Lehrstuhl für Mikrobiologie
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Untersuchungen zur Phylogenie und Verbreitung Ammoniak oxidierender Bakterien

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meinen Eltern

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Abkürzungsverzeichnis

A	Adenin
AMO	Ammoniakmonooxygenase
ARB	*arbor* (lat. “der Baum”; Software-Paket für phylogenetische Datenanalyse)
ATP	Adenosintriphosphat
beta-AOB	Ammoniak oxidierende Bakterien der Klasse *Betaproteobacteria*
bp	Basenpaare
C	Cytosin
CLSM	konfokale Laserscanning Mikroskopie/konfokales Laserscanning Mikroskop
DGGE	denaturierende Gradienten-Gelelektrophorese
DMSO	Dimethylsulfoxid
DNS	Desoxyribonukleinsäure
ERG	Eppendorfreaktionsgefäß
et al.	*et. alii* (lat. “und andere”)
FA	fluoreszente Antikörper
FISH	Fluoreszenz *in situ* Hybridisierung
G	Guanin
HAO	Hydroxylaminoxidoreduktase
HRT	hydraulic retention time (hydraulische Verweilzeit)
ICM	intracytoplasmatische Membran(en)
Min.	Minute(n)
MOB	Methan oxidierende Bakterien
MPN	most probable number (wahrscheinlichste Zahl bei Zellzahlbestimmung)
N	chem. Zeichen für Stickstoff
NAD(P)	Nikotinamidadenindinukleotid(phosphat)
Nc.	*Nitrosococcus*
Nm.	*Nitrosomonas*
NOB	Nitrit oxidierende Bakterien
PCR	polymerase chain reaction (Polymerase-Kettenreaktion)
PFA	Paraformaldehyd
pMMO	partikuläre Metanmonooxygenase
RNS	Ribonukleinsäure
rRNS	ribosomale Ribonukleinsäure
Sek.	Sekunden
T	Thymin
T-RFLP	terminaler Restriktionsfragment-Längenpolymorphismus
UV	ultraviolett

EINLEITUNG

Der Stickstoffkreislauf

Stickstoff bildet neben den Elementen Kohlenstoff, Wasserstoff, Sauerstoff, Schwefel und Phosphor die Grundlage für Leben in der Form, wie wir es heute kennen. Die größte Menge an Stickstoff ($3{,}8 \cdot 10^{17}$ t) liegt als Elementargas (N_2) in der Atmosphäre vor. In der Biosphäre dagegen bildet tote organische Substanz die Haupt-Stickstoffreserve ($8{,}5 \cdot 10^{11}$ t) (Brock *et al.*, 1997). Der Großteil dieser Reserven ist jedoch für das Wachstum von Pflanzen nicht verfügbar, da diese Stickstoff nur in Form von Nitrat oder –seltener– Ammonium aufnehmen können. In die Nahrungskette gelangt der lebensnotwendige Stickstoff erst durch die Aktivität von Mikroorganismen, die in der Lage sind, assimilierbare N-Verbindungen zu produzieren. Daher kommt dem Stickstoffkreislauf, d. h. der kontinuierlichen Umwandlung der verschiedenen Stickstoffverbindungen ineinander (Abbildung 1), in der Natur eine zentrale Bedeutung zu. Essentiell ist dabei die Oxidation von Ammoniak (NH_3) zu Nitrit (NO_2^-). Aerobe, chemolithoautotrophe, Ammoniak oxidierende Bakterien (AOB) sind als einzige Organismen in der Lage, aus dieser Umsetzung Energie zu gewinnen (Hooper, 1969). Das entstandene toxische Nitrit wird in der Natur in Anwesenheit von Sauerstoff von einer weiteren chemolithotrophen Organismengruppe, den Nitrit oxidierenden Bakterien (NOB), rasch zu Nitrat umgewandelt (El-Demerdash und Ottow, 1983; Schmidt, 1982). Ammoniak- und Nitritoxidation sind daher eng verknüpft und werden unter dem Begriff Nitrifikation zusammengefasst. Ohne Nitrifikanten wäre die Aufrechterhaltung eines geschlossenen Stickstoffkreislaufes (Abbildung 1) nicht gewährleistet.

Zu der Umsetzung $NH_3 \rightarrow NO_2^-$ sind neben den AOB noch einige heterotrophe Bakterien, Pilze und Algen befähigt. Diese „heterotrophe Nitrifikation“ ist jedoch nicht mit Energiegewinn verbunden (Wood, 1988). Welchen Anteil sie an der Gesamt-Nitrifikation hat, ist umstritten. (Barraclough und Puri, 1995; Killham, 1986; Robertson und Kuenen, 1988; Stams *et al.*, 1990).

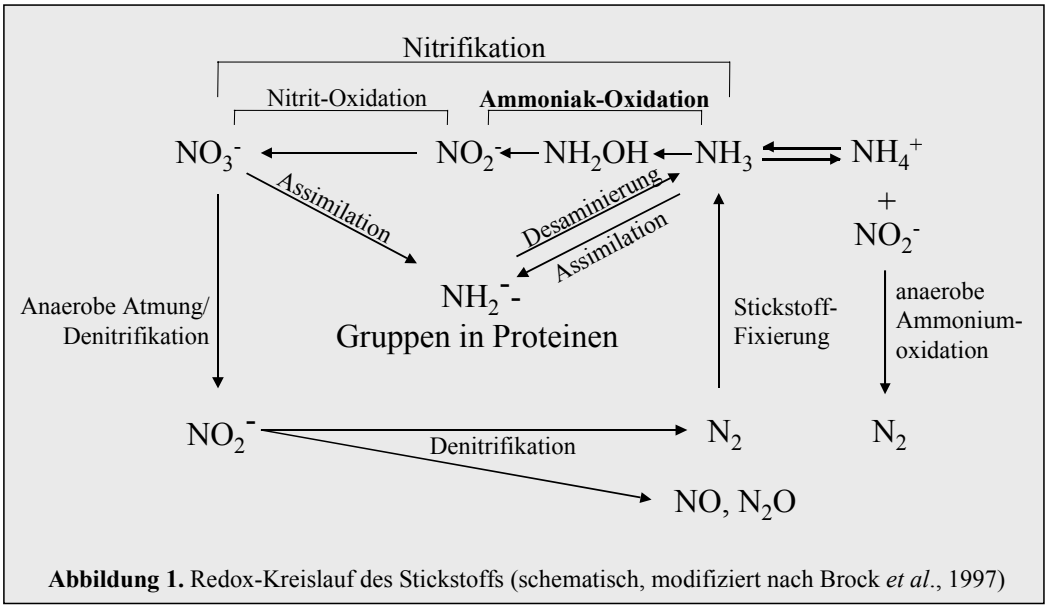


Abbildung 1. Redox-Kreislauf des Stickstoffs (schematisch, modifiziert nach Brock *et al*., 1997)

Biochemie der Ammoniakoxidation

Bei der autotrophen Oxidation von Ammoniak zu Nitrit handelt es sich um eine zweistufige Reaktion, ausgehend von NH_3 (nicht NH_4^+; Drozd, 1976; Suzuki *et al.*, 1974), mit Hydroxylamin als Zwischenprodukt. Verantwortlich für diese Umsetzung sind die Enzyme Ammoniakmonooxygenase (AMO) (Hollocher *et al.*, 1981) und Hydroxylamin-oxidoreduktase (HAO) (Wood, 1986):

$$\text{AMO:} \quad NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \qquad (1)$$

$$\text{HAO:} \quad NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \qquad (2)$$

Die AMO (Abbildung 2) ist ein kupferhaltiges, membranassoziiertes Enzym mit breitem Substratspektrum: Neben Ammoniak können eine Reihe apolarer Verbindungen wie Methan, Kohlenmonoxid, sowie einige aliphatische und aromatische Kohlenwasserstoffen oxidiert werden (Hooper *et al.*, 1997). Andererseits kann die AMO eine extrem hohe enantiomere Selektivität entwickeln (Hooper *et al.*, 1997; Vannelli und Hooper, 1995). Einige Verbindungen inhibieren die Ammoniakoxidation irreversibel (Acetylen) (Hyman und Wood, 1985) oder kompetitiv (Ethylen) (Hyman und Wood, 1984; Keener *et al.*, 1998). Eine spezifische Hemmung wird zudem durch metallbindende Agenzien (Allyl-Thioharnstoff) (Hooper und Terry, 1973) sowie Licht (Hooper und Terry, 1974) erreicht. Alle Substrate und kompetitive Inhibitoren sind unpolar, was auf ein hydrophobes aktives Zentrum schließen lässt (Hooper *et al.*, 1997).
Die AMO ist homolog zu der membrangebundenen, partikulären Methan-Monooxygenase (pMMO) der Methan oxidierenden Bakterien (MOB) (Bedard und Knowles, 1989) und besteht aus den Untereinheiten AmoC, AmoA, und AmoB. Diese werden jeweils von den Genen *amoC*, *amoA* und *amoB* codiert, die zusammen ein Operon bilden (in einigen AOB findet sich außerhalb dieses Operons zusätzlich eine weitere *amoC*-Kopie). Das *upstream* von *amoA* und *amoB* gelegene *amoC*-Gen codiert möglicherweise ein Chaperon, das die korrekte Integration der beiden übrigen Untereinheiten in die Membran unterstützt (Klotz *et al.*, 1997). Die spezifische Bindung des Suizid-Substrats Acetylen an die AmoA-Untereinheit lässt darauf schließen, dass diese das aktive Zentrum trägt (Ensign *et al.*, 1993).

Die HAO ist ein trimeres Multihäm-Protein und –im Gegensatz zur AMO– im Periplasma lokalisiert (Abbildung 2). Der Enzymkomplex beinhaltet 24 Häm-Zentren in drei äquivalenten Untereinheiten. Jedes Monomer enthält neben sieben c-Typ Häm-Zentren das Häm P460 (Igarashi *et al.*, 1997). Die HAO wird durch H_2O_2 inhibiert (Hooper und Terry, 1997) und kann als alternatives Substrat Hydrazin (N_2H_4) verwerten (Hooper und Nason, 1965).

Sowohl das *amoCAB*-Operon als auch das *hao*-Gen liegen bei einigen AOB (z. B. *Nitrosomonas europaea*) in mehreren Kopien vor (Hommes *et al.*, 1996, 1998, 2001; Stein *et al.*, 2000).

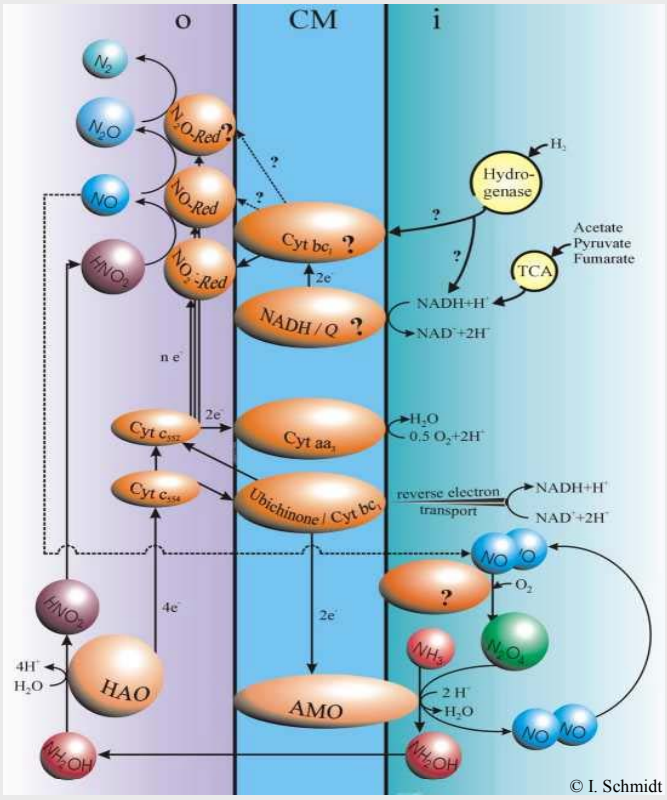


Abbildung 2. Modell des Elektronenflusses in ammoniakoxidierenden Bakterien. CM = Cytoplasmamembran, i= innerhalb der Zelle, o = außerhalb der Zelle, TCA = Tricarboxylsäurezyklus (aus Bock und Wagner, 2001).

Neben der AMO und HAO wurden bei einigen AOB auch *nirK*-Gene, codierend für eine kupferhaltige dissimilatorische Nitrit-Reduktase, nachgewiesen (Casciotti und Ward, 2001; Dispirito *et al.*, 1985; Miller und Nicholas, 1985). Mit Hilfe dieses Enzyms können AOB unter Sauerstoff-limitierten Bedingungen (mit Nitrit als Elektronen-Akzeptor und Ammoniak oder Wasserstoff als Elektronen-Donor) geringe Mengen Stickstoffmonoxid und Distickstoffoxid produzieren (Hooper, 1968; Remde und Conrad, 1990; Stüven *et al.*, 1992). Ob die denitrifizierende Aktivität der Energieerhaltung oder der Entgiftung (durch die Umwandlung des toxischen Nitrits) dient, ist bisher nicht geklärt (Shapleigh, 2002).

Physiologie Ammoniak oxidierender Organismen

1890 gelang Winogradsky bei Versuchen mit AOB der endgültige Nachweis autotrophen Wachstums bei chemolithotrophen Mikroorganismen (Winogradsky, 1890). Im Zuge dieser Untersuchungen isolierte er –wie kurz zuvor bereits Frankland und Frankland (Frankland und Frankland, 1890)– AOB in rein mineralischem Medium mit Ammoniak als Elektronen-Donor und CO_2 als einziger Kohlenstoffquelle.
Auffallend ist die ungewöhnlich lange Generationszeit dieser Organismen in Reinkulturen (Minimum: 7h). Dieses extrem langsame Wachstum ist vor allem auf die hohen Redoxpotentiale der Redoxpaare NH_2OH/NH_3 (+899 mV) und NO_2^-/NH_2OH (+66 mV) zurückzuführen. Der erste Oxidationsschritt ist endergonisch und die gesamte Energieausbeute pro Mol oxidierten Ammoniaks beträgt lediglich 120kJ. Das entspricht ca. 4% des Energiegewinns, den heterotrophe Organismen bei der Oxidation eines Mols Glucose erzielen und hat zur Folge, dass AOB pro Stunde bis zu 1/3 ihres Zellgewichtes Ammoniak umsetzen. Zudem müssen die Reduktionsäquivalente NAD(P)H aufgrund der hohen Redoxpotentiale der Redoxpaare über reversen Elektronentransport regeneriert werden (Aleem, 1966; Bock et al., 1992). Schließlich fließen bis zu 80% des gewonnen ATP in die energieaufwendige CO_2-Fixierung über den Calvin-Benson-Zyklus (Belser, 1984; Harms *et al.*, 1981; Wallace und Nicholas, 1969). Alternativ kann Kohlendioxid über das Enzym Phosphoenolpyruvat-Carboxylase fixiert werden (Takahashi *et al.*, 1993).

Verbreitung Ammoniak oxidierender Organismen

AOB kommen in zahlreichen aeroben Habitaten vor. Man findet sie sowohl in natürlichen Lebensräumen –Böden, Fels- und Gebäudeoberflächen, Süß- und Brackwasser, Weltmeere, etc. (Koops und Pommerening-Röser, 2001)– als auch in künstlichen Systemen wie z.B. Kläranlagen (Juretschko *et al.*, 1998; Purkhold *et al.*, 2000; Wagner *et al.*, 1995), Aquarien (Burrell *et al.*, 2001), oder Trinkwasseraufbereitungsanlagen (Regan *et al.*, 2002). AOB mit Verbreitung in allen potentiellen Habitaten sind bisher jedoch nicht bekannt (Koops und Pommerening-Röser, 2001); bei den einzelnen AOB handelt es sich eher um Spezialisten als Generalisten. Signifikante Unterschiede zwischen den Vertretern der unterschiedlichen ökophysiologischen Gruppen zeigen sich z. B. bezüglich der Sensitivität gegenüber Ammoniak (*Nitrosomonas oligotropha*: 50 mM, *N. eutropha*: 600 mM) sowie in der Salztoleranz (*N. oligotropha*: 50 mM, *N. halophila*: 900 mM NaCl) (Koops *et al.*, in Vorbereitung). Geringe Ammoniaktoleranz geht meist mit hoher Substrat-Affinität (niedrige Ks-Werte des Ammoniak oxidierenden Systems) und der Fähigkeit zur Harnstoffspaltung einher (Koops und Pommerening-Röser, 2001); beide Merkmale werden als Anpassung an oligotrophe Habitate interpretiert. Des weiteren hat der pH-Wert großen Einfluss auf die Populationsstruktur der AOB. Dies liegt unter anderem daran, dass sich mit sinkendem pH

das Säure-Base-Gleichgewicht NH_4^+/NH_3 aufgrund des hohen pKs-Wertes von Ammoniak (9,25) in Richtung NH_4^+ verschiebt. Da der AMO Ammoniak und nicht Ammonium als Substrat dient (Suzuki *et al.*, 1974), sind im leicht sauren Milieu die AOB begünstigt, welche im Besitz einer Urease sind. Dieses Enzym ist im Zellinneren lokalisiert und stellt mit dem Ammoniak aus der Harnstoffspaltung zusätzliches Substrat für die AMO zur Verfügung (De Boer und Laanbroek, 1989).
Neben den oben genannten Faktoren beeinflussen auch Temperatur (Golovacheva, 1976; Jones und Morita, 1985), Sauerstoffverfügbarkeit (Goreau *et al.*, 1980) und Lichtintensität (Hooper und Terry, 1974) die AOB-Populationsstruktur.

Bedeutung Ammoniak oxidierender Organismen

Wie alle Lebewesen stehen auch die AOB in steter Wechselwirkung mit ihrer Umwelt, und nehmen durch die von ihnen katalysierten biochemischen Vorgänge direkt oder indirekt Einfluss auf ihre Umgebung.
Beispielsweise führt die Umsetzung des Ammoniak-Anteils in Düngemitteln zu verstärktem N-Austrag aus landwirtschaftlichen Flächen. Grund dafür sind die unterschiedlichen Ladungseigenschaften von NH_4^+/NH_3 und NO_2^- bzw. NO_3^-. Positiv geladene Verbindungen werden aufgrund der ausgeprägten Adhäsion an Bodenpartikel weniger leicht ausgewaschen als die wasserlöslichen Verbindungen, was zur Eutrophierung der umliegenden Gewässer (Schwoerbel, 1993) sowie zum Eintrag von Nitrat/Nitrit ins Grundwasser führen kann. In ungepufferten Böden bedingt die Versauerung in manchen Fällen die Freisetzung phytotoxischer Kationen, wie beispielsweise Al^{3+} (Spieck und Bock, 1998). Darüber hinaus trägt die denitrifizierende Aktivität von AOB durch die Bildung flüchtiger Stickoxide zu weiterem Stickstoffverlust aus Böden, sowie einer erhöhten Konzentration des Treibhausgases N_2O, bei (Bouwman *et al.*, 1993; Hooper, 1968; Remde und Conrad, 1990; Stüven *et al.*, 1992). Zudem nehmen historische Bauten und andere Gebäude Schaden durch die Auflösung der Carbonat-haltigen Bestandteile des Mauerwerks durch die Säuren (salpetrige und Salpetersäure). Diese entstehen aus der Oxidation von Harnstoff und Ammoniak in Vogelkot oder N-haltigen Abgasen (Bock und Sand, 1993).
AOB haben jedoch nicht ausschließlich unerwünschte Wirkungen. In Kläranlagen stellt die Nitrifikation eine effiziente und kostengünstige Möglichkeit dar, Ammoniak, Harnstoff und Nitrit aus Abwässern zu eliminieren (Eighmy und Bishop, 1989; Painter, 1986). Dies ist für den Umweltschutz aufgrund der Toxizität von Ammoniak und Nitrit (Arthur *et al.*, 1987) sowie der eutrophierenden Wirkung von Ammoniak (Schwoerbel, 1993) von herausragender Bedeutung. Eine stabile Nitrifikation in Kläranlagen ist jedoch aufgrund der Sensitivität von AOB gegenüber niedrigen Temperaturen, pH-Schwankungen sowie toxischen Substanzen nicht immer gegeben (Painter, 1986; Rittmann und Whiteman, 1994).

Kürzlich wurde eine weitere Organismengruppe identifiziert, die Energie aus der Verwertung von Ammonium gewinnt: Die sogenannten ANAMMOX-Organismen setzen Ammonium unter anaeroben Bedingungen direkt zu N_2 um (Schmid *et al.*, 2000; Strous *et al.*, 1999) und bieten in Zukunft möglicherweise eine Alternative bei der Stickstoffentfernung aus Abwässern (Jetten *et al.*, 2001).

Untersuchung komplexer Biozönosen

Um Einblick in komplexe ökologische Zusammenhänge zu erlangen, werden zunächst Informationen bezüglich Identität und Abundanz der vertretenen Arten benötigt. Schon Winogradsky studierte 1890 mit Hilfe unterschiedlicher Kultivierungsmethoden die natürliche Diversität der AOB. Kultivierungsabhängige Ansätze, wie beispielsweise die MPN (*most probable number*)-Technik, bringen jedoch Nachteile mit sich. Häufig kommt es bei der Erfassung der Populationsstruktur zu quantitativen und qualitativen Verschiebungen der Resultate. Um diese Schwierigkeiten zumindest teilweise zu umgehen, wurde bis heute eine Vielzahl molekularer Methoden entwickelt. Fingerprinting-Techniken wie DGGE (denaturierende Gradienten-Gelelektrophorese; Kowalchuk *et al.*, 1997, 2000a, 2000b), oder T-RFLP (terminaler Restriktionsfragment-Längenpolymorphismus; Horz *et al.*, 2000) werden vielfach für Diversitätsanalysen Ammoniak oxidierender Populationen eingesetzt. Spezifische Antikörper (Pinck *et al.*, 2001) sowie FISH (Fluoreszenz-*in situ*-Hybridisierung; Juretschko *et al.*, 1998; Wagner *et al.*, 1995) erlauben den direkten Nachweis von AOB *in situ*, und Mikrochip-basierende Methoden (Guschin *et al.*, 1997; Wu *et al.*, 2001) versprechen hohen Proben-Durchsatz. Auch die quantitative Erfassung einzelner Populationen ist mittels FISH (Daims *et al.*, 2001; Schmid *et al.*, 2000) oder DotBlot (Mobarry *et al.*, 1996) realisierbar.

Klassifizierung Ammoniak oxidierender Organismen

Die große Vielfalt der Prokaryonten stellt Mikrobiologen vor das Problem, ein System zu schaffen, welches einerseits die Wiedererkennung bereits beschriebener Arten, und andererseits eine sinnvolle Zuordnung neuer Spezies erlaubt. Klassischerweise wurde hierzu als ein Kriterium die Morphologie von Bakterien (Zellgröße und -form, Beweglichkeit, Art der Begeißelung) herangezogen. Im Falle der autotrophen Ammoniak oxidierenden Organismen resultierte daraus eine Unterteilung in die fünf Gattungen *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, *Nitrosovibrio*, und *Nitrosospira* (Watson, 1974). Auch innerhalb der Zelle sind bei vielen AOB charakteristische Strukturen zu beobachten: Einige Spezies weisen unterschiedlich angeordnete intracytoplasmatische Membranstapel (ICM) (Koops und Möller, 1992), sowie Carboxysomen auf (kristalline, polyedrische Formen des Enzyms

Ribulose-1,5-bisphospat-Carboxylase; Wullenweber *et al.*, 1977; Nicholas und Rao, 1964) (Abbildung 3).

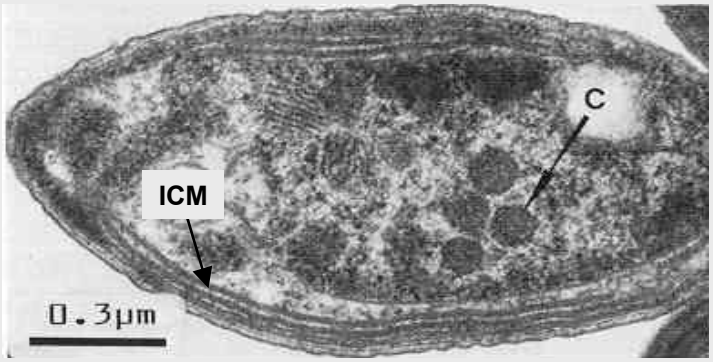


Abbildung 3. Elektronenmikroskopische Aufnahme eines Dünnschnittes durch eine *Nitrosomonas*-Zelle. C: Carboxysom, ICM: intracytoplasmatische Membranen (aus Koops *et al.*, 1992)

Diese morphologischen Merkmale spiegeln jedoch nicht zwingend die phylogenetischen Beziehungen der Bakterien wider. Das Verständnis der verwandtschaftlichen Verhältnisse innerhalb der Gruppe der Ammoniak oxidierenden Bakterien beruht heute vor allem auf der vergleichenden Sequenzanalyse zweier Markermoleküle: der RNS der kleinen ribosomalen Untereinheit (Woese, 1987; Woese *et al.*, 1985) sowie dem *amoA*-Gen, codierend für die aktive Untereinheit des funktionellen Schlüsselenzyms Ammoniakmonooxygenase (AMO) (Rotthauwe *et al.*, 1997). Teil- oder Vollsequenzen beider Gene sind jedoch nicht für alle beschriebenen AOB-Spezies publiziert (Alzerreca *et al.*, 1999; Head *et al.*, 1993; McTavish *et al.*, 1993; Teske *et al.*, 1994). Gemäß den Ergebnissen vergleichender 16S rRNS-Sequenzanalyse gehören alle untersuchten AOB zwei jeweils monophyletischen Entwicklungslinien innerhalb der *Proteobacteria* an (Abbildung 4). Eine bildet die marine Gattung *Nitrosococcus* (bestehend aus den beiden Arten *Nc. oceani* und *Nc. halophilus*) innerhalb der *Gammaproteobacteria*. Alle anderen AOB gehören zu den *Betaproteobacteria* (Head *et al.*, 1993; Pommerening-Röser *et al.*, 1996; Stehr *et al.*, 1995; Teske *et al.*, 1994; Utaaker *et al.*, 1995; Woese *et al.*, 1984) (Abbildung 4). Diese Zuordnung deckt sich weitgehend mit den Ergebnissen *amoA*-basierender phylogenetischer Studien (Rotthauwe *et al.*, 1997).

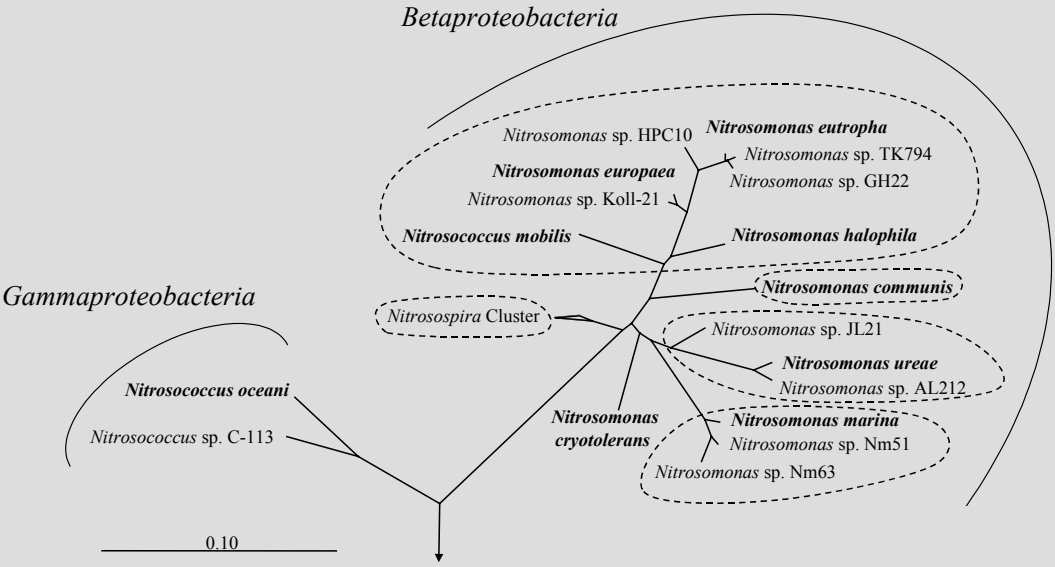


Abbildung 4. 16S rRNS-basierender phylogenetischer Baum mit allen im Jahr 1998 verfügbaren AOB-Reinkultur-Sequenzen (Länge >1000bp). Gestrichelte Linien deuten die von Pommerening-Röser *et al*. (1996) vorgeschlagenen Cluster an. Der Balken entspricht einem Sequenzunterschied von 10%.

Ziel dieser Arbeit

Neben Arbeiten, in denen die *amoA*- bzw. 16S rRNS-Gensequenzen beschriebener Spezies und Isolate ermittelt wurden, existieren zahlreiche Studien, in denen *amoA*- bzw. 16SrRNS-Genfragmente aus unterschiedlichsten Umwelthabitaten amplifiziert wurden (z.B. Stephen *et al.*, 1996; Bruns *et al.*, 1999). Im Zuge dieser Arbeit sollten bestehende Sequenz-Datenbanken mit allen publizierten *amoA*- bzw. AOB-16S rRNS-Sequenzen vervollständigt und kontinuierlich aktualisiert werden. Diese Datenbanken sollten als Grundlage für eingehende Untersuchungen zur Phylogenie von Ammoniak oxidierenden Bakterien dienen. Ziel war es zudem, den Datensatz durch die Ermittlung weiterer *amoA*- und 16S rRNS-Sequenzen wesentlich zu erweitern. Diese sollten nicht nur direkt aus der Umwelt, sondern vor allem aus bisher diesbezüglich nicht untersuchten AOB-Isolaten gewonnen werden, um das Raster für die Zuordnung bisher nicht identifizierbarer Umweltsequenzen zu verfeinern. Ein weiteres Ziel war die Entwicklung eines *amoA*-basierenden „Genospezies-Konzeptes“, welches erlauben sollte, die, anhand neuartiger *amoA*-Sequenzen vielfach postulierte, Existenz unbekannter AOB-Spezies in der Umwelt zu überprüfen. Zudem sollte die Sensitivität und Spezifität bestehender Gensonden und Primer anhand der gesammelten Sequenzinformation neu bewertet werden.

Grundlegendes Ziel dieser Arbeit war damit, einen Beitrag zum Verständnis der verwandtschaftlichen Verhältnisse innerhalb der AOB zu leisten, natürliche Verbreitungsmuster aufzudecken und die Voraussetzung für weitergehende Untersuchungen zur Ökologie dieser faszinierenden Bakteriengruppe zu schaffen.

MATERIAL UND METHODEN

Verwendete Organismen

Die untersuchten AOB sind in Tabelle 1 zusammengefasst. Sie entstammten der Stammsammlung der Mikrobiologische Abteilung der Universität Hamburg und wurden freundlicherweise von H.-P. Koops zur Verfügung gestellt. Die Anzucht erfolgte durch Gabriele Timmermann (nach Koops *et al.,* 1991).

Tabelle1. In dieser Arbeit verwendete Stämme.

Organismus	Referenz	Herkunft
Nitrosococcus mobilis Nc2 ^{T}	Koops *et al.*, 1976	Nordsee, Deutschland
Nitrosococcus halophila Nc4 ^{T}	Koops *et al*., 1990	Salz-Lagune, Sardinien, Italien
Nitrosomonas aestuarii Nm36 ^{T}	Koops *et al.*, 1991	Brackwasser, Nordsee, DK
Nitrosomonas cryotolerans Nm55 ^{T}	Koops *et al.*, 1991	Kasitsna Bucht, Alaska
Nitrosomonas communis Nm2 ^{T}	Koops *et al.*, 1991	Boden, Korfu, Griechenland
Nitrosomonas halophila Nm1 ^{T}	Koops *et al.*, 1991	Nordsee
Nitrosomonas marina Nm22 ^{T}	Koops *et al.*, 1991	Großes Barriere-Riff, Australien
Nitrosomonas nitrosa Nm90 ^{T}	Koops *et al.*, 1991	Kläranlage, Deutschland
Nitrosomonas oligotropha Nm45 ^{T}	Koops *et al.*, 1991	Boden, Deutschland
Nitrosomonas sp. Nm41	Koops *et al.*, 1991	Boden, Russland
Nitrosomonas sp. Nm51	Koops *et al.*, 1991	Meerwasser, Peru
Nitrosomonas ureae Nm10 ^{T}	Koops *et al.*, 1991	Boden, Sardinien, Italien
Nitrosomonas sp. Nm47	H.-P. Koops, unveröffentlicht	Kläranlage, Deutschland
Nitrosomonas europaea Nm50^{T}	Winogradsky, 1890	Boden, USA
Nitrosomonas eutropha Nm57^{T}	Koops und Harms, 1985	Kläranlage, USA
Nitrosomonas sp. Nm58	Stehr *et al.*, 1995	Elbsediment, Deutschland
Nitrosomonas sp. Nm59	S. Sowitzki, unveröffentlicht	Kläranlage, Deutschland
Nitrosomonas sp. Nm84	Stehr *et al.*, 1995	Schwebstoffe, Elbe, Deutschland
Nitrosomonas sp. Nm86	Stehr *et al.*, 1995	Elbe, Deutschland
Nitrosomonas sp. Nm93	Juretschko *et al*., 1998	Kläranlage, Deutschland
Nitrosomonas sp. Nm103	Juretschko *et al*., 1998	Kläranlage, Deutschland
Nitrosomonas sp. Nm104	Purkhold *et al.*, 2002	Kläranlage, Deutschland
Nitrosomonas sp. Nm107	Purkhold *et al.*, 2002	Kläranlage, Deutschland
Nitrosomonas sp. Nm143	H.-P. Koops, unveröffentlicht	Meerwasser, Dom. Republik
Nitrosomonas sp. Nm148	H.-P. Koops, unveröffentlicht	Heiße Quelle, Santorin, GR
Nitrosospira sp. Nsp1	Koops und Harms, 1985	Boden, Sardinien, Italien
Nitrosospira sp. Nsp2	Koops und Harms, 1985	Boden, Deutschland
Nitrosospira sp. Nsp5	Koops und Harms, 1985	Höhlensee, Sardinien, Italien
Nitrosospira briensis Nsp10^{T}	Koops und Harms, 1985	Boden, Kreta, Griechenland
Nitrosospira sp. Nsp12	Koops und Harms, 1985	Boden, Deutschland
Nitrosospira sp. Nsp17	Koops und Harms, 1985	Boden, Island
Nitrosospira sp. Nsp40	H.-P. Koops, unveröffentlicht	Boden, Deutschland
Nitrosospira sp. Nsp41	H.-P. Koops, unveröffentlicht	Boden, Malta
Nitrosospira sp. Nsp57	H.-P. Koops, unveröffentlicht	Mauerwerk, Deutschland
Nitrosospira sp. Nsp58	H.-P. Koops, unveröffentlicht	Mauerwerk, Deutschland
Nitrosospira sp. Nsp62	H.-P. Koops, unveröffentlicht	Mauerwerk, Deutschland
Nitrosospira sp. Nsp65	H.-P. Koops, unveröffentlicht	Mauerwerk, Deutschland
Nitrosospira tenuis Nv1^{T}	Koops und Harms, 1985	Boden, Hawaii
Nitrosospira sp. Nv6	Koops und Harms, 1985	Boden, New Guinea
Nitrosospira sp. NL5	Koops und Harms, 1985	Kläranlage, Saudi-Arabien
Nitrosospira multiformis NL13^{T}	Koops und Harms, 1985	Boden, Indien
Nitrosospira sp. L115	Utaaker *et al.*, 1995	Torfmoor, Finnland
Nitrosospira sp. III7	Utaaker und Nes, 1998	Fichtenwald, Norwegen
Nitrosospira sp. Ka3	Aakra *et al.*, 1999	Boden, Norwegen

^{T}: Typstamm

DNS-Extraktion

Die pelletierten Zellen wurden mit Hilfe eines BeadBeaters (BIO101, Vista, Ca.) und der „Lysing Matrix E“ (BIO101, Vista, Ca.) aufgeschlossen. Die Extraktion der Nukleinsäuren erfolgte durch Ausschütteln mit Phenol/Chloroform, gefällt wurde mit Isopropanol. Die Ausbeute an DNS war bei wenig Ausgangs-Zellmaterial oft sehr gering. Durch die Zugabe von Glycogen (Ambion Inc., Austin, Tex.) wurde die Präzipitation erleichtert (Helms *et al.*, 1985) und dadurch die Ausbeute an Nukleinsäuren deutlich verbessert. Die einzelnen Schritte der DNS-Isolierung sind Anhang II zu entnehmen.

PCR-Amplifikation

PCR-Amplifikation der 16S rRNS-Gene. Die Amplifikation der 16S rRNS-Gene erfolgte in einem Primus PCR-Gerät (MWG Biotech, Germany) mit 50 µl Standardreaktionsansatz (20 mM $MgCl_2$) und dem Primerpaar 616V/630R (616V: 5‘-AGA GTT TGA TYM TGG CTC AG-3‘; 630R: 5‘-CAK AAA GGA GGT GAT CC-3‘). Folgende Reaktionsbedingungen wurden verwendet: einleitende Denaturierung: 94°C, 3 Min.; danach 30 Zyklen mit Denaturierung: 94°C, 30 Sek., Annealing: 63°C, 30 Sek. und Elongation: 72°C, 90 Sek.; abschließende Elongation: 72°C, 3 Min..

PCR-Amplifikation der *amoA*-Gene. Die Amplifikation der *amoA*-Fragmente erfolgte in einem Primus PCR-Gerät (MWG Biotech, Germany) mit 50 µl Standardreaktionsansatz und dem Primerpaar amoA1F/amoA2R (amoA1F: 5‘-GGG GHT TYT ACT GGT GGT-3‘; amoA2R: 5‘-CCC CTC KGS AAA GCC TTC TTC-3‘, Rotthauwe *et al.*, 1997 ; modifiziert nach Stephen *et al.*, 1999). Folgende Reaktionsbedingungen wurden verwendet: einleitende Denaturierung: 94°C, 60 Sek., danach 30 Zyklen mit Denaturierung: 94°C, 15 Sek., Annealing: 50°C, 20 Sek. und Elongation: 72°C, 30 Sek.; abschließende Elongation: 72°C, 3 Min..

Auftrennung der *amoA*-Amplikons über Gelretardation

Die aus Umweltproben amplifizierten *amoA*-Fragmente wurden vor dem Klonierungsschritt in einem retardierenden Agarose-Gel aufgetrennt (Wawer *et al.*, 1995). Das Gel enthielt einen hochmolekularen DNS-Liganden (HA Yellow, Hanse Analytik, Deutschland), der vorzugsweise an AT-reiche Regionen der Amplifikate bindet. Die Wanderungsgeschwindigkeit der unterschiedlichen Amplifikate hing somit von GC-Gehalt und Verteilung der GC-Regionen im PCR-Fragment ab. Diese Methode ermöglichte die Auftrennung heterogener Amplifikate der gleichen Länge nach ihrem GC-Gehalt. Die

resultierenden Banden wurden einzeln aus dem Gel ausgestochen und in unabhängigen Ansätzen kloniert (s.u.). Dadurch war es möglich, die Verzerrung der Ergebnisse, hervorgerufen durch die bevorzugte PCR-Amplifikation bestimmter Sequenzen, zu reduzieren. Eine detaillierte Beschreibung der Methode findet sich bei Schmid *et al.* (2000).

Klonierung und Plasmid-Extraktion

Nach Größenbestimmung der 16S rRNS- und *amoA*-Amplikons über Gelelektrophorese (Präparatives Gel: 2% low melting SeaPlaque Agarose) wurden die Bande der erwarteten Größe mit Kapillaren ausgestochen, in ein ERG überführt, im Wasserbad (70-80°C) aufgeschmolzen, und zuletzt mit 100 µl sterilem H_2O_{bidest} versetzt. Von dieser Lösung wurden 10 µl in die Ligation in den Vektor pCR^{TM}2.1 eingesetzt. Die weiteren Klonierungsschritte (TOPO TA, Invitrogen) erfolgten nach Herstellerangaben, mit der Ausnahme, dass für die Transformation 5 µl Ligationsansatz (statt 3 µl) verwendet wurden. Die Plasmide wurden wie vom Hersteller angegeben mit dem SpinPlasmid Kit (Qiagen, Hilden, Deutschland) isoliert. Die Insertgröße wurde durch Restriktionsverdau (EcoRI) mit anschließender Agarose-Gelelektrophorese kontrolliert.

