plant colonized spontaneously the biodiscs of the pilot plant.

Nitrification started between the days 12 and 18 from the start-up of the pilot plant. From that moment percentages of cells hybridizing with Nso190 and Nit3 on total DAPI counts increased concomitantly with ammonium oxidation rate. After two months, 50% of the cells hybridized with Nso190 probe, 30% with probe Nit3, and ammonium oxidation rate set on 2 g N m^{-2} day^{-1}. Steady state was achieved four months after the start-up, with 40 and 20% cells hybridized with Nso190 and Nit3 respectively, and an ammonium oxidation rate of 1 g N m^{-2} day^{-1} in all the stages. Proportions between percentages of cells hybridizing with both nitrifyiers-targeted probes were constant in the steady state, probably due to the syntrophic relationship of ammonium- and nitrite-oxidizing groups. Colonization of the biodiscs was sequential: NOB (cells oxidizing nitrite) colonized the biofilms after a first appearance of cells hybridizing with Nso190 probe (AOB). The arrangement in series of the four stages of the pilot plant caused a delay in the steady state achievement in the third and fourth stages. Nso190 and Nit3 hybridized cells were found principally forming dense aggregates with variable morphology.

A decrease in ammonium input caused a reduction in the percentages of cells hybridizing with Nso190 and Nit3 probes on total DAPI counts. Moreover, filamentous eubacterial cells, non β-Proteobacteria, were detected under these circumstances. Ammonium oxidation rate was only altered in the two last stages, where ammonium was insufficient.

Fluorescence in situ hybridization technique proved to be useful for assessing quantitative studies in environmental samples. Nevertheless, since the probe Nso190 used hereby detects only previously known ammonia oxidizers from the β- Proteobacteria, and does not take into consideration other AOB subgroups, other techniques should be used to ensure a whole coverage of the ammonia oxidizer bacteria group. Although this technique allowed *in situ* detection and quantification of the AOB in our samples, it presented several disadvantages such as irregularity of the background and signal autofluorescence, and even material from the same slide has been shown to be unevenly fluorescent as previously reported by Nederlof *et al.* (1992). Moreover, since not all the sequences present in our samples are known, the possibility that this probe hybridizes with non-ammonia oxidizers must also be considered. The same analysis is applicable to the results obtained with probe Nit3. Besides, FISH is not useful for phylogenetic or taxonomical purposes, which lead us to the development and application of other sequence-based techniques.

4 Nso1225 PROBE AS A PRIMER FOR FINGERPRINT ANALYSIS OF AOB COMMUNITIES

4.1 INTRODUCTION

Phylogenetically, the cultured ammonia–oxidizing bacteria are found in both the β– and the γ–subdivisions of the *Proteobacteria*. The difficulty in obtaining pure cultures and the low growth rates of the autotrophic ammonia oxidizers have, however, hindered the characterisation of their natural populations.

Known ammonia oxidizers comprise two monophyletic groups based on the comparison of 16S rDNA sequences from cultured strains. One of these consists of strains of *Nitrosococcus oceanus* within the γ–subdivision of the *Proteobacteria*, while the other contains the genera *Nitrosomonas* and *Nitrosospira* within the β-subdivision of the *Proteobacteria*. *Nitrosococcus mobilis* is enclosed within the *Nitrosomonas*. Besides, the *Nitrosospira* group also now encompasses the genera *Nitrosolobus* and *Nitrosovibrio* (Head *et al.*, 1993; Teske *et al.*, 1994). Furthermore, *Nitrosolobus* has recently been suggested to be a separate genus from *Nitrosospira* (Aakra *et al.*, 2001).

Culture–independent techniques based on the analysis of 16S rRNA sequences have been used to examine the diversity of the ammonia–oxidizing bacteria of the β–*Proteobacteria*, in both terrestrial and marine habitats (Voytek and Ward, 1995; Stephen *et al.*, 1996; Juretschko *et al.*, 1998; Bothe *et al.*, 2000; Kowalchuk *et al.*, 2000). While 16S rDNA sequences are standard for taxonomic and phylogenetic studies, insight into the phenotypic characteristics of the ammonia oxidizers requires further characterization at the physiological and molecular levels. Recently 16S rRNA and functional genes (*amoA*) have been compared as targets for the molecular analysis of ammonia–oxidizing bacteria in natural environments (Bothe *et al.*, 2000; Purkhold *et al.*, 2000).

Several studies have used the 16S rDNA sequence information to assess the diversity and distribution of the β–subdivision ammonia oxidizers in natural environments, using specific or semispecific PCR amplification of 16S rDNA followed by direct or cloning assisted sequence analysis (McCaig *et al.*, 1994; Hiorns *et al.*, 1995; Stephen *et al.*, 1996; Hovanec *et al.*, 1996). New sequences have been discovered and identified through phylogenetic analysis, but this approach is time–consuming and not well suited to the analysis of multiple samples in ecological studies. Recently the *amoA* gene sequence has also been used for the same purpose (Purkhold *et al.*, 2000).

Hybridization with oligonucleotide probes of directly extracted rRNA or 16S rDNA fragments recovered by PCR (Hiorns *et al.*, 1995; Voytek and Ward, 1995; Hovanec *et al.*, 1996) is quicker, but does not allow the identification of novel sequences or the detection of sequence variation outside of the probe target region. Whole–cell hybridization with fluorescent oligonucleotide probes provides precise spatial information (Wagner *et al.*, 1995; Mobarry *et al.*, 1996) although real–time PCR perhaps offers the best long–term potential for the accurate quantification of cell numbers (Hermansson and Lindgren, 2001).

Only a few studies have used fingerprinting techniques based on 16S rDNA sequences using the previously published primers NITA and NITB, CTO189f and CTO654r, CTO178f and CTO637r or β–AOBf and β––AOBr (McCaig *et al.*, 1994; Voytek and Ward, 1995; Kowalchuck *et al.*, 1997) to assess AOB diversity. In some cases, nested PCR must be used while, in other cases, the use of degenerate 16S rDNA targeted primers produces heterogeneous PCR products and hence multiple banding from single templates in the DGGE (Kowalchuck *et al.*, 1998; McCaig *et al.*, 1999; Ward *et al.*, 2000; Regan *et al.*, 2002).

According to Mobarry *et al.*, (1996; 1997), the broad range probe Nso1225, recognises only the 16S rRNA molecules from the AOB of the β–subgroup of the *Proteobacteria* with the exception of the closely related freshwater bacterium *Gallionella ferruginea* (Mobarry *et al.*, 1996). The Nso1225 FISH probe is widely used to perform specific AOB bacterial–counts from composite samples (Juretschko *et al.*, 1998; Daims *et al.*, 2001; Gieseke *et al.*, 2001).

The aim of this work was to find a new PCR-based fingerprinting tool for the assessment of ammonia oxidizers diversity in environmental samples by using 16S rRNA-targeted probes as primers. The high specificity of these oligonucleotides for the AOB makes most of them excellent candidates for improving the strictness and rapidity of the AOB-specific bacterial diversity analyses.

4.2 RESULTS

4.2.1 Probes considered to be used as primers

Initially the probes considered for being used as primers were Nso190, Nso1225, NmV, and Nsv443 (Table 2.4). Nso190 and Nso1225 were designed by Mobarry *et al.* (1996) for detecting all β-AOB in a sample. These probes have different capability of detecting some of the species of AOB. Thus, for example with Nso1225 we can detect *Nitrosomonas communis* and *Nitrosomonas eutropha*, whereas the use of Nso190 allows the detection of *Nitrosococcus mobilis*, the only cocci, ammonia oxidizer, enclosed within the β-subgroup of the Proteobacteria (Table 4.1). The probe NmV (Juretschko *et al.*, 1998), specific for *Nitrosococcus mobilis*, has been used to discard the presence of these cocci in our environmental samples. Besides, Mobarry *et al.* (1996) also designed Nsv443, a probe with specificity for the members of the *Nitrosospira* lineage.

Using an AOB-specific primer together with a universal primer for eubacteria should yield an AOB-specific PCR product with appropriate size and characteristics for being run in a denaturing gradient gel electrophoresis. The primer sets tested in this work were: Eub787f with Nso1225r, Eub27f with Nsv443, and NmVf with Eub907r (section 2.3.4.2).

In order to test the potential range of detection and specificity of the probes to use as primers, an online analysis ("probe match" and "sequence match" analyses) was performed in the Ribosomal Database Project web (HYPERLINK "http://35.8.164.52" http://rdp8.cme.msu.edu/). As a result, we obtained a list of the species detected by the original probes, and a list of the species detected with one mismatch, one insertion, and one deletion in the sequence of the probes (Table 4.1).

Table 4.1 Main characteristics of the probes selected for being used as a primers. Specificity with one mismatch, deletion or insertion is indicated (from RDP (http://rdp.cme.msu.edu/html).

	Nso190	Nso1225	NmV	Nsv443
Reference	Mobarry *et al.*, 1996	Mobarry *et al.*, 1996	Juretschko *et al.*, 1998	Mobarry *et al.*, 1996
Sequence (5'-3')	**CGATCCCCTGCTTTTCTCC**	**CGCCATTGTATTACGTGTGA**	**TCCTCAGAGACTACGCGG**	**CCGTGACCGTTTCGTTCCG**
Target position in agreement with *E. coli* numeration	190-208	1225-1244	174-191	444-462
Specificity	*Nitrosospira tenuis* *Nitrosospira multiformis* *Nitrosospira briensis* *Nitrosospira sp.* *Nitrosomonas europaea* *Nitrosomonas halophila* *Nitrosomonas marina* *Nitrosomonas cryotolerans* *Nitrosococcus mobilis*	*Gallionella ferruginea* *Nitrosospira tenuis* *Nitrosospira multiformis* *Nitrosospira briensis* *Nitrosospira sp.* *Nitrosomonas communis* *Nitrosomonas europaea* *Nitrosomonas eutropha* *Nitrosomonas halophila* *Nitrosomonas marina* *Nitrosomonas cryotolerans* *Nitrosomonas ureae*	*Nitrosococcus mobilis*	*Nitrosospira tenuis* *Nitrosospira multiformis* *Nitrosospira briensis* *Nitrosospira* sp.
Specificity with 1 mismatch	No changes	*Chlamydomonas* spp. *Hydrogenophilus* spp. *Nitrosococcus mobilis*	*Legionella* spp. *Pasteurella* spp.	No changes
Specificity with 1 insertion	No changes	No changes	No changes	No changes
Specificity with 1 deletion	No changes	*Methylophilus methylotrophus* *Spirillum volutans* *Methylobacter* spp.	No changes	No changes

4.2.1.1 Eubacterial diversity of the samples

Eubacterial abundance and diversity in the samples from the biodiscs of Campdorà (section 2.1.1.1) were analyzed by using the universal primers Eub1492r and Eub984f_GC (Lane 1991) in a PCR+DGGE analysis (see sections 2.3.4 and 2.3.5.4). As a result the gel presented in Figure 4.1 was obtained.

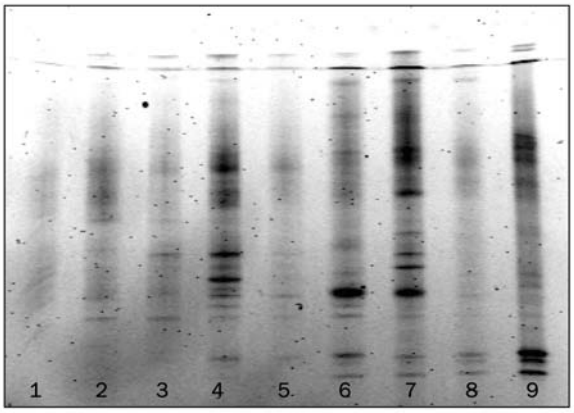


Figure 4.1 Denaturing gradient gel electrophoresis with the PCR products obtained by using the primers Eub984f and Eub1492r. The lanes contain different samples from the biodiscs of Campdorà (section 2.1.1.1): 1. 051998_Stage1; 2. 080698_B2; . 020499_Stage1; 4. 061499_Stage1; 5. 082099_Stage2; 6. 080698_Stage1; 7. 020499__Stage2; 8. 061499_Stage2; 9. 082099_Stage1.

The great number of bands obtained indicated high eubacterial diversity in the samples. Most of the bands were present in all the lanes of the gel, which indicated a similar microbial composition of the several samples analyzed in this study. The differences in the intensity of these bands points to distinct abundances of the members of these communities. Nevertheless, since band intensities may be due to biases in the DNA purification or in the PCR, they cannot be used for quantification. The considerable number and their tightness in the gel impeded the recovery of single bands.

4.2.1.2 Testing the presence of *Nitrosococcus mobilis*

With the goal of either detecting or discarding the presence of *Nitrosococcus mobilis* in the samples, a PCR reaction was prepared as previously specified (see section 2.3.4). The primers NmVf and Eub907r, specific for *Nc mobilis*,

and for all eubacterial 16S rDNA molecules, respectively, were used. The presence of this organism was discarded since no product was detected when running the PCR products in an agarose gel (section 2.3.5.2). Thus, the sequence of the probe Nso1225, which detects more species of *Nitrosomonas* but presents a 3' end mismatch with the sequence of *Nc mobilis*, was chosen instead of Nso190 (Tables 4.1, and 4.3).

4.2.1.3 First approaches with Nsv443 and Nso1225

As a first approach, some of the environmental samples were amplified by using the sets of primers Eub27f-Nsv443r and Eub787f-Nso1225r with a GC clamp on the 5' end (see section 2.3.5.4).

The PCR products obtained in both cases were run in a denaturing gradient gel electrophoresis, and the obtained DNA bands were recovered from the gel as specified in section 2.3.5.4.

In the case of Eub27f-Nsv443r, the resulting bands presented very low intensities (Figure 4.2). Although 5 bands were cut, it was impossible to recover the DNA from the acrylamide, and reamplificate it by PCR. A poor presence of the lineage of *Nitrosospira* in the sample, could explain these difficulties.

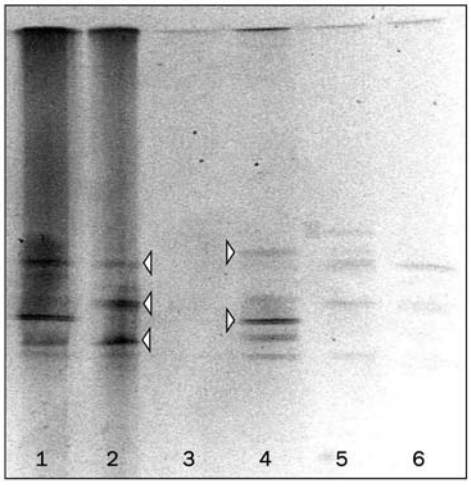


Figure 4.2. Denaturing gradient gel electrophoresis with the PCR products obtained by using the primers Nsv443rand Eub27f. The lanes contain different samples from the biodiscs of Campdorà (section 2.1.1.1): 1. 061499_ Stage1; 2. 082099_Stage1; 3. 080698_Stage4; 4. 020499__ Stage4; 5. 061499_Stage4; 6. 082099_Stage4. Bands cut from the gel are indicated.

In contrast, the use of Eub787f and Nso1225r resulted in sharp, well defined bands (Figure 4.3). The two bands cut from the positive controls run on the gel were reamplified and sequenced (see sections 2.3.5.4, 2.3.4.4, and 2.3.7). Sequence similarity Blast analysis (section2.3.8.2) demonstrated the two obtained sequences to belong to the 16S rDNA gene of *Nitrosomonas eutropha* strain Nm57, and *Nitrosospira* sp. strain Nv6, respectively.

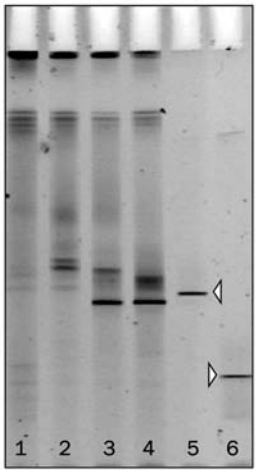


Figure 4.3. Denaturing gradient gel electrophoresis with the PCR products obtained by using the primers Eub787f and Nso1225r. The lanes contain different samples from the biodiscs of Campdorà (section 2.1.1.1), and two positive controls: 1. 051998_Stage3; 2. 080698_Stage4; 3. 020499_Stage2; 4. 082099_Stage1; 5. *Nitrosomonas eutropha* strain Nm57; 6. *Nitrosospira* sp. strain Nv6.

Although the use of Eub27f and Nsv443r was ruled out, further efforts should be made in order to set the proper conditions for these primers to detect and amplify *Nitrosospira's* 16S rDNA.

Besides, since the pair of primers Eub787f-Nso1225r enclosed most of the β-ammonia oxidizing bacteria, and seemed to work better than the pair Eub27f-Nsv443r, we decided to perform specificity analyses, and find the best PCR conditions for further usage in a broader study with environmental samples from several origins.

4.2.2 Eub787f-Nso1225r primers specificity

The specificity of primers Nso1225 and Eub787f was tested against a series of DNA template concentrations starting at 10 ng from 16 strains of β–AOB, β–MOB *Methylophilus methylotrophus* DSM 46235 and γ–MOB *Methylomonas methanica* NCIMB 11130 (Table 4.2).

Strain	Relevant characteristics	Accession number	Source or reference
Nm europaea Nm50	Soil (USA)	M96399	Type strain NCIMB11850
Nm europaea		ATCC19781	ATCC 19718
Nm eutropha Nm57	Municipal sewage (Chicago)	AJ298739	Koops and Harms, 1985
Nm sp. Nm33	Soil (Japan)		Koops *et al.*, 1991
Nm communis *Nm2*	Soil (Corfu)	AJ298732	Koops *et al.*, 1991
Nm aestuarii Nm36	Sea water (North Sea)		Koops *et al.*, 1991
Ns sp. 40KI	Loam (Norway)	X84656	Utåker *et al.*, 1995
Ns sp. Ka4	Lead contaminated soil (Norway)		Aakra *et al.*, 1999 a,b
Ns sp. B6	Sewage treatment column (Norway)	X84656	Utåker *et al.*, 1995
Ns sp. Nv6	Soil, Danlo Pas (Papua New Guinea)		Koops and Harms, 1985
Ns sp. Nsp1	Soil (Sardinia)	AJ298744	Koops and Harms, 1985
Ns multiforme NI13	Soil (Surinam) NCIMB 11849	L35509	Koops and Harms, 1985
Ns sp. Nsp17	Soil (Iceland)	AJ298743	Koops and Harms, 1985
Ns sp. Nsp2	Soil (Hamburg)	AJ298745	Koops and Harms, 1985
Ns sp. A4	Agricultural soil (Norway)	AJ005543	Aakra *et al.*, 1999 a,b
Ns sp. AF	Sandy Loam (Zambia)	X84658	Utåker *et al.*, 1995
Methylophilus methylotrophus	β–subgroup *Proteobacteria*	DSM 46235	DSM 46235
Methylomonas methanica	γ–subgroup *Proteobacteria*	NCIMB 11130	NCIMB 11130

When performing the PCR at low Tm (52ºC), products of the expected size were obtained from all the templates, including *Methylomonas methanica,* a γ–MOB that has four mismatches with the Nso1225 sequence (Figure 4.4 and Table 4.3). An increased Tm of 56ºC resulted in no product formation from this γ–MOB, whereas the amplification still worked for all the other templates (Figure 4.4). Genomic DNA of *Escherichia coli* ATCC12407 was used as a negative control in all cases.

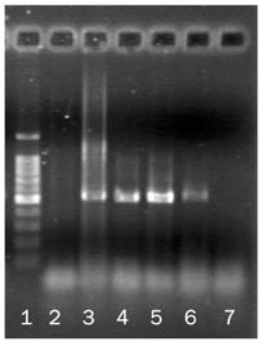


Figure 4.4 Amplification of 16S rRNA genes with Eub27f-Nso1225r by using Tm of 52ºC, and 56ºC. 1. 100bp molecular weight ladder; 2. *Methylomonas methanica* NCIMB11130 at Tm=56ºC; 3. *Nitrosomonas eutropha* strain Nm57 at Tm=56ºC; 4. *Nitrosospira* sp. strain Nv6 at Tm=52ºC; 5. *Nitrosomonas eutropha* strain Nm57 at Tm=52ºC; 6. *Methylomonas methanica* NCIMB11130 at Tm=52ºC; 7. Non template control.