DNS-Sequenzanalyse

Die Sequenzierung der klonierten PCR-Produkte erfolgte an einem automatisierten DNS-Sequenziergerät (LI-COR, Lincoln, USA) nach dem *cycle-sequencing* Prinzip, einer Kombination aus der enzymatischen Didesoxy-Ketten-Abbruchmethode (Sanger *et al*., 1977) und der Polymerase-Kettenreaktion (Saiki *et al.*, 1988). Das Bandenmuster wurde über die Infrarot-markierten (IRD-800) Primer UniV (-21) (5´-TGT AAA ACG ACG GCC AGT-3) und UniR (-29) (5´-CAG GAA ACA GCT ATG ACC-3´) detektiert. Sie sind komplementär zu den Zielsequenzen auf dem zur Klonierung verwendeten Vektor pCR^{TM} 2.1. Auffallend war, dass *amoA*-Sequenzen, die mit dem Primer UniR amplifiziert wurden, auf dem Gel besser zu analysieren waren als bei Verwendung von UniV. Bei 16S rRNS-Sequenzen waren keine derartigen Unterschiede festzustellen. Als hilfreich war die Zugabe von Dimethylsulfoxid (1,5 µl DMSO in 25 µl Standardreaktionsansatz), da es die Auflösung schwer sequenzierbarer Sekundärstrukturen unterstützt.

Phylogenetische Analyse der Sequenzdaten

Vergleichende Sequenzanordnung (Alignment). Grundlage für sinnvolle phylogenetische Analysen ist das korrekte Alignment. Um dies zu gewährleisten, wurden neu ermittelte oder in Datenbanken veröffentlichte 16S rRNS- oder *amoA*-Sequenzen zu bereits bestehenden Datensätzen addiert und derart positioniert, dass sich homologe Basen an der gleichen Stelle befanden. Für diesen Zweck wurde das ARB-Software-Paket (W. Ludwig *et al.*, http://www.arb-home.de) verwendet. Es enthält Programme (ARB-EDIT), die das Alignment automatisch vornehmen (bei 16S rRNS-Sequenzen unter Berücksichtigung der Sekundärstruktur). Meist waren jedoch zusätzlich manuelle Korrekturen erforderlich.

Qualität der 16S rRNS- und *amoA*-Sequenzen von Reinkulturen. Für jede Reinkultur wurden nach Klonierung die Inserts (16S rRNS- bzw. *amoA*-Fragmente) von mindestens zwei verschiedenen Plasmiden sequenziert und aligned. Unter Verwendung der Primer UniR/UniV (s. o.) wurde der Vorwärts- und Rückwärts-Strang der *amoA*-Fragmente jeweils vollständig sequenziert. Bei 16S rRNS-Gensequenzen wurde die Sequenz am Beginn und Ende der Sequenz (jeweils ca. 300-500 bp) nur für einen Strang ermittelt. Der Vergleich der resultierenden Sequenzen ermöglichte es meist, zweideutige Ergebnisse aus der Sequenzanalyse zu klären. Zudem konnten einige falsche Basen, die aus Fehlern der Polymerase bei der PCR-Amplifikation (Keohavong und Thilly, 1989) entstanden waren, identifiziert werden. Bei unterschiedlichen Basen wurde die wahrscheinlichere als die richtige betrachtet. Wichtige Entscheidungskriterien waren dabei die Sekundärstruktur (16S rRNS), die abgeleitete Aminosäuresequenz (*amoA*) sowie der Konservierungsgrad der jeweiligen Position. Bei *amoA*-Fragmenten traten Fälle auf, in denen eindeutige Unterschiede in der Nukleinsäuresequenz keine oder nur selten Änderungen in der Aminosäuresequenz bedingten. Hier beruhten die unterschiedlichen Nukleinsäuresequenzen wahrscheinlich nicht auf Fehlern, sondern auf der Existenz mehrerer *amo*-Operons in den jeweiligen AOB.

Sequenzlänge. In dieser Arbeit wurden mit Hilfe der Primer 616V/630R (s.o.) in allen Fällen fast vollständige 16S rRNS-Gensequenzen ermittelt (>1494 bp). Bei 16S rRNS-basierenden phylogenetischen Untersuchungen wurden ausschließlich Sequenzen mit einer Länge von mehr als 1000 bp berücksichtigt. Die Position kürzerer Sequenzen in phylogenetischen Bäumen ist aufgrund des geringen Informationsgehalts oft nicht eindeutig zu ermitteln. Zudem können kurze Sequenzen die Gesamt-Baumtopologie verzerren (Ludwig *et al.*, 1998).
Aus dem *amoA*-Gen (829bp von Start- bis Stop-Codon; McTavish *et al.*, 1993) wurde mit Hilfe der Primer amoA1F/amoA2R (s.o.) ein 453 bp Fragment amplifiziert. Dieses bildete die Grundlage für *amoA*-basierende Bäume. Sequenzen mit weniger als 414 bp wurden nicht mit in die Berechnungen aufgenommen.

Verwendung von Konservierungsfiltern. Um den Einfluss hochvariabler Positionen auf das Ergebnis der 16S rRNS-basierenden phylogenetischen Analysen zu minimieren, werden vielfach Konservierungsfilter verwendet. Diese schließen Positionen aus, deren Konservierungsgrad innerhalb des verwendeten Datensatzes unterhalb eines festgesetzten Wertes liegt. Beträgt dieser Wert beispielsweise 50%, werden bei der phylogenetischen Analyse nur Positionen berücksichtigt, an denen mindestens 50% der 16S rRNS-Sequenzen die gleiche Base aufweisen. Im Falle der AOB wurde auf die Verwendung derartiger Filter verzichtet, da die 16S rRNS-Sequenzähnlichkeiten innerhalb der AOB sehr hoch sind und der Ausschluss variabler Positionen den Verzicht auf wichtige Informationen bedeutet hätte.
Bei *amoA*-Sequenzen wurden zum Teil Filter für den Ausschluss der dritten, variablen, Codonposition verwendet. Grundlage für *amoA*/AmoA-basierende Bäume waren Aminosäuresequenz, Nukleinsäuresequenz, sowie Nukleinsäuresequenz ohne die dritte Codonposition.

Verwendete Baumberechnungsverfahren. Für die phylogenetische Analyse wurden Distanz-basierende (Jukes Cantor, FITCH) und Merkmals-basierende (Maximum-Parsimony, Maximum-Likelihood) Verfahren genutzt (für eine ausführliche Beschreibung siehe Ludwig *et al.*, 1998).
Bei Distanz-Matrix-Verfahren wird zunächst aus dem verwendeten Datensatz eine Ähnlichkeitsmatrix erstellt, welche die Sequenzähnlichkeiten aller denkbaren Sequenzpaare enthält. Es wird diejenige Baumtopologie als optimal bewertet, bei der die Abstände der zugrundeliegenden Werte-Matrix am besten mit den jeweiligen Astlängen übereinstimmen. Diese Distanz-Werte werden nachfolgend in phylogenetische Abstände umgerechnet. Vorteil von Distanz-Matrix-Verfahren ist der geringe Rechen- und Zeitaufwand. Zu berücksichtigen ist jedoch, dass bei diesen Methoden die Originaldaten nur indirekt (in Form abgeleiteter Distanzwerte) in die Baumtopologie eingehen.
Maximum-Parsimony-Verfahren basieren auf der Annahme, dass in der Evolutionsgeschichte die Erhaltung eines Merkmals (Nukleotide oder Aminosäuren) wahrscheinlicher ist als die Veränderung (Basen- oder Aminosäureautausch). Im Gegensatz zu Distanz-Matrix Methoden werden hier die eigentlichen Sequenzdaten bewertet. Parsimony-Methoden suchen nach Baum-Topologien mit einer minimalen Gesamt-Baum-Länge und der geringstmöglichen Anzahl an Mutationen. Da diese Methode ursprünglich für morphologische Daten, die sich nur selten ändern, entworfen wurde, scheitert sie oft, wenn die untersuchten Merkmale hochvariabel sind. Des weiteren ergeben sich Probleme, wenn die Phylogenie von Linien mit unterschiedlich hohen Mutationsraten analysiert werden soll.
Maximum-Likelihood-Verfahren stellen den ausgeklügeltsten Ansatz dar. Diesen Verfahren liegen bestimmte Evolutionsmodelle zugrunde, die Parameter wie das Verhältnis Transversion/Transition, der Konservierungsgrad an bestimmten Positionen oder die Wahrscheinlichkeit für Konservierung im Gegensatz zu Mutation beinhalten. Wie bei

Maximum-Parsimony-Verfahren dienen hier die Originalsequenzdaten als Grundlage. Nachteilig ist jedoch der große Bedarf an Rechenleistung und -zeit.

Auswahl der Außengruppe. Eine Außengruppe beinhaltet Organismen, die nicht zu der eigentlich untersuchter Gruppe (=Innengruppe) einer phylogenetischen Analyse gehören. Außengruppen fungieren als Wurzel in den eigentlich ungewurzelten Bäumen. Voraussetzung ist hierbei, dass die untersuchten Gene und die Gene der Außengruppe-Organismen homolog sind, d. h. einen gemeinsamen Vorfahren haben. Bei der Erstellung 16S rRNS-basierender Bäume ergab sich daraus kein Problem, da die rRNS-Gene in allen *Bacteria* vertreten sind. Bei *amoA*/AmoA-basierenden Bäumen wurden für phylogenetische Untersuchungen innerhalb der beta-AOB entweder die *amoA*/AmoA-Sequenzen der gamma-AOB oder der Sequenzen der partikulären Methanmonooxygenase (pMMO) der Methan oxidierenden Bakterien herangezogen. Die letztgenannte Möglichkeit diente meist nur als zusätzliche Kontrolle, da die überwiegende Anzahl der verfügbaren *pmoA*-Genfragmente innerhalb der untersuchten Region nicht vollständig sequenziert ist.
Die Anzahl sowie die Zusammensetzung der Sequenzen innerhalb der Außengruppe hängt von dem verwendeten Baumberechnungsverfahren ab. Für 16S rRNS-basierende Neighbour-Joining-Bäume hat sich eine relativ umfangreiche Außengruppe mit Referenzsequenzen aus allen bekannten Phyla der *Bacteria* und *Archaea* bewährt. Die Außengruppen in Maximum-Likelihood Bäumen sind dagegen durch die zeitaufwendige Berechnung limitiert. Hier wurden die Sequenzen von einem bis wenigen MO verwendet, die zu der untersuchten Organismengruppe relativ nah verwandt waren (im Fall der beta-AOB eignete sich z. B. *Burkholderia cepacia*, ein nicht-AOB innerhalb der *Betaproteobacteria*). Die Zusammensetzung der Außengruppe kann die Baumtopologie beeinflussen (Dalevi *et al.*, 2001). Um das Ausmaß dieses Einflusses abschätzen zu können, wurden die einzelnen Berechnungen jeweils mit unterschiedlichen Außengruppen durchgeführt.

Interpretation der berechneten Stammbäume. Die Baumtopologien, die sich aus den unterschiedlichen Berechnungsverfahren mit den verschiedenen Datensätzen ergaben, wurden in einem „Konsensusbaum“ zusammengefasst. In diesem erscheinen nur Verzweigungen, die mit allen verwendeten Verfahren reproduzierbar waren (Ludwig *et al.*, 1998). Diese Vorgehensweise ist sehr konservativ und resultierte häufig in Vielfachverzweigungen der Äste.
Sogenannte Bootstrap-Werte wurden durch wiederholte Berechnung (100x) der Baumtopologie mit dem gleichen Verfahren (Maximum-Parsimony) ermittelt. Dabei wurde von dem entsprechenden Programm jeweils eine zufällige Auswahl von Daten (Alignmentspalten) aus der gegebenen Datenmenge getroffen. In denen resultierenden Pseudostichproben sind Teile des Alignments mehrfach und andere gar nicht vertreten. Bootstrap-Werte gaben Auskunft, in wieviel Prozent der Pseudostichproben-Bäume eine bestimmte Verzweigung aufgetreten ist (statistische Signifikanz). Abzweigungen mit hohen

Parsimony-Bootstrap-Werten (> 90%) waren meist auch mit anderen Baumverfahren reproduzierbar.

Fluoreszenz *in situ* Hybridisierung (FISH)

FISH mit rRNS-gerichteten Oligonukleotiden (Sonden) stellt eine kultivierungs-unbhängige Methode zur Identifizierung und Visualisierung von Mikroorganismen *in situ* dar (DeLong *et al.*, 1989; Amann, 1995). Die Spezifität der Sonden hängt vom Konservierungsgrad der Zielsequenz ab, und erlaubt eine Identifizierung auf verschiedenen hierarchischen Ebenen (Art, Gattung, ..., Domäne). Durch Kombination von hierarchischen Sonden mit drei unterschiedlichen Fluoreszenzmarkierungen ist die simultane Detektion von bis zu sieben unterschiedlichen Populationen möglich (Amann *et al.*, 1996). Der Fluoreszenz-Nachweis erfolgt über Epifluoreszenzmikroskopie oder mit Hilfe konfokaler Laser Scanning Mikroskopie, jeweils mit geeigneten Filtersätzen. Voraussetzung für eine erfolgreiche Durchführung der FISH ist die chemische Fixierung der Zellen. Da es sich bei AOB um gram-negative Organismen handelt, wurde 4%ige PFA-Lösung (Verhältnis PFA:Probe = 3:1) verwendet (Amann, 1995). Um die Adhäsion des Probenmaterials an das Glas zu verbessern war eine vorhergehende Beschichtung der Objektträger mit Gelatine (0,1%) geeignet.

Konfokale Laser Scanning Mikroskopie (CLSM)

Die konfokale Laser Scanning Mikroskopie ermöglicht Objekterfassung ohne störende Fluoreszenz außerhalb der eingestellten Fokusebene. Dies ist vor allem dann vorteilhaft, wenn das Präparat eine starke Autofluoreszenz zeigt oder eine große räumliche Ausdehnung besitzt (z.B. Biofilme), die mit der konventionellen Epifluoreszenzmikroskopie nicht adäquat dargestellt werden kann. Emittiertes Fluoreszenzlicht aus Brennebenen, die über oder unter der Fokusebene liegen, kann die Bildschärfe erheblich reduzieren. Dieser Effekt wird durch das konfokale Prinzip (Abbildung 5) vermieden. Essentiell ist die im Lichtweg eingeschaltete Lochblende, welche bewirkt, dass nur Licht aus einer definierten Brennebene detektiert und Streulicht ausgeblendet wird. Durch wiederholtes Abrastern verschiedener Fokusebenen werden optische Schnitte durch das Präparat gelegt, die zusammen die dreidimensionale Struktur wiedergeben. Digitale Bildverarbeitung ermöglicht die Darstellung der räumlichen Information in Form von Projektionen, Schnitten oder farbcodierten Tiefenprofilen. Darüber hinaus können mit entsprechender Software sowohl Signalintensitäten als auch Flächen quantitativ erfasst werden (Daims *et al.*, 2000; Daims *et al.*, 2001; Schmid *et al.*, 2000).

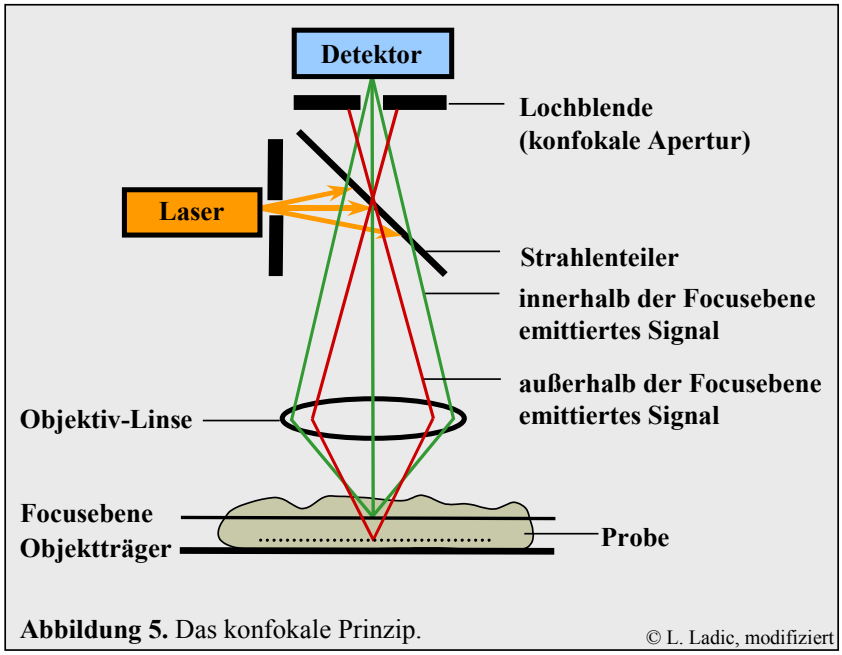


Abbildung 5. Das konfokale Prinzip.

ERGEBNISSE UND DISKUSSION

Ammoniak oxidierende Bakterien (AOB) spielen eine wichtige Rolle im globalen Stickstoffkreislauf, bei der Abwasserreinigung in Kläranlagen, sowie in der Landwirtschaft. Da die Kultivierung von AOB sehr aufwendig ist, bieten die verwendeten kultivierungsunabhängigen, molekularen Methoden große Vorteile bei der Untersuchung dieser Organismen. Schwerpunkte der vorliegenden Arbeit waren i) die Rekonstruktion der verwandtschaftlichen Verhältnisse innerhalb der AOB, ii) die Reevaluierung AOB-spezifischer Nachweismethoden und iii) Untersuchungen zur Diversität und Verbreitung von AOB in der Umwelt.

Die Phylogenie kultivierter Ammoniakoxidanten der Klasse *Betaproteobacteria*

Das Verständnis der Verwandtschaft Ammoniak oxidierender Bakterien basiert heute weitgehend auf der vergleichenden Analyse von 16S rRNS-Sequenzen. Für alle 14 beschriebenen AOB-Arten innerhalb der *Betaproteobacteria* stehen mittlerweile nahezu vollständige 16S rRNS-Gensequenzen zur Verfügung (Purkhold *et al.,* 2000/Anhang I; Purkhold *et al.,* 2002/Anhang II). Darüber hinaus sind die 16S rRNS-Gene von mehr als 100 weiteren AOB-Isolaten sequenziert (davon 44 in dieser Arbeit ermittelt), die zusammen mit den beschriebenen Arten innerhalb der *Betaproteobacteria* eine monophyletische Entwicklungslinie bilden (Purkhold *et al.,* 2000/Anhang I; Purkhold *et al.*, 2002/Anhang II; Head *et al.*, 1993; Teske *et al.*, 1994). Die minimale 16S rRNS-Sequenzähnlichkeit innerhalb dieser Linie beträgt 89,4% (*Nitrosococcus mobilis*/*Nitrosomonas nitrosa*). Abbildung 6 zeigt einen phylogenetischen, 16S rRNS-basierenden Baum mit 42 AOB-Isolaten (19 Nitrosospiren und 23 Nitrosomonaden), die alle bekannten Genospezies dieser Bakteriengruppe repräsentieren (nachgewiesen durch eine DNS-DNS-Ähnlichkeit von weniger als 60%). Die Genera *Nitrosospira*, *Nitrosolobus* und *Nitrosovibrio* bilden zusammen ein stabiles Cluster (Purkhold *et al.,* 2000/Anhang I; Purkhold *et al.*, 2002/Anhang II; Pommerening-Röser, 1996). Die Unterteilung dieses Clusters in drei verschiedene Gattungen wird zwar durch charakteristische morphologische und ökophysiologische Merkmale der jeweiligen Vertreter unterstützt (Koops, persönl. Mitteilung), spiegelt sich jedoch in der 16S rRNS-Phylogenie nicht wider. Es wurde daher vorgeschlagen, das Cluster zu einer einzigen Gattung (*Nitrosospira*) zusammenzufassen (Head *et al.*, 1993). Im Gegensatz zu den Nitrosospiren konnten die kultivierten Nitrosomonaden im Rahmen dieser Arbeit anhand der 16S rRNS-Phylogenie eindeutig in sechs Linien unterteilt werden. Diese Unterteilung ist mit verschiedenen Baumberechnungsverfahren reproduzierbar und wird darüber hinaus durch hohe Parsimony-Bootstrap-Werte (>90%) unterstützt (Abbildung 7). Die Topologie bleibt auch dann erhalten, wenn alle verfügbaren 16S rRNS-Sequenzen von beta-AOB-Isolaten (48 Nitrosospiren und 56 Nitrosomonaden, davon insgesamt 44 in dieser Arbeit ermittelt) in die Analysen mit einbezogen werden (Abbildungen 7 und 8). Die Nomenklatur der Linien

entspricht den bestehenden Klassifikations-Schemata (Pommerening-Röser *et al.*, 1996; Stephen *et al.*, 1996).
Obwohl die 16S rRNS-Phylogenie keine reproduzierbaren Hinweise auf eine Substruktur innerhalb der Nitrosospiren liefert (diese Arbeit), schlugen Stephen *et al.* (1996) vor, dieser Gruppe in drei Cluster zu unterteilen (Cluster 2, 3, 4; –Cluster 1 enthält keine kultivierten Spezies, Abbildung 8). Dieses System wurde nachfolgend von Purkhold *et al.* (2000, Anhang I) übernommen und mit dem Vorschlag zur Einführung von Cluster 0 erweitert. Wie gezeigt wurde (Purkhold *et al.*, 2000/Anhang I; Purkhold *et al.*, 2002/Anhang II), sind diese Cluster mit allen Baumverfahren zu finden, jedoch nicht immer von hohen Bootstrap-Werten unterstützt. Grund dafür ist die hohe Sequenzähnlichkeit innerhalb der Nitrosospiren, die mit >96,1% größer ist als die innerhalb der einzelnen *Nitrosomonas*-Cluster (Abbildung 7). Darüber hinaus weisen die Ergebnisse von DNS-DNS-Hybridisierungen darauf hin, dass Cluster 0, 2 und 4 jeweils aus einer einzigen Genospezies bestehen (Koops, unveröffentlicht). Insgesamt ist somit fraglich, ob die beschriebene oder auch andere Unterteilungen (Aakra *et al.*, 2001) der *Nitrosospira*-Gruppe sinnvoll sind.

Aakra *et al.* (2001) führten zudem phylogenetische Analysen Ammoniak oxidierender Bakterien basierend auf Sequenzen der 16S-23S rDNS intergenische Spacer Region (ISR) durch (Aakra *et al.*, 2001). Sie postulierten eine im Vergleich zu 16S rRNS-Sequenzen bessere Auflösung, sowie übereinstimmende ISR- und 16S rRNS-Baumtopologien. Bei einer Überprüfung dieser Aussage waren jedoch signifikante Unterschiede in den Topologien der publizierten 16S rRNS- und ISR-basierenden Bäume nicht zu übersehen. Des weiteren ergab der Vergleich von ISR-Sequenzen in einer im Verlauf dieser Arbeit erstellten AOB-ISR-Datenbank, dass für die ISR aufgrund der hohen Sequenzvariabilität ein zuverlässiges Alignment nur für ISR-Sequenzen sehr nah verwandter Stämme möglich ist (Angehörige derselben Genospezies). Die ISR ist somit als Grundlage für phylogenetische Untersuchungen über die Artebene hinaus nicht geeignet.

Abbildung 6. 16S rRNS-basierender phylogenetischer Baum betaproteobakterieller AOB. In die Analyse aufgenommen wurden nur AOB, bei denen es sich erwiesenermaßen (DNS-DNS-Homologie < 60%) um unterschiedliche Genospezies handelt und für die 16S rRNS-Gensequenzen mit einer Länge von mehr als 1000 bp zur Verfügung standen. Weitere Vertreter der gleichen Genospezies sind in Klammern angegeben. Die 44 im Rahmen dieser Arbeit ermittelten Sequenzen sind fett markiert. Maximum-Likelihood-, Maximum-Parsimony- sowie Neighbour-Joining-Bäume wurden zu einem Konsensusbaum zusammengefasst. Mehrfachverzweigungen verbinden Äste, deren relative Anordnung bei Verwendung verschiedener Baumverfahren nicht zweifelsfrei bestimmt werden konnte. Ausgefüllte und leere Kreise zeigen Boostrap-Werte (100 Wiederholungen) von >90% bzw. >70% an. Der Balken repräsentiert einen Sequenzunterschied von 10%.

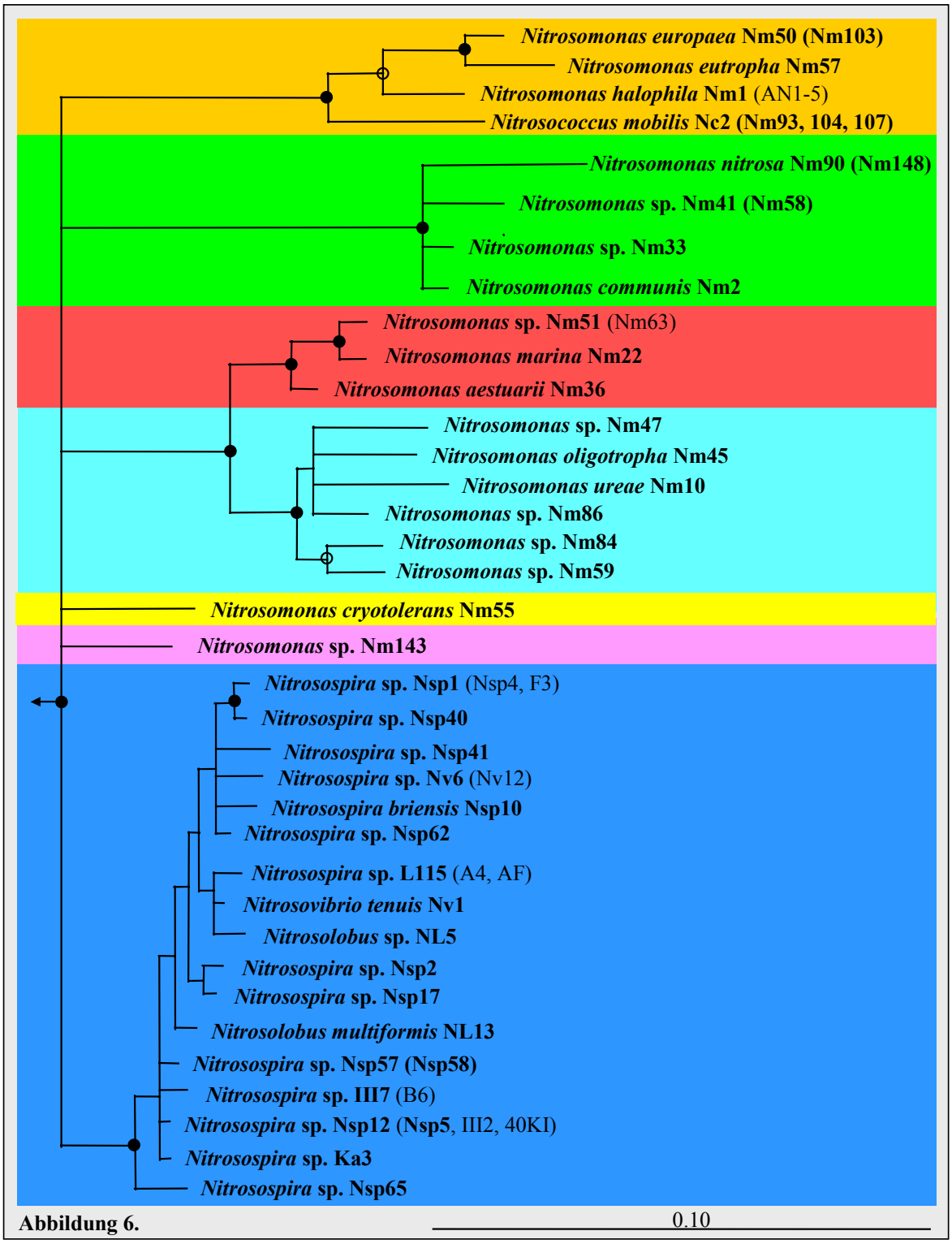

Nitrosomonas europaea **Nm50 (Nm103)**
Nitrosomonas eutropha **Nm57**
Nitrosomonas halophila **Nm1** (AN1-5)
Nitrosococcus mobilis **Nc2 (Nm93, 104, 107)**
Nitrosomonas nitrosa **Nm90 (Nm148)**
Nitrosomonas **sp. Nm41 (Nm58)**
Nitrosomonas **sp. Nm33**
Nitrosomonas communis **Nm2**
Nitrosomonas **sp. Nm51** (Nm63)
Nitrosomonas marina **Nm22**
Nitrosomonas aestuarii **Nm36**
Nitrosomonas **sp. Nm47**
Nitrosomonas oligotropha **Nm45**
Nitrosomonas ureae **Nm10**
Nitrosomonas **sp. Nm86**
Nitrosomonas **sp. Nm84**
Nitrosomonas **sp. Nm59**
Nitrosomonas cryotolerans **Nm55**
Nitrosomonas **sp. Nm143**
Nitrosospira **sp. Nsp1** (Nsp4, F3)
Nitrosospira **sp. Nsp40**
Nitrosospira **sp. Nsp41**
Nitrosospira **sp. Nv6** (Nv12)
Nitrosospira briensis **Nsp10**
Nitrosospira **sp. Nsp62**
Nitrosospira **sp. L115** (A4, AF)
Nitrosovibrio tenuis **Nv1**
Nitrosolobus **sp. NL5**
Nitrosospira **sp. Nsp2**
Nitrosospira **sp. Nsp17**
Nitrosolobus multiformis **NL13**
Nitrosospira **sp. Nsp57 (Nsp58)**
Nitrosospira **sp. III7** (B6)
Nitrosospira **sp. Nsp12** (**Nsp5**, III2, 40KI)
Nitrosospira **sp. Ka3**
Nitrosospira **sp. Nsp65**
0.10

Abbildung 6.

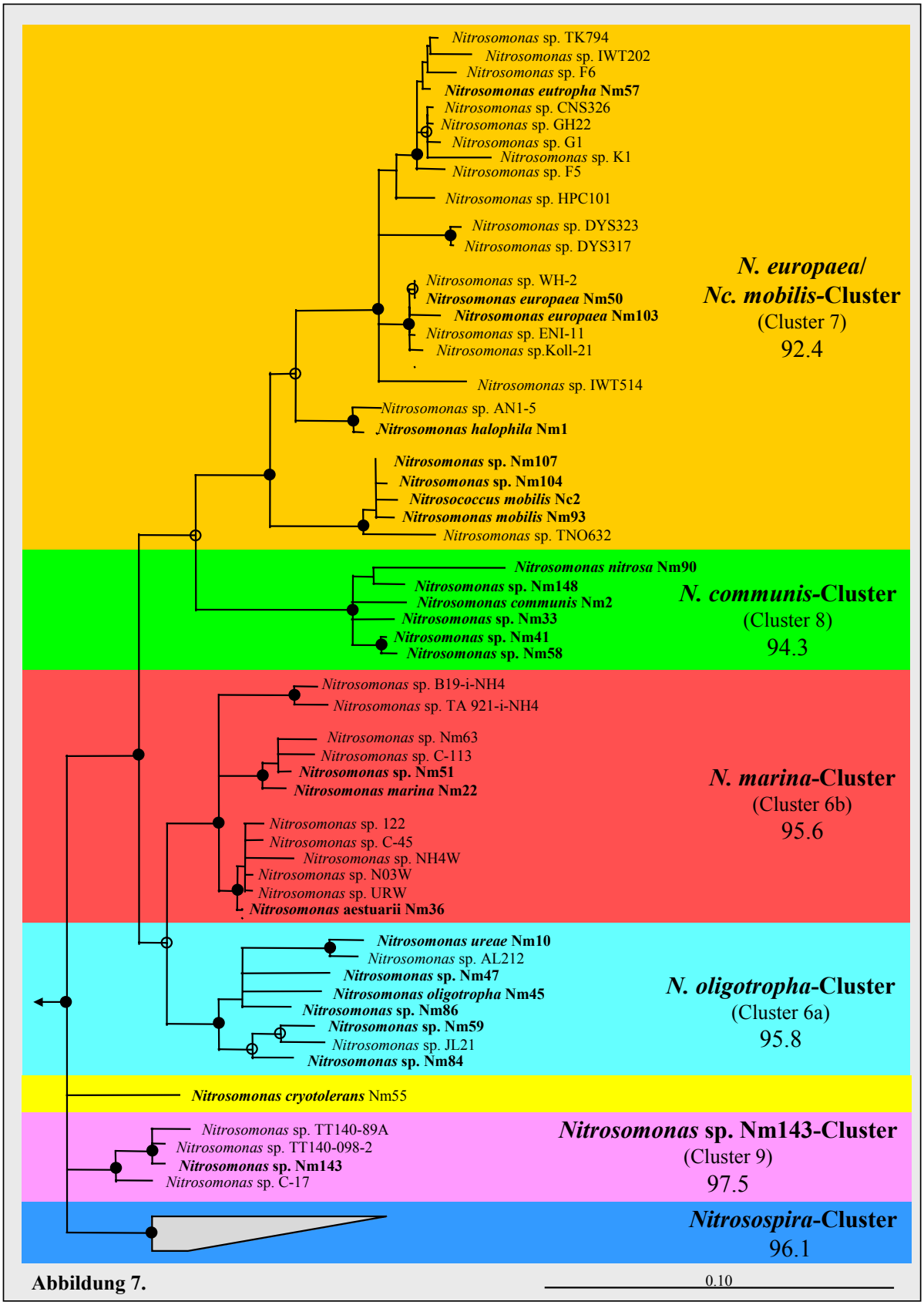

Nitrosomonas sp. TK794
Nitrosomonas sp. IWT202
Nitrosomonas sp. F6
Nitrosomonas eutropha Nm57
Nitrosomonas sp. CNS326
Nitrosomonas sp. GH22
Nitrosomonas sp. G1
Nitrosomonas sp. K1
Nitrosomonas sp. F5
Nitrosomonas sp. HPC101
Nitrosomonas sp. DYS323
Nitrosomonas sp. DYS317
Nitrosomonas sp. WH-2
Nitrosomonas europaea Nm50
Nitrosomonas europaea Nm103
Nitrosomonas sp. ENI-11
Nitrosomonas sp.Koll-21
Nitrosomonas sp. IWT514
Nitrosomonas sp. AN1-5
Nitrosomonas halophila Nm1
Nitrosomonas sp. Nm107
Nitrosomonas sp. Nm104
Nitrosococcus mobilis Nc2
Nitrosomonas mobilis Nm93
Nitrosomonas sp. TNO632
N. europaea/
Nc. mobilis-Cluster
(Cluster 7)
92.4
Nitrosomonas nitrosa Nm90
Nitrosomonas sp. Nm148
Nitrosomonas communis Nm2
Nitrosomonas sp. Nm33
Nitrosomonas sp. Nm41
Nitrosomonas sp. Nm58
N. communis-Cluster
(Cluster 8)
94.3
Nitrosomonas sp. B19-i-NH4
Nitrosomonas sp. TA 921-i-NH4
Nitrosomonas sp. Nm63
Nitrosomonas sp. C-113
Nitrosomonas sp. Nm51
Nitrosomonas marina Nm22
Nitrosomonas sp. 122
Nitrosomonas sp. C-45
Nitrosomonas sp. NH4W
Nitrosomonas sp. N03W
Nitrosomonas sp. URW
Nitrosomonas aestuarii Nm36
N. marina-Cluster
(Cluster 6b)
95.6
Nitrosomonas ureae Nm10
Nitrosomonas sp. AL212
Nitrosomonas sp. Nm47
Nitrosomonas oligotropha Nm45
Nitrosomonas sp. Nm86
Nitrosomonas sp. Nm59
Nitrosomonas sp. JL21
Nitrosomonas sp. Nm84
N. oligotropha-Cluster
(Cluster 6a)
95.8
Nitrosomonas cryotolerans Nm55
Nitrosomonas sp. TT140-89A
Nitrosomonas sp. TT140-098-2
Nitrosomonas sp. Nm143
Nitrosomonas sp. C-17
Nitrosomonas sp. Nm143-Cluster
(Cluster 9)
97.5
Nitrosospira-Cluster
96.1
0.10

Abbildung 7.

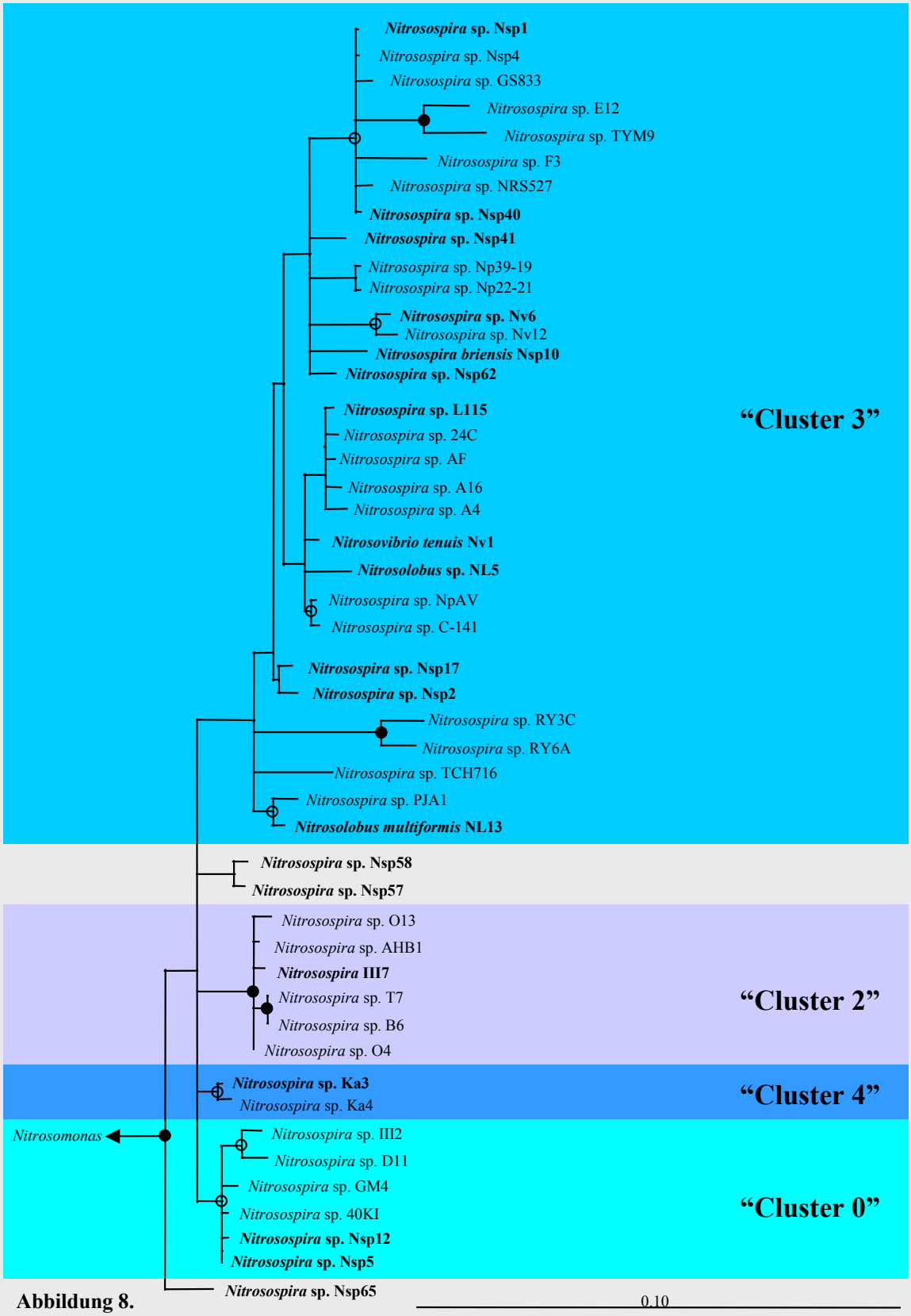

Nitrosospira sp. Nsp1
Nitrosospira sp. Nsp4
Nitrosospira sp. GS833
Nitrosospira sp. E12
Nitrosospira sp. TYM9
Nitrosospira sp. F3
Nitrosospira sp. NRS527
Nitrosospira sp. Nsp40
Nitrosospira sp. Nsp41
Nitrosospira sp. Np39-19
Nitrosospira sp. Np22-21
Nitrosospira sp. Nv6
Nitrosospira sp. Nv12
Nitrosospira briensis Nsp10
Nitrosospira sp. Nsp62
Nitrosospira sp. L115
Nitrosospira sp. 24C
Nitrosospira sp. AF
Nitrosospira sp. A16
Nitrosospira sp. A4
Nitrosovibrio tenuis Nv1
Nitrosolobus sp. NL5
Nitrosospira sp. NpAV
Nitrosospira sp. C-141
Nitrosospira sp. Nsp17
Nitrosospira sp. Nsp2
Nitrosospira sp. RY3C
Nitrosospira sp. RY6A
Nitrosospira sp. TCH716
Nitrosospira sp. PJA1
Nitrosolobus multiformis NL13
Nitrosospira sp. Nsp58
Nitrosospira sp. Nsp57
Nitrosospira sp. O13
Nitrosospira sp. AHB1
Nitrosospira III7
Nitrosospira sp. T7
Nitrosospira sp. B6
Nitrosospira sp. O4
Nitrosospira sp. Ka3
Nitrosospira sp. Ka4
Nitrosomonas
Nitrosospira sp. III2
Nitrosospira sp. D11
Nitrosospira sp. GM4
Nitrosospira sp. 40KI
Nitrosospira sp. Nsp12
Nitrosospira sp. Nsp5
Nitrosospira sp. Nsp65
“Cluster 3”
“Cluster 2”
“Cluster 4”
“Cluster 0”
0.10

Abbildung 8.