Table 4.3 Target sequences of β–AOB, *Methylophilus methylotrophus*, *Methylomonas methanica*, *Thauera terpenica*, *Ralstonia paucula*, *Azoarcus* sp., and *Gallionella ferruginea* for the primer Nso1225.

Nso1225 sequence	5'	CGC CAT TGT ATT ACG TGT GA	3'
Nitrosomonas europaea target	3'	GCG GTA ACA TAA TGC ACA CT	5'
Nitrosomonas eutropha target	3'	... ...	5'
Nitrosospira briensis target	3'	... ...	5'
Nitrosococcus mobilis target	3'	.A.	5'
Methylophilus methylotrophus target	3'	CT.	5'
Methylomonas methanica target	3'	C.. ... ... .CG ... ... .A	5'
Thauera terpenica target	3'	... ..A –G. .G. ..T GG. T.	5'
Ralstonia paucula target	3'	... ..A –G. .G. ..T GG. T.	5'
Azoarcus sp. target	3'	... ..A –G. .G. ..T GG. T.	5'
Gallionella ferruginea target	3'	... ...	5'

4.2.3 DGGE analysis

PCR products obtained from Eub787f-Nso1225r amplification of several environmental samples were run in 20 to 50% denaturing gradient gel together with *Nm europaea* Nm50 NCIMB 11850 and *Nitrosospira* sp. 40KI (Figure 4.5). The denaturing behaviour of 16S rDNA fragments from environmental samples displayed two groups of bands. The first consisted of fragments being denatured between 32.5 to 40%, including standards of AOB. All samples showed between two and five conspicuous bands, which in some cases appeared in more than one lane, but all fingerprints differed from one another. The second group of bands showed up in all samples at approximately the same position (44% denaturant) and would probably be better resolved in a more appropriate gradient.

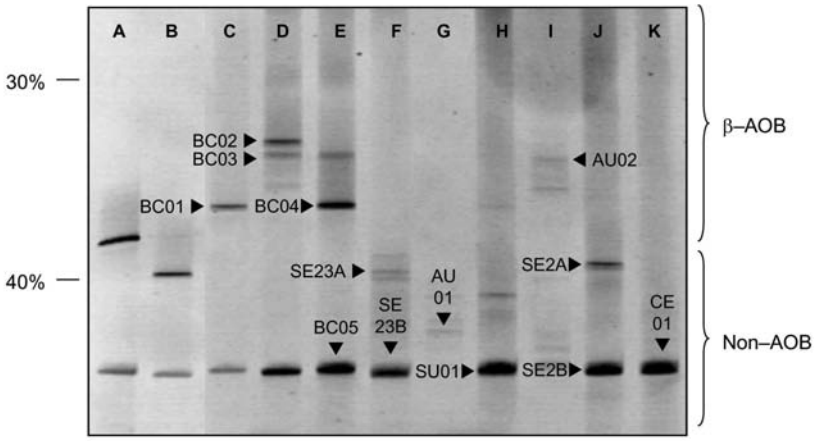


Figure 4.5 Denaturing gradient gel electrophoresis of partial 16S rDNA amplified with primers Nso1225 and Eub787. A. *Nitrosomonas europaea* Nm50; B. *Nitrosospira* sp. 40KI; C., D., E. Sample of a nitrifying biodisc from Campdorà (NE, Spain) (BC); F. SE23; G. Bioreactor (AU); H. Submerged biological contactor (SU); I. AU; J. SE2; K. Carrousel–WWTP of Empuriabrava (NE, Spain) (CE). The excised bands are indicated.

In order to determine the sequence affiliations of the DNA fragments detected, selected DGGE bands were excised for DNA isolation, reamplification and sequence analysis as formerly described (sections 2.3.4, 2.3.5, and 2.3.7).

4.2.4 Sequence analysis of excised bands

In general, the upper set of bands corresponded to AOB. Sequences from the nitrifying biodiscs in Campdorà (BC01 to 05) showed similarity percentages above 92% with known β–AOB (*Nitrosomonas* and *Nitrosospira* genus), and a sequence 97% similar to *Nitrosomonas nitrosa* was recovered from the activated sludge of the aerobic nitrifying reactor AU02 (Table 4.4). As previously observed for Nso1225 primer (Regan *et al.*, 2002), the sequence analyses also revealed the amplification of non–target sequences that generate the same fragment length as AOB after amplification with Nso1225–Eub787f, forming the lower set of bands. All these sequences showed similarity percentages higher than 91% with *Ralstonia* sp., *Thauera* sp., and *Methylophilus* sp. belonging to the β–subgroup of the *Proteobacteria* (Table 4.4).

Table 4.4 NCBI Blast similarity analyses of the sequenced excised bands in the DGGE. Only the highest similarities are indicated.

Band	Accession Number	(% similarity)	NCBI Blast
BC01	gi18447922	97%	*Nitrosomonas* sp. ENI–11
BC02	gi11545279	95%	*Nitrosomonas* sp. Nm33
BC03	gi545274	93%	*Nitrosomonas ureae*
BC04	gi18447922	98%	*Nitrosomonas* sp. ENI–11
BC05	gi24474438	92%	*Nitrosospira* sp. III7
SE2A	gi15419929	94%	*Ralstonia taiwanensis* str. LMG19424
SE2B	gi27884823	91%	*Pseudomonas rhizosphaerae*
SE23A	gi25264693	95%	*Methylophilus freyburgensis*
SE23B	gi9856923	91%	Agricultural soil bacterium clone SC–39
AU01	gi3820652	93%	β–proteobacterium pCyN2
AU02	gi11545285	97%	*Nitrosomonas nitrosa*
SU01	gi3123694	93%	*Thauera terpenica*
CE01	gi15811599	90%	Uncultured bacterium clone P30B–49

Phylogenetic analyses of the sequences derived from the DGGE were performed (Figure 4.6). Six of the 13 sequenced bands corresponded to the β–AOB group, one was similar to the β–MOB *Methylophilus methylotrophus*, and the rest (6) were found to belong to β–non AOB. Most of the sequences clustered congruently with the predicted BLAST similarities. Interestingly the highest similarities corresponded to sequences from AOB strains and not from any of the environmental clones in the databases. Moreover, the distances of the BC02, BC03 and BC05 from their closest relatives were relevant, particularly the latter whose affiliation appears to be unclear between the *Nm cryotolerans* and *Nm marina* clusters. In contrast, BC01 and BC04, as well as AU02, were found to be very similar to their cluster representatives *Nm europaea* and *Nm communis,* respectively. Sequence BC03 was 93% similar to *Nitrosomonas ureae* and clearly grouped within the *Nm oligotropha/Nm ureae* cluster. The highest similarities of sequences AU02 and BC02 corresponded to *Nm nitrosa* (97%) and *Nitrosomonas* sp. Nm33 (95%), respectively, both of which are included within the *Nm communis* cluster. Finally, sequences BC01 and BC04 showed identities of 97 and 98%, respectively with *Nitrosomonas* sp. ENI–11, and fell within the *Nm europaea/Nc mobilis* cluster.

The set of primers used provided a number of unspecific amplifications. Thus, sequence SE23A proved to be closely related (95% similarity) to *Methylophilus freyburgensis* of the β–MOB group. The rest of the β–non AOB sequences (SE2A, SE23B, CE01, AU01, and SU01) were clearly distinguishable in the DGGE gel. In particular CE01, SE2A and SE23B form a separate cluster of uncertain phylogenetic affiliation.

The SE2B sequence was found to be the sequence most distant from the target group, showing high similarity (91%) with *Pseudomonas* sp.

4.2.5 Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper were annotated and have been deposited in the EMBL database under accession numbers AJ555428 through AJ555440.

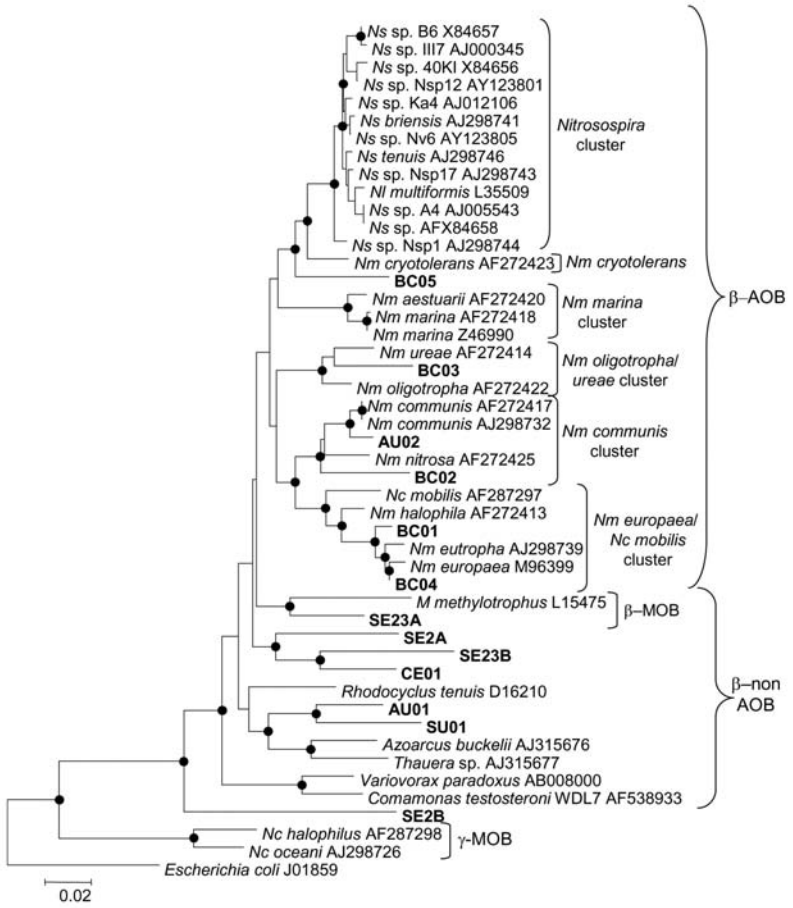


Figure 4.6 Neighbor–joining tree generated from an alignment of partial 16S rDNA sequences from AOB and several other non AOB β–Proteobacteria (*Methylophilus methylotrophus*, *Variovorax paradoxus*, *Comamonas testosteroni*, *Azoarcus buckelii*, *Thauera* sp., *Rhodocyclus tenuis*). AOB genus abbreviations are as follows; Nm: *Nitrosomonas*, Ns: *Nitrosospira*, and Nc: *Nitrosococcus*. Sequences corresponding to excised bands from the DGGE are highlighted. Branch points supported (bootstrap values >50%) by distance, parsimony and maximum likelihood analyses are indicated by solid circles. Scale represents 0.02% relative distance. Several groups have been indicated according to the clusters described by Purkhold et al. (2000). Two members of the γ–AOB, *Nitrosococcus halophilus* and *Nitrosococcus oceani*, and *Escherichia coli* were used as outgroups

4.3 DISCUSSION

Most studies of AOB diversity using 16S rDNA targeted primers have been performed in natural environments such as agricultural plots, marine sediments (Stephen *et al.*, 1996; Kowalchuck *et al.*, 1998), and the water column of lakes (Ward *et al.*, 2000) or the open central Arctic Ocean (Bano and Hollibaugh, 2000). Some works focus on the sediments of ecologically altered systems such as fish farms (McCaig *et al.*, 1999), but little work has been done so far on artificial systems such as WWTPs, reactors, and wetlands that are subjected to highly active nitrogen regimes. In addition, the methods used to date have mostly been based on PCR–amplification, followed by cloning and sequencing. In these studies either the degeneracy of the primers or the size of the amplified fragment do not permit the use of high resolution fingerprinting techniques such as DGGE, and the routine monitoring of AOB populations is not feasible. Here, we used the FISH probe Nso1225 as a primer because of its high specificity for β–AOB, and because it allows us to generate an adequate sized PCR fragment for DGGE. In combination with the eubacterial primer Eub787 (Lane 1991), a PCR fragment of ca. 500 bp can be obtained, which is sufficiently informative to follow up with phylogenetic inferences. Utåker and Nes (1998) concluded that Nso1225 was a good broad spectrum probe for the detection of β–AOB in environmental samples and suggested its use as a reverse primer in a PCR. However, even under optimal hybridization conditions, this probe also recognises some non–AOB bacteria such as *Gallionella ferruginea* (Mobarry *et al.*, 1996). This has to be taken into consideration when using it as a reverse primer, though it can be partially solved by selecting the appropriate annealing temperature in the PCR program. Specificity and sensitivity tests indicated that a Tm of 56ºC in the PCR program prevented non–specific amplifications, and produced good yields when using β–AOB as a template.

A further advantage of using Nso1225 as a primer is its greater sensitivity compared to that of the 16S rDNA targeted primers. In our analyses direct PCR products were obtained from genomic DNA. By contrast, previous studies report the need to perform nested PCR for most of the β–AOB specific primers previously described (Kowalchuk *et al.*1997, 1998; Regan *et al.*, 2002). The protocol used for DNA extraction (Moore, 1996) should also be taken into consideration since it leads to humic substances–free templates, which might partially explain the performance of our primers.

The bands in the DGGE gel displayed two types of denaturing behaviour. The 6 β–AOB related bands were observed between 32.5 and 37.5% denaturant, while the mobility of non–AOB bands ranged from 37.5 to 45% denaturant.

This two–zone denaturing pattern may be useful in discriminating between the ammonia oxidizers and the other organisms in a given sample. The bands observed at 44% denaturant in all the samples produced several sequences, most of which were found to belong to β–non AOB groups. These high G+C content bands would probably be better resolved in a stronger denaturing gradient, but since they were not β–AOB, the denaturing gradient used (20 to 50% urea/formamide) was considered suitable for the purposes of this study. With the exception of AU01 and CE01, the sequences phylogenetically included in non–AOB clusters were retrieved from oxygen–restricted wastewater treatment environments. These results suggest that although non β–AOB have also been detected in nitrifying oxygenated environments, the applicability of this technique to environments where oxygen is restricted might lead to non–specific amplifications.

Remarkably all the β–AOB related sequences recovered from the environments studied here were assigned to the genus *Nitrosomonas*, and no *Nitrosospira*–related sequences were detected. Since Nso1225–Eub787 primers were satisfactorily tested against standard *Nitrosospira* sp. genomic DNA, the absence of this group shows that it was undetectable in the samples studied.

Furthermore, specific FISH AOB cell counts using Nso1225 need to be interpreted in accordance with the origin of the samples. Thus, in anaerobic environments this group might be overestimated due to unspecific hybridization.

The protocol presented here provided a rapid and sensitive screening approach for assessing the diversity of ammonia–oxidizing bacteria, oriented to the monitoring of AOB communities in WWTP. As stated above, however, care must be taken when dealing with low–oxygenated samples. DGGE allows the detection of AOB in a few days and is not subjected to culturability biases. This represents a remarkable improvement over the minimum 3–week incubation period required for MPN enumeration (Matulewich *et al.*, 1975). Although the method is not quantitative in its present form, it could be applied as a simple presence/absence tool for the detection of ammonia–oxidizing bacteria in the plant.

5 MOLECULAR ANALYSIS OF NITROGEN–RELATED MICROORGANISMS IN A SBR PILOT PLANT

5.1 INTRODUCTION

The diversity and enumeration studies of AOB in environmental samples by conventional cultivation techniques have been shown to be time-consuming and unreliable due to both the isolation difficulties, and the slowness of their growth. Molecular methods provide rapid and reliable tools for the study of AOB in the environment.

The gene *amoA* has been recently used as an alternative target for the molecular analysis of ammonia oxidizing bacteria (Sinigalliano *et al.*, 1995; Rotthauwe *et al.*, 1997; Juretschko *et al.*, 1998; Stephen *et al.*, 1999; Norton *et al.*, 2002). This gene is integrated into the AMO operon, which encodes the membrane–bound multiple subunit enzyme ammonia monooxygenase. Since this enzyme is responsible for the oxidation of ammonia to hydroxilamine, is present in all the ammonia-oxidizers (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998). Thus, the study of the *amo* operon genes guarantees the enclosure of the whole physiological group of ammonia-oxidizing bacteria. In contrast, the study of 16S rDNA with specific primers is restricted to specific phylogenetic groups, overlooking other AOB (Calvó *et al.*, 2004).

In SBR technology (Sequencing Batch Reactor, based on a Fill-Reaction-Draw strategy) different operational conditions and cell residence time, applied ammonia load or aerobic-anoxic-anaerobic conditions can be set by programming a reaction phase with different DO set-points, raw wastewater loads or mixing conditions in order to achieve high nitrification and denitrification efficiencies.

Anaerobic ammonium oxidation (anammox) is a very recent addition to the understanding of the biological nitrogen cycle (Kuenen *et al.*, 2001; Strous *et al.*, 1999). Discovered recently, it so far is the most unexplored part of the cycle, and to date, only five new deep–branching Planctomycetes have been identified as the microorganisms responsible for "anammox": Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensis,* Candidatus *Scalindua brodae*, Candidatus *Scalindua wagneri*, and Candidatus *Scalindua sorokinii* (Egli *et al.*, 2001; Schmid *et al.*, 2000; Schmid *et al.*, 2003; Kuypers *et al.*2003).

The aim of this work was to study the nitrogen cycling-related microbial groups in a pilot plant SBR operating with high ammonia concentrations, and alternating different aerobic-anoxic-anaerobic conditions for nitrogen removal. Therefore, the diversity of ammonia oxidizing bacteria (AOB) was studied through partial *amoA* gene cloning. The presence of bacteria performing anaerobic ammonium oxidation (anammox) was tested by cloning the 16S rDNA planctomycete genes present in the sample.

5.2 RESULTS AND DISCUSSION

5.2.1 Ammonia Oxidizers in the SBR

After the DNA extraction, the PCR–amplified *amoA* fragments were cloned and transformed into *E. coli* DH5α high–efficient cells (section 2.3.6). Plasmid DNA from transformants was reamplified, and 46 cloned fragments were screened by digestion with the restriction enzymes AfaI and AluI (section 2.3.6.6). Four different types of restriction patterns were observed in diverse abundances, and were classified from A to D (Figure 1). Pattern A was only detected in one of the digested clones. Besides, 10% of the clones displayed pattern B, 14% exhibited pattern C and, finally, the rest of the clones (74%) showed pattern D.

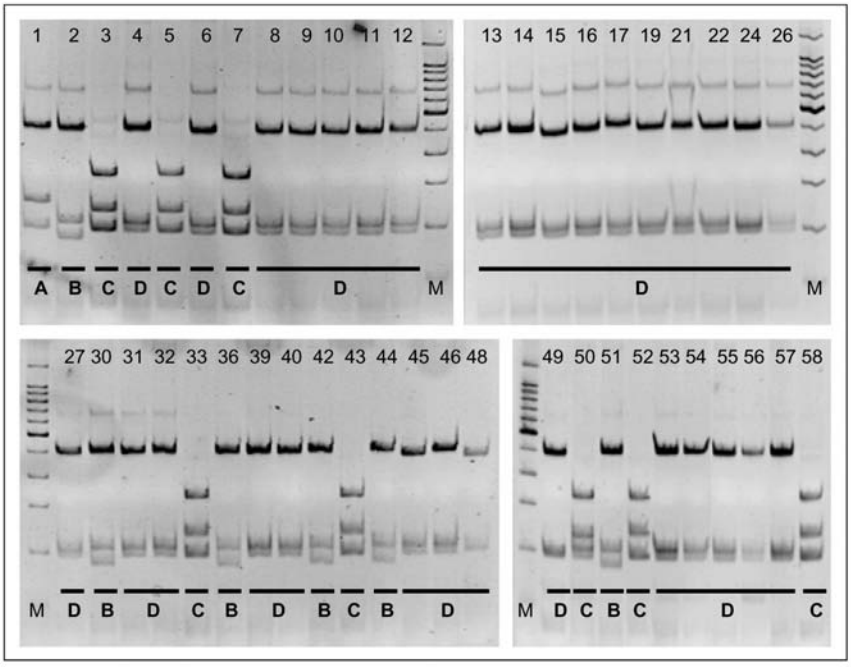


Figure 5.1 Acrylamide gel of restriction fragments of PCR-amplified partial *amoA* gene with primers amoA–1F and amoA–2R for SBR samples digested with restriction enzymes AfaI and AluI. Lanes M contain DNA size marker of 100 bp. All other lanes contain the indicated clones. The distinct restriction digest patterns, named A, B, C and D respectively, are indicated.

In order to check the reliability of our screening, a proportional number of clones representing each one of the restriction patterns were sequenced. A total of 12 high quality nucleotide sequences were obtained. Multiple sequence alignment and comparison of *amoA* partial sequences (section 2.3.8) revealed that the two sequences obtained for the B restriction pattern were identical. The same was observed for the three pattern–C sequences analyzed.

To reveal the closest phylogenetic relatives of the sequences hereby obtained, a consensus tree was built by using all three methods of inference: maximum parsimony, maximum likelihood and distance–minimal evolution (Figure 2) (section 2.3.8.3). Representative partial *amoA* sequences from the EMBL database were included in the analyses. As expected, the general topology of the tree obtained was similar to those based on 16S rDNA, 16S–23S rDNA intergenic spacer region (ISR), and previous partial *amoA* gene sequences studies (Norton *et al.*, 2002; Aakra *et al.*, 2001; Purkhold *et al.*, 2000) in that

the β– and γ–proteobacterial nitrifiers group in separate clades, as well as *Nitrosomonas* and *Nitrosospira* clusters.

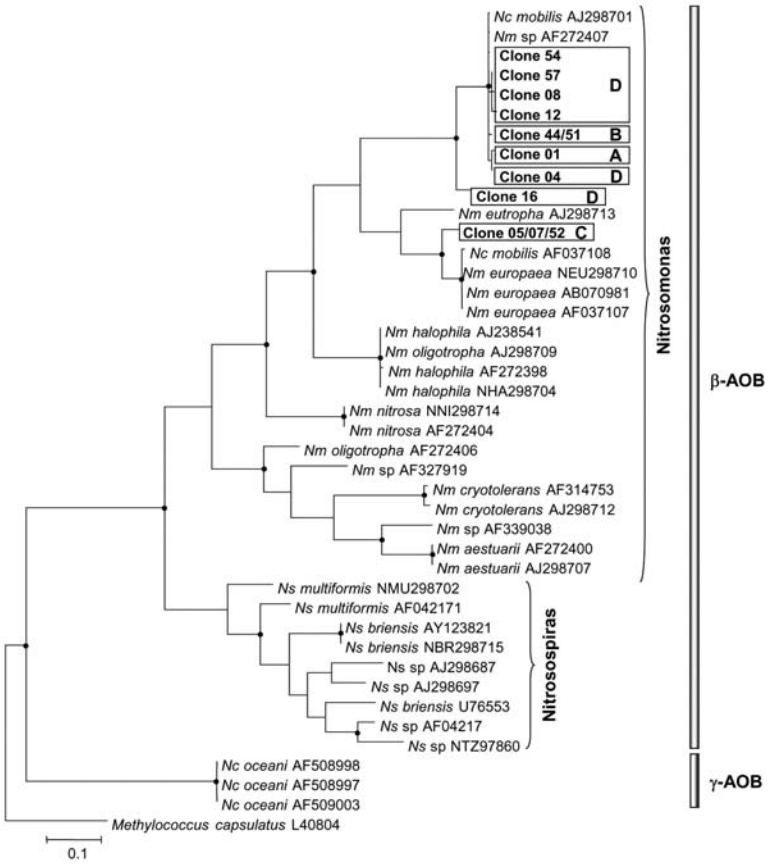


Figure 5.2 Neighbor–joining tree generated from an alignment of partial *amoA* sequences from AOB. Sequences obtained in this report are highlighted, and their restriction pattern group is indicated with A; B, C or D. Branch points supported (bootstrap values >90%) by distance, parsimony and maximum likelihood analyses are indicated by solid circles. Partial *pmoA* sequence from *Methylococcus capsulatus L40804* has been used as outgroup.

The small phylogenetic distances among the partial *amoA* gene cloned sequences seemed to indicate that, although they displayed four different restriction patterns when digesting them with AluI and AfaI, they are all close relatives. They all clearly group within the *Nitrosomonas* cluster, particularly

within the *Nm europaea / Nm eutropha / Nc mobilis* subgroup, as supported by high bootstrap values.

High quality partial *amoA* nucleotide sequences were used to deduce amino acid sequences (Figure 3) (section 2.3.9). Most of the changes in the DNA sequences affected the third position of the codon, with no translational effects. This is consistent with the fact that the *amoA* domain is thought to contain the active site of the enzyme ammonia-monooxygenase.

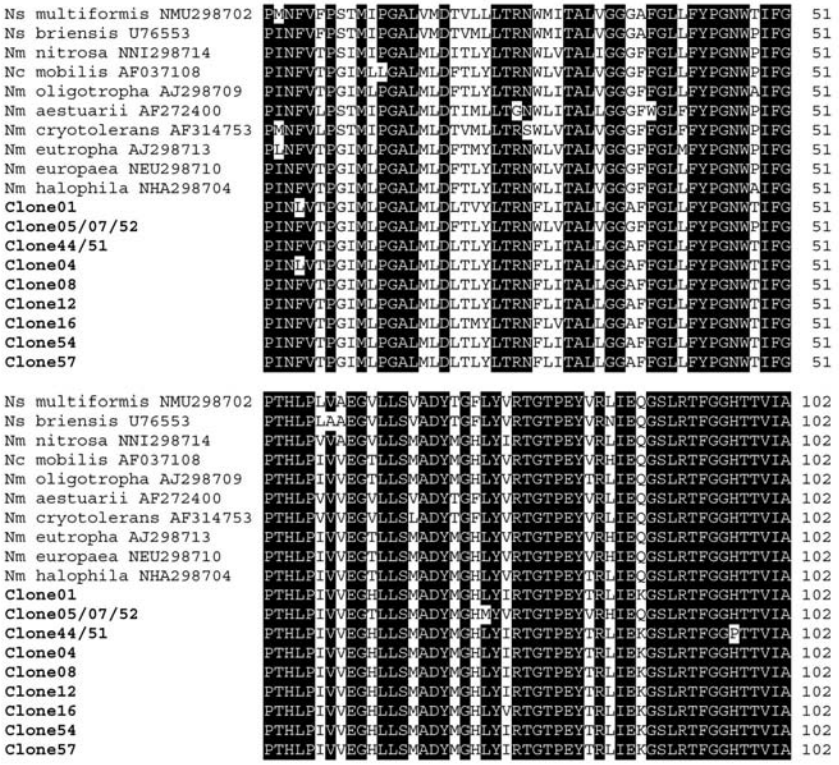


Figure 5.3 Deduced partial AmoA peptide sequences aligned. The titles of the sequences obtained in this report are in boldface. Inverse printed residues indicate conserved aminoacids in the alignment.

Only four amino acid changes were found among the sequences corresponding to the pattern D (Clones 04, 08, 12, 16, 54, and 57), and all of them were conservative. This indicates that although pattern D split into several clusters

when analyzing the DNA sequences, the sequence of the functional peptide is almost identical within the whole group. Nevertheless, some significant changes could be observed within the whole group of partial AmoA peptide sequences hereby presented, i.e. the change of an alanine to a glycine, and a threonine/alanine to a proline in positions 36 and 48 respectively (Fig 3) which could cause a change in the AmoA domain structure. In addition, some changes to hydrophobic from hydrophilic amino acids were also found.

5.2.2 Planctomycetales in the SBR

In the case of the partial 16S rDNA specific for Planctomycetes obtained by PCR with the primers Amx820 and Pla46, only one restriction pattern was observed after the digestion of the cloned inserts with AfaI and AluI. Eight randomly selected clones were sequenced.

To test whether the sequences obtained could or not be phylogenetically related to any sequence associated with “anammox” activity, several sequences from Planctomycetales were obtained from the EMBL database, and added to the phylogenetic analyses. Another consensus tree was built by using all three methods of inference: maximum parsimony, maximum likelihood and distance–minimal evolution analyses (Figure 4). Two major clusters supported by bootstrap values above 90% can be distinguished in the tree, clearly discriminating the “anammox” planctomycetes from the rest of the Planctomycetales. The sequences obtained from the SBR group were situated within the Planctomycetale 16S rDNA sequences from the databases, far from the “anammox” related cluster. Interestingly, although we detected only one restriction pattern within the sequences obtained for the Planctomycetes in the SBR, two different clusters, separated by considerable phylogenetic distance, could be seen in the tree. This sheds some doubts about the usefulness of RFLP techniques to distinguish between closely related phylogenetic sequences. Nevertheless, both of them clearly grouped apart from the “anammox” cluster; which allows us to conclude that no “anammox” related sequence was detected in the SBR.

Nucleotide sequences reported in this paper have been annotated and deposited in the EMBL database under accession numbers AJ575112 through AJ575128.

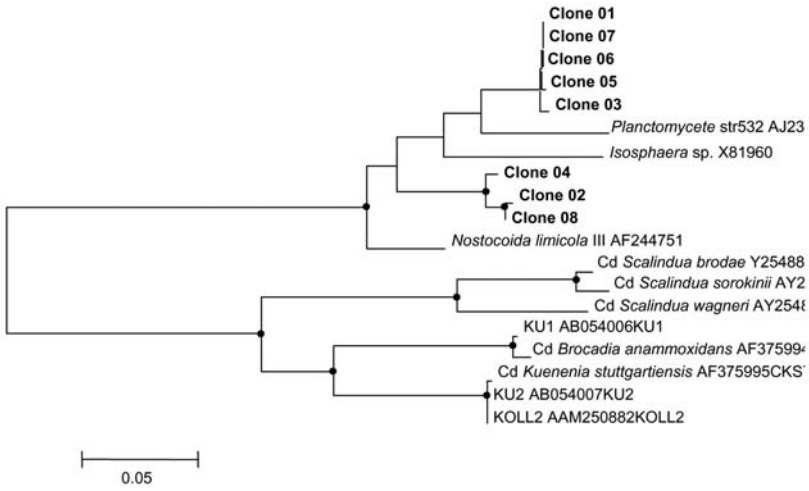


Figure 5.4 Neighbor–joining tree generated from an alignment of partial 16S rDNA sequences from Planctomycetes. Sequences obtained in this report are in boldface. Branch points supported (bootstrap values >90%) by distance, parsimony and maximum likelihood analyses are indicated by solid circles. The abbreviations are as follows: KU1 for uncultured anoxic sludge bacterium KU1, KU2 for Uncultured anoxic sludge bacterium KU1, KOLL2 for Anaerobic ammonium-oxidizing planctomycete KOLL2a, and Cd for *Candidatus*.

5.3 CONCLUDING REMARKS

As previously reported the use of the *amoA* gene as a molecular marker, has been shown to provide a sensitive screening for performing diversity studies in ammonia oxidizing communities Rotthauwe, 1997; Stephen *et al.*, 1999). Nevertheless, the process of cloning is time consuming, which is a handicap when multiple environmental samples have to be quickly analyzed. Moreover, when used for phylogeny inference the resolution obtained is still higher with 16S rDNA gene sequences than with *amoA*. Therefore, a new molecular marker is still needed, for obtaining a faster analytical tool and providing rigorous phylogenetic information.

6 USE OF *amoB* AS A NEW MOLECULAR MARKER FOR AOB

6.1 INTRODUCTION

In the last decades the number of ammonium-rich environments has increased drastically due to human activities. Therefore nitrification has recently become a widespread process affecting the global nitrogen cycling, of which the ammonia-oxidizing bacteria are an essential component. Due to their extremely slow growth, AOB are usually found in low numbers in natural environments, which makes them difficult to detect by traditional microbiological culture-dependent methods (Ward 1986).

In the last decade, culture independent molecular tools based on 16S rDNA have been developed to characterize the ammonia–oxidizing bacteria, and several authors have studied the diversity of the ammonia-oxidizers from both terrestrial and marine habitats (Voytek and Ward, 1995; Stephen *et al.*, 1996; Juretschko *et al.*, 1998; Bothe *et al.*, 2000; Kowalchuk *et al.*, 2000). In these studies, high similarities of partial 16S rDNA sequences of ammonia-oxidizers have been observed when dealing with environmental samples, suggesting that physiology and phenotype should also be considered to distinguish among all the strains of ammonia-oxidizers (Stephen *et al.*, 1996; Purkhold *et al.*, 2000).

AOB are obligate chemolitotrophs that obtain energy and reducing power from oxidation of ammonia (Bock *et al.*, 1986; Hooper 1989), starting at the conversion of ammonia to hydroxylamine. This step is catalyzed by the enzyme ammonia monooxygenase (AMO) (Hyman and Arp, 1992), exclusive to the ammonia-oxidizers. The genes encoding for AMO have been recently used as an alternative target for the molecular analysis of ammonia–oxidizing bacteria (Sinigalliano *et al.*, 1995; Rotthauwe *et al.*, 1997; Juretschko *et al.*, 1998;

Norton *et al.*, 2002). The enzyme AMO is composed of three subunits: AmoA, a 27– to 30–KDa membrane–bound protein containing the active site of AMO (Hyman and Arp, 1992), AmoB, of 38– to 43–KDa (Bergmann and Hooper, 1994)), and AmoC (Klotz *et al.*, 1997).

The AMO enzyme in ammonia–oxidizing bacteria is evolutionarily related to the particulate methane monooxygenase (pMMO), which is found in methane–oxidizing bacteria (Holmes *et al.*, 1995; Klotz and Norton, 1998). Both enzymes are encoded by a three-gene operon (Zahn and Dispirito, 1996; Nguyen *et al.*, 1998; Stolyar *et al.*, 1999; Gilbert *et al.*, 2000). The *amo* operon genes, such as *amoA*, are present in all the ammonia-oxidizers (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998) and their study ensures the enclosure of the whole physiological group of ammonia-oxidizing bacteria. In contrast, the study of 16S rDNA with specific primers is only possible within certain phylogenetic groups belonging to the *Proteobacteria*, overlooking other AOB subgroups (Calvó *et al.*, 2004). In this case the gene *amoA* appears to be less resolutive than 16S rDNA when used for phylogeny inference (Purkhold *et al.*, 2003). Thus, new molecular markers based on a functional approach are required. Both the suitable size and the essential role of *amoB*, suggested it might be a good candidate for phylogeny studies of ammonia-oxidizing bacteria. Since the full length sequences of both *amoA* and *amoB* genes of *Nitrosomonas europaea* were first published (McTavish *et al.*, 1993; Bergmann and Hooper, 1994), additional full length sequences of either *amoA* or *amoB* have been added and are currently available in GenBank.

We report here the design of primers for the partial PCR amplification and sequencing of the *amoB* gene from pure cultures, and the subsequent comparative analyses of some relevant ammonia oxidizing bacteria lineages. Moreover, the same marker *amoB* has been used to develop a new pair of primers to quantify members of the genus *Nitrosococcus* by Real-Time PCR.

6.2 RESULTS AND DISCUSSION

A total of 16 AOB strains of the β– and γ–subclasses of *Proteobacteria* (Table 6.1) were used in this study. Two methane oxidizers, one from the β– and one from the γ–subclass of *Proteobacteria,* respectively, were also used.

Table 6.1 Strains used in this study

Strain	Source	Reference
Nm europaea Nm50	Soil (USA)	NCIMB 11850
Nm europaea	ATCC 19781	ATCC 19781
Nm eutropha Nm57	Municipal sewage (Chicago)	Koops and Harms, 1985
Nm sp. Nm33	Soil (Japan)	Koops *et al.*, 1991
Nm aestuarii Nm36	Sea water (North Sea)	Koops *et al.*, 1991
Ns briensis	ATCC 25971	ATCC 25971
Ns sp. 40KI	Loam (Norway)	Utåker *et al.*, 1995
Ns sp. Ka4	Lead contaminated soil (Norway)	Aakra *et al.*, 1999 a,b
Ns sp. B6	Sewage treatment column (Norway)	Utåker *et al.*, 1995
Ns sp. Nv6	Soil, Goroko (Danlo Pas)	Koops and Harms, 1985
Ns sp. Nsp1	Soil (Sardinia)	Koops and Harms, 1985
Ns multiformis NI13	Soil (Surinam) NCIMB 11849	Koops and Harms, 1985
Ns sp. Nsp17	Soil (Iceland)	Koops and Harms, 1985
Ns sp. Nsp2	Soil (Hamburg)	Koops and Harms, 1985
Ns sp. AF	Sandy Loam (Zambia)	Utåker *et al.*, 1995
Nc halophilus Nc4	γ–AOB	Purkhold *et al.*, 2000
Methylophilus methylotrophus	β–MOB	DSM 46235
Methylomonas methanica	γ–AOB	NCIMB 11130

6.2.1 Primer Design

A new set of primers targeting the *amoB* gene was designed using all *amoB* sequences published to date (Figure 6.1). The *amoB* sequences were aligned and consensus regions were used to design a degenerate set of primers which

was evaluated with the NetPrimer software (PREMIER Biosoft International, Palo Alto, California). As a result, the following primers were chosen: amoBMf (5'–TGGTAYGACATKAWATGG–3') and the reverse primer amoBMr (5'–RCGSGGCARGAACATSGG–3'). Degenerate positions are indicated according to IUB notation: Y=C, T; K=G, T; W=A, T; R=A, G; S=G, C.

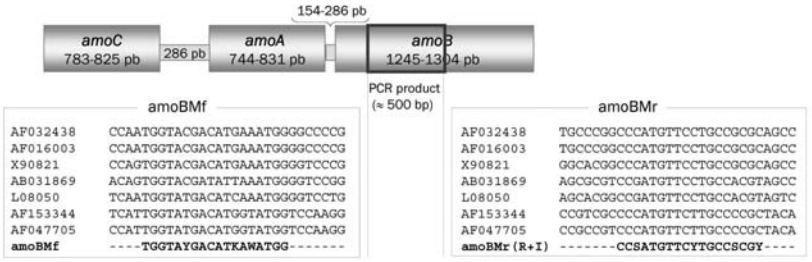


Figure 6.1 Primer location in the CAB operon of AOB, and source organisms of the *amoB* sequences used for the design of the degenerate primers set. AF032438: *Nitrosospira* sp. (Norton *et al.*, 1996); AF016003: *Nitrosospira* sp. (Norton *et al.*, 1996); X90821: *Nitrosospira* sp. (Rotthawe *et al.*, 1995); AB031869: *Nitrosomonas* sp. (Yokoyama, unpublished); L08050: *Nitrosomonas europaea* (McTavish *et al.*, 1993); AF153344: *Nitrosococcus* sp. (Norton *et al.*, 2002); AF047705: *Nitrosococcus oceani* (Norton *et al.*, 2002).

6.2.2 PCR conditions

PCR was carried out in 50 µl (total volume) reaction mixtures by using a thermal cycler (model 9600 P.E. Applied Biosystems, Forster City, CA, USA). Tm of the reaction was determined theoretically by using the software NetPrimer (PREMIER Biosoft International, Palo Alto, California), the equation: [(A+T) x 2] + [(G+C) x 4], and experimentally by performing a polymerase chain reaction by using a range of temperatures from 51.5°C to 62.5°C (Figure 6.2).

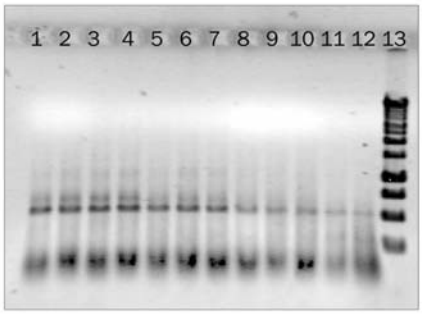


Figure 6.2 1.5% agarose gel containing PCR products obtained with primers amoBM at different

Tm. Lane 1: 51.5ºC; 2: 51.7ºC; 3: 52.5ºC; 4: 53.7ºC; 5: 55.0ºC; 6: 56.3ºC; 7: 57.6ºC; 8: 58.9ºC; 9: 60.2ºC; 10: 61.4ºC; 11: 62.2ºC; 12: 62.5ºC.

Maximum specificity and yield was obtained above 57ºC (figure 6.2, lane 7). Therefore the melting temperature chosen for polymerase chain reaction with the primers amoBMf and amoBMr was 58ºC. Thus, PCR conditions were 94ºC for 5 min; 42 cycles consisting of 94ºC for 45 sec, 58ºC for 1 min and 72ºC for 1 min; and a final extension step consisting of 72ºC for 7 min. Optimum concentrations were 2mM $MgCl_2$ and 0.5 µM for the primers.