Abbildung 7. 16S rRNS-basierender phylogenetischer Baum der Nitrosomonaden. Der Baum enthält alle Isolate, für die 16S rRNS-Gensequenzen mit einer Länge von mehr als 1000 Nukleotiden verfügbar sind. Im Rahmen dieser Arbeit ermittelte Sequenzen sind fett markiert. Maximum-Likelihood-, Maximum-Parsimony- sowie Neighbour-Joining-Bäume wurden zu einem Konsensusbaum zusammengefasst. Mehrfachverzweigungen verbinden Äste, deren relative Anordnung bei Verwendung verschiedener Baumverfahren nicht zweifelsfrei bestimmt werden konnte. Ausgefüllte und leere Kreise zeigen Boostrap-Werte (100 Wiederholungen) von >90% bzw. >70% an. Für jedes Cluster ist die minimale Sequenzähnlichkeit angegeben. 25 der 56 dargestellten Sequenzen wurden in dieser Arbeit ermittelt. Zudem wurden Sequenzen aus folgenden Publikationen in die Analysen einbezogen: Aakra *et al.* (1999a, 1999b), Head *et al.* (1993), Juretschko *et al.* (1998), Sorokin *et al.* (1998), Suwa *et al.* (1997), and Yamagata *et al.* (1999). Sequenzen der Stämme CNS326, G1, K1, IWT202, TK94, WH-2, Koll-21, DYS317, DYS323, IWT514, TNO632, C-45, NH4W, 122, URW, NO3W, C-113, TT140-098-2, TT140-98A, TA 921-i-NH4, B19-i-NH4 und C-17 sind unveröffentlicht, jedoch über GenBank verfügbar. Der Balken repräsentiert einen Sequenzunterschied von 10%.

Abbildung 8. 16S rRNS-basierender phylogenetischer Baum der nah verwandten Gattungen *Nitrosospira*, *Nitrosovibrio* und *Nitrosolobus*. Der Baum enthält alle Isolate, für die 16S rRNS-Gensequenzen mit einer Länge von mehr als 1000 Nukleotiden verfügbar sind. Im Rahmen dieser Arbeit ermittelte Sequenzen sind fett markiert. Maximum-Likelihood, Maximum-Parsimony sowie Neighbour-Joining-Bäume wurden zu einem Konsensusbaum zusammengefasst. Mehrfachverzweigungen verbinden Äste, deren relative Anordnung bei Verwendung verschiedener Baumverfahren nicht zweifelsfrei bestimmt werden konnte. Ausgefüllte und leere Kreise zeigen Boostrap-Werte (100 Wiederholungen) von >90% bzw. >70% an. Für jedes Cluster ist die minimale Sequenzähnlichkeit angegeben. 19 der 48 dargestellten Sequenzen wurden in dieser Arbeit ermittelt. Zudem wurden Sequenzen aus folgenden Publikationen in die Analysen einbezogen: Aakra *et al.* (2001, 1999a, 1999b), Head *et al.* (1993), Teske *et al.* (1994), Tokuyama *et al.* (1997) und Utaaker *et al.* (1995). Sequenzen der Stämme GS833, E12, NRS527, NpAV, RY6A, RY3C, TCH716, and PJA1 sind unveröffentlicht, jedoch über GenBank verfügbar. Der Balken repräsentiert einen Sequenzunterschied von 10%.

1997 schlugen Rotthauwe *et al.* für AOB das *amoA*-Gen als zusätzliches phylogenetisches Markermolekül vor. Seither wurde dieses Makromolekül in zahlreichen Studien erfolgreich für Untersuchungen zur Phylogenie und Diversität von AOB verwendet (Aakra *et al.*, 2001; Alzerreca *et al.*, 1999; Casciotti und Ward, 2001; Hommes *et al.*, 1998; Horz *et al.*, 2000; Klotz und Norton, 1995; McTavish *et al.*, 1993; Rotthauwe *et al.*, 1995; Suwa *et al.*, 1997; Yamagata *et al.*, 1999; Anhang I und II). *AmoA* kodiert die Untereinheit der Ammoniakmonooxygenase mit dem aktiven Zentrum. Es wurden mehrere PCR-Primer veröffentlicht (Holmes *et al.*, 1995; Mendum *et al.*, 1999; Rotthauwe *et al.*, 1995; Sinigalliano *et al.*, 1995; Webster *et al.*, 2002), welche die Amplifikation von *amoA*-Fragmenten erlauben.

Am weitesten verbreitet ist das Paar von Rotthauwe *et al.* (1997) (modifiziert von Stephen *et al.* 1999; Fragmentgröße: 453 bp; in dieser Arbeit verwendet). Die Ergebnisse der phylogenetischen Untersuchungen der vorliegenden Arbeit, die auf der abgeleiteten Aminosäuresequenz der *amoA*-Gene basieren, entsprechen insgesamt denen der 16S rRNS-Phylogenie: Angehörige der Gattungen *Nitrosospira*, *Nitrosolobus* und *Nitrosovibrio* bilden eine eng verwandte monophyletische Gruppe ohne erkennbare Substrukturen. Innerhalb der Nitrosomonaden ist das *N. europaea/Nc. mobilis*-Cluster und das *N. marina*-Cluster mit allen Baumverfahren zu finden. Dagegen sind das *N. communis*- und *N. oligotropha*-Cluster jeweils nicht immer monophyletisch (Abbildung 9). Werden statt der AmoA-Aminosäure- die *amoA*-Nukleinsäuresequenzen als phylogenetische Basis herangezogen, ergibt sich im Wesentlichen das gleiche Bild, mit der Ausnahme, dass Cluster 7 nicht länger monophyletisch ist und *Nitrosomonas* sp. Nm143 eine eigenständige Linie bildet. Im Vergleich zur 16S rRNS-basierenden Phylogenie bietet die AmoA-Analyse eine geringere Auflösung, was auf die Tatsache zurückzuführen ist, dass nur ein relativ kurzer (151 Aminosäuren) und hochkonservierter Bereich (in >98% der beta-AOB sind an 93 Alignment-Positionen die Aminosäuren identisch) als Marker genutzt wird. Sinnvoll wäre in Zukunft sicherlich die Amplifikation des kompletten *amoA*-Gens, beispielsweise mit Hilfe der Primer von Norton *et al.* (2002).

Abbildung 9. AmoA-basierender phylogenetischer Baum der betaproteobakteriellen AOB. Im Rahmen dieser Arbeit ermittelte Sequenzen sind fett markiert. Das 453 bp-Fragment aus der Amplifikation mit dem Primerpaar von Rotthauwe *et al.* (1997) diente als Grundlage der phylogenetischen Untersuchungen dieser Arbeit. AmoA-Sequenzen kürzer als 414 Aminosäuren wurden nicht berücksichtigt. Protein-Maximum-Likelihood-, Maximum-Parsimony-, Neighbour-Joining- und FITCH-Bäume wurden berechnet und gemeinsame Merkmale zusammengefasst. Mehrfachverzweigungen verbinden Äste, deren relative Anordnung bei Verwendung verschiedener Baumverfahren nicht zweifelsfrei bestimmt werden konnte. Ausgefüllte und leere Kreise zeigen Boostrap-Werte (100 Wiederholungen) von >90% bzw. >70% an. Eingerechnete Sequenzen wurden von Aakra *et al.* (2001), Casciotti *et al.* (2001), Holmes *et al.* (1995), McTavish *et al.* (1993), Norton *et al.* (2002), Rotthauwe *et al.* (1995), Sorokin *et al.* (2001), Suwa *et al.* (1997), and Yamagata *et al.* (1999) publiziert oder in dieser Arbeit bestimmt. Sequenzen der Stämme GS833, E12, NRS527, NpAV, RY6A, RY3C, TCH716, and PJA1 (im *Nitrosospira*-Cluster) sind unveröffentlicht, jedoch über GenBank verfügbar. Der Balken repräsentiert einen Sequenzunterschied von 10%.

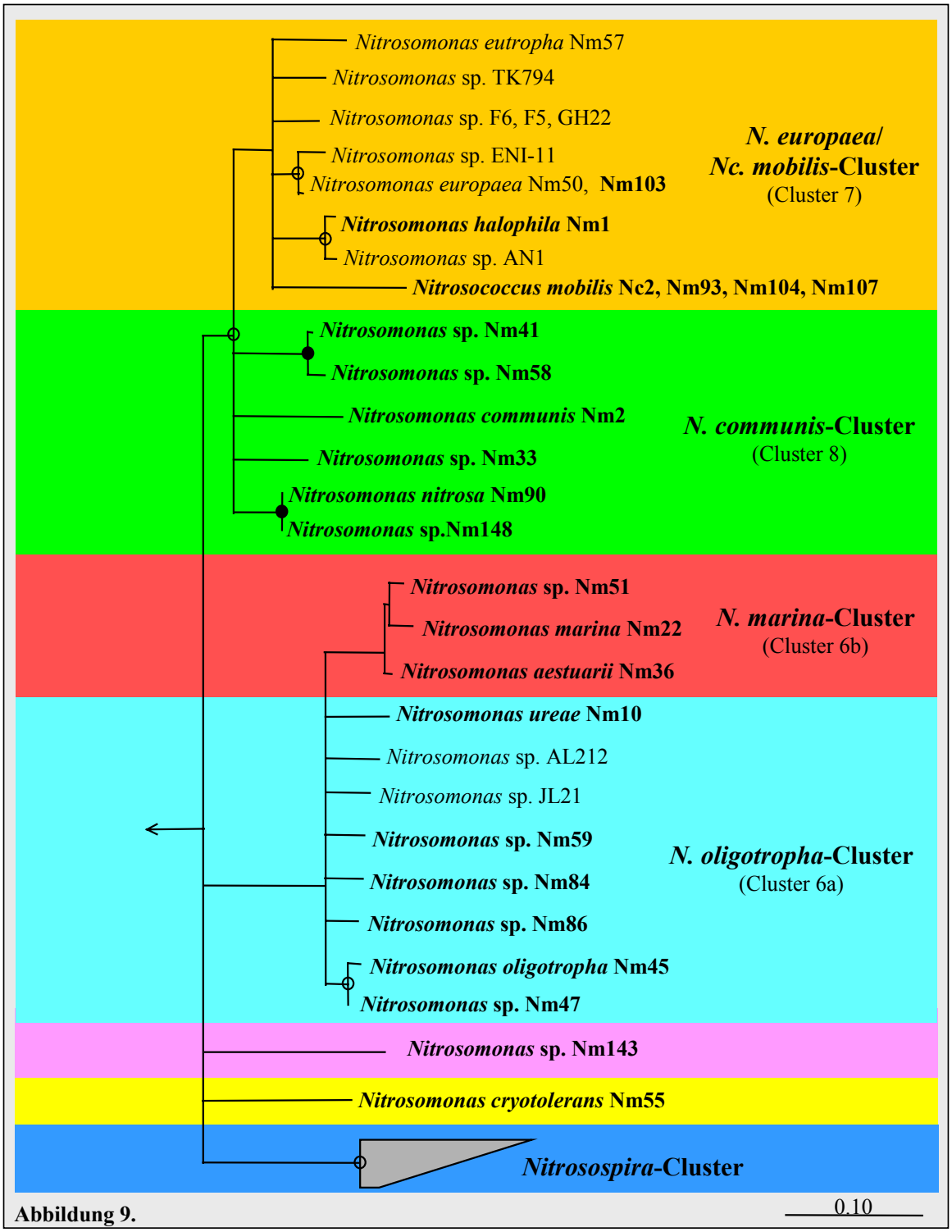


Abbildung 9.

Die graphische Gegenüberstellung von *amoA*- bzw. AmoA-Ähnlichkeiten aller möglichen betaproteobakteriellen Genospeziespaare mit den entsprechenden 16S rRNS-Ähnlichkeiten (Abbildung 10) zeigt i), dass die 16S rRNS einen höheren Konservierungsgrad aufweist als die *amoA* oder AmoA und ii), dass AOB Paare mit weniger als 80% Nukleinsäure-Ähnlichkeit (bzw. 85% Aminosäure-Ähnlichkeit) gleichzeitig immer eine 16S rRNS-Ähnlichkeit unter 97,5% haben (Grenzwert für die Zuordnung zur gleichen Genospezies; Stackebrandt und Goebel, 1994). Somit weisen z. B. mittels PCR aus Umweltproben

amplifizierte *amoA*-Sequenzen mit weniger als 80% Nukleinsäure- bzw. 85% Aminosäure-Ähnlichkeit zu bekannten AOB-Isolaten auf bisher nicht identifizierte Genospezies hin. *AmoA*- bzw. AmoA-Sequenzen mit einer höheren Ähnlichkeit können multiple Genkopien, verschiedene Stämme der gleichen Genospezies, oder neue AOB-Arten repräsentieren.

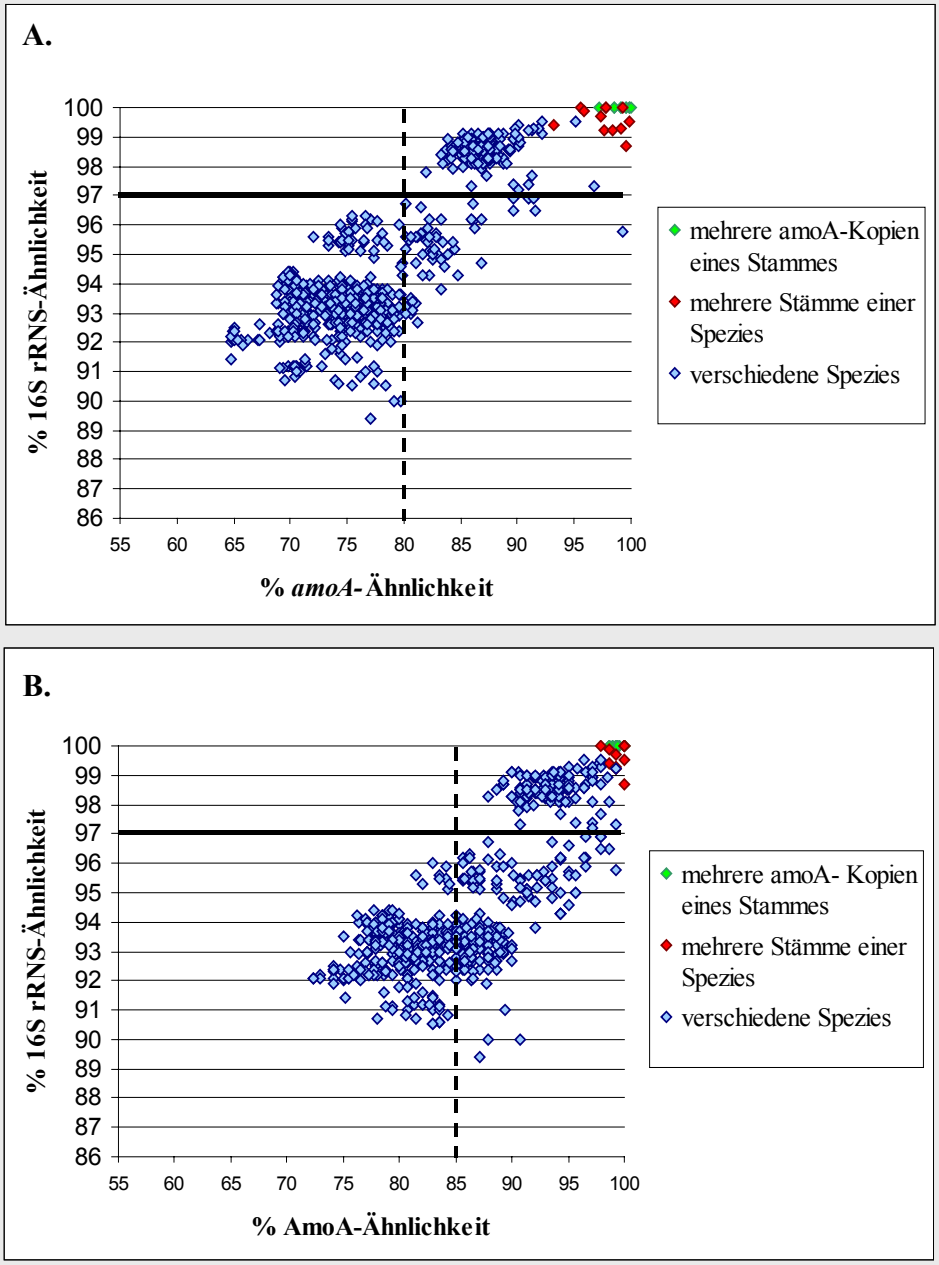


Abbildung 10. Graphische Darstellung der Korrelation von 16S rRNS-Ähnlichkeit und (A) *amoA*- bzw. (B) AmoA- Ähnlichkeit. Durchgezogenen Linien markieren den 16S rRNS-Grenzwert für die Genospeziesdefinition. Gestrichelte Linien deuten die *amoA*- und AmoA-Grenzwerte an, deren Unterschreitung auf die Existenz neuartiger Spezies hinweist.

Kultivierungs-unabhängiger Nachweis von Ammoniakoxidanten in der Umwelt

Traditionell wurde die Abundanz, Diversität und Ökologie Ammoniak oxidierender Organismen mit Hilfe kultivierungsabhängiger Methoden untersucht. In der siebziger Jahren wurde dieser Ansatz durch die Entwicklung der Immunfluoreszenz-Technik ergänzt, die es erstmals ermöglichte AOB *in situ* nachzuweisen (Belser, 1979).

Auf die Einführung der 16S rRNS-basierenden Phylogenie kultivierter AOB (Head *et al.*, 1993; Pommerening-Röser *et al.*, 1996; Teske *et al.*, 1994) folgte die Entwicklung zahlreicher 16S rRNS-gerichteter PCR-Primer und Gensonden für dot blot oder Fluoreszenz *in situ* Hybridisierung (FISH) (Tabelle 2). Am häufigsten wurde die natürliche Diversität von AOB mit Hilfe vergleichender Sequenzanalyse klonierter 16S rRNS-Genfragmente untersucht. Die Spezifität der eingesetzten Primer hat erwiesenermaßen dramatischen Einfluss auf das Ergebnis derartiger molekularer Diversitätsstudien. Die Neubewertung der Primer/Sonden Spezifität (Tabelle 3 und 4) auf Basis der im Rahmen dieser Arbeit erstellten, umfassenden 16S rRNS-Gendatenbank macht deutlich, dass die daraus ableitbare Spezifität der Primer/Sonden zum Teil signifikant von der angegebenen (beabsichtigten) Spezifität in den Originalpublikationen abweicht. Beispielsweise weist der PCR-Primer NitA, der häufig für AOB-Diversitätsuntersuchungen eingesetzt wird (Abd El Haleem *et al.*, 2000; Hollibaugh *et al.*, 2002; Ward *et al.*, 2000), mindestens eine Basenfehlpaarung zu den Isolaten des *N. communis*- und *N. oligotropha*-Clusters, sowie zu den meisten Spezies innerhalb des *N. marina*-Clusters auf. Um verfälschte Resultate zu vermeiden empfiehlt es sich, PCR-Primer mit hoher Sensitivität (Erfassung der meisten beta-AOB) jedoch vergleichsweise geringer Sensitivität (kein vollständiger Ausschluss von Nicht-Ziel-Organismen) zu verwenden. Darüber hinaus muss das amplifizierte 16S rRNS-Genfragment eine Länge von mehr als 1000 Nukleotiden aufweisen, um eine verlässliche phylogenetische Auswertung zu ermöglichen (Ludwig *et al.*, 1998). Die erwähnten Bedingungen werden gegenwärtig am besten von dem Primerpaar βAMOf/βAMOr erfüllt. Die Verwendung dieser Primer nimmt die Amplifikation von nicht-AOB-16S rRNS-Genfragmenten in Kauf, die nachfolgend durch phylogenetische Analyse oder Hybridisierung mit hochspezifischen Sonden (z. B. Nso1225) identifiziert werden müssen.

Tabelle 2. Zusammenfassung publizierter 16S rRNS-gerichteter Sonden und Primer.

Sonde/Primer	Zielregion	beabsichtigte Spezifität	Referenz
NM-75	67-86	terrestrische *Nitrosomonas* spp./ *Nitrosococcus mobilis*	Hiorns *et al.*, 1995
NS-85	76-95	*Nitrosospira* spp.	Hiorns *et al.*, 1995
NmII[§]	120-139	*Nitrosomonas communis* Linie	Pommerening-Röser *et al.*, 1996
NSMR32f	125-141	*Nitrosospira tenuis*-ähnliche AOB	Burrell *et al.*, 2001
NSMR71f	126-143	*Nitrosomonas marina*-ähnliche AOB	Burrell *et al.*, 2001
NSMR34	131-149	*Nitrosospira tenuis*-ähnliche AOB	Burrell *et al.*, 2001
NSMR76[§]	132-149	*Nitrosomonas marina*-ähnliche AOB	Burrell *et al.*, 2001
NitA	136-158	beta-AOB	Voytek und Ward, 1995
ßAMOf	142-162	beta-AOB	McCaig *et al.*, 1994
NSPM	145-162	beta-AOB	Silyn-Roberts und Lewis, 2001

Tabelle 2. (Fortsetzung)

Sonde/Primer	Zielregion	beabsichtigte Spezifität	Referenz
Nm0	148-165	*Nitrosomonas* spp.	Pommerening-Röser *et al.*, 1996
Nsm 156§	155-173	*Nitrosomonas* spp./ *Nitrosococcus mobilis*	Mobarry *et al.*, 1996
NmV§	174-191	*Nitrosococcus mobilis*	Pommerening-Röser *et al.*, 1996
CTO189f A/B-GC	189-207	beta-AOB	Kowalchuk *et al.*, 1997
CTO189f C-GC	189-207	beta-AOB	Kowalchuk *et al.*, 1997
Nso 190§	189-207	beta-AOB	Mobarry *et al.*, 1996
Noli191§	191-208	*Nitrosomonas*-Cluster 6a	Rath, 1996
TAOfwd	192-208	terrestrische AOB	Chandler *et al.*, 1997
NM198	198-218	*Nitrosomonas ureae* + *Nitrosomonas* sp. AL212	Suwa *et al.*, 1997
NmoCL6a_205	205-221	*Nitrosomonas*-Cluster 6a	Stephen *et al.*, 1998
NmI	210-225	*Nitrosomonas europaea*-Linie	Pommerening-Röser *et al.*, 1996
Nmo218§	218-236	*Nitrosomonas oligotropha*- Linie	Gieseke *et al.*, 2001
TMP1	226-253	beta-AOB	Hermansson und Lindgren, 2001
ß-AO233	233-249	beta-AOB	Stephen *et al.*, 1998
NspCL1_249	249-266	*Nitrosospira*-Cluster 1	Stephen *et al.*, 1998
Nmo254a	254-271	alle Nitrosomonaden	Stephen *et al.*, 1998
Nmo254	254-271	alle Nitrosomonaden	Stephen *et al.*, 1998
RT1r	283-304	beta-AOB	Hermansson und Lindgren, 2001
AAO258	258-277	terrestrische beta-AOB	Hiorns *et al.*, 1995
primer 356f	356-372	nested PCR in NitAB Amplifikaten	Hollibaugh *et al.*, 2002
NmoCL6b_376	376-392	*Nitrosomonas*-Cluster 6b	Stephen *et al.*, 1998
Nsp436	436-453	alle Nitrosospiren	Stephen *et al.*, 1998
NmoCL7_439	439-456	*Nitrosomonas*-Cluster 7	Stephen *et al.*, 1998
Nm439	439-459	*Nitrosomonas ureae* + *Nitrosomonas* sp. AL212	Suwa *et al.*, 1997
NitD	439-461	*Nitrosomonas europaea*	Ward *et al.*, 1997
NMOB1f	442-461	*Nitrosococcus mobilis*-ähnliche AOB	Burrell *et al.*, 2001
NSMR52f	443-461	*Nitrosomonas europaea*-ähnliche AOB	Burrell *et al.*, 2001
Nsv 443§	443-461	*Nitrosospira* spp.	Mobarry *et al.*, 1996
NspCL4_446	446-463	*Nitrosospira*-Cluster 4	Stephen *et al.*, 1998
Nsp0	452-469	*Nitrosospira* spp.	Pommerening-Röser *et al.*, 1996
NspCL3_454	454-471	*Nitrosospira*-Cluster 3	Stephen *et al.*, 1998
NspCL2_458	458-475	*Nitrosospira*-Cluster 2	Stephen *et al.*, 1998
Nlm 459r	458-477	*Nitrosospira multiformis*/ *Nitrosospira* sp. C-141	Hastings *et al.*, 1997
NSM1B	478-494	*Nitrosomonas europaea*-Linie/ *Nitrosococcus mobilis*	Hovanec und DeLong, 1996
primer 517r	517-533	nested PCR in NitAB Amplifikaten	Hollibaugh *et al.*, 2002
TAOrev	632-649	terrestrische AOB	Chandler *et al.*, 1997
CTO654r	632-653	beta-AOB	Kowalchuk *et al.*, 1997
NITROSO4E§	638-657	beta-AOB	Hovanec und DeLong, 1996
NEU§	651-668	die meisten halophilen und halotoleranten Nitrosomonaden	Wagner *et al.*, 1995
Amß	738-758	beta-AOB	Utaaker und Nes, 1998
NitF	844-862	beta-AOB	Ward *et al.*, 1997
NitC	846-862	beta-AOB	Voytek und Ward, 1995
NmIII	998-1018	*Nitrosomonas marina*-Linie	Pommerening-Röser *et al.*, 1996
NSMR53r	999-1017	*Nitrosomonas europaea*-ähnliche AOB	Burrell *et al.*, 2001
NSMR74r	1000-1017	*Nitrosomonas marina*- ähnliche AOB	Burrell *et al.*, 2001
NMOB1r	1006-1026	*Nitrosococcus mobilis*- ähnliche AOB	Burrell *et al.*, 2001
NSMR33r	1006-1021	*Nitrosospira tenuis*- ähnliche AOB	Burrell *et al.*, 2001
RNM-1007	1005-1028	terrestrial *Nitrosomonas* spp.	Hiorns *et al.*, 1995
NS-1009	1007-1026	*Nitrosospira* spp.	Hiorns *et al.*, 1995
NmIV§	1004-1022	*Nitrosomonas cryotolerans*-Linie	Pommerening-Röser *et al.*, 1996
NitB	1213-1233	beta-AOB	Voytek und Ward, 1995
Nso 1225§	1224-1243	beta-AOB	Mobarry *et al.*, 1996
ßAMOr	1295-1314	beta-AOB	McCaig *et al.*, 1994
Nse 1472§	1472-1489	*Nitrosomonas europaea*-Linie	Juretschko *et al.*, 1998

§: Sonden wurden erfolgreich in FISH eingesetzt.

Tabelle 3. Spezifität und Sensitivität publizierter 16S rDNS/RNS gerichteter PCR-Primer und Gensonden für Nitrosospiren. NTO: Anzahl von Nicht-Ziel-Organismen außerhalb der AOB mit 0 oder 1 Basenfehlpaarung zu dem jeweiligen Oligonukleotid. Die Farbcodierung visualisiert die Anzahl der Basenfehlpaarungen der verschiedenen *Nitrosospira*-Isolate mit den unterschiedlichen Sonden/Primern. Grün, gelb und rot stehen für 0, 1 oder 2 Basenfehlpaarung(en). Blaue Kästchen bedeuten mehr als 2 Basenfehlpaarungen. Weiße Vierecke zeigen an, dass für den entsprechenden Organismus keine Sequenzinformation bezüglich der Sondenbindestelle verfügbar ist. Falls keine eindeutige Sequenzinformation für einzelne Nukleotide vorhanden ist, ist die Anzahl als Nummer in dem jeweiligen Kästchen angegeben. Derartige Nukleotide sowie offensichtliche Sequenzierfehler wurden bei der Farbcodierung nicht berücksichtigt. Sonden, die in der Literatur erfolgreich für FISH eingesetzt wurden, sind grau hinterlegt.

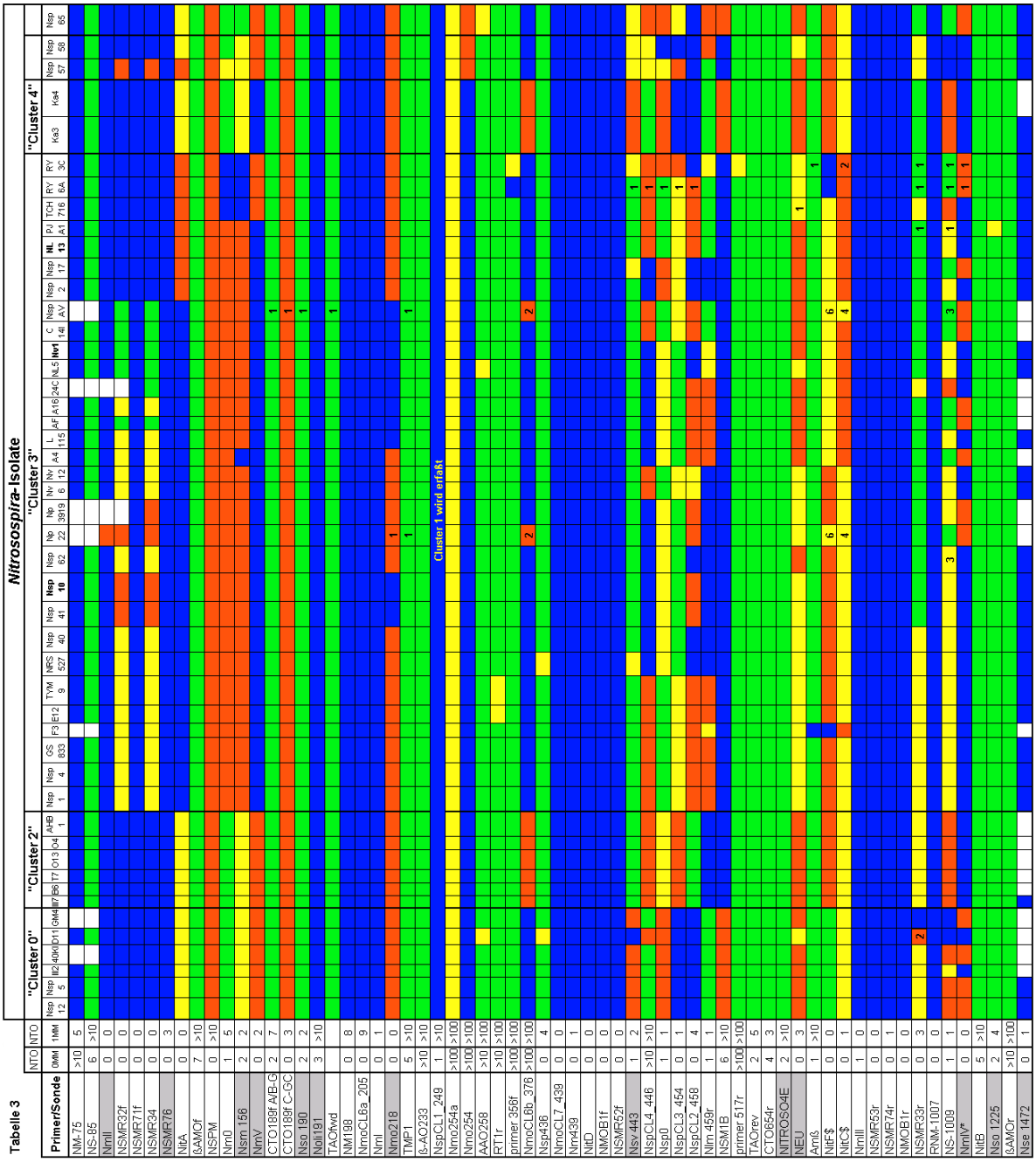


Tabelle 4

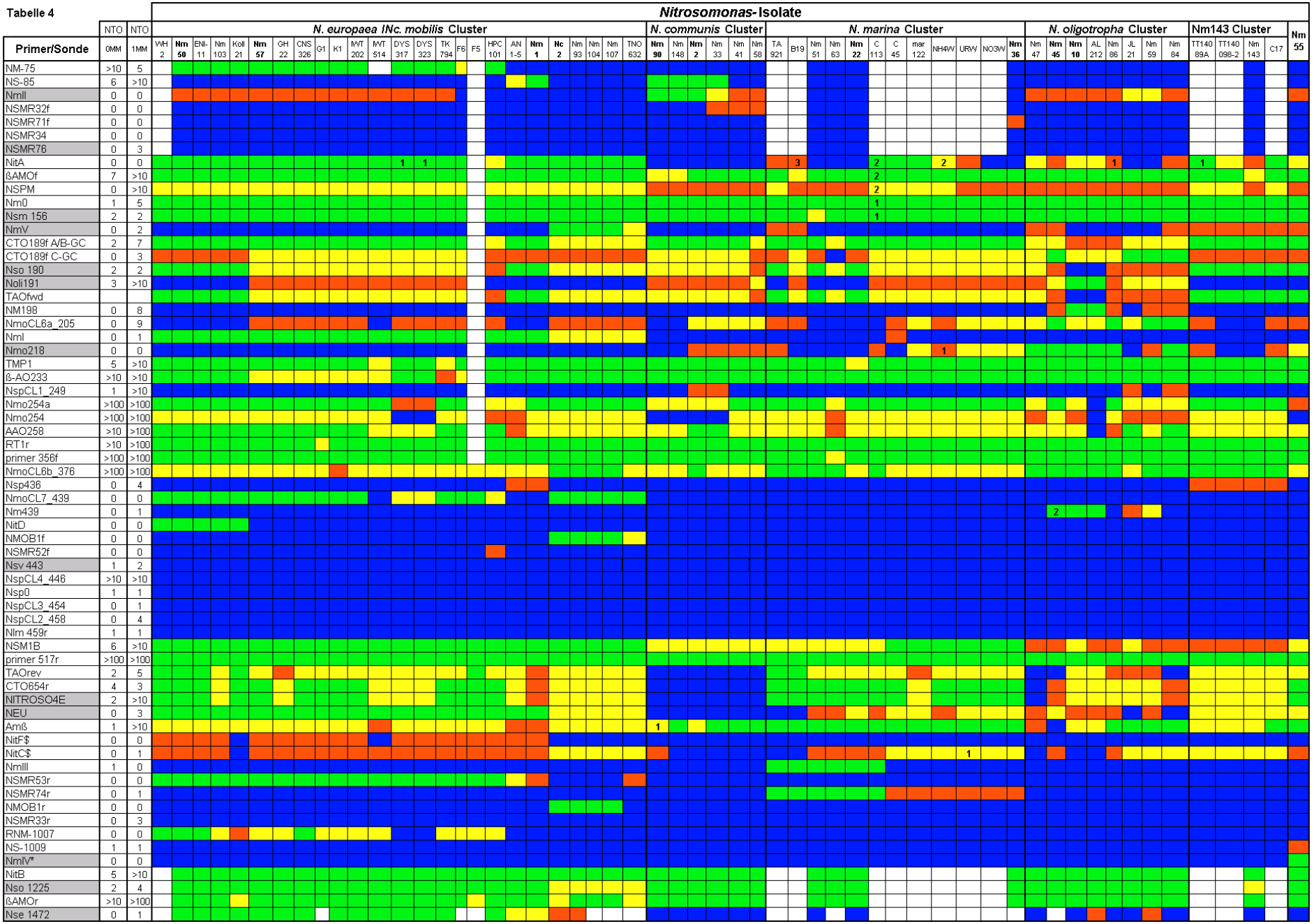

Nitrosomonas-Isolate
N. europaea /Nc. mobilis Cluster
N. communis Cluster
N. marina Cluster
N. oligotropha Cluster
Nm143 Cluster
Primer/Sonde
NTO 0MM
NTO 1MM
NM-75 >10 5
NS-85 6 >10
NmII 0 0
NSMR32f 0 0
NSMR71f 0 0
NSMR34 0 0
NSMR76 0 3
NitA 0 0
ßAMOf 7 >10
NSPM 0 >10
Nm0 1 5
Nsm 156 2 2
NmV 0 2
CTO189f A/B-GC 2 7
CTO189f C-GC 0 3
Nso 190 2 2
Noli191 3 >10
TAOfwd
NM198 0 8
NmoCL6a_205 0 9
Nml 0 1
Nmo218 0 0
TMP1 5 >10
ß-AO233 >10 >10
NspCL1_249 1 >10
Nmo254a >100 >100
Nmo254 >100 >100
AAO258 >10 >100
RT1r >10 >100
primer 356f >100 >100
NmoCL6b_376 >100 >100
Nsp436 0 4
NmoCL7_439 0 0
Nm439 0 1
NitD 0 0
NMOB1f 0 0
NSMR52f 0 0
Nsv 443 1 2
NspCL4_446 >10 >10
Nsp0 1 1
NspCL3_454 0 1
NspCL2_458 0 4
Nlm 459r 1 1
NSM1B 6 >10
primer 517r >100 >100
TAOrev 2 5
CTO654r 4 3
NITROSO4E 2 >10
NEU 0 3
Amß 1 >10
NitF$ 0 0
NitC$ 0 1
NmIII 1 0
NSMR53r 0 0
NSMR74r 0 1
NMOB1r 0 0
NSMR33r 0 3
RNM-1007 0 0
NS-1009 1 1
NmV* 0 0
NitB 5 >10
Nso 1225 2 4
ßAMOr >10 >100
Nse 1472 0 1

Tabelle 4. Spezifität und Sensitivität publizierter 16S rDNS/RNS gerichteter PCR-Primer und Gensonden für Nitrosomonaden. NTO: Anzahl von Nicht-Ziel-Organismen außerhalb der AOB mit 0 oder 1 Basenfehlpaarung zu dem jeweiligen Oligonukleotid. Die Farbcodierung visualisiert die Anzahl der Basenfehlpaarungen der verschiedenen *Nitrosomonas*-Isolate mit den unterschiedlichen Sonden/Primern. Grün, gelb und rot stehen für 0, 1 oder 2 Basenfehlpaarung(en). Blaue Kästchen bedeuten mehr als 2 Basenfehlpaarungen. Weiße Vierecke zeigen an, dass für den entsprechenden Organismus keine Sequenzinformation bezüglich der Sondenbindestelle verfügbar ist. Falls keine eindeutige Sequenzinformation für einzelne Nukleotide vorhanden ist, ist die Anzahl als Nummer in dem jeweiligen Kästchen angegeben. Derartige Nukleotide sowie offensichtliche Sequenzierfehler wurden bei der Farbcodierung nicht berücksichtigt. Sonden, die in der Literatur erfolgreich für FISH eingesetzt wurden, sind grau hinterlegt.