The primer set of choice generated a single specific PCR product of ca. 500 bp, and two products of ca. 400 and 200 bp, when genomic DNA from AOB and methane oxidizers, respectively, was used (see Figure 6.3, lane 16). The primers were also tested with DNA from various representatives of the bacterial lineages. None of the cultures tested produced any unique 500bp PCR amplification when genomic DNA from neither–ammonia– nor methane–oxidizing bacteria was used as template (Figure 6.3).

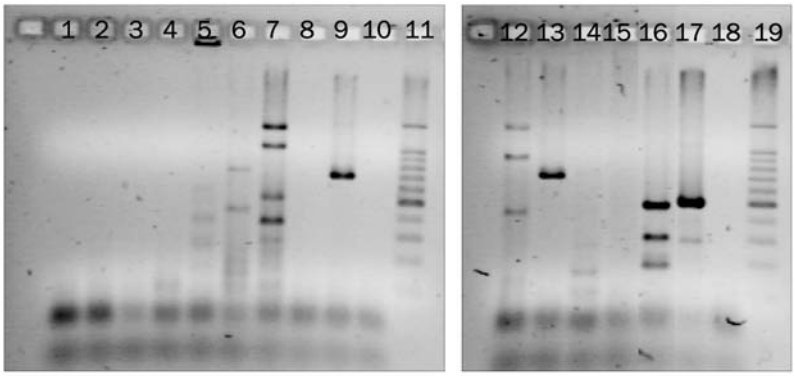


Figure 6.3 PCR products obtained by amplification with amoBM set of primers in a 1.5% agarose gel. Lane 1. *Bacillus cereus;* 2. *Streptococcus faecalis;* 3. *Pseudomonas aeruginosa;* 4. *Corynebacterium;* 5. *Rhodococcus;* 6. *Chlorobium vibrioforme* EII; 7. *Escherichia coli;* 8. *Mycobacterium tuberculosis;* 9. *Erythromyces longus;* 10. *Streptomyces griseus;* 11, 19. Molecular weight ladder 100bp; 12. *Erwinia herbicola;* 13. *Flavobacterium;* 14. *Chlorobium phaeobacteroides* 2430; 15. *Campylobacter jejunii;* 16. *Methylomonas methanica* NCIMB11130; 17. *Nitrosospira* sp. NspB6; 18. Negative control.

A PCR product of ca. 500 bp was obtained for all the ammonia–oxidizing bacteria tested. Two shorter PCR products were obtained when using genomic DNA of either β– or γ–methane oxidizers as a template. This helps to distinguish the methane monooxygenase B gene of the MOB from the ammonia monooxygen-

ase B gene of the AOB, whose similarity has been previously stated (Holmes *et al.*, 1995; Nold *et al.*, 2000).

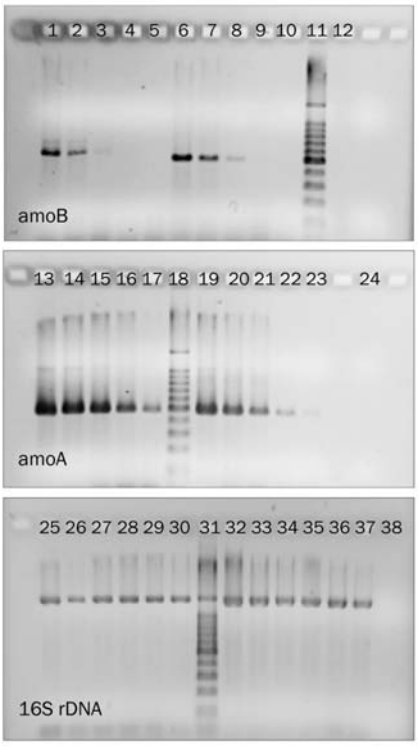
6.2.3 Identification of *amoB*

PCR products from *Nitrosomonas europaea* ATCC 19781 and *Nitrosospira briensis* ATCC 25971 were sequenced and compared to the previously published sequences of *amoB* to corroborate the identification of our amplification products as *amoB*. The sequence corresponding to *Nm europaea* ATCC19781 was identical to the reported *Nitrosomonas europaea* L08050 whereas the sequence obtained from the amplification of *Ns briensis* and the reported sequences of *Nitrosospira* sp showed similarity percentages above 91%.

6.2.4 Sensitivity of *amoB* detection

Genomic DNA from *Nitrosomonas europaea* strain Nm50 NCIMB11850 and from *Nitrosospira* sp str. B6 was used as a target over a range of DNA concentrations (Fig. 6.4). PCR amplification could be observed from as little as 20 pg of target genomic DNA (Figure 6.4). Assuming the bacterial chromosome size of *Nitrosomonas europaea* to be 2.8×10^6 bp, this would represent approximately 6.5×10^3 genomic copies of the *amoB* gene.

The sensitivity of the degenerated *amoB* set of primers was also compared with that of the *amoA* specific primers amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997), and that of the universal 16SrDNA primers Eub27f/Eub1493r (Lane 1991). For the PCR detection of *amoB* 10 times more template was required than with the *amoA* and 16S rDNA primers tested (Figure 6.4).



1. NspB6 3 ng/µl
2. NspB6 0,3 ng/µl
3. NspB6 0,03 ng/µl
4. NspB6 0,003 ng/µl
5. NspB6 0,0003 ng/µl
6. Nm50 2 ng/µl
7. Nm50 0,2 ng/µl
8. Nm50 0,02 ng/µl
9. Nm50 0,002 ng/µl
10. Nm50 0,0002 ng/µl
13. NspB6 30 ng/µl
14. NspB6 3 ng/µl
15. NspB6 0,3 ng/µl
16. NspB6 0,03 ng/µl
17. NspB6 0,003 ng/µl
19. Nm50 20 ng/µl
20. Nm50 2 ng/µl
21. Nm50 0,2 ng/µl
22. Nm50 0,02 ng/µl
23. Nm50 0,002 ng/µl
25. NspB6 150 ng/µl
26. NspB6 30 ng/µl
27. NspB6 6 ng/µl
28. NspB6 1,2 ng/µl
29. NspB6 0,24 ng/µl
30. NspB6 0,048 ng/µl
32. Nm50 100 ng/µl
33. Nm50 20 ng/µl
34. Nm50 4 ng/µl
35. Nm50 0,8 ng/µl
36. Nm50 0,16 ng/µl
37. Nm50 0,032 ng/µl
11, 18, 31. DNA molecular weight ladder 100 bp
12, 24, 38. Negative control

Figure 6.4 Sensitivity test. Agarose gel showing the *amoB*, *amoA* and 16S rDNA amplification for a range of *Nitrosomonas europaea* NCIMB11850 str Nm50 and *Nitrosospira* sp str B6 target DNA concentrations.

Nucleotide sequences obtained by using the new pair of degenerated primers targeting the gene *amoB* are presented in Table 6.2, and have been annotated and deposited in the DDBJ/EMBL/GenBank databases under accession numbers AJ555494 through AJ555509

6.2.5 Construction of a partial *amoB* sequences database. Phylogenetic and topology analyses

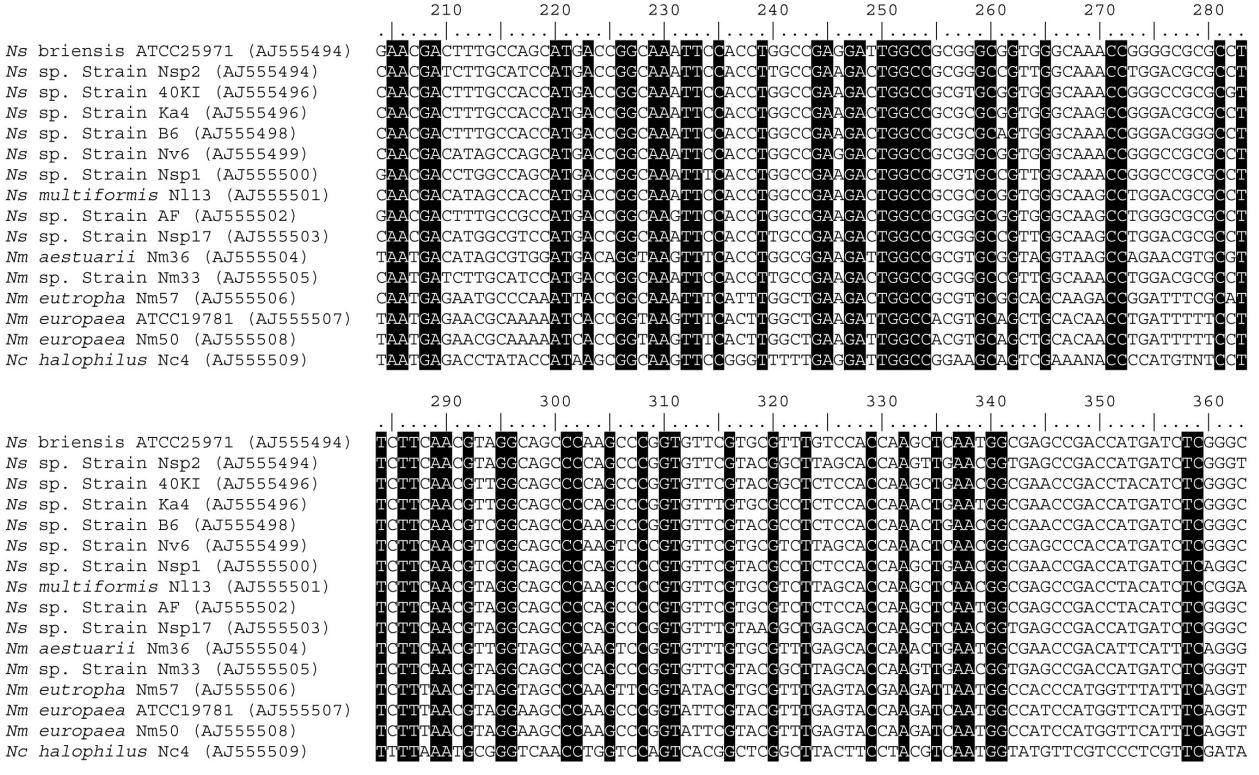
Multiple sequence alignment of partial sequences was carried out with ClustalW (Thompson *et al.*, 1994) and was further refined manually. Sequence similarities were checked using the NCBI "Blastn" program (Altschul *et al.*, 1997). By using all three methods of inference, i.e. maximum parsimony (MP), maximum likelihood (ML) and distance–minimal evolution (D), and analyses of partial *amoB* sequences, a consensus tree was generated.

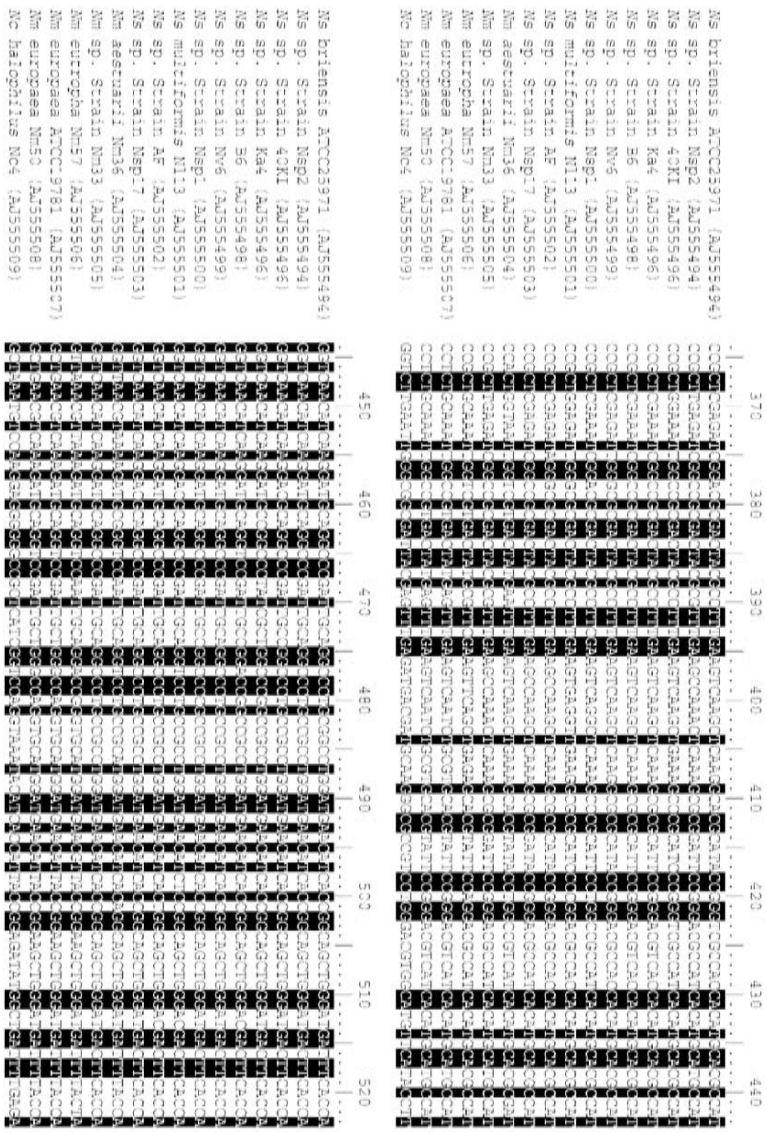
The two branches of Nitrosomonads grouped together and the γ–proteobacterial Nitrosococci branched separately from the γ–proteobacterial *Methylocystis* sp U81596 and *Methylosinus trichosporium* AF186586 used as outgroups (Figure 6.5). In general, the tree topology is similar to those based on 16S rDNA, 16S–23S rDNA intergenic spacer region (ISR), and partial *amoA* gene sequences (Purkhold *et al.*, 2000; Aakra *et al.*, 2001; Norton *et al.*, 2002). Using all these markers, the β– and γ–proteobacterial nitrifiers group in separate clades, as well as *Nitrosomonas* and *Nitrosospira* clusters. With only few exceptions, the phylogenetic clustering obtained using *amoB* partial sequences was in agreement with that previously defined based on 16S rDNA and 16S–23S rDNA sequences (Aakra *et al.*, 2001).

However, regarding the positions of some members of the *Nitrosospira* group, and the *Nitrosomonas* group, respectively, our *amoB* based tree is different from trees based on 16S–23S rDNA ISR sequences and on partial *amoA* peptide sequences (Purkhold *et al.*, 2000; Aakra *et al.*, 2001).

For example, in our work, *Nitrosomonas* str. Nm33 appeared to be closely related to Nsp17 and Nsp2. This is in disagreement with previous works which defined this strain as a *Nitrosomonas* sp., and classified it into the *Nitrosomonas communis* cluster. All this evidence might suggest a possible lateral gene transfer of *amoB* from *Nitrosospira* to *Nitrosomonas* (Koops *et al.*, 1991; Aakra *et al.*, 2001).

Table 6.2 Partial *amoB* gene sequences obtained in this study aligned




Ns briensis ATCC25971 (AJ555494)
Ns sp. Strain Nsp2 (AJ555494)
Ns sp. Strain 40KI (AJ555496)
Ns sp. Strain Ka4 (AJ555496)
Ns sp. Strain B6 (AJ555498)
Ns sp. Strain Nsp6 (AJ555499)
Ns sp. Strain Nsp7 (AJ555500)
Ns multiformis Nl13 (AJ555501)
Ns sp. Strain AE (AJ555502)
Ns sp. Strain Nsp17 (AJ555503)
Nm aeestuarii Nm36 (AJ555504)
Nm sp. Strain Nm33 (AJ555505)
Nm europha Nm57 (AJ555506)
Nm europaea ATCC19781 (AJ555507)
NC halophilus Nc4 (AJ555509)
370
380
390
400
410
420
430
440
450
460
470
480
490
500
510
520

530
540
550
560
570
580
590
Ns briensis ATCC25971 (AJ555494)
Ns sp. Strain Nsp2 (AJ555494)
Ns sp. Strain 40KI (AJ555496)
Ns sp. Strain Ka4 (AJ555496)
Ns sp. Strain B6 (AJ555498)
Ns sp. Strain Nv6 (AJ555499)
Ns sp. Strain Nsp1 (AJ555500)
Ns multiformis N113 (AJ555501)
Ns sp. Strain AF (AJ555502)
Ns sp. Strain Nsp17 (AJ555503)
Nm aestuarii Nm36 (AJ555504)
Nm sp. Strain Nm33 (AJ555505)
Nm eutropha Nm57 (AJ555506)
Nm europaea ATCC19781 (AJ555507)
Nm europaea Nm50 (AJ555508)
Nc halophilus Nc4 (AJ555509)

Nitrosolobus (now included in the *Nitrosospira* genus) was suggested to continue as a genus alongside *Nitrosospira* (Aakra *et al.*, 2001). Our results when analysing the *amoB* gene, strongly support the inclusion of *Nitrosolobus* into the genus *Nitrosospira*.

The great phylogenetic distance observed between the γ– and the β–AOB suggests that the capability of oxidizing ammonia might be an ancient property. Therefore, a possible recent horizontal gene transfer of the ammonia-oxidizing function between these two groups is not feasible.

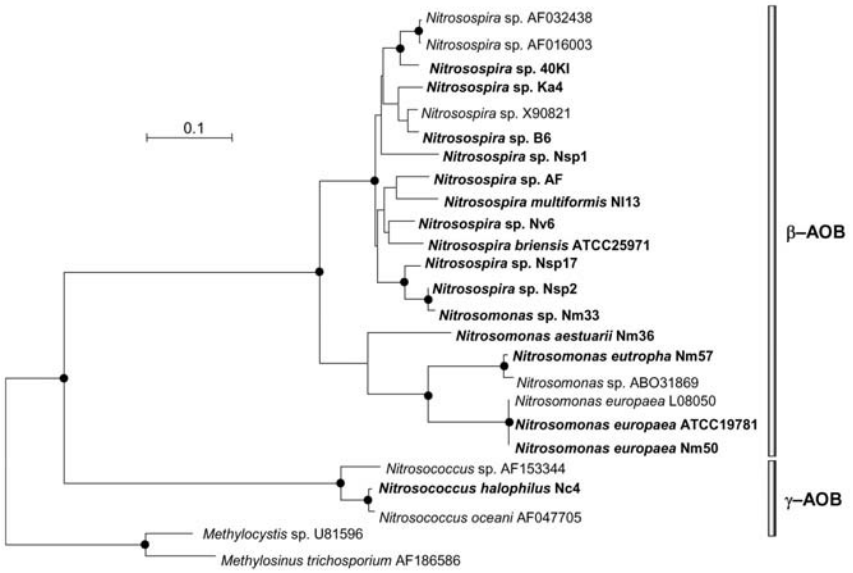


Figure 6.5 Neighbor–joining tree generated from an alignment of partial *amoB* sequences from AOB. Sequences obtained in this report are highlighted. Branch points supported (bootstrap values >90%) by distance, parsimony and maximum likelihood analyses are indicated by solid circles. Scale represents 0.1% relative distance. Partial *pmoB* sequences from *Methylocystis* sp. U81596 and *Methylosinus trichosporium* AF047705 have been used as outgroups.

As previously reported by other authors, based on 16S rDNA, ISR, and *amoA* analyses, *Nitrosomonas* and *Nitrosospira* fall apart into two separate branches of the tree (Purkhold *et al.*, 2000; Aakra *et al.*, 2001; Norton *et al.*, 2002). A remarkable distance separated both clades, highlighting *amoB* sequence as a suitable evolutive molecular marker for the ammonia oxidation capability. Longer distances were observed within the *Nitrosomonas* cluster members

(Figure 6.5), which reflect more changes in the *amoB* gene sequence from the genus *Nitrosomonas* than in that from the *Nitrosospira* genus. The high genetic homogeneity and the low diversity observed between the members forming the *Nitrosospira* cluster (Figure 6.5) give support to this point of view and suggest a recent colonisation of those environments.

6.2.6 AmoB deduced aminoacid sequences

High quality nucleotide sequences from both the β– and the γ– subclasses of the *Proteobacteria* were used to deduce amino acid sequences. As expected, most of the changes affected the third position of the codon, so they had no translational effects. To corroborate whether or not DNA distances were kept in proteins, a neighbor–joining tree was built on a distance matrix calculated by using the Dayhoff PAM method (Fig. 6.6).

As in the tree based on the alignment of partial *amoB* gene sequences (Fig. 6.5), three major branches, supported by high bootstrap values, corresponding to the two groups of Nitrosomonads and to the γ–AOB respectively, were obtained. In addition, there is a strong relationship between the AOB belonging to the β–subgroup of the *Proteobacteria* (i.e. *Nitrosomonas and Nitrosospira* clusters), whereas *Nitrosococcus* cluster appears separated (Fig. 6.6).