Die Übersicht macht klar, dass die Entwicklung neuer Sonden auf Basis des erweiterten Sondensatzes nötig ist. Beispielsweise steht für den spezifischen Nachweis von Cluster 6a keine geeignete FISH-Sonde zur Verfügung. Wie die im Rahmen dieser Dissertation durchgeführten *amoA*-Studien ergaben, spielen jedoch Vertreter dieses Clusters in Kläranlagen (z.B. Klärwerk München II, Gut Marienhof) eine wichtige Rolle. Auch die Herkunft von Isolaten und 16S rDNS-Sequenzen aus Umweltproben weist auf eine weite Verbreitung dieser Gruppe in Kläranlagen hin (Abbildung 13). Daher wurde zusätzlich zur Sonde Nmo218 (Gieseke *et al.*, 2000/Anhang IV), die den Nachteil hat, neben Cluster 6a noch einige Vertreter von Cluster 6b zu erfassen, die Sonde 6a192 (5'- CTT TCG ATC CCC TAC TTT CC -3') entwickelt. Um die unspezifische Detektion von *N. eutropha* und anderen Nicht-Ziel-AOB zu vermeiden, muss bei der Hybridisierung (35% FA) ein unmarkierter Competitor (5'- CTT TCG ATC CCC GAC TTT CC -3') zugegeben werden. Nach Kontrolle der Spezifität mit Hilfe von Reinkulturen konnten Vertreter des Clusters 6a erfolgreich in Belebtschlamm nachgewiesen werden (Abbildung 11).

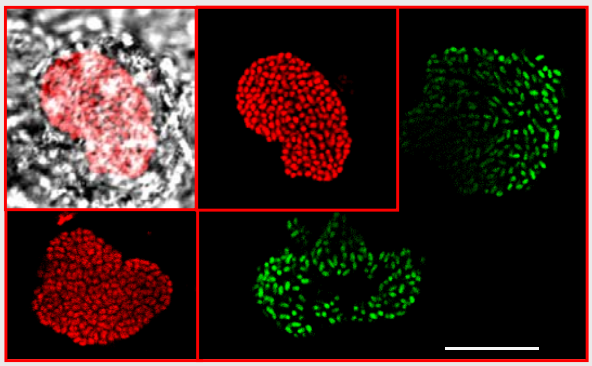


Abbildung 11. FISH-Nachweis von AOB-Mikrokolonien im nitrifizierenden Belebtschlamm aus dem Klärwerk München II. Die Detektion der Sondensignale (Sonde 6a192) erfolgte mit Hilfe konfokaler Laser Scanning Mikroskopie. Der Balken entspricht 10 µm.

Quantitative Studien zur Abundanz und Populationsstruktur von AOB in Umweltproben erfordern die Anwendung von Methoden, die frei von PCR-bedingten Verzerrungen (Wintzigerode und Goebel, 1997) sind. Zu diesem Zweck wurden sowohl dot blot als auch FISH-Methoden entwickelt und bei AOB-Diversitätsstudien eingesetzt (Juretschko *et al.*, 1998; Mobarry *et al.*, 1996; Okabe *et al.*, 1999; Schramm *et al.*, 1996; Wagner *et al.*, 1995). FISH erlaubt gleichzeitig die Identifizierung und Visualisierung der Zielzellen *in situ* und lässt somit Rückschlüsse auf die räumliche Anordnung der AOB zu. Für die Erhebung quantitativer Daten lassen sich absolute Zellzahlen (Daims *et al.*, 2001) oder die relativen Häufigkeiten einzelner Populationen (Nogueira *et al.*, 2000/Anhang III, siehe unten) ermitteln. Untersuchungen zum physiologischen Status (Aktivität) von AOB hingegen erfordern die Kombination von FISH z. B. mit Mikroautoradiogaphie (Lee *et al.*, 1999) oder Mikrosensoren (Gieseke *et al.*, 2000/Anhang IV, siehe unten). Rückschlüsse vom rRNS-Gehalt auf die Aktivität sind bei AOB nicht möglich, da hier der rRNS-Gehalt nicht, wie bei vielen anderen MO, mit geringer Stoffwechselaktivität abnimmt, sondern große Mengen rRNS auch in inaktiven oder inhibierten Zellen nachweisbar sind (Morgenroth *et al.*, 2000; Wagner *et al.*, 1995).

AOB spielen bei der Entfernung von Stickstoffverbindungen aus Abwässern eine Schlüsselrolle, jedoch haben Kläranlagen häufig mit Einbrüchen in der Nitrifikationsleistung zu kämpfen. Verantwortlich dafür sind die lange Generationszeit von AOB, sowie ihre Anfälligkeit gegenüber verschiedenen Chemikalien, pH- und Temperaturschwankungen. Im Rahmen dieser Arbeit wurde mit Hilfe der beschriebenen Methoden in zwei Studien die AOB-Populationsstruktur verschiedener nitrifizierender Labor-Reaktoren analysiert, um den Einfluss der Reaktorführung zu untersuchen, sowie weitere Erkenntnisse bezüglich der Ökologie von AOB zu gewinnen. Vorgehensweise und Ergebnisse der beiden Studien sind im Folgenden kurz zusammengefasst.

Veränderungen in der Zusammensetzung nitrifizierender und heterotropher Populationen in Biofilm-Reaktoren: Einfluss der hydraulischen Verweilzeit sowie der Verfügbarkeit einer organischen Kohlenstoffquelle (Nogueira et al., 2002/Anhang III)

In dieser Studie wurde die Populationsstruktur im Biofilm zweier nitrifizierender Umlauf-Festbett-Reaktoren (R_1, R_2) mit unterschiedlich langen hydraulischen Verweilzeiten (hydraulic retention time, HRT) verglichen. Die Probenahme erfolgte in beiden Reaktoren über mehrere Wochen vor, nach und während der Zugabe einer organischen Kohlenstoff-Quelle (Acetat).

Für die Erfassung sowie zur Beobachtung von Verschiebungen in der Populationsstruktur wurde eine quantitative FISH-Methode verwendet. Dabei wurden mit Hilfe konfokaler Laserscanning Mikroskopie und digitaler Bildanalyse in den einzelnen Proben die relativen Anteile verschiedener Bakteriengruppen an der Gesamtbakterienpopulation bestimmt. Quantifiziert wurden Vertreter des *Cytophaga-Flavobacterium*-Cluster, des *Nitrospira-*

Phylums, der *Alpha- Beta* und *Gammaproteobacteria*, sowie der AOB. Ergänzend wurde die AOB-Ausgangspopulation unter Verwendung des *amoA*-Ansatzes untersucht. Die Nitrifikationsleistung wurde anhand chemischer Parameter verfolgt.

In Übereinstimmung mit den Ergebnissen der *amoA*-Studie ergab die Analyse der AOB-Populationsstruktur mit FISH in allen Proben die Existenz zweier unterschiedlicher AOB-Populationen, von denen die eine nah verwandt zu *N. europaea/eutropha* war. Die andere konnte anhand der *amoA*-Sequenzen keiner bekannten Genospezies zugeordnet werden. Sequenzen dieser Population zeigen jedoch hohe Ähnlichkeit zu Sequenzen, die aus anderen Kläranlagen amplifiziert wurden (Purkhold *et al.,* 2000/Anhang I) und nach dem vorgeschlagenen Grenzwert (siehe oben/Abbildung 10) eine neue Genospezies repräsentieren. Als Vertreter der Nitrit oxidierenden Bakterien konnte in allen Proben *Nitrospira* nachgewiesen werden. *Nitrobacter* war dagegen in geringen Mengen nur im Biofilm des Reaktor mit langer HRT (R_2) während der Acetat-Zugabe präsent. Dieses Ergebnis ist konsistent mit der Hypothese, dass es sich bei *Nitrospira* um k-Strategen handelt, die an niedrige Nitrit-Konzentrationen angepasst sind. *Nitrobacter* als r-Stratege dagegen kann nur bei relativ hohen Nitritkonzentrationen erfolgreich konkurrieren (Schramm *et al.*, 1999). Die Diversität der nitrifizierenden MO wurde insgesamt von der Länge der hydraulischen Verweilzeit nicht beeinflusst.

Die Populationsstruktur heterotropher Mikroorganismen dagegen war in den beiden Reaktoren zum Teil sehr unterschiedlich und somit stark von der hydraulischen Verweilzeit abhängig. Während der Versuchsphase ohne Zugabe von Acetat war in R_2 auf dem autotrophen Biofilm der Aufwuchs von heterotrophen Bakterien, die der Klasse *Alphaproteobacteria* zugeordnet wurden, zu beobachten. Höchstwahrscheinlich bezog diese Population den benötigten Kohlenstoff aus löslichen, mikrobiellen Produkten, die aktive Nitrifizierer produziert hatten (Rittmann *et al.*, 1994). Nach der Zugabe von Acetat verschwand diese Population und wurde von heterotrophen *Betaproteobacteria* abgelöst. Diese traten –im Gegensatz zu den *Alphaproteobacteria*– in beiden Reaktoren auf. In dem Reaktor mit der langen HRT war jedoch der Aufwuchs so stark (60% aller *Bacteria*), dass es durch die resultierende Sauerstofflimitierung der Nitrifizierer am Ende zum vollständigen Zusammenbruch der Nitrifikation kam. Diese Entwicklung steht im Gegensatz zu den Ergebnissen anderer Studien (van Benthum *et al.*, 1997), in denen dieser Effekt gerade durch eine lange HRT vermieden werden konnte, da mit hoher hydraulischer Verweildauer eine räumliche Trennung der konkurrierenden Populationen eintrat und die schnell wachsenden Heterotrophen vor allem in Suspension, die langsam wachsenden autotrophen Nitrifizierer dagegen im Biofilm zu finden waren. Die Verlängerung der HRT allein scheint demnach eine stabile Nitrifikationsleistung in Anwesenheit von organischem Kohlenstoff nicht zu gewährleisten. Vielmehr scheint die Konzeption der Reaktoren (Aufbau, Art der Belüftung) eine Rolle zu spielen.

Populationsstruktur und Veränderungen der Aktivität nitrifizierender Bakterien in einem Phosphat-eliminierenden Biofilm (Gieseke et al., 2001/Anhang IV)

In dieser Studie wurden Struktur und Aktivität der nitrifizierenden Bakteriengesellschaft in einem Biofilm Reaktor (Sequencing Batch Biofilm Reactor, SBBR) mit kombinierter Phosphat-Eliminierung untersucht. Sauerstoff-, Nitrit- und Nitrat-Profile wurden zu verschiedenen Zeitpunkten der Reaktorzyklen (belüftet/unbelüftet) mit Mikrosensoren aufgenommen. Damit waren Rückschlüsse auf die Aktivität der Nitrifikanten möglich. Die Populationsstruktur der nitrifizierenden Gemeinschaft wurde mit Hilfe von 16S rRNS- und *amoA*-Genbanken sowie FISH ermittelt.

Nitrat und Nitrit konnten jeweils erst 90 Minuten nach Start der Belüftung nachgewiesen werden. Das verzögerte Einsetzen der Nitrifikation sowie die Akkumulation von Nitrit trotz hoher Nitrit oxidierender Bakterien (NOB) Zellzahlen reflektierte die eingeschränkte Versorgung der Nitrifizierer mit Sauerstoff. Wegen ihrer hohen K_m-Werte (O_2) sind AOB und NOB im Vergleich zu Heterotrophen in der Konkurrenz um Sauerstoff benachteiligt, so dass der verfügbare Sauerstoff zu Beginn der Belüftungsperiode vermutlich zunächst von aeroben Heterotrophen aufgenommen wurde.

Bei der Analyse der Populationsstruktur ergab die Kombination von FISH, 16S rRNS- und *amoA*-Analysen die Anwesenheit von mindestens drei unterschiedlichen AOB-Linien. Es handelte sich um Vertreter des *Nm. europaea/eutropha*-, *N. ureae/oligotropha*- und *N. communis*-Clusters. Die *amoA*-Analyse lieferte darüber hinaus Hinweise auf das Vorkommen einer bislang nicht isolierten AOB-Genospezies, deren *amoA*-Sequenzen bereits aus anderen Kläranlagen und Labor-Reaktoren amplifiziert wurden (Purkhold *et al.*, 2000/Anhang I; Nogueira *et al.*, 2002/Anhang III). FISH Analysen ergaben, dass ca. 95% aller detektierbaren AOB in den obersten 200 µm des Biofilms lokalisiert waren. Vertreter des *N. communis*-Clusters kamen nur in geringen Zellzahlen und ausschließlich an der Oberfläche des Biofilms vor. *N. europaea/eutropha*-Ähnliche sowie Angehörige des *N. ureae/oligotropha* Clusters bildeten die zwei dominanten AOB-Populationen. Beide waren an der Oberfläche in annähernd gleichen Zellzahlen anzutreffen. In tieferen Schichten (200 µm bis 400 µm) dominierten dagegen eindeutig *N. ureae/oligotropha*-Zellen. Diese offensichtliche Koexistenz dreier verschiedener AOB-Populationen war überraschend, da für viele nitrifizierende Systeme nur eine einzige dominante AOB-Linie beschrieben wurde (Juretschko *et al.*, 1998; Okabe *et al.*, 1999; Schramm *et al.*, 1998). In dem untersuchten System ließe sich die Koexistenz durch die Art der Reaktorführung erklären. Durch den Wechsel zwischen Belüftung und anaeroben Phasen war möglicherweise die Hauptaktivität der einzelnen Linien zeitlich getrennt. Unter der Annahme, dass *N. ureae/oligotropha* besser an niedrige O_2-Konzentrationen angepasst ist (niedrigere $K_m[O_2]$) als *N. europaea/eutropha*, wird *N. ureae/oligotropha* nach Start der Belüftung schneller beginnen, Ammoniak umzusetzen. Erst wenn nach einer gewissen Zeit der Sauerstoff nicht länger limitierend ist, sind auch für *N. europaea/eutropha* optimale Wachstumsbedingungen gegeben. Ein weiterer Grund für die beobachtete Koexistenz ist vermutlich die räumliche Trennung der beiden

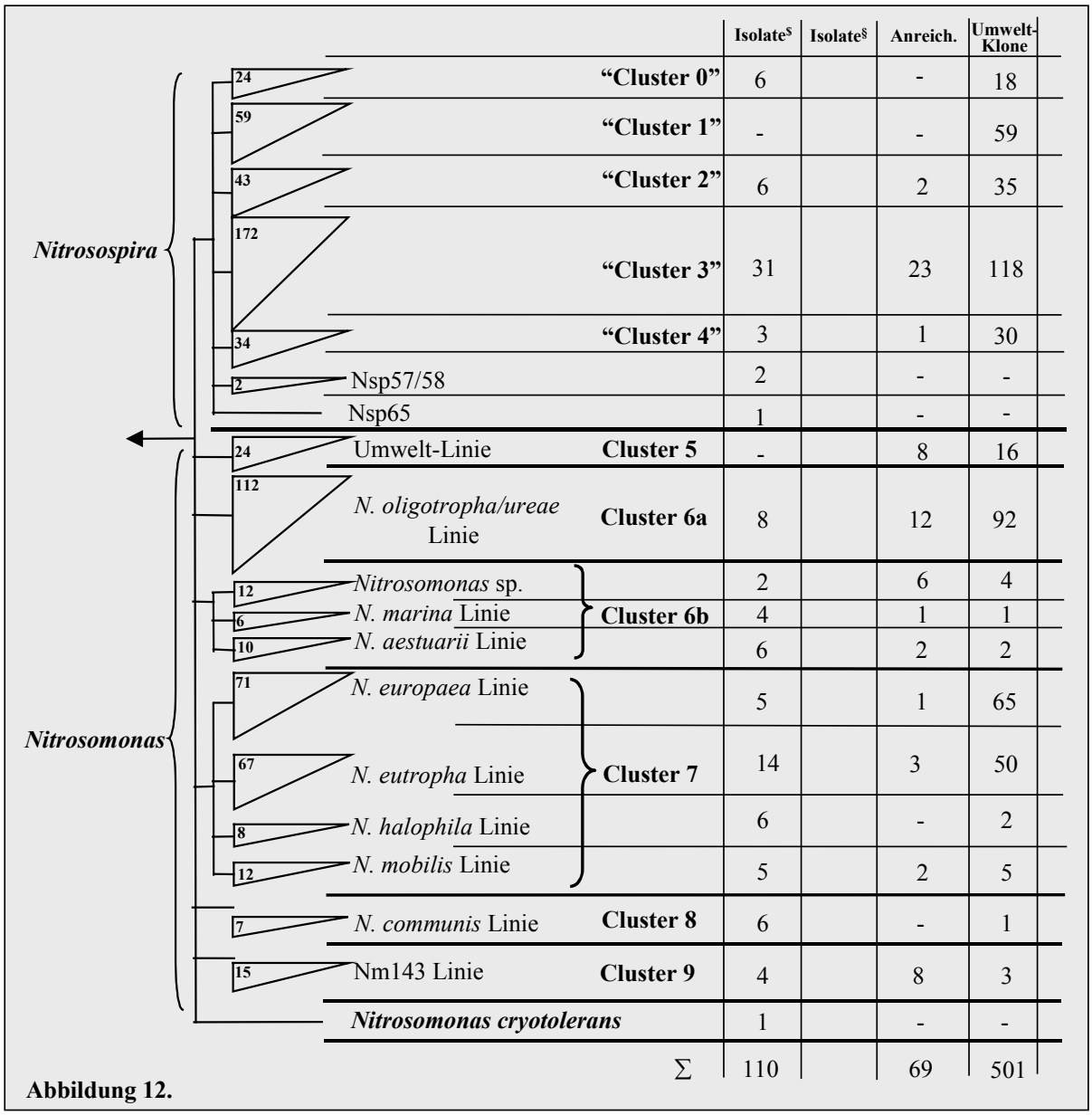
dominanten Populationen. Die Tatsache, dass in tieferen Schichten ausschließlich *N. ureae/oligotropha* vorkommt stützt zudem die Annahme, dass diese Population bei niedrigen Sauerstoff-Konzentrationen im Vorteil ist.
Um die Nitrifikationsleistung in Kläranlagen dauerhaft zu stabilisieren, müssen zukünftige Studien zunächst zeigen, ob eine höhere AOB-Diversität tatsächliche eine stabilere Nitrifikation bedingt (Hinweise darauf liefern z. B. die Resultate von Juretschko *et al.*, 1998 und Daims *et al.*, 2001). Im nächsten Schritt muss dann geklärt werden, welche Parameter die AOB-Diversität in Kläranlagen bestimmen.

Die natürliche Diversität Ammoniak oxidierender Bakterien der Klasse *Betaproteobacteria*

Stephen *et al.* veröffentlichten 1996 die erste AOB-Diversitätsstudie, die auf 16S rRNS-Sequenzen aus der Umwelt beruht. Seither wurden die Artenvielfalt Ammoniak oxidierender Bakterien mit Hilfe des 16S rRNS-Ansatzes in zahlreichen natürlichen und technischen Systemen untersucht. Im Rahmen dieser Arbeit durchgeführte Untersuchungen auf Basis der vervollständigten 16S rRNS-Datenbank ergaben, dass von den mehr als 500 16S rRNS-Umweltklonen innerhalb der beta-AOB mehr als 80% nah verwandt zu kultivierten Vertretern dieser Gruppe sind (Abbildung 12). Wie die phylogenetischen Analysen dieser Arbeit zeigen, bilden die übrigen Klone zwei neue Entwicklungslinien; eine innerhalb der Nitrosomonaden (Cluster 5) und eine innerhalb der Nitrosospiren (Cluster 1) (Abbildung 12).
Aus den zahlreichen AOB-Diversitätsstudien lassen sich Rückschlüsse auf das natürliche Verbreitungsmuster der verschiedenen AOB-Cluster ziehen (Abbildung 13). Nach den in dieser Arbeit aktualisierten Datensätzen zu schließen, kommen Angehörige der Cluster 0, 2, 3 und 4 vor allem in terrestrischen Habitaten vor. Zudem wurden Genfragmente dieser Organismen in Süßwasser-Habitaten nachgewiesen. Ob diese tatsächlich von autochthonen Süßwasser- Bewohnern oder aber von eingetragenen Bodenbakterien stammen, kann noch nicht abschließend beurteilt werden. Nitrosospiren aus Cluster 1 wurden in Böden, weit häufiger jedoch in marinen Systemen gefunden. In den untersuchten Kläranlagen waren Nitrosospiren nur in Einzelfällen nachweisbar. In diesen Fällen handelt es sich entweder um Angehörige des Clusters 2 oder 3. Eine Ausnahme bilden offensichtlich Pflanzenkläranlagen, da aus einer derartigen Anlage überwiegend *Nitrosospira*-ähnliche 16S rRNS-Sequenzen gewonnen wurden (Abd El Haleem *et al.*, 2000). Nsp57, Nsp58 und Nsp65, die vom Mauerwerk verschiedener Bauwerke isoliert wurden, sind die einzigen Nitrosospiren-Isolate, die keine direkte Verwandtschaft zu Umwelt-Klonen aufweisen. Innerhalb der Nitrosomonaden wurden Angehörige der Cluster 5, 6b und 9 noch nie im Boden nachgewiesen; sie scheinen vorzugsweise marine Habitate zu besiedeln. *Nitrosomonas*-Cluster 6a, 7 und 8 sind häufig in Kläranlagen, jedoch auch in vielen anderen Ökosystemen

anzutreffen. Der einzige Vertreter dieser Gattung, der mit Hilfe molekularer Techniken in der Umwelt noch nicht nachgewiesen werden konnte, ist *Nitrosomonas cryotolerans.*
Die Interpretation dieser Daten sollte jedoch unter Vorbehalt erfolgen, da der bloße Nachweis einer 16S rRNS-Sequenz weder die Abundanz noch die physiologische Aktivität des jeweiligen Organismus beweist. Insbesondere AOB sind befähigt, lange Zeiträume unter ungünstigen Bedingungen zu überdauern (Johnstone und Jones, 1988; Pinck *et al.*, 2001; Wilhelm *et al.*, 1998). Darüber hinaus ist die Unterscheidung zwischen autochthonen und allochthonen AOB eines bestimmten Ökosystems extrem schwierig. Auch die Wahl einer repräsentativen Probenahmestelle ist essentiell für eine sinnvolle Daten-Auswertung. Möglicherweise sind AOB, die typischerweise im Belebtschlamm vorkommen auch in Fließgewässern nachweisbar, wenn die Probenahme z. B. in der Nähe eines Kläranlagen-Abflusses erfolgt.

Abbildung 12. Schematische, 16S rRNS-basierende, phylogenetische Einteilung der beta-AOB. Mehrfachverzweigungen verbinden Äste, deren relative Anordnung bei Verwendung verschiedener Baumverfahren nicht zweifelsfrei bestimmt werden konnte. Die Höhe der Vierecke repräsentiert die Anzahl der Sequenzen in dem jeweiligen Cluster. 16S rRNS-Sequenzen mit einer Länge von weniger als 1000 Nukleotiden wurden nur berücksichtigt, wenn eine eindeutige Zuordnung zu einem bestimmten Cluster möglich war. Verwendet wurden die 44 in dieser Arbeit ermittelten 16S rRNS-Sequenzen von AOB-Isolaten sowie 16S rRNS-Sequenzen aus Reinkulturen und Umweltproben publiziert von folgenden Autoren: Aakra *et al.* (2001, 2000; 1999a, 1999b), Abd el Haleem *et al.* (2000), Bano *et al.* (2000), Bollmann *et al.* (2001), Bruns *et al.* (1999), Burrell *et al.* (2001), Chang *et al.* (2001), de Bie *et al.* (2001) , Daims *et al.* (2001), Gieseke *et al.* (2001), Head *et al.* (1993), Hollibaugh *et al.* (2002), Juretschko *et al.* (1998), Kowalchuk *et al.* (1997, 1998, 2000a, 2000b), Logemann *et al.* (1998), McCaig *et al.* (1994, 1999), Mendum *et al.* (1999), Pedersen *et al.* (1996), Philipps *et al.* (1999, 2000), Princic *et al.* (1998), Radeva *et al.* (1999), Regan *et al.* (2002), Rotthauwe *et al.* (1995) , Smith *et al.* (2001) , Sorokin *et al.* (2001) , Speksnijder *et al.* (1998), Stephen *et al.* (1996)., Suwa *et al.* (1997), Takahashi *et al.* (1992) , Teske *et al.* (1994) , Tokuyama *et al.* (1997) , Utaaker *et al.* (1995) , Ward *et al.* (2000), Whitby *et al.* (1999, 2001a, 2001b), and Yamagata *et al.* (1999). Außerdem wurden in GenBank abgelegte 16S rRNS-Sequenzen miteinbezogen (Zugangsnummern: AF107527, AJ441258-AJ441285, AF034139-AF034144, AF034147, AJ318197, U57617, AF510862-AF510865, AJ245751-AJ245760, AJ431350, AJ431351, AF414581, Y10128, Y10127, AJ224941, AF353155-AF353159, AF353161-AF353164, AF359341, AF363287, AF363289- AF363293, and AY036898). $: Anzahl der AOB-Isolate mit verfügbarer 16S rRNS-Sequenz, §: Anzahl der AOB-Isolate ohne publizierte 16S rRNS-Sequenz.



	Isolate§	Isolate§	Anreich.	Umwelt-Klone
"Cluster 0"	6		-	18
"Cluster 1"	-		-	59
"Cluster 2"	6		2	35
"Cluster 3"	31		23	118
"Cluster 4"	3		1	30
Nsp57/58	2		-	-
Nsp65	1		-	-
Umwelt-Linie, Cluster 5	-		8	16
N. oligotropha/ureae Linie, Cluster 6a	8		12	92
Nitrosomonas sp., Cluster 6b	2		6	4
N. marina Linie, Cluster 6b	4		1	1
N. aestuarii Linie, Cluster 6b	6		2	2
N. europaea Linie	5		1	65
N. eutropha Linie, Cluster 7	14		3	50
N. halophila Linie, Cluster 7	6		-	2
N. mobilis Linie, Cluster 7	5		2	5
N. communis Linie, Cluster 8	6		-	1
Nm143 Linie, Cluster 9	4		8	3
Nitrosomonas cryotolerans	1		-	-
Σ	110		69	501

Abbildung 12.

Abbildung 13. Verbreitung der AOB-Cluster in der Umwelt. Für jedes Cluster wurde die Herkunft (Probenahmestelle) der einzelnen 16S rRNS-Sequenzen sowie die Anzahl der Probenahmestellen, an denen Sequenzen eines bestimmten Clusters gefunden wurden, bestimmt („Habitate pro Cluster"). Die verschiedenen Probenahmestellen wurden den Kategorien Kläranlage, Boden (beinhaltet eine Pflanzenkläranlage), Süßwasser (einschließlich Sediment), marin (einschließlich Sediment), Ästuar, und Sanddüne zugeordnet. Die übrigen Habitate wurden unter „andere" zusammengefasst. Die Anzahl der Habitate pro Cluster wurde 100% gesetzt und der Anteil der jeweiligen Kategorien in den einzelnen Clustern dargestellt. Die resultierenden Verteilungen wurden zum einen für Isolate und zum anderen für Sequenzen, die aus Anreicherungen bzw. direkt aus der Umwelt gewonnen wurden bestimmt und gegenübergestellt. Die Anzahl der Habitate pro Kategorie, der die untersuchten Sequenzen entstammen, ist mit n (Isolate) bzw. m (Anreicherungen/Umwelt) angegeben.

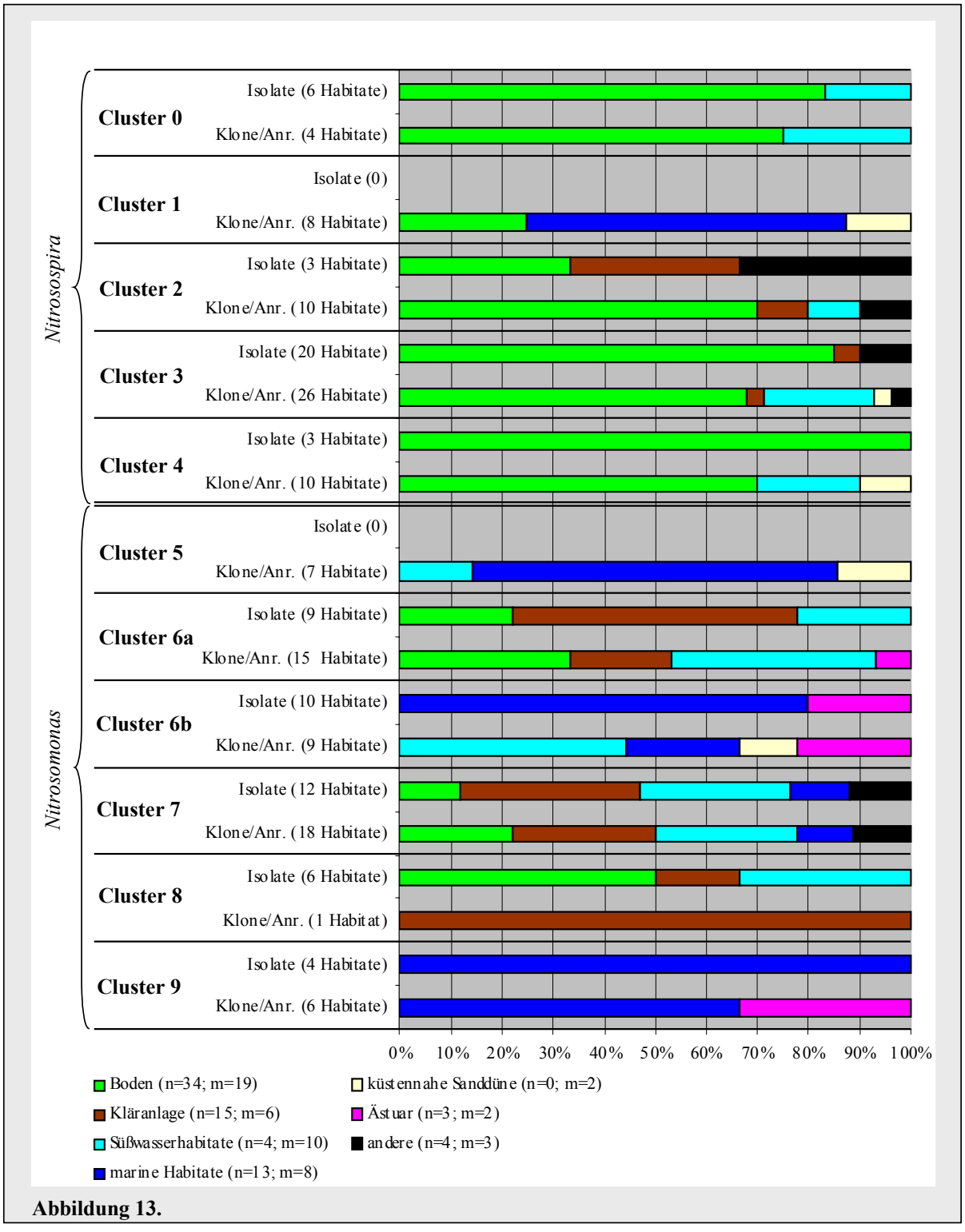


Abbildung 13.

Die natürliche Diversität Ammoniak oxidierender Organismen wurde des weiteren in dieser Arbeit (Purkhold *et al.*, 2000/Anhang I) sowie in zahlreichen anderen Studien (Baribeau *et al.*, 2000; Chang *et al.*, 2001; Dionisi *et al.*, 2002; Gieseke *et al.*, 2001; Helmer *et al.*, 1999; Horz *et al.*, 2000; Ivanova *et al.*, 2000; Nold *et al.*, 2000; Oved *et al.*, 2001; Rotthauwe *et al.*, 1997; Sakano *et al.*, 2002; Schmid *et al.*, 2000; Stephen *et al.*, 1999) mit Hilfe vergleichender Sequenzanalyse von *amoA*-Umweltklonen untersucht. Aus diesen Studien sind heute 387

amoA-Klone verfügbar (davon > 100 in dieser Arbeit ermittelt), die mit allen verfügbaren AOB-Reinkultur-Sequenzen in einer *amoA*-Datenbank zusammengefasst wurden. Wie auch bei 16S rRNS-basierenden Diversitätsstudien ist die überwiegende Anzahl der *amoA*-Umweltklone Clustern mit kultivierten Vertretern zuzuordnen (Tabelle 5). Die aus AmoA-Analysen abgeleiteten natürlichen Verbreitungsmuster (Abbildung 14) bestätigen die Ergebnisse der 16S rRNS-Studie (Abbildung 13), obwohl das geringere Auflösungspotential von AmoA die Unterteilung in einzelne *Nitrosospira*-Cluster und die Abgrenzung von Cluster 6a/6b nicht erlaubt. Gemäß den Grenzwerten aus dem Vergleich der 16S rRNS/*amoA*-Ähnlichkeiten (Abbildung 10) sind nur zwei *amoA*-Sequenzen bekannt (Klon GLII-9 aus einer Kläranlage, sowie Klon Plußsee aus dem gleichnamigen Gewässer), die auf die Existenz bisher nicht kultivierter Genospezies schließen lassen. Diese Beobachtungen bestätigen, dass die AOB in Stammsammlungen die natürliche Diversität dieser Gruppe erstaunlich gut abdecken. Allerdings sind die aus der Umwelt amplifizierten 16S rRNS- oder *amoA*-Sequenzen nur in Ausnahmefällen identisch mit denen kultivierter AOB. Da unkultivierte AOB naturgemäß für die Erhebung von DNS-DNS-Hybridisierungsdaten nicht zur Verfügung stehen, ist nach der gültigen Genospezies-Definition in der Mikrobiologie (Stackebrandt und Goebel, 1994) für sehr nah verwandte Sequenzen eine Unterscheidung zwischen neuartigen und bereits bekannten Arten nicht möglich. Nicht zu unterschätzen sind auch durch PCR und Klonierung bedingte Artefakte (Speksnijder *et al.*, 2001), die zumindest teilweise für das hohe Ausmaß an Mikroheterogenität der 16S rRNS- und *amoA*-Sequenzen der AOB verantwortlich sein können.

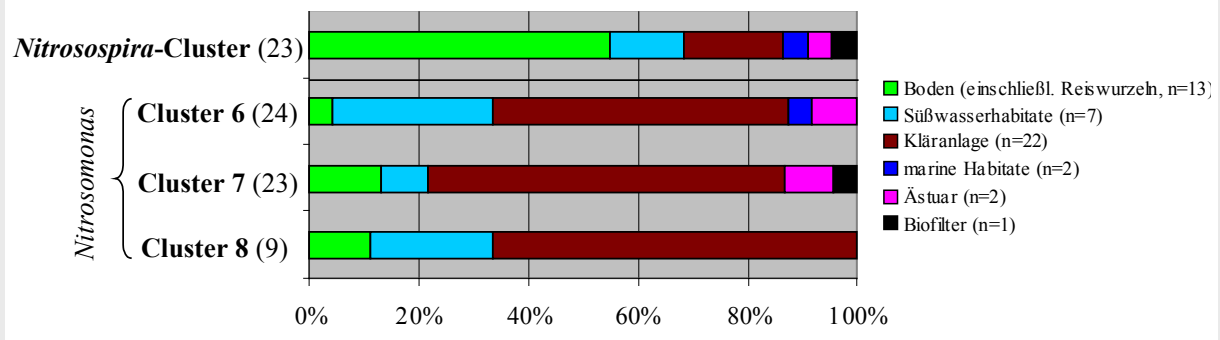


Abbildung 14. Verbreitung der AOB-Cluster in der Umwelt basierend auf *amoA*-Sequenzen. Für jedes Cluster wurde die Herkunft (Probenahmestelle) der einzelnen Sequenzen sowie die Anzahl der Probenahmestellen, an denen Sequenzen eins bestimmten Clusters gefunden wurden, bestimmt. Diese Zahl (in Klammern hinter dem Cluster-Namen) wurde 100% gesetzt und die verschiedenen Probenahmestellen den Kategorien Kläranlage, Boden (beinhaltet eine Pflanzenkläranlage), Süßwasser (einschließlich Sediment), und marin (einschließlich Sediment) zugeordnet. Dargestellt ist der Anteil der jeweiligen Kategorien in den einzelnen Clustern. Die Analyse umfaßt nur *amoA*-Sequenzen, die aus Anreicherungen oder direkt aus Umweltproben amplifiziert wurden.

Tabelle 5.

	gesamt	Isolate	Anreich.	Umwelt	Herkunft
Nitrosospira-Cluster	152	37	3	112	Boden, Kläranlage, Biofilter, Süßwasser, Reiswurzeln, Meerwasser, Ästuar
Cluster 6	116	16	-	100	Boden, Kläranlage, Süßwasser, Ästuar, Süßwassersediment, marines Sediment
Cluster 7	156	16	-	140	Boden, Kläranlage, Biofilter, Süßwasser, Ästuar
Cluster 8	37	6	-	31	Boden, Kläranlage, Süßwasser
Nitrosomonas sp. 143	1	1	-	-	Meerwasser
N. cryotolerans	1	1	-	-	Meerwasser
∑	463	77	3	383	

Tabelle 5. Übersicht über publizierte AmoA-Sequenzen von Isolaten, Anreicherungen und aus der Umwelt. Angegeben ist die phylogenetische Verwandtschaft sowie Anzahl und Herkunft (Probenahmestelle) der Sequenzen. Mehr als 100 der analysierten Sequenzen wurden im Zuge dieser Arbeit bestimmt. Weiter Sequenzen entstammen folgenden Publikationen: Baribeau *et al.* (2000), Chang *et al.* (2001), Dionisi *et al.* (2002), Ivanova *et al.* (2000), Helmer *et al.* (1999), Henckel *et al.* (1999), Holmes *et al.* 1999, Horz *et al.* (2000), Nold *et al.* (2000), Oved *et al.* (2001), Rotthauwe *et al.* (1997), Sakano *et al.* (2002), Schmid *et al.* (2000), and Stephen *et al.* (1999). Außerdem wurden in GenBank abgelegte *amoA*-Sequenzen mit einbezogen (Zugangsnummern: AJ277459, AF338319, AF338320, AF239878-AF239884, AJ388568-AJ388583, AJ388588, und AJ388589).

ZUSAMMENFASSUNG

Der erste Schritt zum Verständnis von Ökologie und Funktion mikrobieller Biozönosen ist die Analyse der Populationsstruktur. Ammoniak oxidierende Bakterien (AOB) sind von besonderem Interesse, da sie in Kläranlagen, in der Landwirtschaft und im globalen N-Kreislauf eine wichtige Rolle spielen. AOB sind jedoch aufgrund ihrer langen Generationszeit schwer zu kultivieren. Daher sind gerade bei Untersuchungen dieser Bakteriengruppe kultivierungsunabhängige molekulare Methoden von großem Vorteil. Mit Hilfe vergleichender Sequenzanalyse der 16S rRNS- und *amoA*-Gene (letztere kodieren die Untereinheit des Schlüsselenzyms Ammoniakmonooxygenase, die das aktive Zentrum trägt) besteht die Möglichkeit, AOB direkt in komplexen Umweltproben nachzuweisen. Grundvoraussetzung für eine erfolgreiche Identifizierung derartiger Umweltklone sind jedoch umfassende Sequenzdatensätze, welche die Sequenzen aller isolierten AOB einschließen.
Im Verlauf dieser Arbeit wurden *amoA*- und 16S rRNS-Datenbanken erstellt, die alle zum gegenwärtigen Zeitpunkt veröffentlichten 700 16S rRNS- bzw. 500 *amoA*-Sequenzen von AOB beinhalten. Diese Sequenzen waren jedoch überwiegend aus Umwelthabitaten amplifiziert worden, lediglich 70 (16S rRNS) bzw. 40 (*amoA*) stammten aus AOB-Reinkulturen. Daher wurden in dieser Arbeit die Sequenzen beider Markermoleküle von 44 weiteren AOB-Isolaten (einschließlich aller 14 beschriebenen Arten) ermittelt und damit das Raster für die Zuordnung bisher nicht identifizierbarer Umweltsequenzen verfeinert.

Mit den aktualisierten Datensätzen als Basis wurden umfassende phylogenetische Analysen Ammoniak oxidierender Bakterien durchgeführt. Der Vergleich der 16S rRNS-basierenden mit den *amoA*-basierenden Ergebnissen ergab eine weitgehende Übereinstimmung der Baumtopologien. *AmoA* ist als phylogenetischer Marker jedoch nur bedingt geeignet, da die Auflösung aufgrund des hohen Konservierungsgrades und der geringen Länge des analysierten Fragmentes stark eingeschränkt ist.

Um die Existenz bislang nicht identifizierter AOB in Umweltproben zu untersuchen, wurde ein *amoA*-basierendes „Genospezies-Konzept“ entworfen. Eine Überprüfung der *amoA*-Datenbank gemäß den erarbeiteten Kriterien lieferte lediglich in zwei Fällen eindeutige Hinweise für das Vorkommen neuartiger Genospezies in der Umwelt. Der Großteil der natürlichen Diversität scheint durch die bereits isolierten Spezies abgedeckt zu sein.

Anhand 16S rRNS-basierender phylogenetischer Analysen wurden die AOB der Klasse *Betaproteobacteria* in sieben verschiedene Cluster gegliedert. Darunter befanden sich zwei Cluster, die nur aus Umweltklonen bestehen und zu denen keine verwandten Isolate existieren, sowie der *Nitrosomonas* sp. Nm143-Cluster, der in dieser Arbeit erstmals beschrieben wurde. Wie bei den *amoA*-basierenden Studien weisen diese Ergebnisse darauf hin, dass die natürliche AOB-Diversität durch die in Stammsammlungen abgelegten Isolaten weitgehend abgedeckt ist. Die phylogenetischen Studien machten zudem deutlich, dass eine in der Literatur häufig zu findende Unterteilung des *Nitrosospira*-Clusters in Subcluster nicht sinnvoll ist.

Unter Berücksichtigung der Herkunft der verschiedenen Sequenzen wurden Verbreitungsmuster für die einzelnen AOB-Cluster erstellt. Dabei wurde klar, dass einige Cluster auf ein definiertes Habitat beschränkt sind, wohingegen andere weiter verbreitet zu sein scheinen. In keinem Fall waren Sequenzen eines bestimmten Cluster in allen untersuchten Habitaten zu finden.

Zudem wurde die Spezifität aller veröffentlichten Gensonden und Primer für den Nachweis von AOB anhand der neu gewonnenen Daten überprüft. Zum Teil ergaben sich signifikante Unterschiede zur beabsichtigten Spezifität. Die erstellte Übersicht erleichtert nun zum einen die Bewertung der Ergebnisse bereits abgeschlossener Studien. Zum anderen bietet sie eine wertvolle Entscheidungshilfe, welche Sonden/Primer sinnvollerweise in zukünftigen molekularen Diversitätsstudien eingesetzt werden sollten.

Abschließend lässt sich sagen, dass die Phylogenie innerhalb der AOB heute weitgehend verstanden ist. Es besteht jedoch Bedarf an weiteren Studien zur natürlichen Verbreitung dieser Organismengruppe. Vor allem die Umweltfaktoren, welche die Verbreitung, die Populationsstruktur und mögliche Koexistenz Ammoniak oxidierender Organismen bestimmen, müssen in künftigen Untersuchungen identifiziert werden.

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ANHANG I

Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys.

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Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys

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The current perception of evolutionary relationships and the natural diversity of ammonia-oxidizing bacteria (AOB) is mainly based on comparative sequence analyses of their genes encoding the 16S rRNA and the active site polypeptide of the ammonia monooxygenase (AmoA). However, only partial 16S rRNA sequences are available for many AOB species and most AOB have not yet been analyzed on the *amoA* level. In this study, the 16S rDNA sequence data of 10 *Nitrosomonas* species and *Nitrosococcus mobilis* were completed. Furthermore, previously unavailable 16S rRNA sequences were determined for three *Nitrosomonas* sp. isolates and for the gamma-subclass proteobacterium *Nitrosococcus halophilus*. These data were used to revaluate the specificities of published oligonucleotide primers and probes for AOB. In addition, partial *amoA* sequences of 17 AOB, including the above-mentioned 15 AOB, were obtained. Comparative phylogenetic analyses suggested similar but not identical evolutionary relationships of AOB by using 16S rRNA and AmoA as marker molecules, respectively. The presented 16S rRNA and *amoA* and AmoA sequence data from all recognized AOB species significantly extend the currently used molecular classification schemes for AOB and now provide a more robust phylogenetic framework for molecular diversity inventories of AOB. For 16S rRNA-independent evaluation of AOB species-level diversity in environmental samples, *amoA* and AmoA sequence similarity threshold values were determined which can be used to tentatively identify novel species based on cloned *amoA* sequences. Subsequently, 122 *amoA* sequences were obtained from 11 nitrifying wastewater treatment plants. Phylogenetic analyses of the molecular isolates showed that in all but two plants only nitrosomonads could be detected. Although several of the obtained *amoA* sequences were only relatively distantly related to known AOB, none of these sequences unequivocally suggested the existence of previously unrecognized species in the wastewater treatment environments examined.

Chemolithoautotrophic ammonia-oxidizing bacteria (AOB) play a central role in the natural cycling of nitrogen by aerobically transforming ammonia to nitrite. From an anthropocentric point of view, the activity of AOB is considered to be both detrimental and beneficial. AOB oxidize urea and ammonia fertilizers to nitrite and, in conjunction with nitrite oxidizers which subsequently convert nitrite to nitrate, thus contribute to fertilizer loss from agricultural soils by producing compounds which are easily washed out or used as electron acceptors for denitrification (42). The former process is also responsible for significant pollution of water supplies with nitrite and nitrate. Furthermore, AOB can produce greenhouse gases (8, 74) and corrode, because of the produced acid, stonework and concrete (46). On the other hand, AOB activity is encouraged in wastewater treatment plants to reduce the ammonia content of sewage before discharge into the receiving waters (49). Reduction of ammonia releases into aquatic environments reduces the risk of local oxygen depletion, helps to prevent eutrophication (15), and protects aquatic life (6).

After the first reports on successful isolation of chemolithoautotrophic ammonia oxidizers at the end of the 19th century (14, 88), researchers have continued to investigate the diversity of AOB in natural and engineered environments by applying enrichment and isolation techniques. These efforts resulted in the description of 16 AOB species (27, 30, 32, 34, 84). Furthermore, DNA-DNA hybridization studies provided evidence for the existence of at least 15 additional species (30, 31, 67). However, low maximum growth rates and growth yields of AOB render cultivation-based analysis of their environmental diversity extremely time-consuming and tedious. Furthermore, all culture techniques are potentially selective and thus bear the risk of incomplete coverage of the actually existing bacterial diversity (5, 28, 79).

Comparative 16S rRNA sequence analyses of cultured AOB revealed that members of this physiological group are confined to two monophyletic lineages within the *Proteobacteria*. *Nitrosococcus oceani* (75, 84) is affiliated with the gamma-subclass of the class *Proteobacteria*, while members of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* form a closely related grouping within the beta-subclass of *Proteobacteria* (17, 52, 67, 73, 76, 92). It has been suggested (17) and subsequently questioned (73) that the latter three genera should be reclassified in the single genus *Nitrosospira*.

The availability of 16S rRNA sequences also provided a basis for the development of cultivation-independent methods to investigate the diversity and community composition of these microorganisms in complex environments. PCR-mediated preferential amplification of AOB 16S rDNA and subsequent cloning and sequencing have been extensively applied to create phylogenetic inventories of various environments (7, 35,

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TABLE 1. Pure cultures of AOB used in this study[a]

Organism[b]	Reference	Origin
Nitrosococcus halophilus Nc4^T	34	Salt lagoon, Sardinia, Italy
Nitrosococcus mobilis Nc2^T	32	North Sea, Harbour of Husum, Germany
Nitrosococcus sp. strain Nm 93	28	Activated-sludge, rendering plant Kraftisried, Germany
Nitrosococcus sp. strain Nm 104	This study	Activated-sludge, rendering plant Kraftisried, Germany
Nitrosococcus sp. strain Nm 107	This study	Activated-sludge, rendering plant Kraftisried, Germany
Nitrosomonas aestuarii Nm36^T	30	Brackish water, North Sea, Denmark
Nitrosomonas communis Nm2^T	30	Soil, isle of Korfu, Greece
Nitrosomonas cryotolerans Nm55^T	27	Kasitsna Bay, Alaska
Nitrosomonas europaea Nm50^T, ATCC 25978	88, 91	Soil, United States
Nitrosomonas halophila Nm1^T	30	North Sea
Nitrosomonas marina Nm22^T	30	Shell grit, great barrier reef, Australia
Nitrosomonas nitrosa Nm90^T	30	Activated-sludge, chemical processing facility, Germany
Nitrosomonas oligotropha Nm45^T	30	Soil, Hamburg, Germany
Nitrosomonas sp. strain Nm33	30	Soil, Japan
Nitrosomonas sp. strain Nm41	30	Soil, Leningrad, Russia
Nitrosomonas sp. strain Nm51, ATCC 25981	30, 87	Seawater, off Peru
Nitrosomonas sp. strain Nm103	28	Activated-sludge, rendering plant Kraftisried, Germany
Nitrosomonas ureae Nm10^T	30	Soil, Sardinia, Italy

[a] AOB were obtained from the culture collection of the Institut für Allgemeine Botanik der Universität Hamburg, Mikrobiologische Abteilung, Germany.
[b] T, type strain; ATCC, American Type Culture Collection.

37, 38, 44, 47, 50, 65, 87), which led to the recognition of seven 16S rRNA beta-subclass AOB sequence clusters. Recently, the battery of molecular tools to infer the presence of AOB in the environment has been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural gene *amoA* (22, 47, 56, 64). While environmental 16S rDNA and *amoA* libraries significantly extended our knowledge on the natural diversity of AOB, biases introduced by DNA extraction, PCR amplification, and cloning methods (10, 12, 51, 54, 71, 72, 90) blur quantitative information on the community composition. Furthermore, due to long-term stability of extracellular DNA and frequent passive dispersal of microbial cells over long distances, the detection of DNA from a certain AOB is inadequate to prove that this organism is part of the autochthonous microbial community. In contrast to PCR-based methods, quantitative information on AOB population structure and dynamics in the environment is obtainable via membrane or in situ hybridization techniques in combination with AOB-specific oligonucleotide probes (28, 40, 48, 61, 62, 80, 81). The latter approach also allows one to directly relate community structure with the morphology and spatial distribution of the detected organisms.

The application of molecular tools already provided exciting new insights into the diversity and community composition of AOB in various environments. However, incomplete coverage of cultured AOB in the current 16S rRNA and *amoA* data sets hampers the design and evaluation of specific primers and probes and renders it impossible to decide whether a novel environmentally retrieved 16S rRNA or *amoA* sequence represents a previously not cultured AOB or is identical to an already isolated AOB which is not yet included in the respective database. One goal of the present study was to complete the 16S rDNA and *amoA* sequence databases in regard to described AOB species. A thorough phylogenetic analysis including all available 16S rRNA and *amoA* sequences of AOB was conducted in order to establish robust phylogenetic frameworks for molecular surveys of the natural diversity of AOB. Furthermore, the specificity of all published AOB-specific 16S rRNA and *amoA*-targeting primers was reevaluated. These analyses helped to resolve several inconsistent results in the literature. Subsequently, the diversity of AOB occurring in wastewater treatment plants was analyzed by assigning more than 100 cloned *amoA* sequences from 11 nitrifying treatment plants to the established *amoA* framework.

MATERIALS AND METHODS

Pure cultures of AOB and sampled wastewater treatment plants. Table 1 summarizes the AOB investigated in this study. AOB were cultured using the

TABLE 2. Characteristics of 11 German nitrifying wastewater treatment plants analyzed[a]

Type of treatment plant, location	System	PE	Sewage type
Semitechnical, Ingolstadt, SBBR1	B	1,800	Concentrated sewage from sludge dewatering
Semitechnical, Ingolstadt, SBBR2	B	50	Municipal
Semitechnical, Ingolstadt, BIOFOR1	B	500	Municipal
Semitechnical, Ingolstadt, BIOFOR2	B	500	Municipal
Full-scale, Poing	AS	105,000	Municipal
Full-scale, Munich I, Großlappen	AS	1,200,000	Municipal
Full-scale, Kraftisried	AS	6,000	Rendering plant effluent
Full-scale, Plattling	AS	26,000	Rendering plant effluent
Full-scale, Sünching, Plant A	AS	ND	Municipal
Full-scale, Sünching, Plant B	AS	ND	Industrial
Semitechnical, Stuttgart, trickling filter 1	B	ND	Semisynthetic

[a] B, biofilm; AS, activated sludge; PE, population equivalent (1 PE = 60 g of biological oxygen demand d^{-1} [26]); SBBR, sequencing batch biofilm reactor; BIOFOR, biological fixed oxygen reactor ND, not determined.

media and conditions described previously (30). *Nitrosococcus* sp. strains Nm 104 and Nm 107 were isolated from the industrial wastewater treatment plant Kraftisried by using the enrichment and isolation procedures (with 10 to 100 mM NH_4Cl and 10 to 200 mM NaCl) described by Juretschko et al. (28). Samples of 11 different wastewater treatment plants were collected between 1997 and 1999 (Table 2).

DNA extraction. AOB were harvested from 10 liters of exponentially growing cultures by continuous-flow centrifugation (20,000 × *g*, 400 ml min^{-1}). Activated-sludge samples (2 ml each) were pelleted by centrifugation (5 min, 10,000 × *g*). Biofilm samples were detached from their substratum by swirling in a suitable volume of DNA extraction buffer (see below). After removal of the substratum, biofilm material was harvested by centrifugation (5 min, 10,000 × *g*). Total genomic DNA was extracted according to the following protocol. A 0.25-g (wet weight) pellet of each sample was resuspended in a 2-ml polypropylene tube with a screw top with 625 μl of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% cetyltrimethylammonium bromide). After addition of 50 μl of enzyme mixture I (lysozyme [66,200 U mg^{-1}; Fluka, Buchs, Switzerland], lipase type 7 [2,000 U mg^{-1}; Sigma, Deisenhofen, Germany], pectinase [1,200 U mg^{-1}; Roth, Karlsruhe, Germany], and β-glucuronidase [120,000 U mg^{-1}; Sigma] each at 10 mg ml^{-1}), the mixture was incubated for 30 min at 37°C. Subsequently, 50 μl of enzyme mixture II (proteinase K [20 U mg^{-1}; Boehringer Mannheim], protease typ9 [1 U mg^{-1}; Sigma], and pronase P [20,000 U mg^{-1}; Serva, Heidelberg, Germany], each at 10 mg ml^{-1}) was added and the mixture was incubated again for 30 min at 37°C. After addition of 75 μl of 20% sodium dodecyl sulfate and incubation at 65°C for 2 h, cell lysis was completed by addition of 600 μl of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and 20 min of incubation at 65°C. After vortexing, the mixture was centrifuged for 10 min at 10,000 × *g* at room temperature. The aqueous phase was carefully transferred to a fresh tube, mixed with 1 volume of chloroform-isoamyl alcohol (24:1), and centrifuged for another 10 min at 10,000 × *g*. The aqueous phase was transferred to a fresh tube, and nucleic acids were precipitated by incubation with 0.6 volumes of isopropanol for 1 h at room temperature and subsequent centrifugation for 20 min at 10,000 × *g*. Pellets were washed with 1 ml of 70% ethanol, dried, and finally resuspended in 30 to 50 μl of elution buffer (10 mM Tris-HCl [pH 8.5]). The amount and purity of DNA were determined spectrophotometrically by determining the optical densities at 260 and 280 nm (58).

PCR amplification of the 16S rDNA. Almost-complete 16S rDNA gene fragments (1,461 to 1,502 bp after deletion of the primer sequences) were amplified from pure cultures of AOB by using the 616V-630R primer pair as described previously (28). Positive controls containing purified DNA from *Escherichia coli* were included in all of the amplification sets along with negative controls (no DNA added). The presence and sizes of the amplification products were determined by agarose (1%) gel electrophoresis of the reaction product. Ethidium bromide stained bands were digitally recorded with a video documentation system (Cybertech, Hamburg, Germany).

PCR amplification of the *amoA* gene fragment. For AOB of the beta-subclass of *Proteobacteria*, a 453-bp fragment (without primers) of the *amoA* gene was amplified from 100 ng of DNA by using the primers *amoA*-1F and *amoA*-2R (targeting positions 332 to 349 and 802 to 822 of the *Nitrosomonas europaea amoA* gene [56]) for PCR with a capillary cycler (Idaho Technology). A 507-bp *amoA-amoB* fragment was amplified from *Nitrosococcus halophilus* by using the newly designed primers *amoA*-3F (5′-GGT GAG TGG GYT AAC MG-3′, positions 295 to 310 of the *amoA* gene of *Nitrosomonas europaea* [45]) and *amoB*-4R (5′-GCT AGC CAC TTT CTG G-3′, positions 30 to 44 of the *amoB* gene of *Nitrosococcus oceani* C-107 [4]), which are complementary to target regions in the *amoA* and *amoB* genes of *Nitrosococcus oceani* and *Nitrosococcus* sp. strain C-113 [4]). Reaction mixtures containing 15 pM concentrations of each primer were prepared in accordance with the manufacturer's recommendations in a total volume of 50 μl by using 20 mM $MgCl_2$ reaction buffer and 1.5 U of *Taq* polymerase (Promega, Madison, Wis.). Thermal cycling was carried out by an initial denaturation step at 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55 or 48°C (*amoA*-1F and *amoA*-2R at 55°C and *amoA*-3F and *amoB*-4R at 48°C) for 20 s, and elongation at 72°C for 40 s. Cycling was completed by a final elongation step at 72°C for 1 min.

Positive controls containing purified DNA from *Nitrosomonas europaea* Nm50 were included in all of the amplification sets along with negative controls (no DNA added). Examination of the amplification products was performed as described above.

Cloning, sequencing, and phylogeny inference. *amoA* PCR products were ligated according to the manufacturer's recommendations into the cloning vector pCR2.1 supplied with the TOPO TA cloning kit (Invitrogen Corp., San Diego, Calif.). Nucleotide sequences were determined for both strands by the dideoxynucleotide method (59) by cycle sequencing of purified plasmid preparations (Qiagen, Hilden, Germany) with a Thermo Sequenase Cycle sequencing kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) and an infrared automated DNA sequencer (Li-Cor, Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. Dye-labeled (IRD 800) M13-targeted sequencing primers were used. 16S rDNA PCR amplificates (approximately 80 to 100 ng) obtained from AOB pure cultures were sequenced directly using primers targeting conserved regions. The new 16S rRNA sequences were added to an alignment of about 18,000 homologous primary structures from bacteria using

TABLE 3. 16S rRNA sequence similarities of beta-subclass AOB[a]

Strain	% Sequence similarity																				
	Nitrosomonas communis cluster				*Nitrosomonas marina* cluster				*Nitrosomonas oligotropha* cluster		*Nitrosomonas cryotolerans* cluster (Nm55)	*Nitrosomonas europaea-Nitrosococcus mobilis* cluster						*Nitrospira* cluster		
	Nm2	Nm33	Nm41	Nm90	Nm22	Nm63	Nm51	Nm36	Nm45	Nm10		Nm50	Nm57	Nm1	Nc2	Nm104	Nm107	C128	C71	Nv12
Nitrosomonas communis Nm2																				
Nitrosomonas sp. Nm33	98.2																			
Nitrosomonas sp. Nm41	97.2	98.1																		
Nitrosomonas nitrosa Nm90	94.9	95.3	**94.6**																	
Nitrosomonas marina Nm22	93.3	93.3	93.4	90.6																
Nitrosomonas sp. Nm63	92.2	92.0	92.4	91.8	98.1															
Nitrosomonas sp. Nm51	93.3	93.2	93.4	90.6	98.9	98.8														
Nitrosomonas aestuarii Nm36	93.7	93.7	93.9	92.0	98.1	**97.1**	98.1													
Nitrosomonas oligotropha Nm45	93.3	93.1	93.0	91.5	94.8	94.1	94.9	95.6												
Nitrosomonas ureae Nm10	93.6	93.3	93.0	90.9	94.2	93.4	94.3	94.9	**96.7**											
Nitrosomonas cryotolerans Nm55	93.8	94.0	93.7	91.4	95.2	94.5	95.3	95.9	95.1	94.7										
Nitrosomonas europaea Nm50	92.9	93.2	92.4	90.8	92.4	91.8	92.5	92.6	92.3	91.6	93.4									
Nitrosomonas eutropha Nm57	93.0	93.0	92.9	92.4	92.2	91.4	92.0	92.7	91.9	91.6	93.2	98.0								
Nitrosomonas halophila Nm1	93.9	94.3	93.6	91.1	93.5	92.6	93.4	93.1	92.5	92.1	94.1	96.4	95.3							
Nitrococcus mobilis Nc2	92.4	92.4	91.9	89.5	93.0	92.6	93.2	93.2	92.9	92.1	93.7	95.0	**94.1**	94.9						
Nitrosomonas sp. Nm104	92.5	92.7	92.1	90.8	93.3	92.9	93.5	93.6	93.1	92.6	94.0	95.4	94.7	95.3	99.5					
Nitrosomonas sp. Nm107	92.5	92.5	92.2	90.7	93.2	92.9	93.3	93.5	93.0	92.5	93.9	95.3	94.7	95.3	99.3	99.9				
Nitrospira sp. C128	94.0	93.7	93.1	91.9	93.4	92.3	93.3	93.7	92.5	92.6	95.3	92.0	92.1	93.8	92.0	92.5	92.5			
Nitrospira multiformis C71	93.8	93.8	93.3	91.8	93.0	92.2	93.1	93.4	92.7	92.8	95.5	92.4	92.5	94.0	92.1	92.5	92.5	98.8		
Nitrospira tenuis Nv12	93.5	93.3	93.0	92.2	93.0	91.6	92.7	93.4	92.1	92.5	95.1	92.1	92.1	93.6	91.7	92.1	92.2	98.8	**98.3**	
Nitrospira sp. NpAV	93.7	93.7	93.5	93.2	93.8	92.8	93.4	94.2	92.4	93.0	96.1	93.2	92.3	94.4	92.4	92.8	92.9	99.0	98.9	98.5

[a] The lowest sequence similarity within a cluster is in bold.

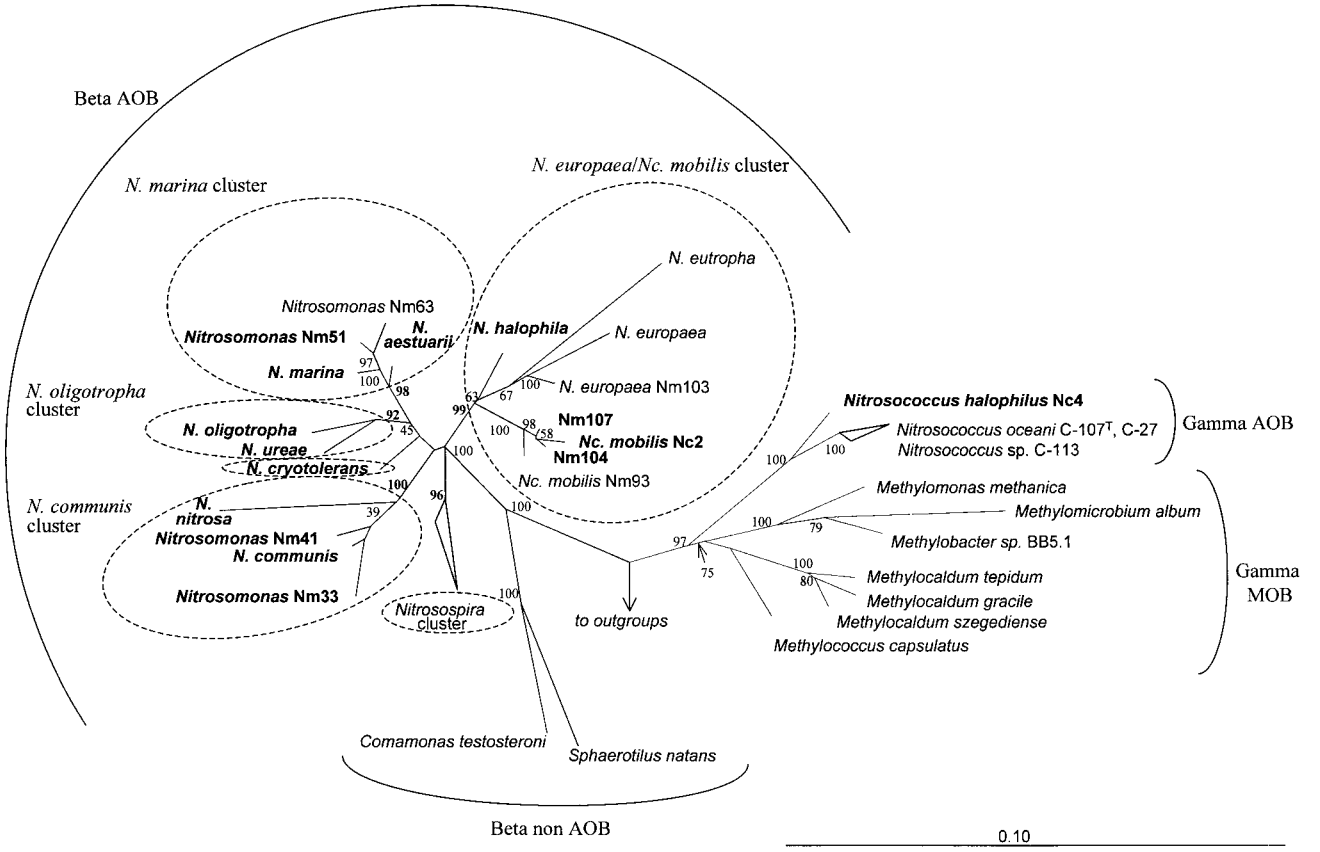

FIG. 1. Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms. The tree is based on results of neighbor-joining analysis using a 50% conservation filter for the *Bacteria*. An encompassing collection of organisms representing all major lineages of the *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. Missing bootstrap values indicate that the branch in question was not recovered in the majority of bootstrap replicates by the parsimony method. The bar indicates 10% estimated sequence divergence. MOB, methane-oxidizing bacteria.

the alignment tool of the ARB program package (O. Strunk and W. Ludwig, http://www.biol.chemie.tu-muenchen.de/pub/ARB). Alignments were refined by visual inspection. Phylogenetic analyses were performed by applying distance-matrix, maximum-parsimony, and maximum-likelihood methods using the respective tools of the ARB and PHYLIP (Phylogeny Inference Package, version 3.57c; J. Felsenstein, Department of Genetics, University of Washington, Seattle) program packages and the fastDNAml program (39). The composition of the data sets varied with respect to the reference sequences and the alignment positions included. Variabilities of the individual alignment positions were determined using the ARB package and were used as criteria for removing or including variable positions for phylogenetic analyses.

The new *amoA* sequences were added to an ARB *amoA* sequence database which contains all publicly available *amoA* sequences. Deduced amino acid sequences were aligned using the editor GDE 2.2 (S. W. Smith, C. Wang, P. M. Gillevet, and W. Gilbert, Genetic Data Environment and the Harvard Genome Database, Genome Mapping and Sequencing, Cold Spring Harbor Laboratory) implemented in the ARB software package. Nucleic acid sequences were aligned according to the amino acid alignment. To construct phylogenetic trees based on amino acid alignments, protein distances were inferred by using a maximum-likelihood method implemented in the PROTDIST program, with the Dayhoff PAM 001 matrix as the amino acid replacement model. Trees were inferred from the distances by using FITCH with global rearrangements and randomized input order of species (PHYLIP, version 3.57c). In addition, protein maximum-likelihood (using the JTT-f amino acid replacement model, computer science monographs, no. 28, MOLPHY version 2.3; programs for molecular phylogenetics based on maximum likelihood, Institute of Statistics and Mathematics, Tokyo, Japan), protein parsimony (PHYLIP, version 3.57c), and neighbor–joining methods (using the Dayhoff PAM 001 matrix as amino acid replacement model and the respective tool in the ARB program package) were applied. To perform *amoA* phylogenetic analysis on the nucleotide level, filters were constructed which allowed exclusion of the third codon position for phylogenetic analysis. Nucleotide-level phylogenetic analyses were performed by applying distance-matrix, maximum-parsimony, and maximum-likelihood methods using the tools described above.

Bootstrap analysis for protein-level (AmoA) and nucleotide-level (*amoA*, 16S rRNA) phylogenetic analyses were performed for parsimony using the tool in the Phylogeny Inference Package PHYLIP (version 3.57c. Department of Genetics, University of Washington). For each calculation, 100 bootstrap resamplings were analyzed.

The terms nucleic acid similarity and amino acid similarity are used instead of nucleic acid identity and amino acid identity to indicate that, especially at variable positions, "false" identities (plesiomorphies) may result from multiple base changes during the course of evolution (41). It should be noted that the term amino acid similarity does not refer to chemical similarities in this context.

Nucleotide sequence accession numbers. The sequences determined in this study are available in GenBank under accession no. AF272398 to AF272412 and AF272521 (*amoA* and AmoA sequences of reference strains); AF272426 to AF272520 and AF276464 to AF276499 (*amoA* and AmoA sequences of environmental clones); and AF272413 to AF272425, AF287297, and AF287298 (16S rDNA of reference strains). The *amoA* and AmoA sequences of *Nitrosomonas halophila* (AF272389) and *Nitrosomonas nitrosa* (AF272404) are identical with those recently published by Horz et al. (24) (AJ238541 and AJ238495).

RESULTS

AOB phylogeny inferred from 16S rRNA. 16S rDNA sequences (1,461 to 1,502 nucleotides) were determined for *Nitrosomonas halophila*, *Nitrosomonas communis*, *Nitrosomonas ureae*, *Nitrosomonas marina*, *Nitrosomonas aestuarii*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans*, *Nitrosomonas nitrosa*, *Nitrosomonas* sp. strain Nm33, and *Nitrosomonas* sp. strain Nm41. For these strains, only partial 16S rDNA sequences (209 to 1224 nucleotides) were published previously. Ambiguities and errors in the 16S rDNA sequence of *Nitrosococcus mobilis* Nc2 (17) were corrected. In addition, we deter-

TABLE 4. *amoA* and AmoA sequence similarities of beta-subclass AOB[a]

Strain	% *amoA* (AmoA) sequence similarity								
	Nitrosomonas communis cluster				*Nitrosomonas marina* cluster			*Nitrosomonas oligotropha* cluster	
	Nm2	Nm33	Nm41	Nm90	Nm22	Nm51	Nm36	Nm45	Nm10
Nitrosomonas sp. Nm 33	86.2 (**90.1**)								
Nitrosomonas sp. Nm 41	85.3 (90.8)	88.7 (90.8)							
Nitrosomonas nitrosa Nm 90	**80.9** (91.5)	83.8 (90.8)	86.5 (92.2)						
Nitrosomonas marina Nm 22	73.3 (79.4)	74.9 (81.6)	73.7 (82.3)	74 (81.6)					
Nitrosomonas sp. Nm 51	74.8 (81.4)	75.2 (83.6)	74 (84.3)	75 (83.6)	89 (**97.2**)				
Nitrosomonas aestuarii Nm 36	75.5 (80.7)	76.7 (83.0)	76.7 (83.6)	74.8 (83.7)	**86.5** (97.2)	88.5 (98.6)			
Nitrosomonas oligotropha Nm 45	75.5 (79.4)	78.6 (82.3)	78.4 (83.0)	76.7 (83.0)	84.1 (93.7)	82.8 (94.3)	82.8 (95.0)		
Nitrosomonas ureae Nm 10	74.5 (78.0)	75.7 (79.4)	76.4 (81.6)	77.5 (83.0)	81.9 (94.4)	81.6 (94.3)	84.1 (95.7)	**85.8** (**93.7**)	
Nitrosomonas cryotolerans Nm 55	74.5 (80.9)	79.6 (83.7)	76.7 (82.3)	76.5 (83.0)	79.2 (88.7)	79.7 (88.7)	80.4 (90.1)	81.1 (90.8)	80.4 (90.1)
Nitrosomonas europaea Nm 50	79.4 (89.4)	80.8 (88.0)	81.6 (90.1)	80.4 (90.8)	74 (80.1)	71.8 (82.1)	75 (82.3)	75.2 (80.9)	75.7 (82.3)
Nitrosomonas sp. Nm 103	78.8 (88.3)	80 (87.0)	81 (89.1)	79.8 (89.9)	73.5 (78.8)	71.5 (80.9)	74.2 (81.0)	74.7 (79.6)	75 (81.0)
Nitrosomonas eutropha Nm 57	80.1 (90.1)	81.1 (88.7)	81.1 (89.4)	79.9 (88.0)	74.3 (78.7)	73 (80.7)	76.2 (80.1)	75 (80.1)	73.5 (78.7)
Nitrosomonas halophila Nm 1	77.5 (87.2)	79.4 (87.3)	79.6 (88.7)	77.5 (89.4)	74.8 (78.7)	71.3 (80.7)	72.5 (80.9)	76.7 (80.1)	71.3 (79.4)
Nitrosococcus mobilis Nc2A	75.5 (83.0)	77.9 (84.5)	78.1 (85.1)	76.2 (87.3)	70.1 (75.9)	72.1 (77.9)	73.3 (78.0)	72.3 (77.3)	72.5 (76.6)
Nitrosomonas sp. Nm 104	75.5 (83.0)	77.9 (84.5)	78.1 (85.1)	76.2 (87.3)	70.1 (75.9)	72.1 (77.9)	73.3 (78.0)	72.3 (77.3)	72.5 (76.6)
Nitrosomonas sp. Nm 107	75.5 (83.0)	77.9 (84.5)	78.1 (85.1)	76.2 (87.3)	70.1 (75.9)	72.1 (77.9)	73.3 (78.0)	72.3 (77.3)	72.5 (76.6)
Nitrosomonas sp. Nm 93	75.7 (83.0)	78.1 (84.5)	78.4 (85.1)	76.5 (87.3)	70.3 (75.9)	72.3 (77.9)	73.5 (78.0)	72.1 (77.3)	72.8 (76.6)
Nitrosospira sp. C128	69.1 (78.0)	69.0 (78.9)	69.7 (78.0)	68.7 (79.6)	74.0 (82.4)	72.6 (83.0)	72.0 (83.8)	77.3 (87.3)	71.3 (83.8)
Nitrosospira multiformis C71	69.5 (76.6)	71.7 (79.4)	72.3 (80.1)	71.1 (80.9)	77.5 (83.1)	75.0 (83.7)	74.6 (84.4)	77.0 (85.9)	75.7 (85.2)
Nitrosospira tenuis Nv12	71.3 (76.6)	71.5 (78.9)	71.9 (77.3)	69.8 (77.5)	76.6 (81.0)	73.5 (81.6)	74.0 (82.4)	78.4 (85.9)	74.6 (82.4)
Nitrosospira sp. NpAV	69.3 (78.4)	69.0 (79.9)	69.9 (79.1)	70.2 (81.3)	78.8 (85.7)	77.2 (86.4)	75.5 (87.1)	78.6 (89.3)	75.7 (87.2)

[a] Nucleic acid similarities include the third codon position; the lowest sequence similarity within a cluster is bold.

mined almost-full-length 16S rDNA sequences (1,461 to 1,502 nucleotides) for *Nitrosococcus halophilus* (34), *Nitrosomonas* sp. strain Nm51 (30, 85), and two AOB strains (Nm104, Nm107) isolated in this study from the industrial wastewater treatment plant Kraftisried.

The 16S rDNA of *Nitrosococcus halophilus* showed the highest sequence similarity (95.6 and 95.7%) to the 16S rRNAs of the gamma-subclass AOB *Nitrosococcus oceani* strains C-107^T (17, 91) and C-27 (17), respectively. These results confirm that *Nitrosococcus halophilus* should be considered a separate AOB species (34). The 16S rDNA sequences of all other AOB investigated were most similar to AOB sequences of the beta-subclass of *Proteobacteria* (Table 3). Phylogenetic trees for the 16S rDNA of AOB were estimated for data sets differing in regard to selection of outgroup organisms and number of variable positions included by distance, parsimony, and maximum-likelihood methods. Independent of the data set and method used, *Nitrosococcus halophilus* formed a monophyletic lineage together with *Nitrosococcus oceani* (strains C-107^T and C-27) and *Nitrosococcus* sp. strain C-113 (4) within the gamma-subclass *Proteobacteria* while the other AOB analyzed formed a monophyletic grouping with the beta-subclass AOB (Fig. 1). Within the beta-subclass AOB, five stable clusters were revealed using the different treeing methods (Fig. 1). This clustering was also supported by high parsimony bootstrap values (92 to 100%). The nomenclature of the clusters was adopted from a study by Pommerening-Röser et al. (52). The first cluster comprised *Nitrosomonas marina*, *Nitrosomonas aestuarii*, together with two strains of a third species (30), *Nitrosomonas* sp. strain Nm63, and *Nitrosomonas* sp. strain Nm51 (*Nitrosomonas marina* cluster). The second cluster encompassed *Nitrosomonas ureae* and *Nitrosomonas oligotropha* (*Nitrosomonas oligotropha* cluster). Most but not all treeing analyses suggested that these two clusters formed a grouping to the exclusion of all other sequences. The third cluster was represented by *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas halophila*, *Nitrosococcus mobilis*, and the isolates Nm104 and Nm107, which are most probably strains of *Nitrosococcus mobilis* (*Nitrosomonas europaea-Nitrosococcus mobilis* cluster). The fourth cluster allied *Nitrosomonas nitrosa*, *Nitrosomonas communis*, *Nitrosomonas* sp. strain Nm33, and *Nitrosomonas* sp. strain Nm41 (*Nitrosomonas communis* cluster). The fifth cluster contained all published *Nitrosospira*-like 16S rDNA sequences (*Nitrosospira* cluster). The phylogenetic position of *Nitrosomonas cryotolerans* and the specific branching order of the above-mentioned clusters varied dependently on the data set and treeing method used and could thus not unambiguously be resolved. In contrast to previous studies (17, 52, 73), phylogeny inference based on the more complete data set did not support that all nitrosomonads are more closely related with each other than with members of the *Nitrosospira* lineage (Fig. 1).