The topology of the tree is similar to that of *amoA* and *amoB* genes. *Nitrosomonas* sp strain Nm33 groups within *Nitrosospira* clusters, suggesting again that its phylogenetic affiliation is unclear and should be revised, preferably using a polygenic approach. The AmoB deduced aminoacid sequence of *Nitrosospira multiformis* str. NI13 also appears to be closely related to the *Nitrosospira* group, which is in agreement with the results obtained through *amoB* gene sequence analysis (Fig. 6.5), and supporting *Nitrosolobus* to continue being included into the genus Nitrosospira.

Even though no conserved domains for the Monooxygenase-B family have yet been defined, some specific amino acid motifs were found to be characteristic of both the β– and the γ–AOB subunit B ammonia monooxygenases (Table 6.3). The sequences of partial AmoB protein corresponding to *Nitrosomonas* and *Nitrosospira* groups have been found to share c.a. 75% of the amino acidic sequence, whereas, as expected, this percentage dramatically drops to 35% when the partial AmoB from ammonia–oxidizing bacteria of the γ–subgroup of the *Proteobacteria* are included.

The primers used in this study have been shown to be suitable to obtain partial *amoB* gene sequences from all the ammonia–oxidizers tested, both the β– and the γ–subgroups of the *Proteobacteria*. The gene *amoB* is therefore a good molecular marker for AOB, since it can be used for easy detection and discrimination between methane– and ammonia–oxidizing bacteria of both β– and γ–subclass of the *Proteobacteria* in environmental samples. It also provides a higher specificity in the study of AOB at the species level. Moreover, *amoB* can be applied in AOB diversity studies. Finally, the sequences reported, hereby, constitute a starting point for further quantitative and physiological determinations of AOB through the use of either real-time PCR or RT-PCR.

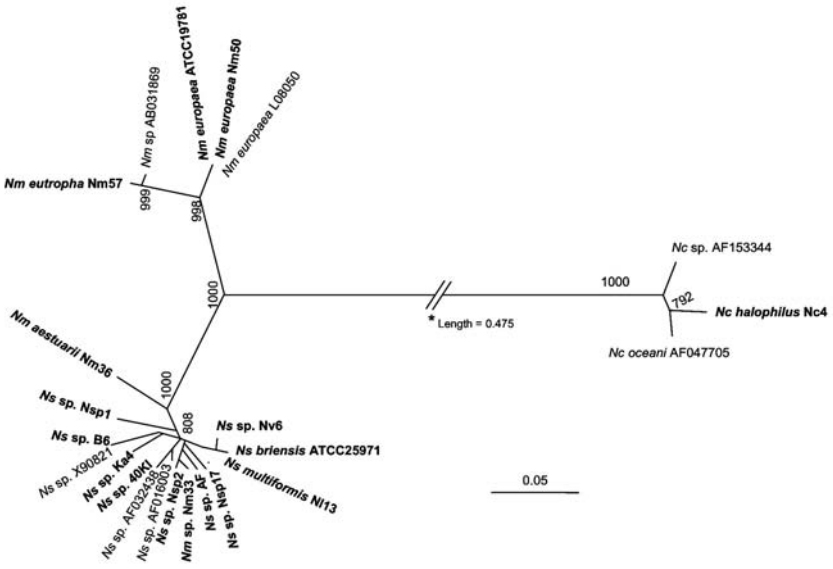


Figure 6.6 Unrooted tree of partial AmoB deduced amino acid sequences from AOB. The tree has been constructed using the Neighbor joining analysis on a distance matrix calculated by using the Dayhoff PAM method. Bootstrap values over 1000 replications are indicated Table 6.3 Alignment of deduced partial *amoB* amino acid sequences from AOB and MOB

6.2.7 Use of *amoB* as a molecular marker for quantitative determination of the genus *Nitrosococcus* by Real–Time PCR

As mentioned above, the sequences reported in this section become a starting point for additional objectives such as applying the gene *amoB* as a molecular marker to quantify ammonia-oxidizing bacteria in natural environments. Nowadays, the need to quantify the existing populations in microbial natural environments is one of the main topics in microbial ecology. In the last years, techniques such as fluorescent in situ hybridization (Biesterfeld *et al.*, 2001; Daims *et al.*, 2001; This work), and density gradient centrifugation combined with FISH (Hesselsoe *et al.*, 2001) have been used in an attempt to estimate the density of some AOB populations overcoming the pitfalls associated to the classical culture-based techniques. Real-Time PCR was first developed in the mid 1990s (Heid *et al.*, 1996), and supposed a revolution in nucleic acid quantification due to its rapidity, and high precision over a wide range of concentrations (Schmittgen *et al.* 2000). This technique has recently been used in the study of ammonia oxidizers. Harms *et al.* (2003) developed and optimized Taqman® and Molecular Beacons® probes to quantify the NOB *Nitrospira* spp. and AOB *Nitrosomonas oligotropha*-like populations by using 16S rDNA– and *amoA*–targeted primers and probes, respectively. Okano *et al.* (2004) have recently presented an *amoA*–targeted real-time PCR assay to estimate *Nitrosomonas* and *Nitrosospira* populations in soil. Besides, Aoi *et al.* (2004) have used competitive RT-PCR to estimate the relationship between the levels of *amoA* mRNA and ammonia oxidation activity.

Here we present an RTi-PCR approach for the quantification of the AOB included into the γ–subgroup of the Proteobacteria. The system targets the gene *amoB*, which has been recently reported as a new and reliable molecular marker for the ammonia oxidizing bacteria (Calvó and Garcia-Gil, 2004).

6.2.7.1 Primer design

The design of the new set of primers targeting the genus *Nitrosococcus* was performed with the help of the software Primer Express v2.0 (Applied Biosystems). Since this software can only find appropriate primers from single sequences, and not in sequence alignments, only one *amoB* gene sequence (*Nitrosococcus halophilus* strain Nc4) was used for this purpose. The resulting primer sets were checked to target conserved regions of the three available *amoB* gene sequences belonging to members of the γ– Proteobacteria *Nitrosococci* (Figure 6.7). As a result, the following primers, which generated a

70
80
90
100
110
120
130
Nm europaea L08050
Nm sp AB031869
Nm eutropha Nm57
Nm europaea ATCC19781
Nm europaea Nm50
Nm aestuarii Nm36
Nm sp. Nm33
Ns sp. AF032438
Ns sp. AF016003
Ns sp. X90821
Ns briensis ATCC25971
Ns sp. Nsp1
Ns sp. Nsp2
Ns sp. 40KI
Ns sp. Ka4
Ns sp. B6
Ns sp. Nv6
Ns multiformis N113
Ns sp. AF
Ns sp. Nsp17
Nc halophilus Nc4
Nc sp. AF153344
Nc oceani AF047705
Nm Ns amoB consensus
amoB consensus

140 150 160 170 180 190
Nm europaea L08050 RIPGRHHMHAMLNVKDAGPIAGPGAWMNITGSWDDFTNPLKLLTGETIDSETFNLSNGIFW
Nm sp. AB031869 RIPGRHHMHAMLNVKDAGPIAGPGAWMNVTGSWDDFTNPLKLLTGETIDSETFNLSNGIFW
Nm eutropha Nm57 RIPGRHHMHAMLNVKDAGPIAGPGAWMNVTGSWDDFTNPLKLLTGETIDSETFNLGNGIFW
Nm europaea ATCC1978 RIPGRHHMHAMLNVKDAGPIAGPGAWMNITGSWDDFTNPLKLLTGETIDSETFNLSNGIFW
Nm europaea Nm50 RIPGRHHMHAMLNVKDAGPIAGPGAWMNITGSWDDFTNPLKLLTGETIDSETFNLSNGIFW
Nm aestuarii Nm36 RIPGRHHMHAMVNVKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFANGIFW
Nm sp. Nm33 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPLKLLTGETIDTETFNFSNGIFW
Ns sp. AF032438 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGIFW
Ns sp. AF016003 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGIFW
Ns sp. X90821 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNFSNGIFW
Ns briensis ATCC25971 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNFSNGIFW
Ns sp. Nsp1 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNLGNGLFW
Ns sp. Nsp2 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNFSNGIFW
Ns sp. 40KI RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNYSNGVFW
Ns sp. Ka4 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNYSNGVFW
Ns sp. B6 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNYSNGIFW
Ns sp. Nv6 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFSNGIFW
Ns multiformis N113 RIPGRHHMHAMVNIKDAGPIAGPAAWMNISGSWDDFTNPVKLLTGETIDTETFNFSNGIFW
Ns sp. AF RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPVKLLTGETIDTETFNFNNGMFW
Ns sp. Nsp17 RIPGRHHMHAMVNIKDAGPIAGPAAWMNITGSWDDFTNPIKLLTGETIDTETFNFSNGIFW
Nc halophilus Nc4 RRPGTWHVHTLLNVQGGGPLIGPGKYITITGDMADFESKITDLTGNTVNLETMATGTVIGW
Nc sp AF153344 RRPGTWHVHTLLNVQGGGPLIGPGKYITITGDMADFENKVTDLTGNTVNLETMATGTVIGW
Nc oceani AF047705 RRPGTWHVHTLLNVQGGGPLIGPGKYITITGDMADFENKITDLTGNTVNLETMATGTVIGW
Nm Ns amoB consensus RIPGRHHMHAM-N-KDAGPIAGP-AWMN--GSWDDFTNP-KLLTGETID-ETFN--NG-FW
amoB consensus R-PG-H-H--N---GP--GP------G---DF------LTG-T---ET-------W

75 bp PCR product, were chosen: Nc337f (5'–AATGGTATGTTCGTCCCTCGTT–3') and Nc412r (5'–CCCTTGCATCGTCATCTCAA–3'). The Tm in both cases was 58ºC, and presented a GC content of 45% and 50%, respectively.

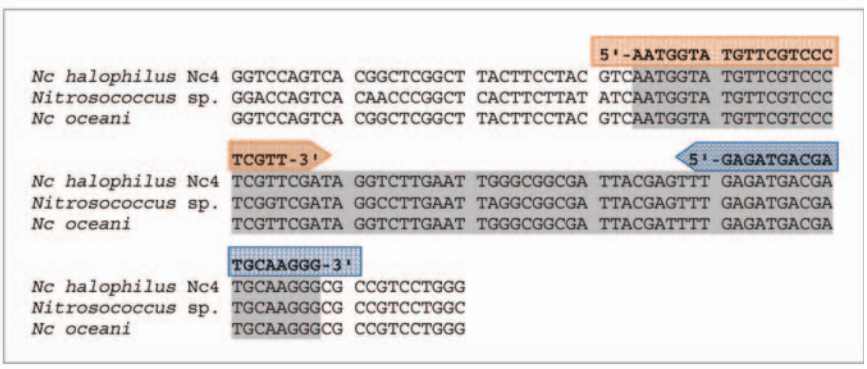


Figure 6.7 Location of the primers Nc337f and Nc412r in consensus regions of the three available *amoB* gene sequences: *Nitrosococcus halophilus* Nc4 (AJ555509), *Nitrosococcus* sp. (AF153344), and *Nitrosococcus oceani* (AF047705).

Besides, the selected primer pair was tested against the databases by using the NCBI "Blastn" application (Altschul *et al.*, 1997) in order to make sure that the selected primers did not match *amoB* gene sequences belonging to any other ammonia oxidizing bacteria. Results (Table 6.4) indicated a high specificity of the designed primers, which targeted only the *amoB* gene sequences of the three known Nitrosococci belonging to the γ–subgroup of the Proteobacteria.

Genomic DNA from a pure culture of *Nitrosococcus oceani* ATCC19707 (see sections 2.2.1 and 2.3.1) was used to optimize the primer concentration to perform RTi-PCR with the new primers. The same genomic DNA serially diluted was used to build the quantification standard curve.

Table 6.4 NCBI Blastn results for the new pair of primers Nc337f and Nc412r.

Nc337f [5'(AATGGTATGTTCGTCCCTCGTT) 3'] (22 letters)

Sequences producing significant alignments:	Score (bits)	E Value
gi|40809671|emb|AJ555509.1|NHA555509 Nitrosococcus halophilus.	44	0.002
gi|3282844|gb|AF047705.1| Nitrosococcus oceanus ammonia mon...	44	0.002
gi|6117982|gb|AF153344.1| Nitrosococcus sp. C-113 strain C-...	40	0.039
gi|34482500|emb|BX571658.1| Wolinella succinogenes, complet...	32	9.4
gi|33569172|emb|BX088547.12| Zebrafish DNA sequence from cl...	32	9.4
gi|21627589|gb|AE003832.3| Drosophila melanogaster chromoso...	32	9.4
gi|3818342|gb|AC005894.1|AC005894 Drosophila melanogaster, ...	32	9.4

Nc412r [5' (CCCTTGCATCGTCATCTCAA) 3'] (20 letters)

Sequences producing significant alignments:	Score (bits)	E Value
gi|40809671|emb|AJ555509.1|NHA555509 Nitrosococcus halophilus.	40	0.019
gi|6117982|gb|AF153344.1| Nitrosococcus sp. C-113 strain	40	0.019
gi|3282844|gb|AF047705.1| Nitrosococcus oceanus ammonia monoo.	40	0.019
gi|37059974|gb|AC107761.9| Mus musculus chromosome 7, clone...	34	1.2
gi|17569024|ref|NM_077630.1| Caenorhabditis elegans metridi...	32	4.7
gi|34101311|gb|AE016910.1| Chromobacterium violaceum ATCC 1...	32	4.7
gi|23396275|gb|AC099536.3| Homo sapiens chromosome 3 clone ...	32	4.7

6.2.7.2 Primer optimization

To set the best conditions to obtain optimal Real-Time PCR results with the new primers, a wide range of both Nc337f and Nc412r concentrations (Table 6.5) were tested.

Table 6.5 Primer optimization protocol. With the exception of the primers, the mix in Table A contained all the reactives needed to perform a PCR. The primers were prepared in a range of concentrations as detailed in table B.

A

Reactive	[Stock]	[Final]	µl/reaction	x 29 reactions
Milli-Q H_2O	-	-	1.00	29.00
TaqMan buffer A	10 x	1 x	2.00	58.00
Mg^{2+}	25 mM	6 mM	4.80	139.20
dNTPs	10 mM (20 mMdUTP)	200 µM (400 µM dUTP)	1.60	46.40
SYBR Green	10 x	0.5 x	1.00	29.00
Taq Gold	5 U/µl	1 U	0.20	5.80
DNA			1.00	29.00
TOTAL			11.60	336.40

B

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	1 µM	50 nM	1.00	3.00
Nc412r	1 µM	50 nM	1.00	3.00
H_2O			6.40	19.20
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	300nM	0.60	1.80
Nc412r	10 µM	300nM	0.60	1.80
H_2O			7.20	21.60
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	1 µM	50 nM	1.00	3.00
Nc412r	10 µM	300 nM	0.60	1.80
H_2O			6.80	20.40
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	300nM	0.60	1.80
Nc412r	10 µM	900 nM	1.80	5.40
H_2O			6.00	18.00
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	1 µM	50 nM	1.00	3.00
Nc412r	10 µM	900 nM	1.80	5.40
H_2O			5.60	16.80
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	900 nM	1.80	5.40
Nc412r	10 µM	300nM	0.60	1.80
H_2O			6.00	18.00
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	300nM	0.60	1.80
Nc412r	1 µM	50 nM	1.00	3.00
H_2O			6.80	20.40
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	900 nM	1.80	5.40
Nc412r	10 µM	900 nM	1.80	5.40
H_2O			4.80	14.40
			8.40	25.20

Primer	[stock]	[Final]	µl/reac.	x 3 replicates
Nc337f	10 µM	900 nM	1.80	5.40
Nc412r	1 µM	50 nM	1.00	3.00
H_2O			5.60	16.80
			8.40	25.20

Previous studies performed in our laboratory demonstrated that the best results were obtained by using the dye SYBR Green at a concentration of 0.5 X in the reactions. Moreover, in some cases, the use of more concentrated SYBR Green solutions caused the inhibition of the PCR reaction (Marta Ros, personal communication). Therefore, this was the concentration used in all the reactions performed with the primers Nc337f and Nc412r.

Data were analyzed by Sequence detector software v 1.7 (Applied Biosystems), and the amplification program used in the ABI Prism 7700 SDS RT-PCR device (Applied Biosystems) is detailed below.

1. Denaturing	95ºC	10 min
2. 50 cycles:	95ºC	15 sec (denaturing)
	60ºC	1 min (annealing and extension)

The amplification of genomic DNA from *Nc. oceani* ATCC19707 by using a series of dilutions of both, forward and reverse, primers (Figure 6.8) resulted in an exponential increase in the fluorescence with all the primers' proportions tested. Nevertheless, the best results were obtained when the primer proportions 300nM Nc337f : 300 nM Nc412r were used.

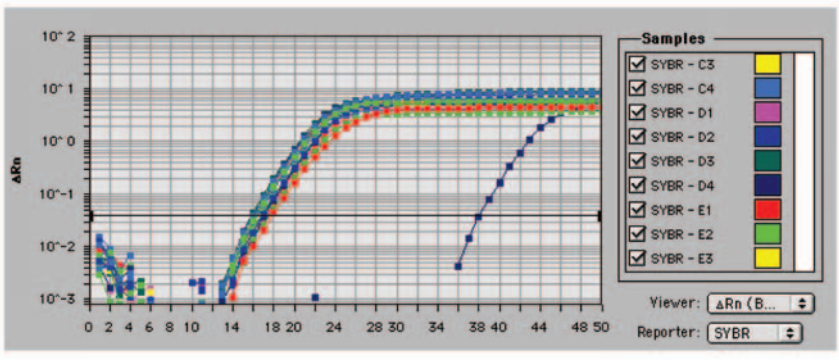


Figure 6.8 Amplification of the *amoB* gene of *Nc. oceani* in serially diluted primer concentrations. The plot shows the log of the increase in fluorescence vs. PCR cycle number. Blue line corresponds to the non template control.

To ensure that the fluorescence detected was entirely produced by the desired DNA fragment, a dissociation curve of the PCR product was performed by applying a temperature ramp from 60ºC to 90ºC. This resulted in the melting of

the DNA at a temperature of 84.2°C. The dissociation plot obtained is presented in figure 6.9.

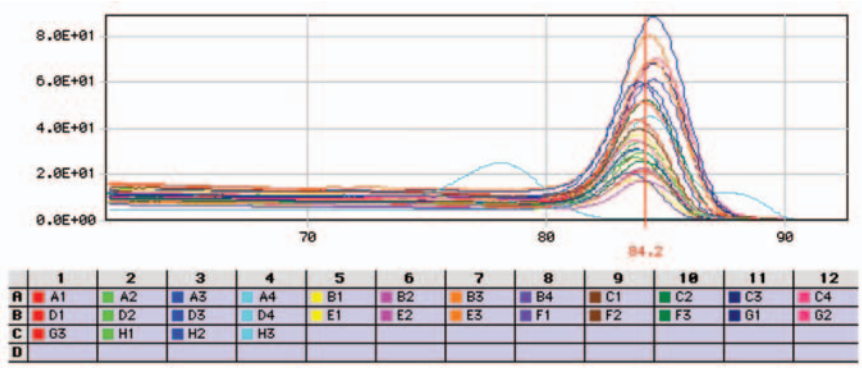


Figure 6.9 Dissociation curve of the amplicons produced after amplification with the primers Nc337f and Nc412r. The plot shows the variation of fluorescence vs. the melting temperature. The light blue line corresponds to the non template control.

6.2.7.3 Quantification of the extracted DNA of *Nitrosococcus oceani* ATCC19707

DNA was purified from the culture of *Nc oceani* (see sections 2.2.1.1 2.3.1.4) and quantified by using Picogreen® (see section 2.3.3.2), which resulted in a DNA concentration of 3.75 ng DNA μl^{-1}. The size of *Nitrosococcus oceani* genome has been reported to be of 3Mb (DOE Joint Genome Institute. U.S. Dept. of Energy: http://www.jgi.doe.gov/sequencing). Since the weight of 1 basepair averages 652 g mol^{-1}, the extracted DNA of *Nitrosococcus oceani* contained 1.15 x 10^6 copies of the genome μl^{-1}.

Ammonia oxidizers belonging to the γ-subgroup of Proteobacteria contain single copies of the *amo* operon as reported by Alzerreca *et al.* (1999), whereas it exists in multiple copies in all the β–AOB examined to date.