AOB phylogeny inferred from *amoA*. Partial (453 bp) *amoA* sequences were determined for *Nitrosococcus mobilis* Nc2, *Nitrosococcus mobilis* Nm93 (28), *Nitrosomonas halophila*, *Nitrosomonas communis*, *Nitrosomonas ureae*, *Nitrosomonas marina*, *Nitrosomonas aestuarii*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans*, *Nitrosomonas nitrosa*, *Nitrosomonas europaea* Nm103 (28), *Nitrosomonas* sp. strain Nm33, *Nitrosomonas* sp. strain Nm41, *Nitrosomonas* sp. strain Nm51, isolate Nm104, and isolate Nm107 after PCR amplification using the primers described by Rotthauwe et al. (56). Since these primers did not amplify an *amoA* fragment of *Nitrosococcus halophilus*, we exploited the complete *amoA* and *amoB* sequence of its closest known relative, *Nitrosococcus oceani* (4), for the design of the new PCR primer pair *amoA*-F3 and

TABLE 4—*Continued*

% *amoA* (AmoA) sequence similarity											
Nitrosomonas cryotolerans cluster Nm55	*Nitrosomonas europaea*/*Nitrosococcus mobilis* cluster								*Nitrosospira* cluster		
	Nm50	Nm103	Nm57	Nm1	Nc2	Nm104	Nm107	Nm93	C128	C71	Nv12
75 (80.9)											
74 (79.6)	99.7 (99.3)										
75 (80.9)	87 (94.4)	87.1 (93.5)									
72.8 (79.4)	81.9 (95.1)	81.3 (94.2)	80.6 (90.8)								
71.8 (75.2)	77 (88.7)	76.5 (87.7)	**76.5 (85.2)**	76.7 (91.5)							
71.8 (75.2)	77 (88.7)	76.5 (87.7)	**76.5 (85.2)**	76.7 (91.5)	99.8 (100)						
71.8 (75.2)	77 (88.7)	76.5 (87.7)	**76.5 (85.2)**	76.7 (91.5)	100 (100)	99.8 (100)					
72.1 (75.2)	77.2 (88.7)	76.8 (87.7)	76.7 (**85.2**)	76.5 (91.5)	99.8 (100)	99.5 (100)	99.8 (100)				
74.2 (85.9)	70.5 (78.2)	69.8 (76.8)	70.1 (76.8)	71.7 (76.8)	65.0 (72.5)	64.9 (72.5)	64.9 (72.5)	64.7 (72.5)			
75.5 (86.6)	72.7 (80.9)	72.3 (79.6)	71.2 (76.6)	73.7 (79.4)	67.6 (75.2)	67.5 (75.2)	67.5 (75.2)	67.8 (75.2)	**83.5** (92.3)		
75.3 (85.9)	71.2 (78.2)	70.5 (77.5)	69.9 (76.8)	73.5 (76.8)	65.6 (71.8)	65.6 (71.8)	65.6 (71.8)	65.8 (71.8)	85.9 (93.0)	85.7 (90.8)	
75.9 (89.3)	70.8 (80.6)	73.2 (84.2)	69.0 (77.7)	72.6 (78.4)	66.2 (74.1)	66.2 (74.1)	66.2 (74.1)	66.4 (74.1)	85.3 (93.6)	85.1 (92.1)	84.2 (**90.7**)

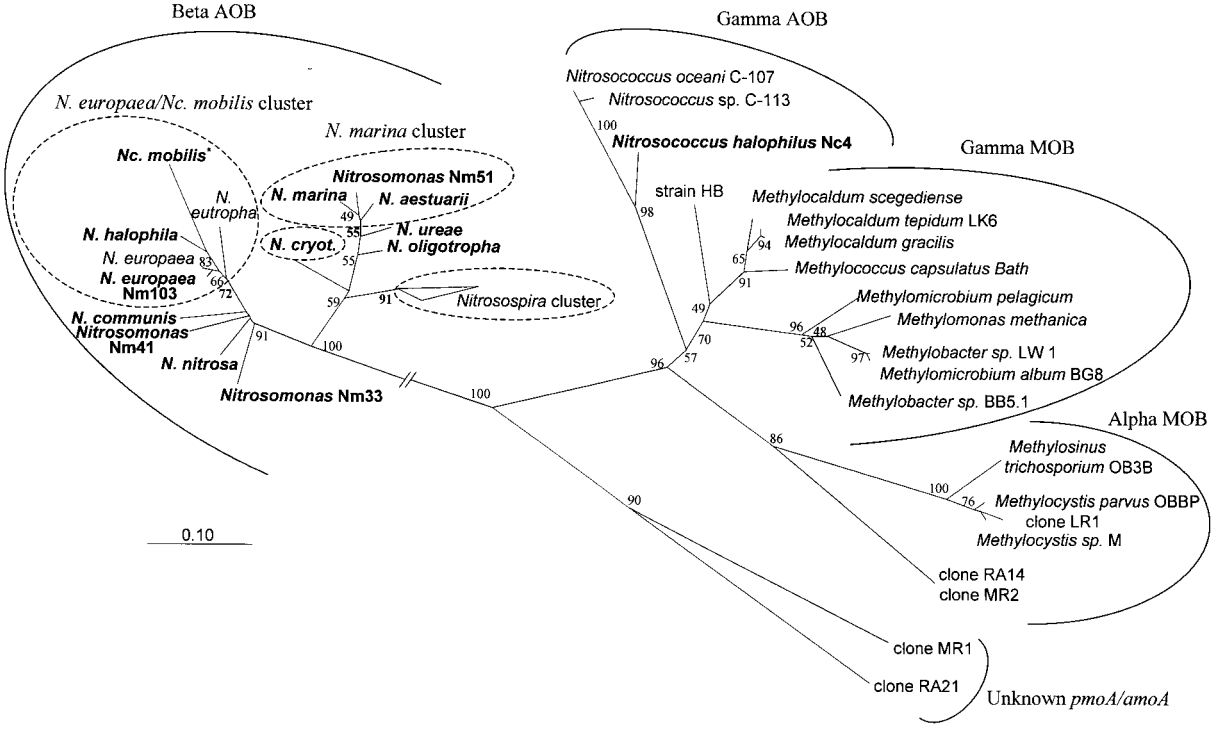

FIG. 2. Phylogenetic Fitch-Margoliash tree (using global rearrangement and randomized input order [7 jumbles]) reflecting the relationships of AOB and methane-oxidizing bacteria (MOB) based on deduced AmoA and PmoA sequences. Parsimony bootstrap values (100 replicates) for branches are reported. Missing bootstrap values indicate that the branch in question was not recovered in the majority of bootstrap replicates by the parsimony method. The bar indicates 10% estimated sequence divergence. Clones RA14 and RA21 (20) and MR1 and MR2 (23) were retrieved in previous studies from soil. Whether clones RA21 and MR1 represent AOB or MOB has not been clarified yet. *, 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.

amoB-R4. These primers were successfully used to amplify the expected *amoA* and *amoB* fragment from *Nitrosococcus halophilus*. In accordance with the 16S rDNA phylogeny, nucleic acid similarities and amino acid similarities were highest between *Nitrosococcus halophilus* and *Nitrosococcus oceani* C-107 (77.8 and 82.5%) and *Nitrosococcus* sp. strain C-113 (77.6 and 81.0%). The *amoA* and AmoA sequences of the other AOB investigated showed highest sequence similarities and similarities to beta-subclass AOB (Table 4).

Phylogenetic trees for *amoA* and AmoA were calculated from the nucleotide and amino acid data sets by distance, parsimony, and maximum-likelihood methods. Overall, highly similar orderings of taxa were found between *amoA* and AmoA and the 16S rRNA trees described above. For all methods with both DNA (with and without the third codon position) and amino acid *amoA* and AmoA data sets, *Nitrosococcus halophilus* grouped together with *Nitrosococcus oceani* and *Nitrosococcus* sp. strain C-113 (Fig. 2). The *amoA* and AmoA sequences of the other AOB investigated clustered together with the beta-subclass AOB *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and the members of the *Nitrosospira* cluster. Three of the five beta-subclass AOB clusters revealed by comparative 16S rRNA analysis were also found in all or most of the *amoA* and AmoA trees (Fig. 2). The monophyly of the *Nitrosospira* cluster, the *Nitrosomonas marina* cluster, and the *Nitrosomonas europaea-Nitrococcus mobilis* cluster was supported by all methods and data sets. However, comparatively low parsimony bootstrap values were calculated for the latter two clusters (55 and 72%). Furthermore, the topology of the *Nitrosomonas europaea* and *Nitrococcus mobilis* cluster differed significantly between the 16S rRNA- and AmoA-based trees, demonstrating the limited phylogenetic resolution provided by these biopolymers for highly related organisms. All methods and data sets suggested a grouping of *Nitrosomonas oligotropha* and *Nitrosomonas ureae* with the *Nitrosomonas marina* cluster. The monophyly of the *Nitrosomonas communis* cluster was supported by the different treeing methods only if a nucleic acid data set including the third codon position was analyzed. Consistent with the 16S rRNA phylogeny, the phylogenetic position of *Nitrosomonas cryotolerans* varied within the beta-subclass AOB dependently on the treeing method and data set used. As for the 16S rRNA, comparative *amoA* and AmoA sequence analysis does not suggest a bifurcation of the beta-subclass AOB into nitrosomonads and nitrosospiras (Fig. 2).

Comparison of AOB DNA-DNA, 16S rRNA, and *amoA*-AmoA similarity. By plotting the 16S rRNA sequence similarity versus the DNA-DNA reassociation values for several bacterial species pairs, Stackebrandt and Goebel demonstrated that at 16S rRNA similarity values below 97%, it is unlikely that two organisms have more than 70% DNA similarity and hence that they are related at no more than the species level (66). We confirmed that the above-mentioned correlation does also apply for beta-subclass AOB species according to published DNA-DNA reassociation values (28, 30, 31, 33, 34, 52) and the 16S rRNA similarities given in Table 3 (Fig. 3A). DNA similarities of AOB species may be as low as 31% at 16S rRNA similarities of 98.1% (*Nitrosomonas marina* Nm22 and *Nitrosomonas aestuarii* Nm36), demonstrating again the superior resolution of DNA-DNA hybridization versus comparative 16S rRNA sequencing for closely related microorganisms.

amoA is increasingly used as phylogenetic marker molecule for molecular diversity inventories of AOB in environmental samples (18, 24, 28, 47, 56, 57, 60, 68; see below). These analyses frequently revealed *amoA* sequences related to but not identical to known AOB species even when the above-presented *amoA* data set containing all validly described AOB

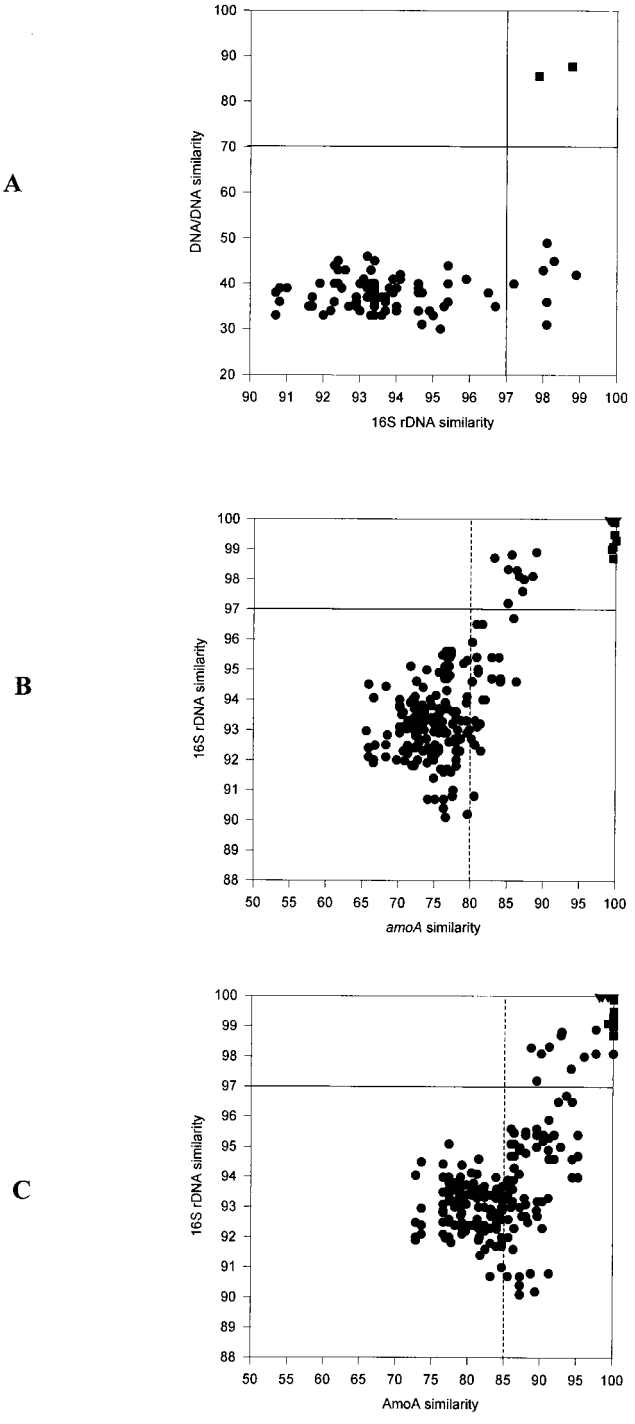

FIG. 3. Correlation plots of DNA-DNA reassociation, 16S rRNA similarity, and *amoA* and AmoA similarity values of AOB. (A) Comparison of 16S rRNA similarity and DNA-DNA similarity values. DNA-DNA hybridization data were obtained from studies by Juretschko et al. (28), Koops et al. (34), Koops et al. (30), Koops and Harms (31), and Pommerening-Röser et al. (52). (B) Comparison of *amoA* similarity and 16S rRNA similarity values. (C) Comparison of AmoA and 16S rRNA similarity values. Sequences of multiple *amoA* gene copies of *Nitrosomonas eutropha* and *Nitrosospira* sp. strain Np39-19 were obtained from GenBank (accession no. AF006692, AF016002, AF042170, U51630, and U72670). Solid lines indicate the DNA and 16S rRNA threshold values for species delineation. Dotted lines indicate the suggested *amoA* and AmoA threshold values below which environmentally retrieved *amoA* and AmoA sequences are indicative of novel AOB species. Circle, pair of different AOB species; square, pair of different strains of a single AOB species; triangle, pair of different *amoA* operons of a single AOB species.

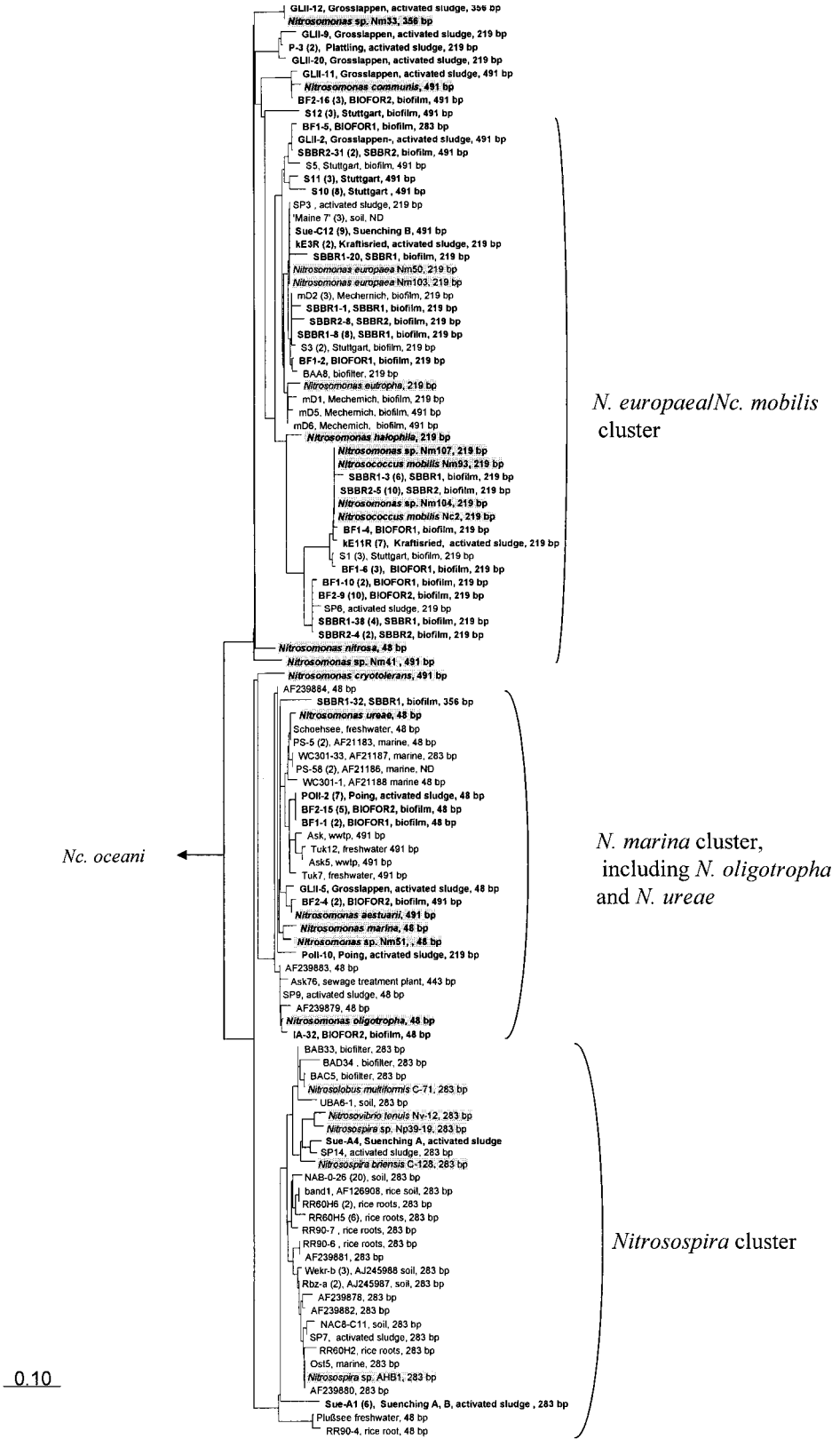

FIG. 4. Phylogenetic Fitch-Margoliash AmoA dendrogram (using global rearrangement and randomized input order [3 jumbles]) showing the positions of cultured ammonia oxidizers (shaded in gray) in relation to environmental sequences recovered from 11 wastewater treatment plants (bold [this study]) and other previously published environmental sequences (18, 19, 23, 24, 56, 57, 60, 68). The bar indicates 10% estimated sequence divergence. The root was determined by using the AmoA sequences of gamma-subclass AOB. Cloned AmoA sequences with amino acid similarities of >99% which originated from the same sample are represented by a single clone—the number in parentheses indicates the number of *amoA* clones for each representative. For each clone, the calculated fragment length in the *Taq*I-based restriction fragment length polymorphism analysis (24) is listed.

TABLE 5. Specificity and sensitivity of published 16S rDNA/RNA targeting PCR primers and hybridization probes for beta-subclass AOB

Primer (OPD nomenclature [3])[a]	Target region[b]	Reference	Intended specificity[c]	No. of mishits with[d]:		Sensitivity[e]						
						Nitrosospira cluster			*Nitrosomonas communis* cluster			
				0MM	1MM	C128	C71	Nv12	Nm2	Nm33	Nm41	Nm90
Nm-75 (S-*-Nsm-0067-a-S-20)	67–86	21	Terrestrial *Nitrosomonas* spp., *Nitrosococcus mobilis*	>10	5	>5	>5	>5	>5	>5	>5	>5
NS-85 (S-G-Nsp-0076-a-S-20)	76–95	21	*Nitrosospira* spp.	6	>10	0	0	0	1I	0	3	0
NmII (S-*-Nsm-0120-a-S-20)	120–139	52	*Nitrosomonas communis* lineage	0	0	3	3	3	0	1	2	0
NitA (S-F-bAOB-0136-a-S-23)	136–158	78	β-AOB	0	0	4	2	4	3	3	3	4
βAMOf (S-F-bAOB-0142-a-S-21)	142–162	43	β-AOB	7	>10	0	0	0	0	0	0	1
Nm0 (S-G-Nsm-0148-a-S-18)	148–165	52	*Nitrosomonas* spp.	1	5	2	2	2	0	0	0	0
Nsm 156 (S-G-Nsm-0155-a-A-19)[f]	155–173	48	*Nitrosomonas* spp., *Nitrosococcus mobilis*	2	2	2	2	2	0	0	0	0
NmV (S-S-Nmob-0174-a-S-18)[f]	174–191	52	*Nitrosococcus mobilis*	0	2	4	3	4	3	2	2	3, 1N
Nso 190 (S-F-bAOB-0189-a-A-19)[f]	189–207	48	β-AOB	2	2	0	0	0	1	1	1	1
CTO189f, A/B-GC (S-F-bAOB-0189-a-S-19)	189–207	37	β-AOB	2	7	0	0	0	1	1	1	1
CTO189f, C-GC (S-F-bAOB-0189-a-S-19)	189–207	37	β-AOB	0	3	2	2	2	1	1	1	1
NmI (S-*-Nsm-0210-a-S-19)	210–225	52	*Nitrosomonas europaea* lineage	0	1	3	3	3	5	5	5	4
AAO258 (S-*-bAOB-0258-a-S-19)	258–277	21	Terrestrial β-AOB	>10	>100	0	0	1N	0	0	1	1
NitD (S-S-Nse-0439-a-S-23)	439–461	83	*Nitrosomonas europaea*	0	0	>5	>5	>5	>5	>5	>5	>5
Nsv 443 (S-G-Nsp-0443-a-S-19)[f]	443–461	48	*Nitrosospira* spp.	1	2	0	0	0	>5	>5	>5	>5
Nsp0 (S-G-Nsp-0452-a-S-18)	452–469	52	*Nitrosospira* spp.	1	1	0	0	0	>5	>5	>5	>5
Nlm 459r (S-*-Nsp-0458-a-A-20)	458–477	16	*Nitrosospira multiformis*, *Nitrosospira* sp. strain C-141	1	1	2	0	3	>5	>5	>5	>5
NSM1B (S-*-Nsm-0478-a-A-17)	478–494	25	*Nitrosomonas europaea* lineage, *Nitrosococcus mobilis*	6	>10	3	3	3	1	1	1	1
TAOrev (S-F-bAOB-0632-a-A-18)	632–649	11	β-AOB	2	5	0	0	1	3	3	4	3
CTO654r (S-F-bAOB-0632-a-A-17)	632–653	37	β-AOB	4	3	0	0	1	3	3	3	3
NITROSO4E (S-F-bAOB-0632-a-A-22)	638–657	25	β-AOB	2	>10	0	0	1	3	3	3	3
NEU (S-*-Nsm-0651-a-A-18)[f]	651–668	80	Most halophilic and halotolerant *Nitrosomonas*	0	3	1	2, 1N	1	4	3	3	3
Amβ (S-F-bAOB-0738-a-S-21)	738–758	77	β-AOB	1	>10	0	0	0	1	0	0	3
NitF (S-F-bAOB-0844-a-A-19)[g]	844–862	83	β-AOB	0	0	2	1	4	3	4	4	3
NitC (S-F-bAOB-0846-a-A-17)[g]	846–862	78	β-AOB	0	1	3	4	5	3	4	4	3
NmIII (S-*-Nsm-0998-a-S-21)	998–1018	52	*Nitrosomonas marina* lineage	1	0	>5	>5	>5	>5	>5	>5	>5
RNM-1007 (S-*-Nsm-1005-a-A-25)	1005–1028	21	Terrestrial *Nitrosomonas* spp.	0	0	>5	>5	>5	>5	>5	>5	>5
NS-1009 (S-G-Nsp-1007-a-A-25)	1007–1026	21	*Nitrosospira* spp.	1	1	1	1, 1N	1	5	>5	>5	>5
NmIV (S-S-Nsm-1004-a-S-19)[f,h]	1004–1022	52	*Nitrosomonas cryotolerans* lineage	0	0	5	3, 1N	4	5	>5	4	>5
NitB (S-F-bAOB-1213-a-A-21)	1213–1233	78	β-AOB	5	>10	0	0	0	0	0	0	0
Nso 1225 (S-F-bAOB-1224-a-A-20)[f]	1224–1243	48	β-AOB	2	4	0	0	0	0	0	0	0
βAMOr (S-F-bAOB-1295-a-A-20)	1295–1314	43	β-AOB	>10	>100	0	0	0	0	0	0	0

[a] OPD, Oligonucleotide Probe Database.
[b] Nucleotide numbers correspond to *E. coli* numbering (9).
[c] Each specificity was given by the respective authors when the primers were published.
[d] Shown are the numbers of non-AOB targeted with zero mismatches (0MM) or one mismatch (1MM). Environmental 16S rDNA clones were not included in this analysis.
[e] D, deletion; I, insertion; N, undetermined base in the target region.
[f] Probe has been demonstrated to be suitable for in situ hybridization.
[g] Corrected sequences were used (77).
[h] Probe NmIV (52) should be modified as follows: T should be replaced by A at position 1 of the probe sequence to eliminate a mismatch to the target region of *Nitrosomonas cryotolerans*.

species was used as a framework (see below). However, it is not possible to estimate whether such an environmental *amoA* sequence represents a different strain of a described species or whether it originates from a novel species. Correlation plots of *amoA* and AmoA similarity (Table 4) versus 16S rRNA similarity (Table 3) of all possible pairs of beta-subclass AOB species demonstrate that (i) 16S rRNA is more conserved than *amoA* and (ii) AOB showing below 83.2% *amoA* nucleic acid similarity (*Nitrosospira* sp. C128 and *Nitrosolobus multiformis*) and 89.1% AmoA amino acid similarity (*Nitrosomonas communis* and *Nitrosomonas* sp. strain Nm41) do possess less than 97% 16S rRNA similarity (Fig. 3B and C). We consequently suggest that environmental *amoA* sequences with lower than 80% nucleic acid similarity (85% amino acid similarity) to described AOB species are indicative of previously undiscovered species. An *amoA* or AmoA sequence with a higher similarity to a described AOB species can represent multiple gene copies, different strains of this species, or a novel AOB species. The latter possibility exists since 16S rRNA similarities between different species can be higher than 97% (the value used to define the *amoA* threshold, see above) (for an example, see reference 13).

***AmoA* sequences from wastewater treatment plants.** Beta-subclass AOB diversity surveys were performed in 11 nitrifying wastewater treatment samples (Table 2). *amoA* PCR products (using the primers amoA-1F and amoA-2R) retrieved from the samples were used for the generation of *amoA* libraries. A total of 122 clones were randomly selected and sequenced. Phylogenetic analysis demonstrated that all clones contained *amoA* sequences affiliated to the beta-subclass AOB (Fig. 4). *Nitrosospira*-related sequences could be detected only in the municipal and industrial plant Sünching (the latter plant was inoculated with sludge from the former plant during start-up). However, all 11 plants investigated harbored nitrosomonads.

TABLE 5—*Continued*

Sensitivity[e]													
Nitrosomonas marina cluster				*Nitrosomonas oligotropha* cluster		*Nitrosomonas cryotolerans* cluster (Nm55)	*Nitrosomonas europaea-Nitrosococcus mobilis* cluster						
Nm22	Nm63	Nm51	Nm36	Nm45	Nm10		Nm50	Nm103	Nm57	Nm1	Nc2	Nm104	Nm107	Nm93
>5	>5	>5	>5	>5	>5	>5	0, IN	0, 1D	0	>5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	0	>5	>5	>5	>5
4	>5	5	4	2	2	2	2	2	2	4	3	3	3	3
4	3	4	3	2	1	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	0	0	4N	0	0	0	0	0
3	3	3	4	2	4	2	2, 1D	2, 1D	3, 1D	3	0	0	0	0
0	1	0	1	2	3	0	0	0	1	0	1	1	1	1
0	1	0	1	2	3	0	0	0	1	0	1	1	1	1
2	3	2	1	0	1	2	2	2	1	2	2	2	2	2
3	3	3	4	5	>5	4	0	0	0	0	1	1	1	1
1	2	1	1	1	1	1	0	0	0	1	1	1	1	2
>5	>5	>5	>5	>5	>5	>5	0	0	4	5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
1	1	1	0	2	1	1	0	0	0	0	0	0	0	0
1	1	1	1	3	1	1	0	0, 3D	1	2	1	1	1	1
0	0	0	0	2	1	0	0	3D	0	2	1	1	1	1
0	0	0	0	2	1	0	0	3D	0	2	1	1	1	1
1	2	2	1	1	2	1	0	0	0	0	1	1	1	1
0	0	0	0	3	1	0	1	1	1	2	1	1	1	1
5	5	5	4	3	4	4	2	2	2	2	4	4	4	4
3	3	3	2	3	4	2	3	3	3	3	2	2	2	2
0	0	0	3	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	>5	0	1	0, 1D, 3N	>5	5	5	5	5
>5	>5	>5	>5	>5	>5	2	>5	>5	>5	>5	>5	>5	>5	>5
>5	>5	>5	>5	>5	>5	1	>5	>5	>5	>5	>5	>5	>5	>5
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
0	0	0	0	0	0	0	0	0	0	1	2	0	0	0

AmoA sequences closely related to those of *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosococcus mobilis*, *Nitrosomonas communis*, *Nitrosomonas* sp. strain Nm33, *Nitrosomonas oligotropha*, *Nitrosomonas ureae*, and the *Nitrosomonas marina* cluster were detected. No indications for the occurrence of *Nitrosomonas* sp. strain Nm41, *Nitrosomonas cryotolerans*, *Nitrosomonas halophila*, and *Nitrosomonas nitrosa* in the analyzed wastewater treatment plants could be obtained.

DISCUSSION

In general, the phylogenetic analyses of the completed 16S rRNA AOB data set supported the previously published perception of AOB phylogeny (17, 52, 73). As expected from DNA-DNA hybridization data (34), the 16S rRNA sequence of *Nitrosococcus halophilus* groups together with the gamma-subclass AOB *Nitrosococcus oceani* (C-107^T, C-27) and *Nitrosococcus* sp. strain C-113, which is most probably a strain of *Nitrosococcus oceani*. The obtained 16S rRNA tree topology of the beta-subclass AOB is overall consistent with the one reported by Pommerening-Röser et al. (52), who suggested six lines of descent among the beta-subclass nitrosomonads. Based on our analyses, however, we suggest grouping the *Nitrosococcus mobilis* cluster together with the *Nitrosomonas europaea* cluster since (i) 16S rRNA similarities between both clusters are comparable to similarities within the other five proposed clusters (Table 3), (ii) both clusters are monophyletic in all treeing analyses, and (iii) no physiological traits separating members of both clusters are known. These facts were considered to be more decisive than the morphological differences between members of both clusters, which obviously evolved relatively recently. We would like to point out again (52, 73) that a taxonomic revision of *Nitrosococcus mobilis* is required to express its phylogenetic affiliation with the genus *Nitrosomonas.*

Based on the completed 16S rRNA sequences of the beta-subclass AOB, we reevaluated the specificity of previously published PCR primers and hybridization probes for the direct detection of these organisms in the environment (Table 5). None of the primers and probes intended to target all beta-subclass AOB showed both 100% sensitivity (targeting all beta-subclass AOB) and 100% specificity (excluding all non-beta-subclass AOB). For general beta-subclass AOB diversity surveys in environmental samples using 16S rDNA libraries (7, 69) or fingerprinting techniques (36, 37) we recommend using PCR primer pairs with high sensitivity [e.g., βAMOf and βAMOr (43) accepting unwanted amplification of non-AOB 16S rDNA fragments which subsequently have to be identified by phylogenetic analysis or hybridization with probes with excellent specificity (e.g., Nso1225 [48]). For AOB community composition analysis, using in situ hybridization (e.g., see references 28, 63, and 80), probes with nested specificity (and

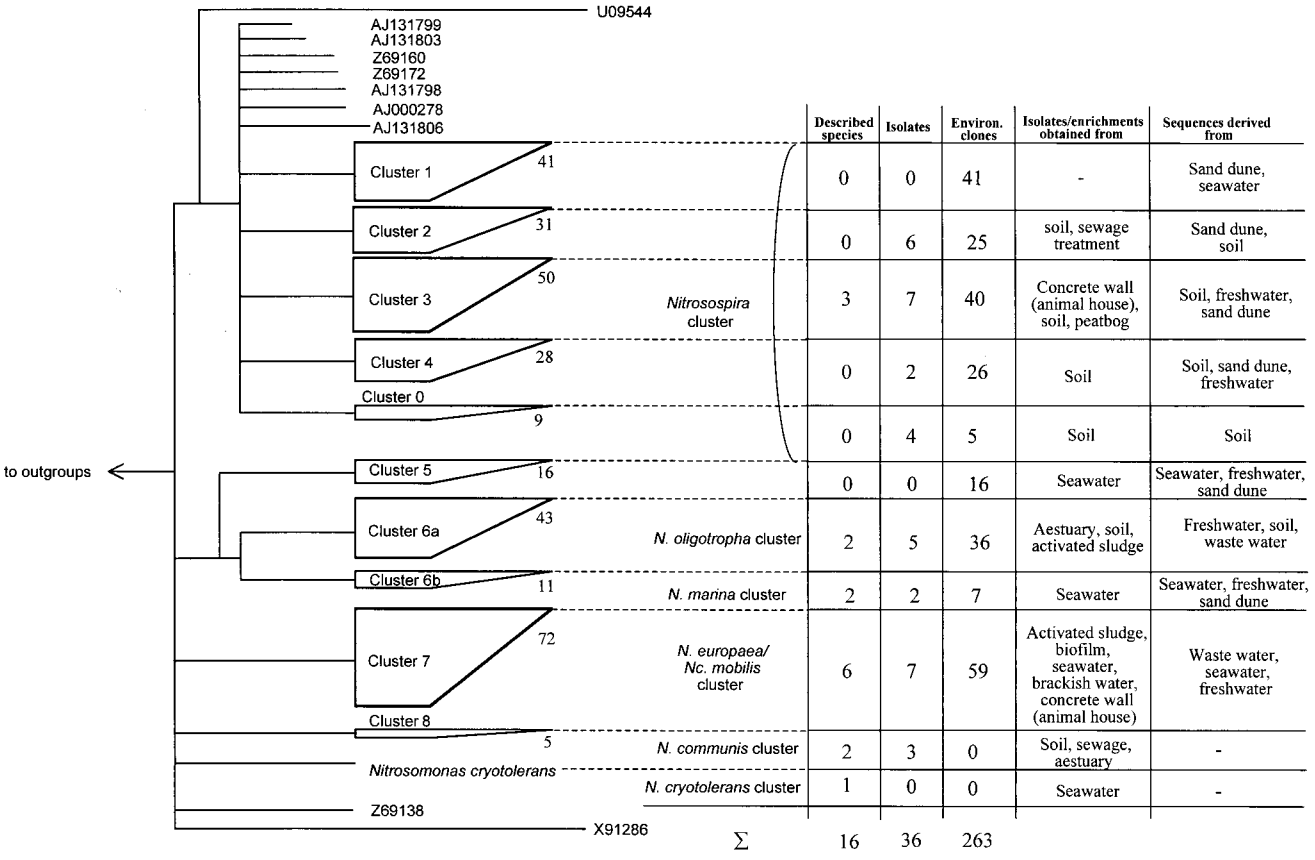

FIG. 5. Schematic 16S rRNA-based phylogenetic classification of the beta-subclass AOB. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The height of each tetragon represents the number of sequences in the cluster. Due to the presence of many published partial 16S rRNA sequences in the clusters, no meaningful estimate of the sequence diversity within a cluster could be inferred. The cluster designations were adopted from those of Stephen et al. (69). We suggest including two additional clusters in the scheme (*Nitrosospira* cluster 0; *Nitrosomonas* cluster 8). Furthermore, cluster 6 should be subdivided into clusters 6a and 6b (see text). In addition to the 16S rRNA sequences determined in this study, 16S rRNA sequences published by Aakra et al. (1, 2), Head et al. (17), Suwa et al. (70), Kowalchuk et al. (35, 37, 38), Logemann et al. (40), McCaig et al. (43), Mendum et al. (47), Phillips et al. (50), Princic et al. (53), Rotthauwe et al. (55), Speksnijder et al. (65), Stehr et al. (67), Stephen et al. (69), Teske et al. (73), Utaker et al. (76), and Whitby et al. (87) as well as unpublished AOB 16S rRNA sequences deposited in GenBank were used to calculate the schematic dendrogram. The composition of each cluster is indicated in the adjacent table. Isolates which have not been analyzed with regard to their species affiliation are as follows: for cluster 2, *Nitrosospira* sp. strains AHB1 (55), O4 and O13 (2), III7 and B6 (1), and T7 (76); for cluster 3, *Nitrosospira* sp. strains NpAV and Np22-21 (43) and F3, L115, AF, A4, and A16 (1); for cluster 4, *Nitrosospira* sp. strains Ka3 and Ka4 (2); for cluster 0, *Nitrosospira* sp. strains III2, D11, GM4, and 40KI (76); for cluster 6, *Nitrosomonas* sp. strains Nm80, Nm84, and Nm86 (67) and AL212 and JL21 (70); for cluster 7, *Nitrosomonas* sp. strains GH22 and HPC101 (71), F5 (1), Koll21 (GenBank accession no. AJ224941), and Nm104 and Nm107 (this study); and for cluster 8, *Nitrosomonas* sp. strains Nm58 (67) and Nm33 and Nm41 (this study).

good sensitivity) should be simultaneously applied (for example, Nso1225, Nsv443, and Nso 156 [48]). However, apparently inconsistent results from simultaneous in situ hybridization experiments with multiple probes can also be indicative of the presence of novel AOB.

Recently, Stephen et al. (69) suggested a 16S rRNA-based phylogenetic classification scheme for beta-subclass AOB consisting of seven clusters, which has found widespread application (7, 35, 37, 38, 44, 47, 50, 65, 87). We reevaluated this scheme using the completed and newly obtained 16S rRNA AOB sequences of this study by using different treeing methods and data sets. The overall tree topology was determined by exclusively using sequences with more than 1,000 nucleotides. More partial 16S rRNA sequences were subsequently added without changing the overall tree topology (Fig. 5). According to Ludwig et al. (41), this procedure produces more reliable trees than calculating a single tree based on only a few hundred aligned nucleotides (37, 69). This is also exemplified in several obviously incorrect tree topologies obtained in previous studies in which only a few hundred informative positions of the 16S rRNA were analyzed. For example, in the trees constructed by different authors (44, 47, 53, 69, 82, 87), *Nitrosococcus mobilis* does not belong to cluster 7 but is incorrectly assigned to cluster 6 or to *Nitrosomonas cryotolerans*.

Our phylogenetic analyses demonstrated that *Nitrosospira* clusters 1 to 4 are supported by some but not by all treeing methods. While cluster 1 is recovered with most methods and data sets, clusters 2, 3, and 4 are less stable. It should also be noted that four *Nitrosospira* isolates (40KI, GM4, D11, and III2 [76, 77]) which form an additional and stable cluster (together with five environmental clones) are not yet included in the current scheme (Fig. 5). Within the nitrosomonads we propose to extend the scheme by the previously excluded *Nitrosomonas communis* cluster, which thus represents cluster 8. Furthermore, we suggest splitting cluster 6 into clusters 6a and 6b, which are represented by members of the *Nitrosomonas oligotropha* cluster and the *Nitrosomonas marina* cluster, respectively (Fig. 5). Most environmental AOB 16S rRNA sequences retrieved so far belong to *Nitrosospira* clusters 1 and 3 and to the *Nitrosomonas europaea-Nitrosococcus mobilis* cluster. However, it should be stressed that the relationships inferred from

very short 16S rRNA sequences, even using the "combined" treeing method applied here, are still of low confidence.

Despite the discussed limitations, several interesting observations can be made from the hitherto performed AOB diversity studies. First, within the nitrosomonads, only cluster 5 clearly represents a missing species within the AOB culture collection with sequence similarities of <96.5% to previously described AOB species (highest similarity was to a 186-bp 16S rRNA fragment of *Nitrosomonas* sp. strain Nm84 [67]). In addition, four 340-bp-long molecular wastewater isolates from a reactor with high ammonium level (clones AI-8H, AI-7K, AI-8B1, and AI-9K3 [53]) might represent a new species within cluster 7 (<96% sequence similarity to previously described AOB species). *Nitrosospira* cluster 1, which does not yet contain a cultured isolate, is nevertheless not demonstrative for the existence of a novel *Nitrosospira* species since all cluster 1 16S rRNA sequences show more than 97% similarity to available *Nitrosospira* pure cultures. In addition, some environmentally retrieved partial 16S rDNA sequences (the majority of them related to nitrosospiras) cannot be unambiguously assigned to one of the clusters (Fig. 5). Due to the short sequence lengths, it is difficult to decide whether these sequences represent putative novel AOB species. Second, none of the environmental AOB sequences retrieved so far in the various studies are affiliated with the *Nitrosomonas communis* cluster (cluster 8), *Nitrosomonas halophila*, or *Nitrosomonas cryotolerans*. This might in part be caused by insufficient coverage of these organisms by some of the "AOB-specific" primers used. However, we could detect *Nitrosomonas communis* and *Nitrosomonas* sp. strain Nm 33 but not *Nitrosomonas halophila* and *Nitrosomonas cryotolerans* in wastewater treatment plants using the *amoA* approach (see below). Future studies will have to show whether *Nitrosomonas halophila* and the *Nitrosomonas communis* and *Nitrosomonas cryotolerans* clusters are of limited environmental distribution or whether methodological biases cause underestimation of their actual abundance.

The gene encoding the active site subunit of the ammonia monooxygenase (*amoA*) has increasingly been exploited as a marker molecule for cultivation-independent analyses of ammonia oxidizer diversity. Different sets of PCR primers for the amplification of *amoA* gene fragments were published (22, 47, 56, 64). In this study, the primers described by Rotthauwe and coworkers (56) were successfully used to amplify the expected *amoA* fragment from all beta-subclass AOB analyzed, demonstrating the excellent sensitivity of this PCR assay. For amplification of an *amoA* fragment of the gamma-subclass AOB *Nitrosococcus halophilus*, a new PCR primer pair was developed. After completion of the *amoA* database, phylogeny inference based on the nucleic acid and amino acid *amoA*-AmoA data sets was, both for the beta- and the gamma-subclasses of AOB, overall consistent with the picture described above derived from the 16S rRNA analysis. It is of importance to note that the *amoA* sequence of *Nitrosococcus* sp. strain Nm93 reported in this study is, as expected, almost identical to the *amoA* sequence of *Nitrosococcus mobilis* Nc2 (99.6% nucleic acid similarity) while we amplified a *Nitrosomonas europaea*-like *amoA* sequence from *Nitrosococcus* sp. strain Nm93 in a previous study (28). Thus, this strain was most likely contaminated at that time with *Nitrosomonas europaea*. Furthermore, the *amoA* sequence of *Nitrosococcus oceani* (C-107, identical with ATCC 19707 and NCIMB 11848) differs significantly in the publications of Holmes et al. (22) and Alzerecca et al. (4) caused by a misidentification of *Methylomicrobium pelagicum* as *Nitrosococcus oceani* in the former publication (now corrected by the authors in a recent update of GenBank accession no. U31652). Consequently, gamma-subclass AOB have a lower level of AmoA similarity (<75.5%) to type I methanotrophs than previously considered (22). The separate clustering of gamma-subclass AOB and type I methanotrophs in the AmoA and 16S rRNA trees might reflect their specialization of using either ammonia or methane as preferred substrate. In accordance with this hypothesis, the deduced AmoA sequences of the gamma-subclass AOB do differ in 4 of the 21 signature amino acids of the particulate methane monooxygenase of type I and type II methanotrophs (23). At one (*Nitrosococcus oceani*; *Nitrosococcus* sp. strain C-113) or two (*Nitrosococcus halophilus*) of these signature positions, the gamma-subclass AOB possess amino acids which are absolutely conserved within the ammonia monooxygenases of beta-subclass AOB, which might indicate that these positions are influencing substrate affinity of the respective monooxygenases.

The completed *amoA* database was also used to perform a specificity check of the primers published by Sinigalliano et al. (64) and Holmes et al. (22). Surprisingly, only *Nitrosomonas europaea* possesses fully complementary target sequences to the Sinigalliano primers. Most likely, the *amoA* sequences from *Nitrosococcus oceani* and *Nitrosomonas cryotolerans* that were amplified by Sinigalliano et al. (64) originated from a contamination with *Nitrosomonas europaea* and were thus reported to be identical with the *amoA* sequence of the latter species. The correct *amoA* sequences of *Nitrosococcus oceani* and *Nitrosomonas cryotolerans* were reported by Alzerecca et al. (4) and in this study, respectively. The Holmes primers do target some beta-subclass AOB and gamma-subclass methanotrophs but possess several mismatches with other beta-subclass AOB and all three gamma-subclass AOB in the database (Table 6). Consequently, conclusions on ecological relevance (19, 20) or diversity of AOB using these primers (57) have to be interpreted with caution.

Comparative sequence analysis of 122 *amoA* clones obtained from 11 activated-sludge and biofilm samples demonstrated that generally nitrosomonads are responsible for ammonia oxidation in wastewater treatment plants and that nitrosospiras occur only sporadically in these systems. This result is consistent with PCR-independent AOB community structure analysis performed by fluorescent in situ hybridization FISH (28, 81) but disagrees with findings of Hiorns et al. (21), who could detect nitrosospiras but not nitrosomonads in an activated-sludge plant. The latter finding, however, was most likely caused by the very limited coverage of nitrosomonads by probe Nm75 (Table 5). Furthermore, it should be noted that, considering the extended *amoA* database, the recently developed terminal-restriction fragment length polymorphism (TRFLP) method for identification of major subgroups AOB (24) will not produce meaningful community fingerprint patterns (Fig. 5).

Using the *amoA* approach, with the exception of *Nitrosomonas cryotolerans*, *Nitrosomonas halophila*, and *Nitrosomonas nitrosa*, sequences related to all recognized *Nitrosomonas* species were obtained from wastewater treatment plants (Fig. 4). *amoA* sequences related to *Nitrosococcus mobilis* were detected in six different wastewater treatment plants, including the industrial plant Kraftisried. In a previous study, Juretschko et al. (28) obtained exclusively *Nitrosomonas europaea*-like *amoA* sequences from this plant by using the primers described by Sinigalliano et al. (64) while FISH clearly demonstrated the in situ dominance of *Nitrosococcus mobilis*. This contradiction was caused by the limited sensitivity of the Sinigalliano primers and was able to be resolved in this study. In different plants, several *amoA* sequences (for example clones S12 and SBBR1-32) which showed only relatively moderate sequence similarities to known beta-subclass AOB species were recovered. Application of the *amoA* and AmoA similarity threshold

TABLE 6. Mismatches of the PCR primers A189 (A-*-MOB-189-a-S-18) and A682 (A-*-MOB-682-a-A-18) (3, 22) with the *amoA* genes of beta- and gamma-subclass AOB

Primer	No. of mismatches																												
	gamma AOB			*Nitrosospira* cluster									*Nitrosomonas europaea/ Nitrosococcus mobilis* cluster						*Nitrosomonas cryotolerans* cluster	*Nitrosomonas communis* cluster				*Nitrosomonas oligotropha* cluster			*Nitrosomonas marina* cluster		
	C107	C113	Nc4	NpAV[a]	Np39-19[a]	Nspl	Np22	AHB1	L13	Nv1	Nv12	C128	C71	Nc2	Nm93	Nm104	Nm107	Nm1	Nm50	Nm103	Nm57[b]	Nm41	Nm2	Nm33	Nm90	Nm45	Nm10	Nm51	Nm22	Nm36
A189	0	1	—[c]	0	0	1	—	0	—	—	1	0	0	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—
A682	4	5	2	0/1/2	3	—	—	0	1	3	3	4	1	6	6	6	6	4	1	1	3	4	4	3	3	3	1	2	1	1	2

[a] Contains three *amoA* operons (29), which differ from *Nitrospira* sp. strain NpAV in sequence at the primer A682 target region.
[b] Contains two *amoA* operons which are identical in sequence at the primer target regions.
[c] —, no sequence information are available.

values indicative of novel AOB species (obtained by *amoA* and AmoA 16S rRNA correlation plots) did not support that these sequences represent previously unrecognized nitrosomonads. However, it is important to clarify that while *amoA* and AmoA similarities below the suggested threshold values are strongly indicative of the existence of novel species, an *amoA* and AmoA sequence with a similarity to a described AOB species above the threshold level can originate from either a novel species or the described AOB species. This problem could be solved if the respective threshold values were inferred from correlation plots of *amoA* and AmoA versus DNA-DNA similarity. However, this analysis has to await the availability of more DNA-DNA hybridization data of cultured AOB.

Different wastewater treatment plants obviously differ significantly in regard to species richness of AOB. While some plants are dominated by a single AOB species (e.g., *Nitrosococcus mobilis* in the Kraftisried plant), other plants harbor at least four different AOB species (e.g., Munich I-Großlappen). A high AOB diversity could increase the resistance of nitrification against perturbation while the presence of a AOB monoculture in a plant might render its nitrification more susceptible.

In conclusion, a robust phylogenetic framework of AOB was established by comparative sequence analysis of all described AOB species based on the 16S rRNA and the *amoA* marker molecule. Reevaluation of the specificity of published primers and probes developed for the detection of both biopolymers in environmental samples demonstrated, in many cases, insufficient specificity. High-resolution assignment of all published environmentally retrieved 16S rRNA sequences only provided evidence for the existence of two yet undescribed beta-subclass AOB species, suggesting that available AOB isolates might be more representative of the natural diversity within this physiological group than previously thought. A similar picture emerged from an *amoA*-based diversity survey of AOB in wastewater treatment plants, which demonstrated that most retrieved molecular isolates were closely related to known nitrosomonads. While almost every *amoA* or 16S rRNA AOB gene library from environmental samples contains many sequences which are not identical to those of cultured AOB, the degree of divergence is, for most of the sequences obtained up to now, insufficient to unequivocally prove the existence of novel AOB species.

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ANHANG II

16S rRNA and *amoA*-based Phylogeny of 12 Novel Betaproteobacterial Ammonia Oxidizing Isolates: Extension of the Data Set and Proposal of a New Cluster within the Nitrosomonads

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Short title: Phylogeny of ammonia-oxidizers

SUMMARY

The phylogenetic relationship of 12 ammonia oxidizing isolates (eight nitrosospiras and four nitrosomonads), for which no gene sequence information was available, was investigated based on their genes encoding for 16S rRNA and the active site subunit of the ammonia monooxygenase (AmoA). Almost full length 16S rRNA gene sequences were determined for the 12 isolates. In addition, previously published 16S rRNA gene sequences of 15 ammonia oxidizing bacteria (AOB) were completed to allow for a more reliable phylogeny inference of members of this guild. Moreover, sequences of 453 bp-fragments of the *amoA*-gene were determined from 15 AOB including the 12 isolates, and completed for 10 additional AOB. 16S rRNA gene and *amoA*- based analyses, including all available sequences of AOB pure cultures, were performed to determine the position of the newly retrieved sequences within the established phylogenetic framework. The resulting 16S rRNA gene and *amoA* tree topologies were similar but not identical and demonstrated a superior resolution of 16S rRNA- versus *amoA*-analysis. While 11 of the 12 isolates could be assigned to different recognized phylogenetic groups within the betaproteobacterial AOB, the estuarine isolate *Nitrosomonas* sp. Nm143 formed a separate sublineage together with three other marine isolates which 16S rRNA sequences have not been published but deposited in public data bases. In addition, 17 previously not assignable environmentally retrieved 16S rRNA gene sequences, all exclusively originating from marine or estuarine sites, clearly belong to this sublineage.

INTRODUCTION

Chemolithoautotrophic ammonia-oxidizing bacteria (AOB) are capable of gaining energy via conversion of ammonia to nitrite and are thus of considerable importance in the global nitrogen cycle. Almost all aerobic environments where organic matter is mineralized are possible habitats for AOB (Bock & Wagner, 2001). They have been detected in a variety of soil, marine, estuarine, and freshwater systems and are crucial for the removal of nitrogen compounds in wastewater treatment plants (Painter, 1986) thus contributing to the impairment of anthropogenic damage to the environment. On the other hand, AOB activity causes deterioration of natural building stones (Bock & Sand, 1993) and enhances nitrogen fertilizer loss from arable soil (MacDonald, 1986). Due to their importance in natural and engineered systems, significant research efforts have been made to characterize the diversity, distribution patterns and ecophysiology of AOB (for reviews see Koops & Pommerening-Röser, 2001; Kowalchuk & Stephen, 2001; Wagner *et al.*, submitted).

The first isolation of AOB has been reported in 1890 (Frankland & Frankland, 1890; Winogradsky, 1890) and since then a considerable number of AOB isolates were obtained from various environments leading to the description of 16 AOB species (Wagner *et al.*, submitted). Comparative 16S rRNA gene sequence analyses of these species showed that *Nitrosococcus halophilus* and *Nitrosococcus oceani* belong to the class *Gammaproteobacteria* while the remaining 14 species form a monophyletic lineage within the class *Betaproteobacteria* (Head *et al.*, 1993; Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stehr *et al.*, 1995a; Teske *et al.*, 1994; Woese *et al.*, 1985; Woese *et al.*, 1984). Betaproteobacterial AOB encompass the genera *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*; Head *et al.*, 1993). Cultured nitrosomonads can further be subdivided into five phylogenetically well-defined sublineages (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996). A similar subdivision system has also been suggested for nitrosospiras and was used to assign cultured nitrosospiras into four “clusters” (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996). However, due to the close phylogenetic relationship of all known nitrosospiras with each other, their subdivision is not well supported by phylogeny inference methods (Purkhold et al., 2000; Wagner *et al.*, submitted). The current perception of AOB phylogeny established by comparative 16S rRNA sequence analysis could independently be confirmed by exploiting the gene *amoA* encoding for the active site subunit of the enzyme ammonia monooxygenase (AMO) as alternative phylogenetic marker (Klotz & Norton, 1995; McTavish *et al.*, 1993; Purkhold *et al.*, 2000; Rotthauwe *et al.*, 1995). Generally, 16S rRNA and *amoA* based trees possess congruent topologies although the fragment of the latter gene which is usually used for phylogeny inference provides less resolution (Wagner *et al.*, submitted).

Cultivation dependent analysis of environmental AOB diversity are time consuming and tedious due to the slow growth rates of these microorganisms. Furthermore, the currently applied enrichment and isolation strategies might fail to recover the entire diversity of this

guild. Triggered by these limitations, the last decade saw an enormous increase of molecular, cultivation-independent diversity surveys of AOB. 16S rRNA gene sequences directly retrieved from environmental samples revealed that, with the exception of two sublineages within the nitrosomonads (Stephen *et al.*, 1996; de Bie *et al.*, 2001) and one "cluster" within the nitrosospiras (Stephen *et al.*, 1996), most environmentally retrieved sequences are closely related to cultured AOB (for a recent review see Purkhold *et al*., 2000). Similar findings were obtained by phylogenetically analyzing environmentally retrieved *amoA* gene fragments (e. g. Casciotti & Ward, 2001; Hommes *et al.*, 1998; Klotz & Norton, 1995; McTavish *et al.*, 1993; Purkhold *et al.*, 2000; Rotthauwe *et al.*, 1995; Yamagata *et al.*, 1999).

In the present study we extended the current 16S rRNA and *amoA*- gene databases of AOB by (i) determining the respective sequences of 12 novel AOB isolates (ii) and improving the length and/or quality of several previously published sequences of other AOB. Based on these data a thorough phylogenetic analysis of betaproteobacterial AOB was performed to obtain a phylogenetic framework which is required for design and specificity evaluation of PCR primers and probes and which allows the assignment of environmentally retrieved sequences. Based on the obtained findings we propose a new sublineage within the nitrosomonads which also encompasses many previously not assignable 16S rRNA gene clones from marine systems.

METHODS

Pure cultures of AOB. Table 1 summarizes the strains investigated in this study. AOB were cultured using the media and conditions described previously (Koops *et al.*, 1991).

DNA-DNA hybridization. DNA similarities were estimated by photometric determination of thermal renaturation rates (DeLey *et al.*, 1970) as described by Koops & Harms (1985).

DNA extraction for PCR. AOB were harvested from 10 l of exponentially growing cultures by continuos flow centrifugation (20,000 g, 400 ml min^{-1}). Total genomic DNA was extracted according to the following protocol: A 0.25 g-pellet (wet weight) of each sample was resuspended in a 2 ml polypropylen tube containing glass beads [Fast DNA Spin Kit for soil (BIO101, Vista, Ca.)] with 500 µl AE buffer (20 mM sodiumacetate, 1mM EDTA, pH 5,5 adjusted with acetic acid), 50 µl 25% sodiumdodecylsulfate and 600 µl phenol-chloroform-isoamyl alcohol (25:24:1). Cells were lysed in a BeadBeater (BIO101, Vista, Ca.; 2x 15 sec at speed setting 4,5) and the mixture subsequently was centrifuged (10 min, 10,000 g, at 4°C). The aqueous phase was carefully transferred to a fresh tube, mixed with 600 µl of chloroform-isoamyl alcohol (24:1), and centrifuged for another 10 min at 10,000 g. The aqueous phase was transferred to a fresh tube, and after addition of 0.1 vol 3 M sodiumacetate nucleic acids were precipitated by incubation with 0.6 volume of isopropanol and 5 µl glycogen (5mg/ml, Ambion Inc., Austin, Tex.) for 1 h at –20°C and subsequently pelleted by centrifugation (20 min, 10,000 g, 4°C). Pellets were washed with 1 ml of ice cold 70% ethanol, dried, and finally resuspended in 30-50 µl elution buffer (10 mM Tris/HCl [pH 8.5]).

PCR amplification of the 16S rDNA. Amplification of 16S rRNA genes was performed as specified by Juretschko *et al.* (Juretschko *et al.*, 1998) and Purkhold *et al.* (Purkhold *et al.*, 2000) using the primers 616F/630R.

PCR amplification of the *amoA* gene fragment. A 453 bp fragment (without primers) of the *amoA* gene was amplified from 100 ng DNA using the optimized (Stephen *et al.*, 1999) primers *amoA*-1F and *amoA*-2R (Rotthauwe *et al.*, 1995) for PCR with a Primus cycler (MWG Biotech, Germany). Reaction mixtures containing 50 pM of each primer were prepared in a total volume of 50 µl by using 20 mM $MgCl_2$ reaction buffer and 1.5 U of *Taq* polymerase (Promega, Madison, Wis.). Thermal cycling was carried out by an initial denaturation step at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, and elongation at 72°C for 40 s. Cycling was completed by a final elongation step at 72°C for 5 min.

Cloning, sequencing, and phylogeny inference. The amplified 16S rRNA- and *amoA* gene fragments were cloned according to the manufacturer's instructions into pCR2.1 TOPO TA vectors (Invitrogen Corp., San Diego, Calif.). After plasmid purification (Qiagen, Hilden, Germany), sequences were determined using a Thermo Sequenase Cycle sequencing kit (Amersham, Little Chalfont, Buckinghamshire, UK), infrared-labeled (IRD 800) primers and an automated DNA sequencer (Li-Cor, Inc., Lincoln, Nebr.). 16S rRNA and *amoA* gene sequences were added to the respective database with the ARB program package (http://www.arb-home.de). The sequences were thoroughly aligned using the implemented tools and subsequently corrected by visual inspection. Phylogenetic analyses were performed based on nucleic acid (16S rRNA, *amoA*) and amino acid (AmoA) sequences applying distance-matrix (Phylip and FITCH), maximum-parsimony, and maximum likelihood methods using the respective tools in the program package. Since the betaproteobacterial AOB encompass a closely related group of microorganisms no conservation filters were applied and all sequence positions were considered in the calculations. For a more detailed description of the phylogeny inference methods applied see Purkhold *et al.* (2000).

Nucleotide sequence accession numbers. The sequences determined in this study are available in GenBank under accession numbers AY123787-AY123813 (16S rRNA gene sequences) and AY123815-AY123840 (*amoA*- and AmoA sequences).

RESULTS AND DISCUSSION

AOB phylogeny inferred from 16S rRNA

In order to establish an encompassing high-quality 16S rRNA gene database for AOB we re-sequenced the respective genes of several AOB isolates for which only incomplete sequences were available. In detail, 16S rRNA gene sequences (1496-1498 nucleotides) were completed for *Nitrosomonas* sp. Nm58, *Nitrosomonas* sp. Nm84 and *Nitrosomonas* sp. Nm86 which represent isolates from river Elbe (Stehr *et al*., 1995a). For these strains, only very short 16S

rRNA gene sequences (186-281 nucleotides) have been published previously (Stehr *et al.*, 1995a). In addition, ambiguities and errors in the 16S rRNA gene sequences of *Nitrosospira* sp. Nsp1, *Nitrosospira* sp. Nsp2, *Nitrosospira briensis* Nsp10, *Nitrosospira* sp. Nsp12, *Nitrosospira* sp. Nsp17, *Nitrosospira* sp. Nv6 (all Aakra *et al.*, 2001b), *Nitrosomonas eutropha* Nm57 (Aakra *et al.*, 2001b; Head *et al.*, 1993), *Nitrosospira* sp. Ka3 (Aakra *et al.*, 1999b), *Nitrosovibrio tenuis* Nv1 (Head *et al.*, 1993), *Nitrosolobus multiformis* Nl13 (Teske *et al.*, 1994), *Nitrosospira* sp. III7 (Utaaker & Nes, 1998), and *Nitrosospira* sp. L115 (Utaaker *et al.*, 1995) were corrected and the sequences were extended by 23-290 bp to almost full length (1497-1498 nucleotides).

Furthermore, we determined almost full-length 16S rRNA gene sequences (1494-1501 nucleotides) for the following 12 AOB isolates which were not characterized at this level previously: *Nitrosomonas* sp. Nm47, *Nitrosomonas* sp. Nm59, *Nitrosomonas* sp. Nm143, *Nitrosomonas* sp. Nm148, *Nitrosospira* sp. Nsp5, *Nitrosospira* sp. Nsp40, *Nitrosospira* sp. Nsp41, *Nitrosospira* sp. Nsp57, *Nitrosospira* sp. Nsp58, *Nitrosospira* sp. Nsp62, *Nitrosospira* sp. Nsp65, and *Nitrosospira* sp. NL5.

As expected, all 16S rRNA gene sequences determined showed highest similarities (96.7-100%) to sequences of AOB belonging to the class *Betaproteobacteria*. (for details see supplementary online material available at http://www.microbial-ecology.de). Phylogenetic inference based on 16S rRNA gene sequences of AOB included distance, parsimony and maximum likelihood methods and only considered sequences more than 1000 nucleotides in length. All analyzed AOB formed a monophyletic group within the *Betaproteobacteria.* Within this group, *Nitrosomonas cryotolerans* forms an independent lineage. In addition, five stable subgroupings (*Nitrosomonas oligotropha* sublineage, *Nitrosomonas marina* sublineage, *Nitrosomonas europaea*/*Nitrosococcus mobilis* sublineage, *Nitrosomonas communis* sublineage, *Nitrosospira* line of descent; Stephen *et al.*, 1996; Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000), as well as a previously not recognized sublineage (including *Nitrosomonas* sp. Nm143) were recovered. All groupings are highly supported by parsimony bootstrap analysis (above 90%) and were found independent from the treeing method applied (Fig. 1). In the following, the phylogenetic affiliation of the 12 newly sequenced AOB isolates is summarized.

Phylogenetic relationship of the newly analyzed nitrosomonads. *Nitrosomonas* isolates Nm47, Nm59, Nm84 and Nm86 are most closely related to organisms within the *N. oligotropha* sublineage (97.0-97.5%). As many other members of this group, they have been isolated either from freshwater or wastewater habitats and are characterized by remarkable low affinity constants for ammonia (Koops & Pommerening-Röser, 2001; Pommerening-Röser *et al.*, 1996; Stehr *et al.*, 1995a; Suwa *et al.*, 1994). Moreover, *Nitrosomonas* sp. Nm84 has been shown to produce significant amounts of exopolymeric substances, especially under ammonia-limited conditions (Stehr *et al.*, 1995b). This property has also been observed for other members of this sublineage (Koops, unpublished results).

Nitrosomonas sp. Nm58 and *Nitrosomonas* sp. Nm148 can both be unambiguously assigned to the *N. communis* sublineage (maximum sequence similarities 99.6% and 98.3 %, respectively; Fig 1). *Nitrosomonas* sp. Nm148 has been isolated from a hot spring, and is a strain of the species *Nitrosomonas nitrosa* (82% DNA-DNA homology) which has been obtained from activated sludge of a wastewater treatment plant connected to chemical processing facilities (Koops *et al.*, 1991). The 16S rRNA gene sequence of *Nitrosomonas* sp. Nm58 is almost identical to the sequence of the soil isolate *Nitrosomonas* sp. Nm41 (99.6%). The close relationship between both isolates is also reflected by their high DNA-DNA homology value (71%). In contrast to the *N. oligotropha* sublineage, the *N. communis* sublineage exhibits a high heterogeneity regarding the ecophysiological traits of its members (Koops & Pommerening-Röser, 2001; Pommerening-Röser *et al.*, 1996).

The only 16S rRNA gene sequence obtained in this study that was not directly related to a published sequence from an AOB isolate was extracted from the estuarine isolate *Nitrosomonas* sp. Nm143. Considering only described species, the 16S rRNA gene of this organism shows highest sequence similarity to *Nitrosomonas cryotolerans* (96.7%). Together with the marine strains C-17, TT140-098-2, and TT140-89A isolated from sediment samples at the Galapagos islands and Washington coast samples (Acc. Nos AF338202, AF338209, AF338208; Ward, 1982; Ward & Carlucci, 1985; maximum sequence similarity 99.0 %) *Nitrosomonas* sp. Nm143 forms a novel sublineage within the betaproteobacterial AOB. This sublineage is recovered with all treeing methods and is highly supported by bootstrapping. It does not only comprise sequences of isolated strains but also harbors 17 16S rRNA gene sequences directly retrieved from different marine habitats (Acc. Nos U09545-U09547, Z69090, AJ132050, AJ132056, AY114346, AY114347, AF489686-AF489689, Z69127, Z69134, Z69136, Z69141, Z69143; de Bie *et al.*, 2001; McCaig *et al.*, 1994; Nicolaisen & Ramsing, 2002; Stephen *et al.*, 1996; Freitag, T. & Prosser, J. I., unpublished). In accordance with the current classification schemes (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996) we propose to designate the new sublineage as *Nitrosomonas* sp. Nm143 sublineage. All isolates and sequences within this group originate from a total of eight distinct estuarine or marine habitats. Within these environments members of the *Nitrosomonas* sp. Nm143 sublineage seem to be widely distributed since the sampling sites range from coastal surface water (McCaig *et al.*, 1994) to polluted (Stephen *et al.*, 1996) or even anoxic sediments (Freitag, T. & Prosser, J. I., unpublished). Nitrogen load and oxygen concentration at the various sampling sites differ significantly [polluted and non-polluted fish farm sediments (Stephen *et al.*, 1996), an eutrophicated estuary (Nicolaisen & Ramsing, 2002), and estuarine sampling sites with ammonium concentrations below 15mM (de Bie *et al.*, 2001) as well as anoxic sediments (Freitag, T. & Prosser, J. I., unpublished) and estuarine sites with O_2 saturation levels around 40% (de Bie *et al.*, 2001)]. A common feature among the investigated sites are salinity values above 10 ppt. Interestingly however, members of this sublineage were not yet detected in the open sea (e.g. Bano & Hollibaugh, 2000 ; Phillips *et al.*, 1999, Hollibaugh *et al.*, 2002).

Phylogenetic relationship among the newly analyzed nitrosospiras. As expected, *Nitrosospira* isolates NL5, Nsp5, Nsp40, Nsp41, Nsp57, Nsp58, Nsp62, and Nsp65 show the highest 16S rRNA gene similarities to sequences within the *Nitrosospira* line of descent (98.6-100%). *Nitrosospira* isolates Nsp57 and Nsp58, which according to DNA-DNA hybridization data are members of the same species (63% homology; please note that AOB strains having more than 60% DNA-DNA homology are considered as members of the same species; Wagner *et al.*, submitted) group together but can neither be assigned to one of the "clusters" within the cultured nitrosospiras nor to the *Nitrosospira* "cluster 1" which entirely exists of environmentally retrieved sequences (Stephen *et al.*, 1996). Similarly *Nitrosospira* sp. Nsp65 forms an independent branch in the *Nitrosospira* line of descent and currently represents the deepest branch within this evolutionary lineage. In contrast, isolate Nsp5 groups with "cluster 0" and isolates Nsp 62, Nsp 41, Nsp 40, and NL5 are most closely related to members of the *Nitrosospira* "cluster 3" which contains the three described species of this genus (Fig. 2). Although three of the twelve *Nitrosospira*-related isolates investigated can not be assigned to previously suggested subdivisions of this line of descent (Purkhold *et al.*, 2000; Stephen *et al.*, 1996) we refrain to propose two novel "clusters" for these AOB because it has been noted that subdivision of nitrosospiras is not well supported by bootstrap analysis (Fig. 2; Purkhold *et al.*, 2000). The failure to recover stable "clusters" within the nitrosospiras reflects that 16S rRNA sequence similarities within the entire *Nitrosospira* line of descent are higher (>96.1%) than those found within each of the described *Nitrosomonas* sublineages. Furthermore, according to DNA-DNA hybridization data of available strains, *Nitrosospira* "clusters" 0 and 2 each currently encompass only strains from a single species. Within "cluster 0" *Nitrosospira* sp. III2, *Nitrosospira* sp. 40KI, *Nitrosospira* sp. Nsp12, and *Nitrosospira* sp. Nsp5 possess DNA-DNA homology values with each other above 67%. Within "cluster 2" *Nitrosospira* sp. III7 and *Nitrosospira* sp. B6 share 76% DNA-DNA homology. These values indicate that for both groupings of strains the proposal of additional taxonomic units ("cluster 0" and "cluster 2") is not justified at that time.

AOB phylogeny inferred from *amoA*

During the last years the gene encoding for the active site subunit of the ammonia monooxygenase (*amoA*) has increasingly been exploited as marker molecule for AOB diversity research in natural and engineered systems (e.g. Baribeau *et al.*, 2000; Gieseke *et al.*, 2001; Horz *et al.*, 2000; Rotthauwe *et al.*, 1997). Initially, previously published *amoA* gene fragment sequences of *Nitrosospira* sp. Nsp1, *Nitrosospira* sp. Nsp2, *Nitrosospira briensis* Nsp10, *Nitrosospira* sp. Nsp12, *Nitrosospira* sp. Nsp17, *Nitrosospira* sp. Nv6, *Nitrosospira* sp. Ka3, *Nitrosovibrio tenuis* Nv1, *Nitrosospira* sp. III7 and *Nitrosospira* sp. L115 (Aakra *et al.*, 2001a) were extended by 39 bp each to 453 bp which represent the complete fragment obtained after PCR amplification using the modified (Stephen *et al.*, 1999) primer set of Rotthauwe *et al.* (Rotthauwe *et al.*, 1995). Additionally, 453bp long *amoA* sequences were determined for the twelve novel AOB isolates *Nitrosomonas* sp. Nm47, *Nitrosomonas* sp.

Nm59, *Nitrosomonas* sp. Nm143, *Nitrosomonas* sp. Nm148, *Nitrosospira* sp. Nsp5, *Nitrosospira* sp. Nsp40, *Nitrosospira* sp. Nsp41, *Nitrosospira* sp. Nsp57, *Nitrosospira* sp. Nsp58, *Nitrosospira* sp. Nsp62, *Nitrosospira* sp. NL5 and *Nitrosospira* sp. Nsp65. In addition the *amoA* gene fragment sequences of the river Elbe isolates *Nitrosomonas* sp. Nm58, *Nitrosomonas* sp. Nm84 and *Nitrosomonas* sp. Nm86 were determined.

All determined *amoA*/AmoA sequences showed the highest similarity (83.2-99.3% and 90.7-100%, respectively) to sequences of AOB belonging to the class *Betaproteobacteria* (for details see supplementary online material available at http://www.microbial-ecology.de). Phylogenetic trees for *amoA*/AmoA were calculated from the nucleotide and amino acid data sets by distance, parsimony, and maximum likelihood methods. In general, topologies of *amoA*/AmoA- and 16S rRNA based trees were highly similar (Figs. 1 and 3). The monophyly of the *Nitrosospira* line of descent, the *N. marina* sublineage, and the *N. europaea*/*Nc. mobilis* sublineage was recovered by all methods, although the bootstrap support for these sublineages was considerably lower than found for 16S rRNA gene trees. In contrast to 16S rRNA trees, the *N. oligotropha* sublineage and *N. communis* sublineage are not always retrieved in *amoA*/AmoA trees as monophyletic assemblages (for details see Purkhold *et al.*, 2000).

For all AOB strains for which the *amoA*/AmoA sequence was newly determined in this study, consistent affiliations were found in the 16S rRNA gene- and *amoA*/AmoA-based trees (Figs. 1 and 3). However, the *amoA*/AmoA sequences of the analyzed nitrosospiras do not provide detailed information because *amoA*/AmoA analyses offer insufficient resolution for any meaningful subdivision of the *Nitrosospira* line of descent. As expected, the *amoA*/AmoA sequence of *Nitrosomonas* sp. Nm143, the organism representing the novel sublineage in the 16S rRNA AOB tree, is not closely related to *amoA*/AmoA sequences of cultured AOB in the database. However, some but not all treeing methods suggest a weak affiliation of the *amoA*/AmoA sequence of *Nitrosomonas* sp. Nm143 with members of the *N. marina* and/or *N. oligotropha* sublineage.

With the extending data set and an increasing number of closely related *amoA*/AmoA-sequences the limitation of the *amoA*/AmoA approach as applied now becomes more apparent. Although using the *amoA* approach AOB pure cultures or AOB in environmental samples can rapidly be asssigned to some phylogenetic subgroups within this guild (see above), the *amoA*/AmoA fragment analyzed does provide less resolution compared to the 16S rRNA, since it is relatively short (453 nucleotide and 151 amino acid positions, respectively) and highly conserved (224/93positions have an identical nucleic/amino acid in at least 98% of the betaproteobacterial AOB). This limitation might be solved in future studies by application of primers allowing the amplification of a longer *amoA*-fragment (Norton *et al.*, 2002).

Inconsistencies between determined sequences and published database entries

To improve the respective databases we re-sequenced in this study several 16S rRNA and *amoA* sequence of defined isolates. Comparison of the newly determined sequences with those sequences previously published by others revealed several inconsistencies which could not be explained by simple sequencing errors.

Firstly, differences in the *amoA*/AmoA sequences (97.6 and 99.2%, respectively) of *Nitrosospira* sp. III7 determined in this study and those published by Aakra *et al.* (2001) were detected. However, both sequences show the same phylogeny and therefore probably represent two different gene copies. The existence of more than one *amoA* gene copy is widely found among betaproteobacterial AOB, and up to four copies were reported to occur within the genomes of some nitrosospiras (Bock & Wagner, 2002).

Secondly, we came across published sequences which were obviously retrieved from a contaminant and have not been extracted from the indicated AOB. The 16S rRNA gene sequence of "*Nitrosospira* sp. Nv1" published by Aakra *et al.* (Aakra *et al.*, 2001b) significantly differs (sequence similarity 98.6%) from the sequences determined in this study (Acc. No. AY123803) and by Head *et al.* (Head *et al.*, 1993; Acc. No. M96404), which are almost identical (99.9%) with each other. It seems likely that the latter two sequences are correct also because the close association of "*Nitrosospira* sp. Nv1" with *Nitrosospira* sp. Nv12 (Head *et al.*, 1993; Acc. No. M96405; 99.8 %) is not supported by DNA-DNA hybridization data which demonstrate the two organisms to represent different species (Pommerening-Röser, Ph.D. thesis). Furthermore, the *amoA*-fragment of "*Nitrosospira* sp. L115" (Aakra *et al.*, 2001a) shows significant sequence differences to the respective sequence determined in this study (nucleic acid: 88.9%, amino acid: 94.6%). We claim our sequence to be correct since (in contrast to the sequence of Aakra and co-workers) the results of its phylogenetic analysis are in accordance with the respective 16S rRNA phylogeny (Fig. 2).

Conclusion

This study significantly extended the current 16S rRNA and *amoA* databases for AOB. For several AOB isolates previously published sequences of both marker molecules were improved in quality and length. Furthermore, gene sequences of both macromolecules were determined for 12 novel AOB isolates. Based on these data a thorough phylogenetic analysis was performed which led to the description of a new 16S rRNA gene sublineage within the nitrosomonads. This sublineage also contains 17 previously unassignable environmental clones demonstrating that at least one of the new AOB lineages discovered during the last years by molecular diversity surveys harbors AOB species which are culturable by traditional techniques.

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TABLE AND FIGURE LEGENDS

Table 1. Pure cultures of AOB used in this study.

Fig. 1. 16S rRNA based phylogenetic tree of the nitrosomonads. The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor joining trees were calculated and merged. Multifurcations connect branches for which a relative order can not be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. For each cluster the minimum 16S rRNA sequence similarity between two of its members is depicted. Sequences included in the analysis were published by Aakra *et al.* (1999a, 1999b), Head *et al.* (1993), Juretschko *et al.* (1998), Purkhold *et al.* (2000), Sorokin *et al.* (1998), Suwa *et al.* (1997), and Yamagata *et al.* (1999). Sequences of strains CNS326, G1, K1, IWT202, TK94, WH-2, Koll-21, DYS317, DYS323, IWT514, TNO632, marine bacteria C-45, NH4W, 122, URW, NO3W, C-113, TT140-098-2, TT140-98A, and estuarine bacteria TA 921-i-NH4, B19-i-NH4, C-17 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

Fig. 2. 16S rRNA based phylogenetic tree of the highly related genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* forming the *Nitrosospira* line of descent (Head *et al.*, 1996). The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Species whose sequences have been determined in this study are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor joining trees were calculated and merged. Multifurcations connect branches for which a relative order can not be unambiguously determined by applying different treeing methods. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively.

Sequences included in the analysis were published by Aakra *et al.* (2001, 1999a, 1999b), Head *et al.* (1993), Teske *et al.* (1994), Tokuyama *et al.* (1997), and Utaaker *et al.* (1995). Sequences of strains GS833, E12, NRS527, NpAV, RY6A, RY3C, TCH716, and PJA1 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

Fig. 3. AmoA based phylogenetic tree of the betaproteobacterial AOB. Species whose sequences have been determined in this study are depicted in bold. The 453 bp gene fragment obtainable with the most commonly used *amoA* PCR primers (Rotthauwe *et al.*, 1995) was used for phylogeny inference. AmoA sequences shorter than 414 nucleotides were excluded from the analysis. Protein maximum likelihood, protein maximum parsimony, neighbor joining, and FITCH trees were calculated and merged. Multifurcations connect branches for which a relative order can not be unambiguously determined by applying different treeing methods. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Sequences included in the analysis were published by Aakra *et al.* (2001), Casciotti *et al.* (2001), Holmes *et al.* (1995), McTavish *et al.* (1993), Norton *et al.* (2002), Purkhold *et al.* (2000), Rotthauwe *et al.* (1995), Sorokin *et al.* (2001), Suwa *et al.* (1997), and Yamagata *et al.* (1999). Sequences of *Nitrosospira* sp. C-57 and *Nitrosomonas* sp. TK794 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

Table 1.