6.2.7.4 Building the standard curve

A calibration curve relating the C_T value and the amount of *amoB* gene copies in the genus *Nitrosococcus* was built by performing the R-Ti PCR reaction with a 10-fold dilution series of *Nitrosococcus oceani* ATCC 19707 genomic DNA. The number of copies of the gene *amoB* used to build the standard curve ranged from 10^6 to 0.1.

The amplification plot obtained (figure 6.10) presented an exponential increase in the fluorescence with all the template concentrations analyzed, and, as expected, the C_T value increases accordingly with the number of *amoB* gene copies initially used.

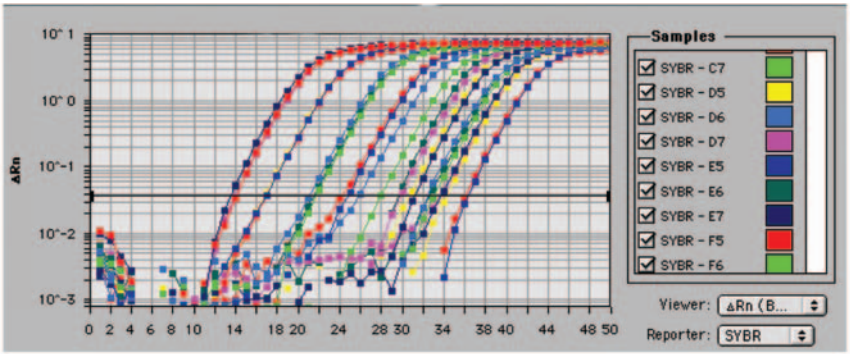


Figure 6.10 Amplification of the *amoB* gene of *Nc. oceani* in ten-fold dilutions from 10^6 to 0.1 copies of this gene. The plot shows the log of the increase in fluorescence vs. PCR cycle number.

The obtained C_T numbers were plotted against the $\log_{10}$ of the starting quantity of genomic DNA used, which resulted in a linear inverse relationship between both parameters. As shown in figure 6.11 there's a strong linear correlation (R^2=0.9943) between the *amoB* gene copy numbers and the threshold cycle over 7 orders of magnitude. The equation that describes this relationship is: $C_T = -3.2339 \times \log_{10}(amoB) + 33.64$.

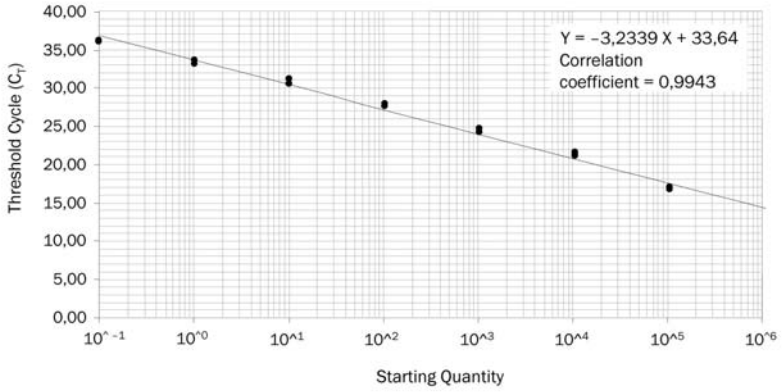


Figure 6.11 Standard curve showing tenfold dilutions from 10^6 to 0.1 *amoB* gene copies. C_T values have been plotted vs. the log of the DNA quantity.

The slope of the calibration curve allows the estimation of the efficiency of the PCR reaction. At an ideal efficiency of a 100%, the template doubles its concentration after each cycle in an exponential behavior. Consequently, reproducible and truthful results will be obtained by reactions having efficiency as close as possible to a 100%. The following equation is used to determine the efficiency of any PCR reaction:

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

Therefore, the efficiency of the reactions performed in this work presented an efficiency of a 103.81%. This value indicated that more DNA is produced in the reaction than expected, which lead us to suspect the presence of unspecific amplifications. In order to test it out, a dissociation curve was performed showing two peaks instead of a single one. To distinguish the sample triplicates containing large amounts of template DNA from the triplicates less concentrated, results were divided in two separate plots (represented as A and B in figure 6.12).

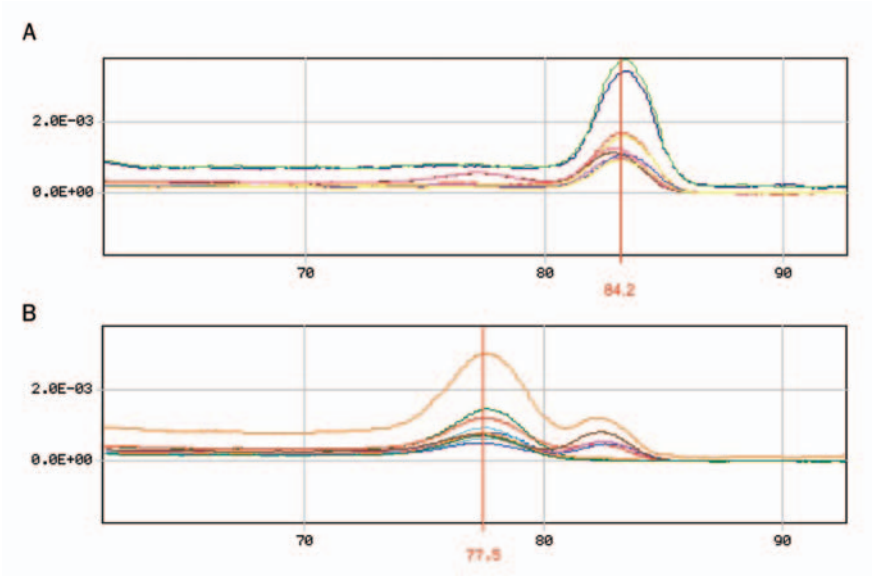


Figure 6.12 Dissociation curve showing the variation of fluorescence vs. the melting temperature. (A) Shows the main peak obtained from the dissociation of the amplicon produced by the primers Nc337f and Nc412r, and (B) shows the secondary peak obtained from the dimmer primer formation when the concentration of template DNA is low.

The expected peak at Tm=84.2ºC corresponding to the denaturation of the generated PCR product is shown in figure 6.12–A, but figure 6.12–B depicts a secondary peak at 77.5ºC, obtained when the DNA template used in the PCR was lower than 10 copies of the gene per reaction. This minor peak is thought to correspond to a primer-dimer formation in the real-time PCR when lowest amounts of initial template are used. The formation of these primer-dimers is probably responsible for, at least the percentage efficiency exceeding 100%. Thus, the detection limit was set in 10 genomes per reaction.

In this study the gene *amoB* has been used for the first time as a target in a quantitative PCR-based assay for a quick, sensitive reliable and broad-ranged enumeration of a whole subgroup of ammonia oxidizing bacteria. In addition, since Aoi *et al.* (2004) demonstrated a long-term relationship between *amoA* mRNA levels and ammonia oxidation activities, this study may constitute a starting point for a new competitive *amoB*-based RT-PCR tool, which would allow estimating the activity of the γ- AOB Proteobacteria.

7 A POLYGENIC APPROACH TO THE CLASSIFICATION OF AOB

7.1 INTRODUCTION

The environmental and biotechnological interest of ammonia oxidizing bacteria (AOB), and their slow growth together with the culture difficulties, have pushed the development of a variety of culture-independent techniques to perform ecological and taxonomical studies (McCaig *et al.*, 1994; Hiorns *et al.*, 1995; Voytek and Ward, 1995; Wagner *et al.*, 1995; Stephen *et al.*, 1996; Juretschko *et al.*, 1998; Hermansson and Lindgren, 2001). Both 16S rDNA and protein encoding genes have been used in the characterization of natural AOB populations (Sinigalliano *et al.*, 1995; Bothe *et al.*, 2000; Kowalchuck *et al.*, 2000; Rowan *et al.*, 2003; Calvó *et al.*, 2004) and in taxonomic and phylogenetic inferences (Purkhold *et al.*, 2000; Aakra *et al.*, 2001 a, b; Purkhold *et al.*, 2003; Calvó and Garcia-Gil, 2004).

Taxonomists have increasingly used 16S rDNA to support bacterial classifications. However, it is widely accepted that although 16S rDNA sequences are suitable for providing a comprehensive long-term evolutionary view of the prokaryotes taxonomy, it fails to discriminate among close relatives such as species within a given group or genus (Rosselló-Mora and Amann, 2001). In turn, considerable variability can be found among organisms with almost identical 16S rDNA genes (Béjà *et al.*, 2002). Thus, although 16S rDNA has proven to be useful to discriminate between *Nitrosococci* and *Nitrosomonads* (Ward *et al.*, 2000; Bruns *et al.*, 1999), the outcome is confusing when looking inside a single genus as for example *Nitrosopira* (Rotthauwe *et al.*, 1997). Protein encoding genes have been added to the comparative tools used by taxonomists and molecular ecologists for diversity studies (Garcia-Gil *et al.*, 2003; Sinigalliano *et al.*, 1995).

The *amoA* gene encodes for the active site of the ammonia monooxygenase enzyme (McTavish *et al.*, 1993) and has been extensively used for the detection and study of ammonia oxidizers, particularly in natural environments (Aakra *et al.*, 2001a; Gieseke *et al.*, 2001; Juretschko *et al.*, 1998). According to Rotthauwe *et al.*, (1997), the *amoA* gene appeared to be more useful at a fine-scale than 16S rDNA. In contrast, Ludwig and Schleifer (1999) stated that 16S rDNA gene constitutes the leading marker to infer phylogenetic relationships, since the topologies derived from 16S rDNA are in accordance with those obtained using markers with rather diverse functions. This has been recently supported by Purkhold *et al.* (2003), who showed a higher resolution of 16S rDNA versus *amoA* within the traditional classification.

Recently, the gene *amoB* has been presented as a suitable molecular marker for the study of AOB, showing a high capacity of resolution within genera. In addition, its phylogeny has resulted to be highly consistent with the current taxonomic outline (Calvó and Garcia-Gil, 2004).

The reconstruction of phylogenetic reationships between closely related species requires the use of markers with significant mutation rates, but then the accumulation of recurrent mutations leads to large amounts of mutational homoplasy integrated in the molecular data (Smouse, 1998). Besides, mutations occurring repeatedly in the same site mask the previous ones, turning the sequences useless for phylogenetic purposes. This phenomenon, known by geneticists as substitution saturation, should be taken into account before proceeding with any analysis (Ford, 2002).

The polymorphisms detected in the sequences of a given population reveal both the mutations experienced by the ancestors and the consequences of evolutionary forces, such as genetic drift or natural selection. It is therefore essential to check whether or not the molecular dataset has been significantly affected by evolutionary pressures. Moreover, the Neutral Theory (Kimura, 1983; Fu and Li, 1993) has become the standard null hypothesis used to approach the study of molecular evolution.

This work was aimed at determining whether a polygenic or single-marker analysis was suitable for taxonomical purposes. Therefore, three markers (*amoA*, *amoB*, and 16S rDNA) have been evaluated independently in view of several genetic tests to obtain information of the amount of useful information contained in their respective sequences. In addition, the phylogenetic trees built from each gene have been weighted against the composite sequence dataset to identify the most useful marker reproducing the information resulting from the polygenic tree.

7.2 RESULTS

7.2.1 Quantitative aspects of gene variation

The total numbers and percentages of variable and conserved positions in the available sequences of the studied genes were calculated. Sequences of *amoA* contained the highest proportion of polymorphic sites (Table 7.1). A 62.15% of the sites in this gene were found to be variable, compared to the 27.42% and 45.80% for the 16S rDNA and *amoB* genes, respectively. Approximately 50% of the nucleotide substitutions in the *amoA* gene affected the third codon position (Table 7.3), while in the case of *amoB* the variable positions were evenly distributed along the gene. Besides, 44.27% of the polymorphisms detected in the third basepair of *amoB* were also silent substitutions (caused no effect in the amino acidic sequence). Sequences used in this study are presented in Table 7.2.

Table 7.1 Quantitative aspects of variation of 16S rDNA, *amoA* and *amoB* genes. In brackets percent relative values.

Gene	Total	Variable Sites silent : effective	Parsimony informative sites
16S rDNA	1014 (100.00)	278 (27.42)	231
amoA	399 (100.00)	45 (11.27) : 203 (50.88)	232
amoB	393 (100.00)	0 (0.00) : 2180 (45.80)	172

Table 7.2 Source sequences used in this study

	16S rDNA		*amoA*		*amoB*	
	Accession Number	Reference	Accession Number	Reference	Accession Number	Reference
Nm europaea Nm50	M96399	Head *et al.*, 1993	AJ298710	Aakra *et al.*, 2001	AJ555508	Calvó and Garcia-Gil, 2004
Nm aestuarii Nm36	AJ298734	Aakra *et al.*, 2001b	AJ298707	Aakra *et al.*, 2001b	AJ555504	Calvó and Garcia-Gil, 2004
Nm eutropha Nm57	AY123795	Purkhold *et al.*, 2000	AJ298713	Aakra *et al.*, 2001	AJ555506	Calvó and Garcia-Gil, 2004
Nm europaea L08050	AB070982	Shinozaki and Fukui, 2002	L08050	McTavish *et al.*, 1993	L08050	McTavish *et al.*, 1993
Nm sp. K794	AB031960	Yokoyama *et al.*, Unpubl.	AB031869	Yokoyama *et al.*, Unpubl.	AB031869	Yokoyama *et al.*, Unpubl.
Ns sp. NpAV – copy1	Y10127	McCaig *et al.*, 1994	AF032438	Klotz and Norton, 1995	AF032438	Norton *et al.*, 2002
Ns sp. NpAV – copy2	Y10127	McCaig *et al.*, 1994	AF016003	Klotz and Norton, 1995	AF016003	Klotz and Norton, 1995
Ns sp. Nsp2	AY123802	Purkhold *et al.*, 2003	AY123822	Purkhold *et al.*, 2003	AJ555494	Calvó and Garcia-Gil, 2004
Ns sp. 40KI	X84656	Utaker *et al.*, 1995	AJ298687	Aakra *et al.*, 2001	AJ555496	Calvó and Garcia-Gil, 2004
Ns sp. Ka4	AJ012106	Aakra *et al.*, Unpublished	AJ298697	Aakra *et al.*, 2001	AJ555497	Calvó and Garcia-Gil, 2004
Ns sp. B6	X84657	Utaker *et al.*, 1995	AJ298690	Aakra *et al.*, 2001	AJ555498	Calvó and Garcia-Gil, 2004
Ns sp. Nv6	AY123805	Purkhold *et al.*, 2003	AY123826	Purkhold *et al.*, 2003	AJ555499	Calvó and Garcia-Gil, 2004
Ns sp. Nsp1	AY123808	Purkhold *et al.*, 2003	AY123828	Purkhold *et al.*, 2003	AJ555500	Calvó and Garcia-Gil, 2004
Ns multiformis Nl13	AY123807	Purkhold *et al.*, 2003	AJ298702	Aakra *et al.*, 2001	AJ555501	Calvó and Garcia-Gil, 2004
Ns sp. AF	X84658	Utaker *et al.*, 1995	AJ298689	Aakra *et al.*, 2001	AJ555502	Calvó and Garcia-Gil, 2004
Ns sp. Nsp17	AY123804	Purkhold *et al.*, 2003	AY123825	Purkhold *et al.*, 2003	AJ555503	Calvó and Garcia-Gil, 2004
Ns sp. AHB1	X90820	Rotthauwe *et al.*, 1995	X90821	Rotthauwe *et al.*, 1995	X90821	Rotthauwe *et al.*, 1995
Nc sp. AF153344	AF153343	Norton *et al.*, 2002	AF153344	Norton *et al.*, 2002	AF153344	Norton *et al.*, 2002
Nc oceani C-107	M96395	Head *et al.*, 1993	AF047705	Norton *et al.*, 2002	AF047705	Norton *et al.*, 2002
Nc halophilus Nc4	AF287298	Purkhold *et al.*, 2000	AF272521	Purkhold *et al.*, 2000	AJ555509	Calvó and Garcia-Gil, 2004
Methylocystis sp. M	U81595	McDonald and Murrell, 1997	U81596	McDonald and Murrell 1997	U81596	McDonald and Murrell 1997
M. trichosporium OB3b	Y18947	Dedysh *et al.*, 2000	U31650	Gilbert *et al.*, 2000	U31650	Gilbert *et al.*, 2000

The information enclosed in the sequence of the three genes was also calculated by using the option "variable substitution rate over sites" implemented in DAMBE software (Figure 7.1). The same analysis was performed for every position of the codon in *amoA* and *amoB* genes (Figure 7.2). The information in both *amoA* and *amoB* genes, seems to be homogeneously distributed along the sequence, whereas 16S rDNA gene presents peaks of information randomly distributed along the molecule. However, when considering the three positions of the codon, the most regular distribution of the information is observed in all *amoB* codon positions.

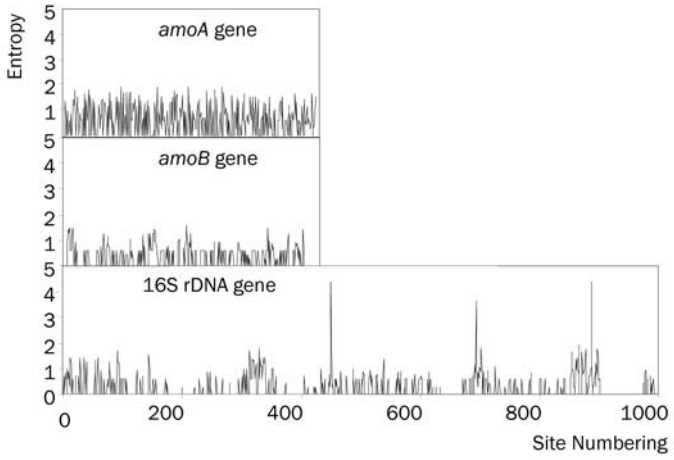


Figure 7.1 Variable substitution rate over sites calculated for *amoA*, *amoB* and 16S rDNA genes.

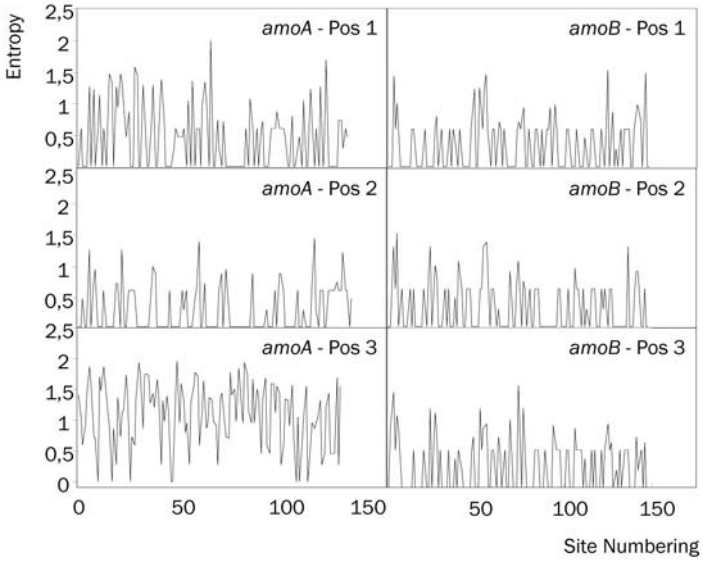


Figure 7.2 Variable substitution rate over sites calculated for the three positions of the codon in *amoA* and *amoB* genes.

7.2.2 Neutrality and substitution saturation

Results of Tajima's D test, which has been used to test the hypothesis of neutrality, indicated no significant skew in the entire sequences of the three markers (Tajima, 1989). Therefore, the dataset appeared to fit the model of neutral molecular evolution. However, Tajima's D statistic revealed a significant excess of polymorphisms in the third position of the codon in *amoA* gene (Table 7.4).

These results agree with the measurements of substitution saturation, which produced a strong signal in the third position of the codon for *amoA* gene (Figure 7.3). The persistent accumulation of changes in these specific sites in *amoA* gene may produce a loss of phylogenetic information. No substitution saturation was detected in the rest of the cases when determined with Xia's index (Xia and Xie, 2001).

Table 7.4 Neutrality results obtained for 16S rDNA, *amoA* and *amoB* partial DNA sequences with Tajima's D test

Gene	16S rDNA	*amoA*				*amoB*			
Position	All	All	Pos 1	Pos. 2	Pos. 3	*All*	Pos. 1	Pos. 2	Pos. 3
Tajima's D	0.487	1.967	1.091	0.315	3.131*	0.532	0.505	0.421	0.623

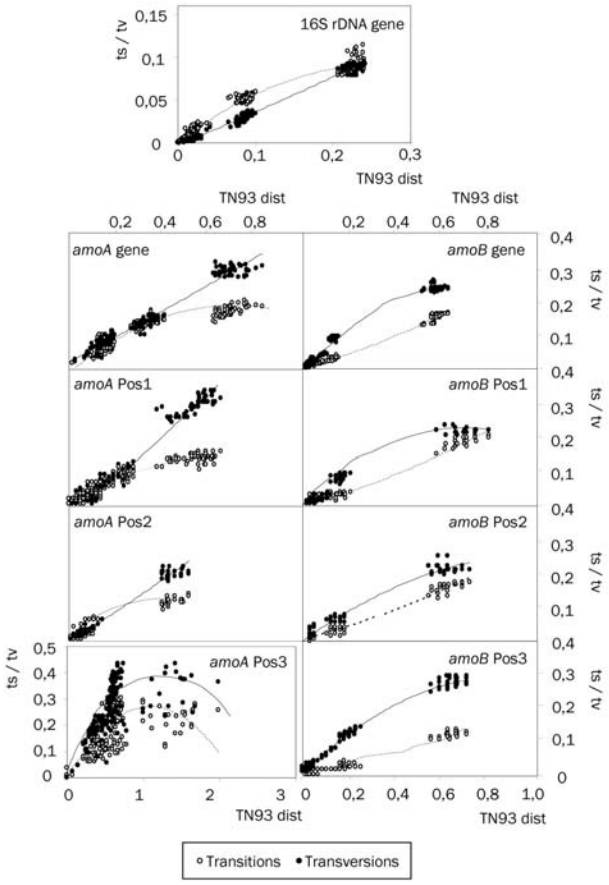


Figure 7.3 Saturation graphics for 16S rDNA, *amoA* and *amoB* genes,and for the three positions of the codon in *amoA* and *amoB*, showing the rate of transitions (Ts) and transversions (Tv) versus Tamura-Nei 93 distance

7.2.3 Phylogenetic and topology analyses

The model of nucleotide substitution best fitting the 16S rDNA, *amoA*, and *amoB* gene sequences were estimated by using the software MODELTEST 3.04 (Posada and Crandall, 1998). Each studied gene evolved under a distinct substitution model (Table7.4). Tamura-Nei 1993 (TrN93) is the nucleotide substitution model including the greatest number of parameters, and the one best fitting the combined dataset. Besides, the models Hasegawa-Kishino-Yano 1985 (HKY) and Felsenstein 1981 (F81), obtained for *amoA* and *amoB* respectively, can be considered simplifications of the model TrN93. We applied this model in all the trees based on genetic distances, with a single transition type and a single substitution rate when the selected models were HKY and F81 respectively.

Table 7.5 Nucleotide substitution models, obtained base frequencies and γ-shape distribution parameters for 16S rDNA, *amoA*, *amoB* genes, and for the polygenic dataset (MODELTEST 3.04 Posada and Crandall, 1998). TrN: Tamura Nei 1993; HKY: Hasegawa-Kishino-Yano 1985; F81: Felsenstein 1981

		16S rDNA	*amoA*	*amoB*	Polygenic
Model Selected		TrN + I + G	HKY + G	F81 + G	TrN+I+G
Base frequencies	A: C: G: T:	0.2668 0.2074 0.3108 0.2150	0.1820 0.2990 0.2546 0.2644	0.2321 0.2502 0.3148 0.2029	0.2258 0.2491 0.3026 0.2225
γ distribution shape parameter		0.5645	0.3546	0.1026	0.3824

A NJ tree was built by for each marker separately, with the appropriate nucleotide substitution models (Table 7.5; Figures 7.4, 7.6 and 7.8). The trees constructed by MP showed similar topologies with their NJ counterparts (Figures 7.5, 7.7 and 7.9). In all cases the *Nitrosomonas* and *Nitrosospira* radiations grouped together, and the γ-proteobacterial Nitrosococci branched separately. This agrees with the classical phylogenetic topology of the AOB. Likewise, two different clusters are distinguishable within the β-subgroup of the ammonia oxidizers, as *Nitrosomonas* and *Nitrosospira* clearly form two separate clades. Nevertheless, the allocation of *Nitrosomonas aestuarii* Nm36 is uncertain depending on the marker used, and groups within the *Nitrosospira* cluster when using *amoB*, whereas it falls within the *Nitrosomonas* group when using 16S rDNA and *amoA*. The ILD test corroborated ($P < 0.001$) the incongruence

between the phylogenetic information provided by the three markers. However, under some circumstances, to combine sequences with different phylogenetic histories can improve the accuracy of phylogenetic analysis (Wiens, 1998).

The consensus polygenic trees generated by MP and NJ are presented in Figure 7.10 and 7.11, respectively. The topology of both trees was similar and consistent with the classical classification of the AOB and with the results previously obtained with the three markers separately. In this polygenic analysis *Nm aestuarii* Nm36 was considered to be the most divergent *Nitrosomonas* within their branch.

Topological evaluations were performed through the modified Kishino and Hasegawa test (Shimodaira and Hasegawa, 1999). All the evaluations (namely likelihood, minimum evolution and parsimony criteria) indicate that the MP tree obtained from the composite dataset displayed the most likely topology (Table 7.6). Besides, with the exception of NJ+TN93 *amoB* approach, the rest of the trees are not significantly worse than the best supported tree.

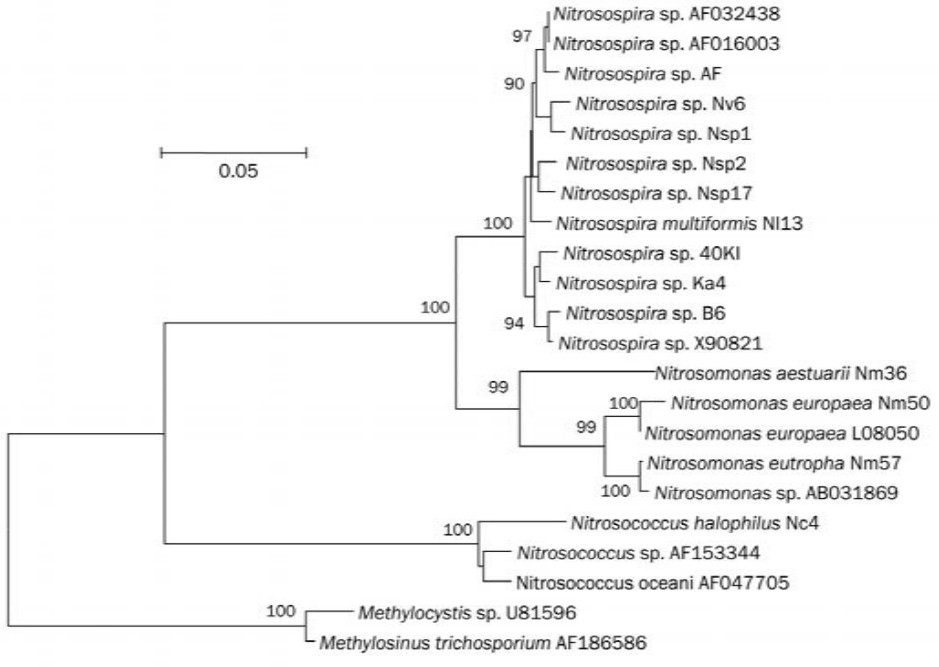


Figure 7.4 Neighbor-Joining (NJ) tree generated from an alignment of 16S rDNA sequences from AOB. The model of nucleotide substitution used was TrNei + I + G as specified in Table 7.4. Bootstrap values above 75% are shown. The scale bar represents 0.01 estimated changes per nucleotide. 16S rDNA sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups

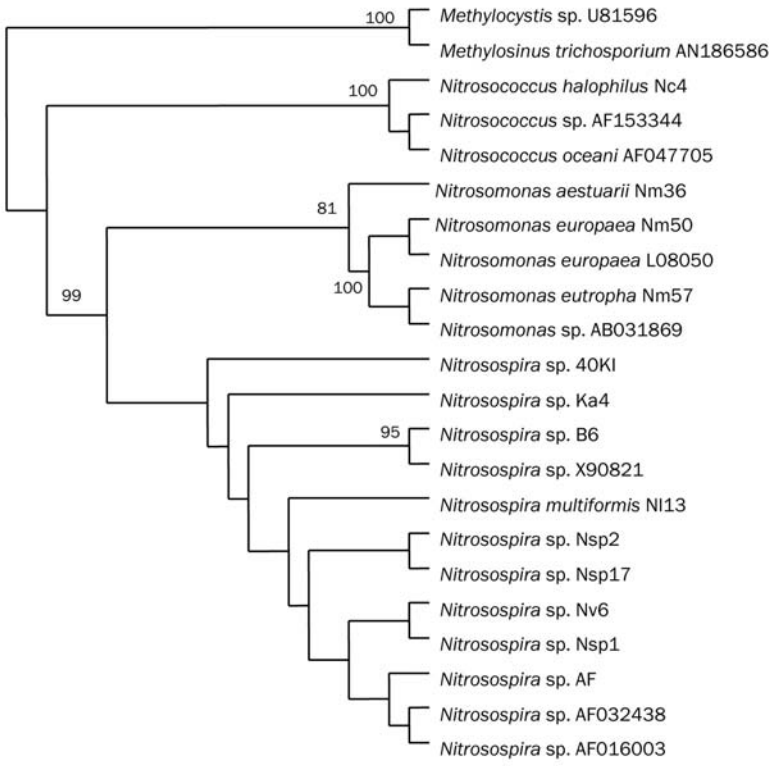


Figure 7.5 Maximum-Parsimony (MP) tree generated from an alignment of 16S rDNA gene sequences from AOB. Bootstrap values (in percentage) are shown. 16S rDNA sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups.

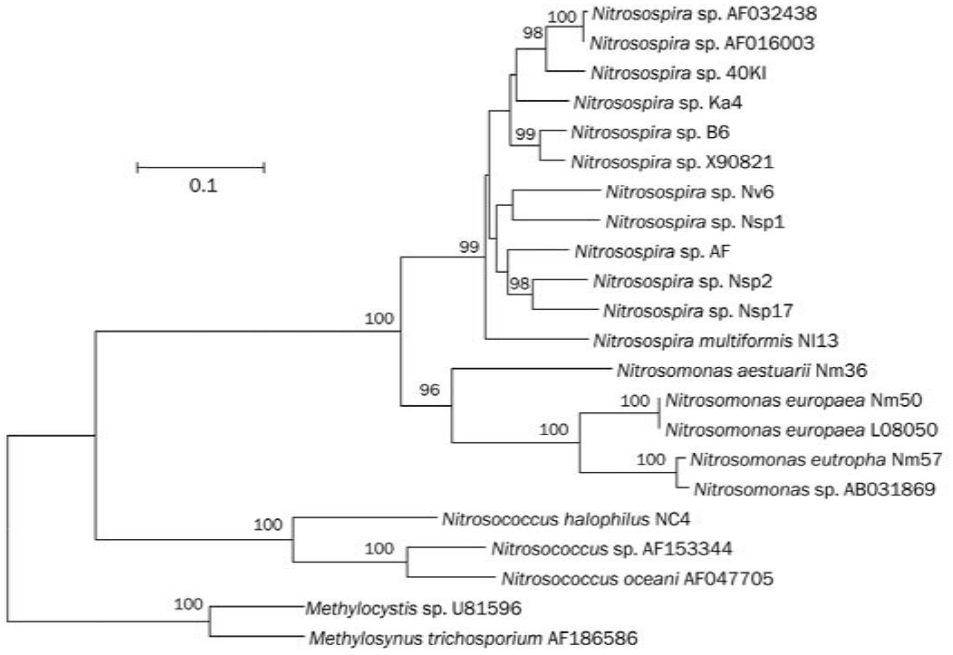


Figure 7.6 Neighbor-Joining (NJ) tree generated from an alignment of partial *amoA* sequences from AOB.The model of nucleotide substitution used was HKY + G as specified in Table 7.4. Bootstrap values above 75% are shown. The scale bar represents 0.01 estimated changes per nucleotide. Partial *pmoA* sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups

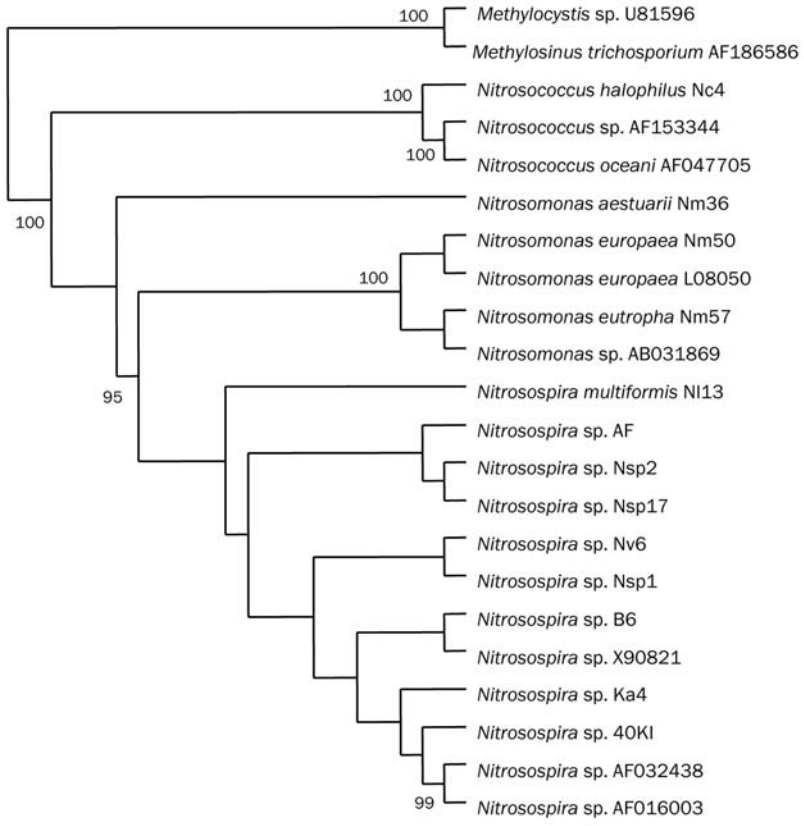


Figure 7.7 Maximum-Parsimony (MP) tree generated from an alignment of partial *amoA* sequences from AOB. Bootstrap values (in percentage) are shown. Partial *pmoA* sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups.

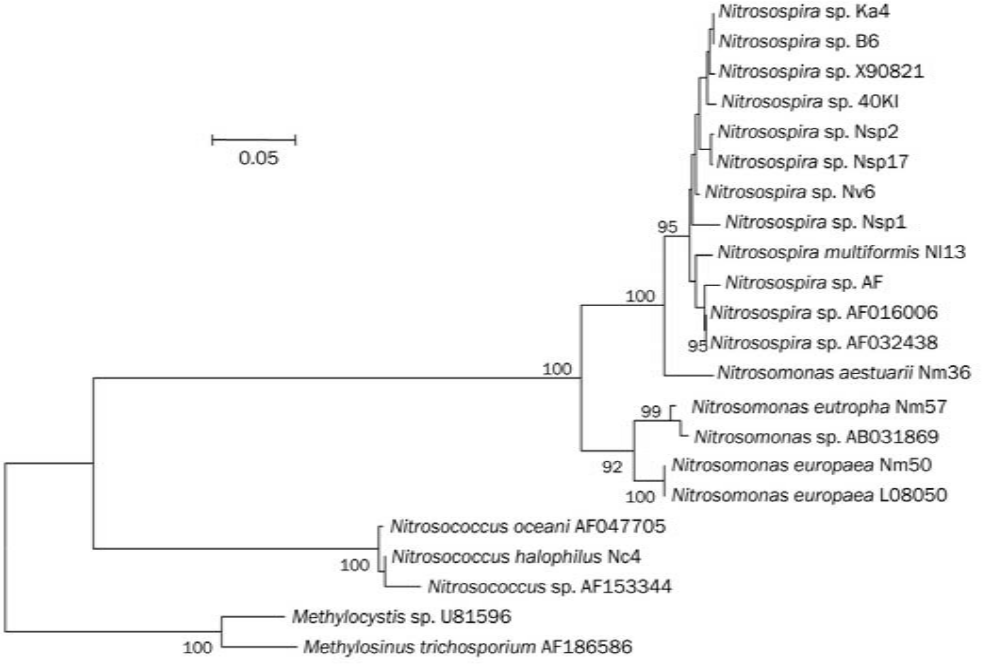


Figure 7.8 Neighbor-Joining (NJ) tree generated from an alignment of partial *amoB* sequences from AOB. The model of nucleotide substitution used was TrNei + I + G as specified in Table 7.4. Bootstrap values above 75% are shown. The scale bar represents 0.01 estimated changes per nucleotide. Partial *pmoB* sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups.

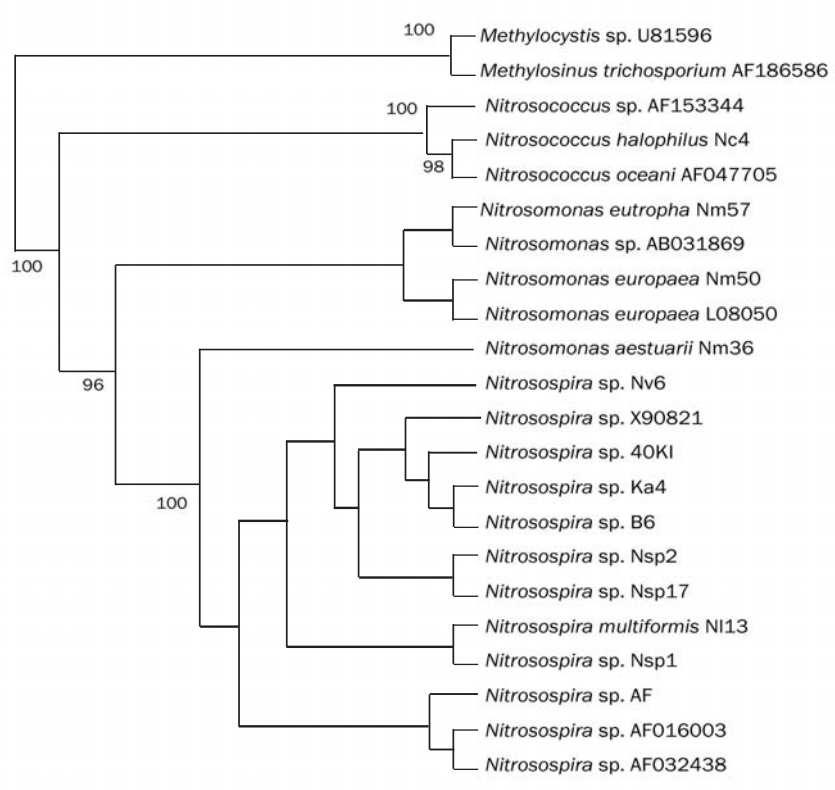


Figure 7.9 Maximum-Parsimony (MP) tree generated from an alignment of partial *amoB* sequences from AOB. Bootstrap values (in percentage) are shown. Partial *pmoB* sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups.

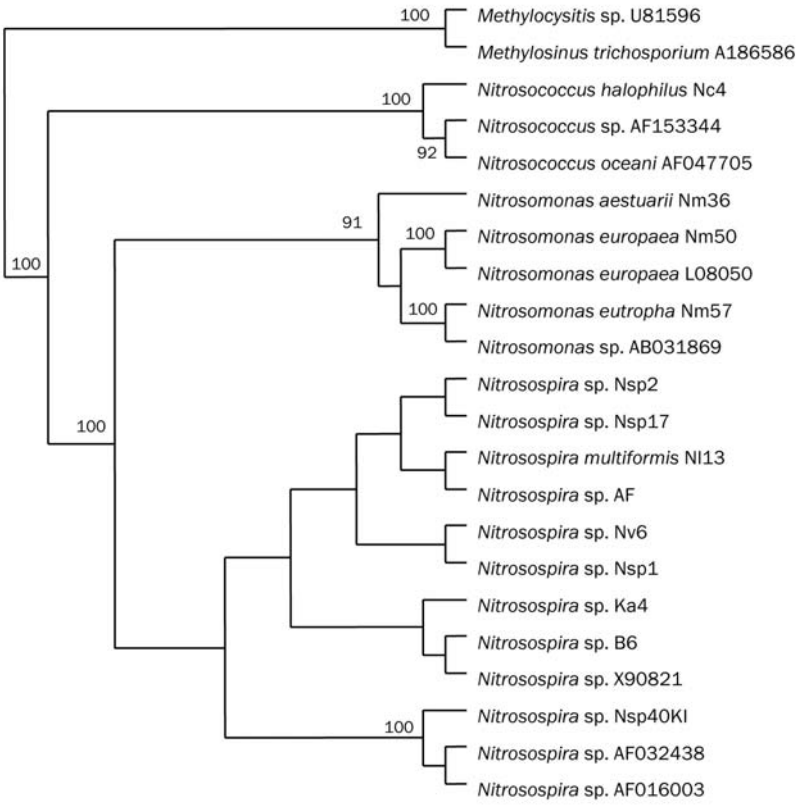


Figure 7.10 Maximum parsimony consensus tree generated from an alignment of partial *amoA*, *amoB* and 16S rDNA sequences from AOB. Bootstrap values (in percentage) are indicated. Partial *pmoA*, *pmoB* and 16S rDNA sequences from *Methylocystis* sp. U81596 and *M. trichosporium* AF047705 have been used as outgroups.

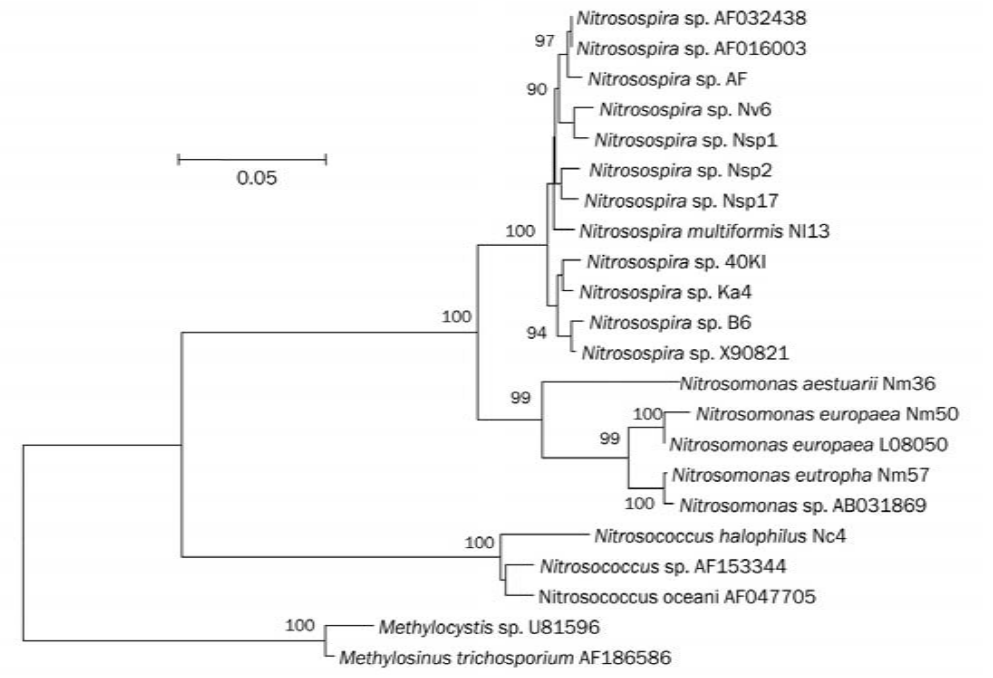


Figure 7.11 Neighbor-Joining (NJ) distance tree generated from an alignment of partial amoA, amoB an 16S rDNA sequences from AOB. Distance matrix was built based on Tamura-Nei, 1993 substitution rate. Bootstrap values (in percentage) are indicated. Partial pmoA, pmoB and 16S rDNA sequences from Methylocystis sp. U81596 and M. trichosporium AF047705 have been used as outgroups

Table 7.6 Comparisons among tree topologies. KHT: Shimodaira and Hasegawa (1999) likelihood analysis (likelihood parameters used are described in Table 7.4); ME: minimum evolution scores reported by PAUP 4.0b (Swofford, 1998); Parsimony length, CI and RI are respectively the number of steps, the consistency index and the retention index of the parsimony analysis for each topology using PAUP 4.0b. * Statistically significant best tree.

	Polygenic NJ+TN93	Polygenic PARS	*amoA* NJ+TN93	*amoA* PARS	*amoB* NJ+TN93	*amoB* PARS	16S rDNA NJ+TN93	16S rDNA PARS
KHT	-12764.5	-12755.1*	-12783.0	-12786.5	-12996.1	-13039.6	-12884.7	-12872.7
ME	1.67865	1.67904*	1.69236	1.75645	1.79980	1.79070	1.69853	1.71277
Parsimony length	2170	2168*	2176	2184	2260	2264	2206	2201
Parsimony CI	0.633	0.633*	0.631	0.629	0.608	0.606	0.622	0.624
Parsimony RI	0.771	0.772*	0.770	0.767	0.746	0.744	0.761	0.762

7.3 DISCUSSION

A correct classification of any bacterial group would require the input of different genetic and phenetic characters, which is not possible in uncultured bacteria from natural environments (Rosselló-Mora and Amann, 2001). Alternatively, a polygenic approach should lead to a more accurate estimation of the diversity and composition of a natural population (Park *et al.*, 2003; Pena *et al.*, 2004), although care must be taken when combining datasets from different markers (Wiens, 1998; Dolphin *et al.*, 2000).

The active site of the enzyme AMO is encoded by *amoA* (Mc Tavish *et al.*, 1993), which hinders the capability of this gene to accept mutations. In *amoA* gene, 50% of mutations were detected in the third basepair (Table 7.3), which evidences a great accumulation of nucleotidic changes at this position. As a consequence, the third position of the codon in the *amoA* gene presents a strong saturation (Figure 7.3) and deviation from the neutrality (Table 7.4,), suggesting that this position has experienced different selective pressures than the others (Klotz and Norton, 1998). In contrast, in the case of *amoB* c.a. 55% of conserved sites were detected in all the positions of the codon (Table 7.3). Moreover, c.a. 70% of the amino acid variations observed in the deduced partial amino acid sequences of AmoB are conservative (data not shown).

The general topology of most of the constructed trees is almost identical, with only the exception of *Nitrosomonas aestuarii* Nm36. This strain groups along with the *Nitrosospira* lineage when using *amoB,* but is affiliated to the *Nitrosomonas* when considering both 16S rDNA and *amoA* genes. Moreover, although included within the *Nitrosospiras* when using *amoB*, *Nm aestuarii* Nm36 can be distinguished by a considerable phylogenetic distance. The ambiguous phylogenetic arrangement of this specie depending on the treeing method employed, and the type of sequences used was previously reported by Purkhold *et al.* (2000).

In turn, in the polygenic tree, *Nm aestuarii* Nm36 falls into the *Nitrosomonas* clusters, respectively. The composite dataset, enclosing *amoA*, *amoB* and 16S rDNA sequences, contain more information than any marker independently, and it is therefore the best source to infer the most accurate classification. Thus, the marker leading to the tree best-fitting the information of the whole dataset should be the one of choice for taxonomical and diversity studies.

As expected, when comparing all the trees with the data using the likelihood, minimum evolution, and parsimony criteria, the best one is the polygenic MP

tree. However, the rest of the trees, with the exception of the NJ *amoB* tree, are not significantly worse, which indicates that phylogeny can be inferred by any of the markers.

Although our results may be biased due to the sequence sizes of the used markers (16S rDNA: 1014bp; *amoA*: 399bp; *amoB*: 393bp) and the number of parsimonic informative sites, they support 16S rDNA as a good phylogenetic marker, especially concerning the avoidance of ambiguous classifications due to lateral gene transfers. Several authors have recently reaffirmed the potential of 16S rDNA sequences for phylogenetic inferences (Ludwig and Schleiffer, 1999; Béjà *et al.*, 2002; Purkhold *et al.*, 2003).

However, obtaining the 16S rDNA gene from environmental samples is a time-consuming and tedious work. It requires the cloning of all the 16S rDNA genes present in the sample, and it is further necessary to distinguish 16S rDNA genes belonging to the AOB from the rest. In contrast, environmental population studies based on the analysis of *amoA* or *amoB* genes present some remarkable advantages: they are AOB-specific, have an adequate size to perform quick fingerprinting techniques of natural communities, and provide a phylogeny consistent with the current taxonomic outlines. Additionally, one extra benefit of using *amoB* in ecophysiological studies is the possibility of distinguishing methane-oxidizing bacteria from the AOB with a simple agarose gel (Calvó and Garcia-Gil, 2004).

7.4 CONCLUDING REMARKS

On the basis of the results hereby presented, and depending on the purposes of the study to perform, we recommend:

For taxonomical purposes 16S rDNA should be the most reliable marker available. Nevertheless, since taxonomical studies are usually carried out with pure cultures, we strongly recommend sequencing amoA and amoB genes as well, and building a polygenic tree. Whenever possible, sequences of *amoA* and *amoB* genes should also be obtained in order to check for possible recent evolution episodes such as lateral gene transfers. The respective trees must be generated by using the appropriate models of nucleotide substitution.

In environmental ecophysiological studies we recommend using either *amoA* or *amoB* genes. Both are AOB specific, provide the possibility of performing fingerprinting techniques such as tRFLP or DGGE, and render a reliable phyloge-

netic profile. Moreover, when using *amoB* as a marker, the methane-oxidizers present in the sample can be quickly and easily distinguished from the AOB, which may be of great help in complex samples.

8 GENERAL DISCUSSION

The first studies dealing with ammonia oxidizing bacteria were published by the end of the XIX century, when Percy Frankland and his wife, as well as Sergei Winogradsky (1890), isolated the first ammonia oxidizers. Although they postulated the existence of numerous species capable of oxidizing ammonium, new ammonia oxidizers were not isolated until 1965 (Watson, 1965).

Traditionally, microbiology studies were based on culture-dependent techniques, but nowadays we know that most of the culture media and conditions used do not appropriately reproduce the natural nitrifying habitats. Therefore, quantification and recovering of most of the bacterial groups in the environment (including AOB) always underestimates the real numbers.

In the 80s, the first phylogenetic analyses revolutioned taxonomy and the understanding of microbial diversity in the environment. The study of DNA revealed huge numbers of unknown species and rendered a new perspective of taxonomy and phylogenetic relationships among microorganisms. Today, comparative 16S rRNA gene sequence analysis (Head *et al.*, 1993; Teske *et al.*, 1994; Utåker *et al.*, 1995; Purkhold *et al.*, 2000) have confirmed and improved the new microbial classification of ammonia oxidizers. Additionally, other molecular markers such as the ISR 16SrDNA–23SrDNA fragment (Aakra et al 1999), the *amoA* gene (Rotthauwe *et al.*, 1997; Stephen *et al.*, 1999), and the *amoB* gene (Calvó and Garcia-Gil, 2004) have been introduced, and are comprehensively used in AOB studies (Aakra *et al.*, 2001; Jiang 1999). Currently, massive numbers of both 16S rDNA and *amoA* sequences can be found in the databases. Furthermore, these molecular markers have been used in the last years in quantitative PCR-based techniques (Harms *et al.*, 2003; Okano *et al.*, 2004; Aoi *et al.*, 2004; This work), which allow obtaining

accurate and reliable estimations of the real density of AOB, and of their activity in a given sample. Besides, the whole genome of several ammonia-oxidizers is being sequenced, and the genome approx 2.2Mb of *Nitrosomonas europaea,* the first AOB ever isolated, has already been sequenced and annotated (Chain *et al.*, 2003).

The main goal of this project was to find and improve functional and reliable tools to identify and analyze the ammonia oxidizing bacteria. The first technique used in this work to detect and quantify the ammonia oxidizer bacteria growing on nitrifying rotating biodiscs was fluorescence in situ hybridization (FISH).

The probe used was Nso190, which detects only previously known ammonia oxidizers from the β- Proteobacteria, and does not take into consideration other AOB subgroups. And in addition, the growth of ammonia oxidizers in microcolonies supposes an additional setback to the use of FISH in the study of natural communities of ammonia oxidizing bacteria. Consequently, other techniques should be used to ensure a whole coverage of the ammonia oxidizers group, as well as to pursue phylogenetic or taxonomical purposes.

The second attempt consisted in the development of a new set of 16S rRNA-targeted primers combined with denaturing gradient gel electrophoresis for fingerprinting analyses of AOB populations. The method presented provided a sensitive screening approach to assess the diversity and to monitor AOB communities. Moreover, improving the tedious and time-consuming culture-based procedures (Matulewich *et al.*, 1975), the new protocol allowed the detection of AOB in only a few days and avoided culturability biases. However, when dealing with low–oxygenated samples, false positives were obtained, and still, the main disadvantage of this approach was the impossibility of simultaneously detecting the whole group of β- and γ-proteobacterial ammonia oxidizers.

Since the analysis of 16S rDNA with specific primers is only possible within certain phylogenetic groups belonging to the *Proteobacteria*, a new molecule, other than the 16S rRNA, was needed to recognize all AOB lineages with a single tool.

The gene *amoA,* integrated into the AMO operon and encoding the active site of the enzyme ammonia monooxygenase, had been used as an alternative target for the molecular analysis of ammonia oxidizing bacteria (Sinigalliano *et al.*, 1995; Rotthauwe *et al.*, 1997; Juretschko et al 1998; Stephen *et al.*, 1999; Norton *et al.*, 2002). Since the ammonia monooxygenase enzyme is responsible for the oxidation of ammonia to hydroxilamine, is present in all

the ammonia-oxidizers (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998), which guaranteed the enclosure of the whole physiological group of ammonia-oxidizers. The use of the *amoA* gene as a molecular marker has been shown to provide a sensitive tool for performing diversity studies in ammonia oxidizing communities (Rotthauwe, 1997; Stephens et al 1999). Nevertheless, the process of cloning is time consuming, which is a handicap when multiple environmental samples have to be quickly analyzed. Besides, some authors optimized the application of an *amoA* PCR-DGGE approach (Oved *et al.*, 2001; Nicolaisen and Ramsen, 2002), but reported the amplification of non-AOB sequences, which, as recently pointed out by Cébron *et al.* (2004), denotes that this technique must be cautiously performed. Moreover, when used for phylogeny inference the resolution obtained is still bigger with 16S rDNA gene sequences than with *amoA* (Purkhold *et al.*, 2003). Therefore, a new molecular marker was required to obtain a faster analytical tool providing rigorous phylogenetic information.

With this aim we developed a new set of primers for the use of partial *amoB* sequences as a molecular marker. The gene *amoB*, is also included into the AMO operon, and is supposed to encode a 43 KDa transmembrane domain of the enzyme ammonia-monooxygenase (Bergmann and Hooper, 1994; Vanelli *et al.*, 1996). *AmoB* was first used to study AOB by Pinck *et al.* (2001). They used polyclonal antibodies targeting the AmoB domain of ammonia monooxygenase, but this technique detected only the four AOB genera belonging to the β–subclass of the Proteobacteria. The *amoB*-targeted primers presented here demonstrated to be suitable to obtain partial *amoB* gene sequences from all β– and γ–ammonia–oxidizers tested.

In order to perform comparative *amoB* sequence analysis for diversity, distribution or phylogenetic studies, the construction of a database of *amoB* sequences was required. Thus, partial *amoB* sequences from the main representatives of each lineage of ammonia oxidizers were obtained. In general, the phylogenetic clustering obtained using *amoB* partial sequences was in agreement with that previously defined based on 16S rDNA and 16S–23S rDNA sequences, which demonstrated that the gene *amoB* was a good molecular marker for AOB, and that the primers presented in this work supposed a new tool for studies involving the whole physiological group of ammonia oxidizers.

Currently, quantitative PCR methods are being developed and applied in the study of ammonia oxidizers (Harms *et al.*, 2003; Okano *et al.*, 2004; Aoi *et al.*, 2004). To date, 16S rDNA and *amoA* have been used as a target

for quantitative PCR studies. In this work we present the first *amoB* targeted RTi-PCR tool, with specificity for the members of the genus *Nitrosococcus*, from the γ-subclass of the Proteobacteria. This study demonstrates the utility of the gene *amoB* as an alternative target for quantitative studies. Pinck *et al.* (2001) detected AMO in great amounts in one year starving cells, which seemed to indicate that the quantity of ammonia monooxygenase cannot be straightly correlated to ammonium oxidation activity. Nevertheless, since Aoi *et al.* (2004) demonstrated a long-term relationship between *amoA* mRNA levels and ammonia oxidation activities, the results hereby presented constitute a preliminary step towards the development of a competitive *amoB*-based RT-PCR tool to estimate the activity the ammonia oxidizers.

Our final goal was to situate *amoB* as a new marker in the global context of the main molecular markers existing for the study of AOB by comparing and evaluating our partial sequences to the rest. Besides, we were aimed to determine whether a polygenic or single-marker analysis was suitable for taxonomical purposes, and to build, if possible, a polygenic tree. Therefore the markers *amoA*, *amoB*, and 16S rDNA were independently evaluated by several genetic tests to obtain information of the quantity of useful information contained in their respective sequences. In addition, the phylogenetic trees built from each gene were weighted against the composite sequence dataset to identify the most useful marker reproducing the information resulting from the polygenic tree. As a result we recommend using different molecular markers in view of the kind of study to perform, thus, for taxonomical purposes 16S rDNA should be the most reliable marker available, although we strongly recommend sequencing *amoA* and *amoB* genes as well, and building a polygenic tree. In contrast, for environmental ecophysiological studies we recommend using either *amoA* or *amoB* genes due to their specificity, and to the possibility of performing fingerprinting techniques rendering reliable phylogenetic profiles. Besides, an advantage of the use of *amoB* as a marker is that it allows discriminating the methane-oxidizers if present in the sample.

9 CONCLUSIONS

1. Although useful for detection and enumeration, FISH is not a suitable technique to either identify or perform microbial ecology studies on AOB. This is mainly due to the fact that available probes only match known sequences, and therefore unknown species might be overlooked.

2. DNA probes targeting 16S rDNA can be used in combination with eubacterial universal oligonucleotides in a semi-specific PCR for community screening by PCR–DGGE.

3. Of all oligonucleotides tested, Nso1225 displayed the highest resolution as it targeted the widest AOB phylogenetic range. However, some β-proteobacterial non-AOB sequences were also retrieved, showing that phylogenetic separation among AOB and closest relatives based on 16SrDNA was not clear-cut.

4. The marker based on *amoA* was useful to assess the AOB community of a SBR, but offered less resolution than 16S rDNA. In addition, the use of *amoA* requires cloning, which considerably increases sample processing times.

5. The gene *amoB*, encoding the transmembrane subunit of ammonia monooxygenase, has been shown to be a good alternative to either *amoA* or 16SrDNA. Main advantages are: (i) Primers were found to be highly specific and sensitive (only 20 pg of target genome DNA are required) (ii) Methane oxidizing bacteria were also detectable, yielding a smaller PCR product which can be used to reveal the presence of this physiological bacterial group in natural samples. (iii) All known AOB were positive for this marker regardless of phylogenetic affiliations.

6. AmoB deduced proteins of *Nitrosomonas* and *Nitrosospira* clusters share 75% homology and are clearly differentiated from *Nitrosococcus* group, which only shares 35% identity. This reveals a long evolutive distance between both *Nitrosomonas / Nitrosospira* and *Nitrosococcus* groups.

7. Partial *amoB* sequences from *Nitrosococcus* are sufficiently conserved to be used as a quantitative molecular tool by real-time PCR. The designed unique (not degenerate) primer set is highly sensitive and specific for this genus.

8. Comparison of AOB phylogeny based on *amoA*, *amoB* and 16S rDNA reveals a consistent grouping that includes *Nitrosomonas* and *Nitrosospira* on one side and *Nitrosococcus* on the other. Polygenic phylogenetic analysis was not different from that obtained from 16S rDNA alone. Grouping for individual markers is also conserved.

9. The marker based on *amoB* is a suitable molecular tool to readily assess diversity and phylogeny of AOB in environmental samples. In contrast, 16S rDNA is indicated for taxonomical and phylogenetic inference from isolates.

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