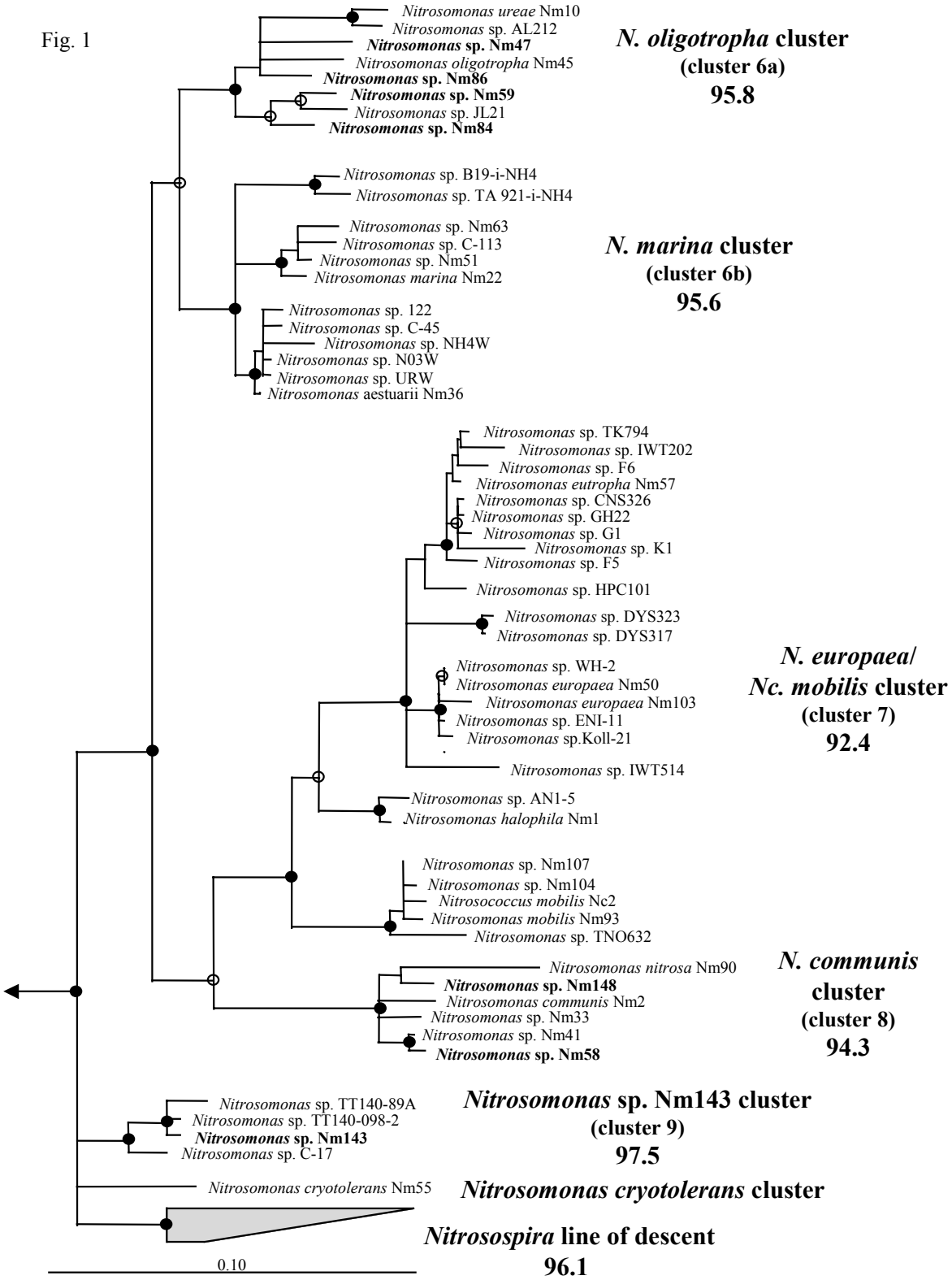
Organism	Reference	Origin
Nitrosomonas sp. Nm47	H.-P. Koops (unpublished)	Waste water treatment plant, Germany
Nitrosomonas eutropha Nm57^T	Koops & Harms (1985)	Waste water treatment plant, USA
Nitrosomonas sp. Nm58	Stehr *et al.* (1995)	Sediment, River Elbe, Germany
Nitrosomonas sp. Nm59	S. Sowitzki (unpublished)	Waste water treatment plant, Germany
Nitrosomonas sp. Nm84	Stehr *et al.* (1995)	Suspended particulate matter, River Elbe, Germany
Nitrosomonas sp. Nm86	Stehr *et al.* (1995)	River Elbe, Germany
Nitrosomonas sp. Nm143	H.-P. Koops (unpublished)	marine estuary, Dominican Republic
Nitrosomonas sp. Nm148	H.-P. Koops (unpublished)	Hot spring, Santorin, Greece
Nitrosospira sp. Nsp1	Koops & Harms (1985)	Soil, Sardinia, Italy
Nitrosospira sp. Nsp2	Koops & Harms (1985)	Soil, Germany
Nitrosospira sp. Nsp5	Koops & Harms (1985)	Freshwater cave lake, Sardinia, Italy
Nitrosospira briensis Nsp10^T	Koops & Harms (1985)	Soil, Crete

Nitrosospira sp. Nsp12	Koops & Harms (1985)	Soil, Germany
Nitrosospira sp. Nsp17	Koops & Harms (1985)	Soil, Iceland
Nitrosospira sp. Nsp40	H.-P. Koops (unpublished)	Soil, Germany
Nitrosospira sp. Nsp41	H.-P. Koops (unpublished)	Soil, Malta
Nitrosospira sp. Nsp57	H.-P. Koops (unpublished)	Masonry, Germany
Nitrosospira sp. Nsp58	H.-P. Koops (unpublished)	Masonry, Germany
Nitrosospira sp. Nsp62	H.-P. Koops (unpublished)	Masonry, Germany
Nitrosospira sp. Nsp65	H.-P. Koops (unpublished)	Masonry, Germany
Nitrosospira tenuis Nv1^T	Koops & Harms (1985)	Soil, Hawaii
Nitrosospira sp. Nv6	Koops & Harms (1985)	Soil, New Guinea
Nitrosospira sp. NL5	Koops & Harms (1985)	Waste water treatment plant, Saudi Arabia
Nitrosospira multiformis. NL13^T	Koops & Harms (1985)	Soil, India
Nitrosospira sp. L115	Utaaker *et al.* (1995)	Peat bog, Finland
Nitrosospira sp. III7	Utaaker & Nes (1998)	Spruce forest, Norway
Nitrosospira sp. Ka3	Aakra *et al.* (1999)	Soil, Norway

^{T}: type strain

AOB were obtained from the culture collection of the Institut für Allgemeine Botanik der Universitaet Hamburg, Mikrobiologische Abteilung, Germany.

Fig. 1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(cluster 6a)
95.8
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Nitrosomonas sp. TA 921-i-NH4
Nitrosomonas sp. Nm63
Nitrosomonas sp. C-113
Nitrosomonas sp. Nm51
Nitrosomonas marina Nm22
N. marina cluster
(cluster 6b)
95.6
Nitrosomonas sp. 122
Nitrosomonas sp. C-45
Nitrosomonas sp. NH4W
Nitrosomonas sp. N03W
Nitrosomonas sp. URW
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Nitrosomonas sp. TK794
Nitrosomonas sp. IWT202
Nitrosomonas sp. F6
Nitrosomonas eutropha Nm57
Nitrosomonas sp. CNS326
Nitrosomonas sp. GH22
Nitrosomonas sp. G1
Nitrosomonas sp. K1
Nitrosomonas sp. F5
Nitrosomonas sp. HPC101
Nitrosomonas sp. DYS323
Nitrosomonas sp. DYS317
Nitrosomonas sp. WH-2
Nitrosomonas europaea Nm50
Nitrosomonas europaea Nm103
Nitrosomonas sp. ENI-11
Nitrosomonas sp.Koll-21
Nitrosomonas sp. IWT514
N. europaea/
Nc. mobilis cluster
(cluster 7)
92.4
Nitrosomonas sp. AN1-5
Nitrosomonas halophila Nm1
Nitrosomonas sp. Nm107
Nitrosomonas sp. Nm104
Nitrosococcus mobilis Nc2
Nitrosomonas mobilis Nm93
Nitrosomonas sp. TNO632
Nitrosomonas nitrosa Nm90
Nitrosomonas sp. Nm148
Nitrosomonas communis Nm2
Nitrosomonas sp. Nm33
Nitrosomonas sp. Nm41
Nitrosomonas sp. Nm58
N. communis
cluster
(cluster 8)
94.3
Nitrosomonas sp. TT140-89A
Nitrosomonas sp. TT140-098-2
Nitrosomonas sp. Nm143
Nitrosomonas sp. C-17
Nitrosomonas sp. Nm143 cluster
(cluster 9)
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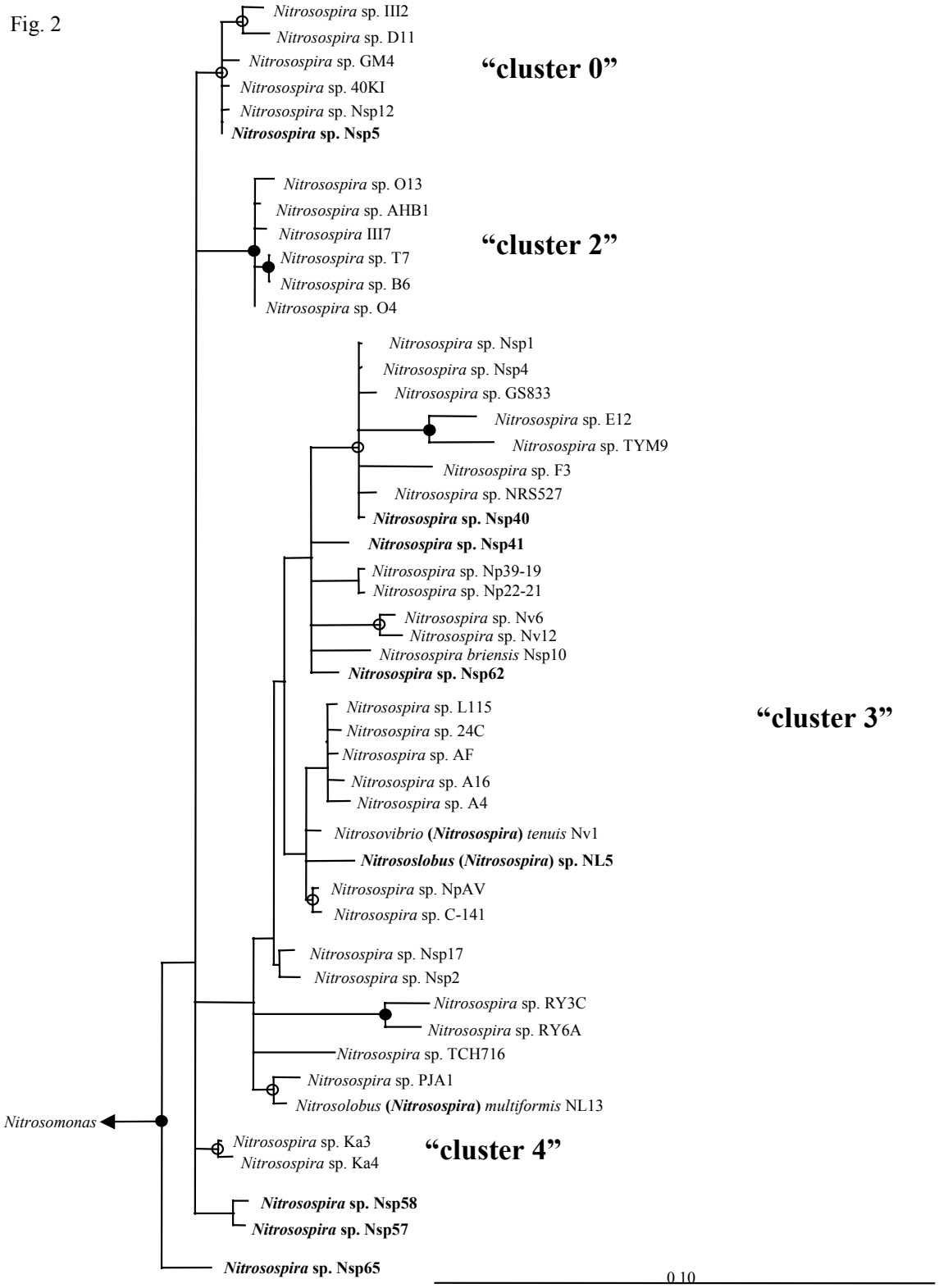
Nitrosospira line of descent
96.1
0.10

Fig. 2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Nitrosospira sp. AHB1
Nitrosospira III7
Nitrosospira sp. T7
Nitrosospira sp. B6
Nitrosospira sp. O4
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Nitrosospira sp. Nsp4
Nitrosospira sp. GS833
Nitrosospira sp. E12
Nitrosospira sp. TYM9
Nitrosospira sp. F3
Nitrosospira sp. NRS527
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Nitrosospira sp. Np22-21
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Nitrosospira sp. Nv12
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Nitrosospira sp. Nsp62
Nitrosospira sp. L115
Nitrosospira sp. 24C
Nitrosospira sp. AF
Nitrosospira sp. A16
Nitrosospira sp. A4
Nitrosovibrio (Nitrosospira) tenuis Nv1
Nitrososlobus (Nitrosospira) sp. NL5
Nitrosospira sp. NpAV
Nitrosospira sp. C-141
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Nitrosospira sp. Nsp2
Nitrosospira sp. RY3C
Nitrosospira sp. RY6A
Nitrosospira sp. TCH716
Nitrosospira sp. PJA1
Nitrosolobus (Nitrosospira) multiformis NL13
Nitrosomonas
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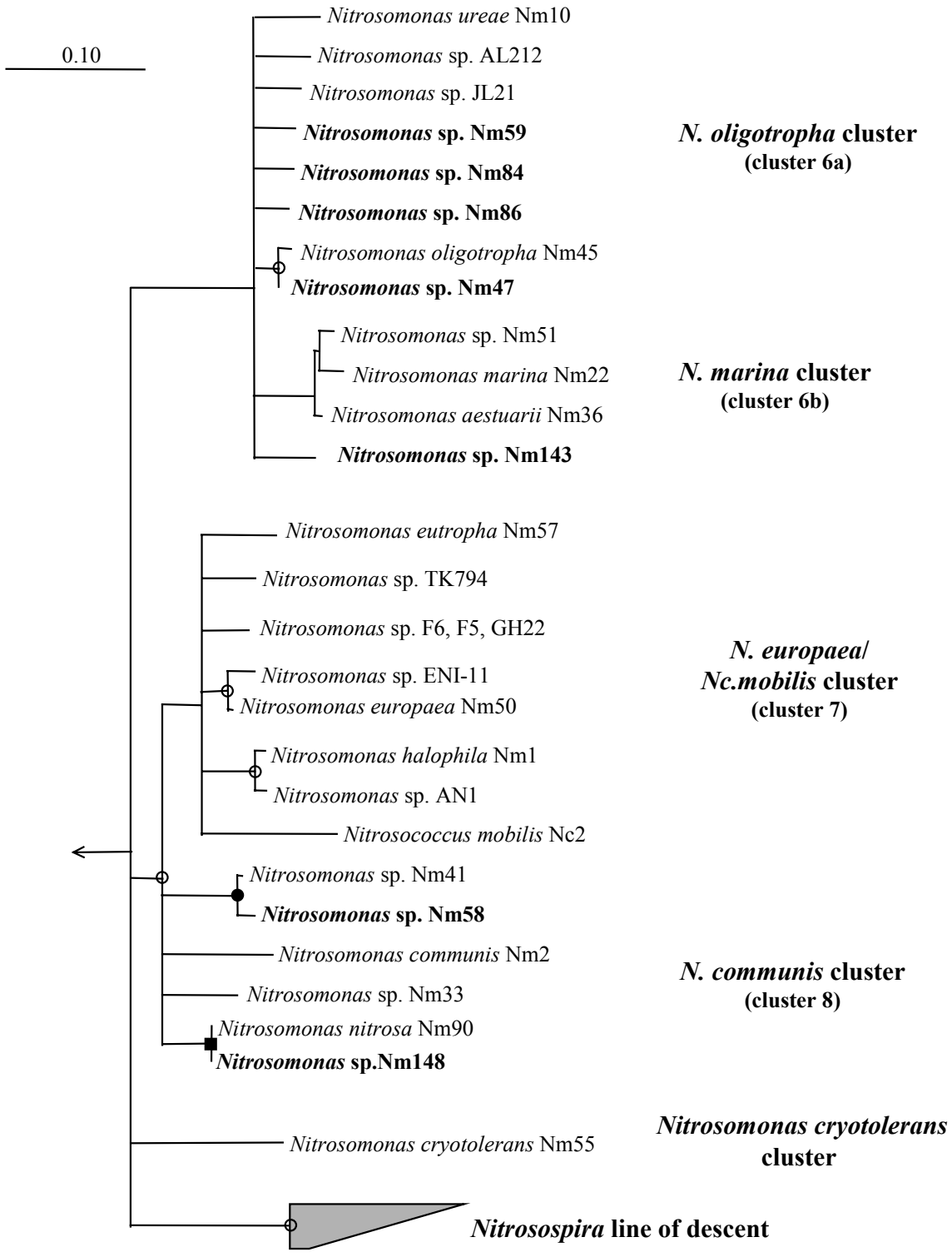
Fig. 3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Nitrosomonas sp. Nm47
N. oligotropha cluster
(cluster 6a)
Nitrosomonas sp. Nm51
Nitrosomonas marina Nm22
Nitrosomonas aestuarii Nm36
Nitrosomonas sp. Nm143
N. marina cluster
(cluster 6b)
Nitrosomonas eutropha Nm57
Nitrosomonas sp. TK794
Nitrosomonas sp. F6, F5, GH22
Nitrosomonas sp. ENI-11
Nitrosomonas europaea Nm50
Nitrosomonas halophila Nm1
Nitrosomonas sp. AN1
Nitrosococcus mobilis Nc2
N. europaea/
Nc.mobilis cluster
(cluster 7)
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Nitrosomonas communis Nm2
Nitrosomonas sp. Nm33
Nitrosomonas nitrosa Nm90
Nitrosomonas sp.Nm148
N. communis cluster
(cluster 8)
Nitrosomonas cryotolerans Nm55
Nitrosomonas cryotolerans
cluster
Nitrosospira line of descent

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ANHANG III

Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon

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Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon

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Abstract

Two biofilm reactors operated with hydraulic retention times of 0.8 and 5.0 h were used to study the links between population dynamics and reactor operation performance during a shift in process operation from pure nitrification to combined nitrification and organic carbon removal. The ammonium and the organic carbon loads were identical for both reactors. The composition and dynamics of the microbial consortia were quantified by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes combined with confocal laser scanning microscopy, and digital image analysis. In contrast to past research, after addition of acetate as organic carbon nitrification performance decreased more drastically in the reactor with longer hydraulic retention time. FISH analysis showed that this effect was caused by the unexpected formation of a heterotrophic microorganism layer on top of the nitrifying biofilm that limited nitrifiers oxygen supply. Our results demonstrate that extension of the hydraulic retention time might be insufficient to improve combined nitrification and organic carbon removal in biofilm reactors.

Keywords: Nitrification; Biofilm; Organic carbon; Hydraulic retention time; Fluorescence in situ hybridization

1. Introduction

The competition between heterotrophic and nitrifying bacteria for substrates (oxygen and ammonia) and space in biofilms is of major practical importance and thus has been the subject of several previous studies (for example [1–3]). According to these investigations, competition in biofilms results in a stratified biofilm structure, the fast growing heterotrophic bacteria being placed in the outer layers, where both substrate concentration and detachment rate are high, while the slow growing nitrifying bacteria stay deeper inside the biofilm. Thus a heterotrophic layer can form above the nitrifiers in the biofilm, which constitutes a disadvantage to them when the bulk liquid oxygen concentration is low. In this case oxygen limitation resulting from consumption and resistance to mass transfer within the heterotrophic layer affects the nitrification performance negatively. As long as the bulk oxygen concentration is high enough to preclude its depletion in the biofilm, however, the heterotrophic layer can also have a positive effect on the nitrifiers by protecting them from detachment [4].

One possible approach to minimize the competition of heterotrophs and nitrifiers for oxygen is their spatial separation into a nitrifying biofilm population and a

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heterotrophic population in suspension. This separation can be achieved by extending the reactor's hydraulic retention time. At comparatively long hydraulic retention times, the fast growing heterotrophic microorganisms (with reciprocal maximum specific growth rates smaller than the selected hydraulic retention time) grow mainly in suspension while the slow growing nitrifiers form biofilms [2]. However, in this study the reactor was operated at a high oxygen concentration of $6\,mg\,l^{-1}$, a condition which did also allow full nitrification in a conventional biofilm reactor (nitrifiers and heterotrophs in the biofilm) [2].

Reactors with a spatial separation of heterotrophic and nitrifying activity for the treatment of effluents with a high chemical oxygen demand (COD)/NH_4^+-N ratio can be very attractive for future practical applications if (i) it can be demonstrated that these reactors allow complete nitrification at a relatively low dissolved oxygen concentration and if (ii) the energy savings in aeration relatively to the traditional process compensate the investment costs in the construction of a bigger reactor required due to its longer retention time. So far the effects of influent composition and operational conditions were studied with respect either to total reactor performance (macroscale studies) or to biofilm composition and structure (microscale studies), but hardly to both simultaneously (for example [3]).

The research subjects of this study were the effects of different hydraulic retention times and changes in the organic carbon dosing on the population dynamics of nitrifying biofilm reactors operated at around $2\,mg\,l^{-1}$ dissolved oxygen. The specific objectives were: (i) to identify and quantify nitrifying and heterotrophic bacteria in the biofilm and in suspension using a set of rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH), (ii) to correlate changes in microbial community composition in the biofilm and suspension with reactor performance during a shift from pure nitrification to combined nitrification and organic carbon removal, and (iii) to asses the recovery of the nitrification process after a shift back to pure nitrification.

2. Materials and methods

2.1. Biofilm reactors

Two laboratory-scale circulating bed reactors (CBR) of 1.2 l each [5] were employed for this study. This type of airlift reactor has a rectangular geometry of 310 mm height and $60\,mm \times 60\,mm$ cross-section and is separated in an up-flow aerated section and a down-flow non-aerated one by a vertical wall (Fig. 1). High density polyethylene granulate with a particle size of 1 mm and a density of $731\,kg\,m^{-3}$ was used as support material for biofilm growth. The superficial air velocity in both

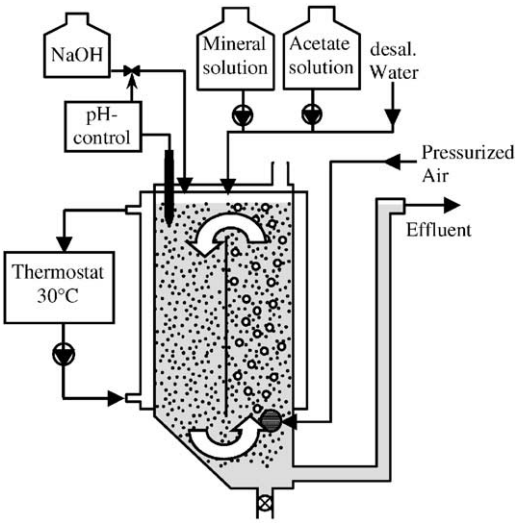

Fig. 1. Schematic diagram of continuous circulating bed reactor set-up.

reactors (defined as the air flow rate divided by the aerated reactor cross-section) was set at $0.003\,m\,s^{-1}$. The temperature was maintained at 30°C, and the pH was kept at 7.5 by adding sodium hydroxide (1 M). The experimental protocol included 4 phases (Fig. 2). The corresponding experimental conditions are summarized in Table 1.

Phase I. A CBR (hereafter reactor R_0) was filled with nitrifying biofilm particles (23 volume percent) obtained from a nitrifying circulating bed reactor, which was maintained at identical operating conditions. The biofilm particles had been stored at 4°C for 90 days prior to inoculation of reactor R_0. An ammonium solution was supplied continuously (N operation mode), with a retention time of 0.70 h. During phase I a stable nitrifying biofilm was established.

Phase II. Half of the biofilm particles from reactor R_0 were transferred to a second identical reactor. Both CBRs (hereafter reactors R_1 and R_2) were operated simultaneously with retention times of 0.8 h for reactor R_1 and 5.0 h for reactor R_2. The ammonium load supplied to each reactor was approximately half of the value used in phase I in order to maintain a constant NH_4^+-N load to biomass ratio ($0.46\,kg\,kg^{-1}\,d^{-1}$), and still no organic carbon was added (N operation mode).

Phase III. Acetate as an organic carbon source was supplied to reactors R_1 and R_2 at the same COD/NH_4^+-N mass ratio (C + N operation mode) in order to investigate the effect of organic carbon on the nitrification performance of reactors operating at different retention times.

Phase IV. Discontinuation of the supply of acetate to reactors R_1 and R_2 in order to study the recovery of the nitrification process (N operation mode).

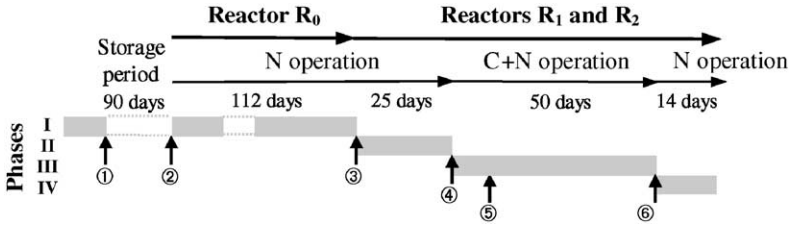

Fig. 2. Schematic diagram describing the experimental phases. The arrows indicate biofilm and suspended biomass sampling times.

Table 1
Operating conditions and performance of reactors R_0, R_1, and R_2. For all parameters average values are given for the different phases of operation

Phase/ mode of operation		Influent		NH_4^+-N load		NH_4^+-N removal (%)	COD load		COD removal (%)	COD/N applied ($g\,g^{-1}$)
		NH_4^+-N	COD ($kg\,m^{-3}$)	Applied	Removed ($kg\,m^{-3}\,d^{-1}$)		Applied	Removed ($kg\,m^{-3}\,d^{-1}$)		
Reactor R_0 hydraulic retention time of 0.7 h										
I	N	0.037	—[a]	1.26	1.20	95	—	—	—	—
Reactor R_1 hydraulic retention time of 0.8 h										
II	N	0.020	0.01	0.61	0.60	98	0.33	0.04	12	0.53
III	C+N	0.022	0.03	0.65	0.45	69	0.85	0.61	72	1.30
IV	N	0.019	0.01	0.57	0.51	90	0.17	0.05	29	0.30
Reactor R_2 hydraulic retention time of 5.0 h										
II	N	0.100	0.02	0.48	0.48	100	0.10	0.04	40	0.21
III	C+N	0.109	0.14	0.53	0.53→0	100→0	0.68	0.61	90	1.28
IV	N	0.117	0.02	0.56	0.53	94	0.08	0.05	63	0.14

[a] — = not determined.

Phases I, II and IV are characteristic of a pure nitrification process, while phase III corresponds to a combined nitrification and organic carbon removal process.

2.2. Media

During the experimental phases I, II and IV (N operation mode) $3.4\,ml\,min^{-1}$ of an ammonium medium was delivered to each reactor. The medium contained $(NH_4)_2SO_4$ ($0.59\,g\,l^{-1}$), $NaHCO_3$ ($0.19\,g\,l^{-1}$), KH_2PO_4 ($0.06\,g\,l^{-1}$), $CaCl_2\,2H_2O$ ($0.014\,g\,l^{-1}$) and trace elements. In reactors R_0 and R_1, also $15.9\,ml\,min^{-1}$ of deionized water was added to the reactors to obtain the desired hydraulic retention time. During phase III (C+N operation mode) $1.7\,ml\,min^{-1}$ of a sterilized acetate solution ($CH_3COONa\,3H_2O$, $0.71\,g\,l^{-1}$) was added separately to reactors R_1 and R_2. In order to maintain the retention time in both reactors constant, the ammonium medium's volumetric flow rate was reduced to half, and its concentration was doubled.

2.3. Overall kinetics

The macroscale reactor performance was evaluated from effluent samples filtered with 0.22 μm membrane filters. COD, ammonia plus ammonium, nitrite and nitrate were determined photometrically (LCK, Dr. Lange). Biofilm and suspended biomass total solids were measured according to APHA [6] using 0.22 μm membrane filters. Prior to this analysis, the biofilm was detached from the support material by an ultrasonic homogenizer (Bandelin electronics D-1000, Berlin) treatment (120 s at 50 W). The dissolved oxygen concentration was measured with an oxygen electrode (WTW, model Oxi 340-A).

2.4. Fluorescence in situ hybridization, microscopy and quantification of probe-targeted bacteria

Microbial population dynamics in biofilm particles and suspended biomass was evaluated using FISH with rRNA-targeted oligonucleotide probes. Samples 1–6

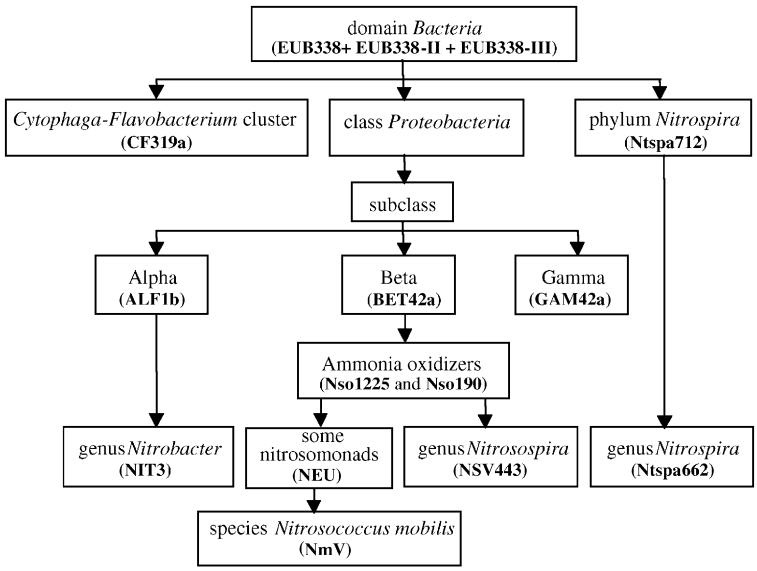

Fig. 3. Specificity of the rRNA-targeted oligonucleotide probes used for in situ identification of nitrifiers and heterotrophic bacteria.

(Fig. 2) were taken from the reactors and immediately fixed with paraformaldehyde. During phases I and II (N operation) only biofilm was sampled since the suspended biomass concentration was very low, while during phase III (acetate addition) both biofilm and suspended biomass samples were taken.

In situ characterization of microbial populations followed a top to bottom approach (Fig. 3). First the samples were hybridized with a probe set (EUB338, EUB338-II, EUB338-III) designed to target almost all bacteria [7], then with previously published group specific probes (Fig. 3; [8,9]). The ammonia-oxidizing and nitrite-oxidizing bacteria were identified using previously published probes (Fig. 3; [10–14]).

Oligonucleotide probes were purchased as derivatives labeled with the fluorescent dyes Cy3, Cy5, and 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS), respectively (Interactiva, Ulm, Germany). FISH was performed using the hybridization and washing buffers as described by Manz et al. [8]. A Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Jena, Germany) was used for image acquisition. For quantification of probe-targeted bacteria, simultaneous hybridizations were performed with Cy3 labeled specific probes and the FLUOS labeled bacterial probe set. The relative biovolume defined as the ratio between the area of probe-targeted bacteria to the area of all bacteria detectable by FISH was determined for each probe in 20 randomly recorded confocal images (thickness 1 μm) using the procedure described by Schmid et al. [15]. Biofilm thickness was determined for fresh, unfixed biofilm samples which were stained with a $0.25\,g\,l^{-1}$ fluorescein isothiocianate solution for 3 h at room temperature, using CLSM optical sectioning in the sagittal (*xz*) direction.

2.5. Comparative sequence analyses of the amoA gene

High resolution analyses of ammonia-oxidizer diversity in reactor R_0 was performed using the gene encoding the catalytic subunit of the ammonia-monooxygenase enzyme (*amoA*) as a marker. Amplification, cloning, sequencing and phylogenetic analyses of the biofilm-derived *amoA* fragments was performed as described by Purkhold et al. [16].

3. Results

3.1. Reactor performance

3.1.1. Phase I

One of the main drawbacks of biological nitrification processes is the requirement of a long start-up period. By using biofilm particles as inoculum, the start-up period of reactor R_0 in phase I could be reduced to 4 days after which the ammonium removal efficiency had reached 95%. Subsequently, R_0's biofilm particles were split between reactors R_1 and R_2 to ensure that both reactors had the same original microbial population composition.

3.1.2. Phases II to IV

Fig. 4 (A–D) depicts the performance of reactors R_1 and R_2 operated with retention times of 0.8 and 5.0 h, respectively, during pure nitrification (phases II and IV) and combined nitrification and organic carbon removal (phase III). Tables 1 and 2 summarize the experimental results obtained during reactors operation and the characterization of biofilm and suspended biomass. Though acetate as organic carbon source was only added during phase III, there was a certain background COD in the influent during phases I, II and IV (Table 1; Figs. 4B and D) deriving from oxidizable matter in the deionized water source. However, during these phases maximal 63% of the incoming COD was removed demonstrating that a significant fraction of these compounds were not degraded in the reactors.

During phase II, both reactors had a stable performance, no nitrite accumulation was observed and the NH_4^+-N effluent concentration was below $1.0\,mg\,l^{-1}$ corresponding to an ammonium removal efficiency higher than 95%. The biofilm characterization at the end of phase II showed similar biofilm thickness (41 and 42 μm), and biofilm mass concentration (2.48 and $2.43\,kg\,m^{-3}$) in reactors R_1 and R_2.

Shortly after the addition of acetate to reactor R_1 (start of phase III), the ammonium removal rate decreased from 0.65 to $0.45\,kg\,m^{-3}\,d^{-1}$ (69%) and hereafter, was constant (Fig. 4A). Due to a mechanical problem in the ammonium dosing pump, reactor R_1 received an ammonium overload 10 days after the start of phase III (gray area in Fig. 4A). The ammonium removal rate, however, remained constant. In reactor R_2

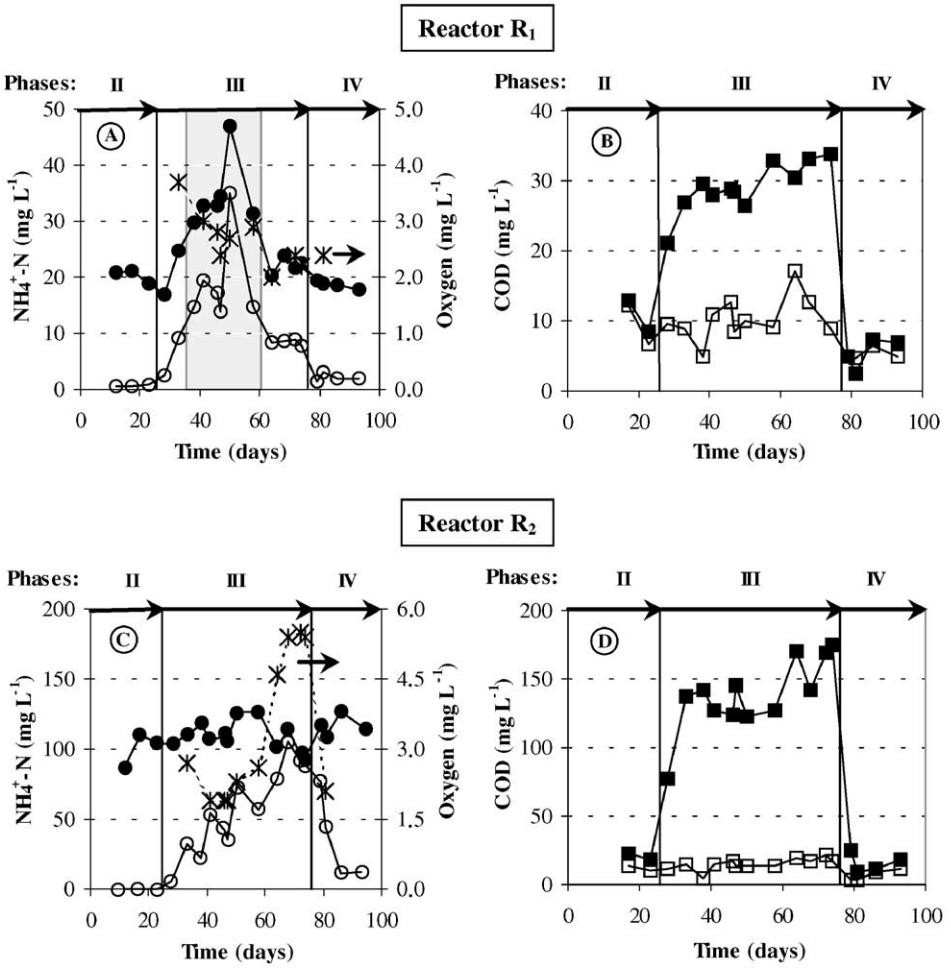

Fig. 4. Time changes of NH_4^+-N, COD and oxygen during operation of reactors R_1 and R_2 with pure nitrification (phases II and IV) and combined nitrification and organic carbon removal (phase III). Closed symbols correspond to influent concentrations and open symbols to effluent concentrations. *Corresponds to oxygen concentrations.

Table 2
Characterization of biofilm and suspended biomass in reactors R_0, R_1, and R_2 during the different phases of operation. Values listed in the table are the average ±95% confidence interval

Mode of operation/ sample		Biofilm mass per reactor volume ($kg\,m^{-3}$)	Suspended solids concentration ($kg\,m^{-3}$)	Biofilm thickness (μm)
Reactor R_0 hydraulic retention time of 0.7 h				
N	3	2.70±0.80	—[a]	33±5
Reactor R_1 hydraulic retention time of 0.8 h				
N	4	2.48±0.37	—	41±2
C+N	5	2.49±0.37	—	—
C+N	6	2.54±0.41	0.46±0.01	44±3
Reactor R_2 Hydraulic retention time of 5.0 h				
N	4	2.43±0.40	—	42±3
C+N	5	2.72±0.40	—	—
C+N	6	3.63±0.38	0.61±0.04	56±5

[a] — = not determined.

the ammonium removal continuously decreased to zero within 50 days (Fig. 4C), while the dissolved oxygen concentration simultaneously increased (Fig. 4B). A constant COD removal rate of $0.61\,kg\,m^{-3}\,d^{-1}$ was reached within 5 days in both reactors (Figs. 4B and D) and no nitrite accumulation was observed during the entire phase III. The increase of biofilm thickness and mass after carbon addition was more pronounced in reactor R_2 than in R_1 (Table 2).

The amount of biomass found in the reactors during phase III was by a factor of 10 to 40 higher than the value expected for theoretical equilibrium between bacteria growth rate and dilution rate (Table 2). This inconsistency was obviously caused by an accumulation of mostly heterotrophic biomass in the reactors' bottom and effluent tubes increasing the actual sludge retention time considerably. In reactor R_2 this effect was supported by the lower hydraulic retention time, leading to a higher biomass accumulation than in R_1. The accumulated biomass was removed once per week and included in the samples for suspended solids quantifications and FISH analyses of suspended cells.

After the discontinuation of acetate addition (phase IV) the ammonium removal in both reactors recovered and after a period of 14 days approx. 90% of the influent ammonium load was nitrified. In reactor R_2 the dissolved oxygen concentration decreased back to the value at the beginning of phase III.

3.2. Diversity of nitrifying bacteria in the reactors

The ammonia-oxidizing cells in all biofilm samples could be labeled simultaneously with probes BET42a, Nso1225 and Nso190. No ammonia-oxidizers belonging to the *Nitrosospira*-cluster were detected. A fraction of the ammonia-oxidizing population was detectable with probe NEU, while *Nitrosococcus mobilis* was absent. The NEU-positive subpopulation of ammonia-oxidizers is most likely affiliated with the *Nitrosomonas europaea/eutropha* group [17]. Comparative sequence analyses of *amoA* clones derived from the biofilm of reactor R_0 independently confirmed the presence of two different groups of ammonia-oxidizers (Fig. 5). One *amoA* sequence cluster is closely related to *Nitrosomonas europaea*, most likely representing the NEU-positive ammonia-oxidizers, while the other *amoA* cluster is not closely related with any described ammonia-oxidizer reference strain. In both reactors the nitrite-oxidizing cells in the biofilm were affiliated with the genus *Nitrospira* during all phases of operation. Members of the genus *Nitrobacter* were only detected in the biofilm from reactor R_2 (5.0 h retention time) during operation with acetate addition (Table 3).

3.3. Diversity of heterotrophic bacteria in the reactors

The heterotrophic bacteria present in the biofilm of reactor R_2 were *Proteobacteria* of the alpha- and beta-subclasses while in reactor R_1 only beta-subclass *Proteobacteria* could be detected (Table 3). In both reactors the microbial populations in the suspended biomass during phase III were *Proteobacteria* of the alpha-, beta- and gamma-subclasses and bacteria belonging to *Cytophaga-Flavobacterium*-cluster (Table 4).

The dominating microbial populations both in biofilm and suspended biomass during combined organic carbon and ammonia oxidation belong to the beta-subclass of *Proteobacteria*. Concerning the two reactor

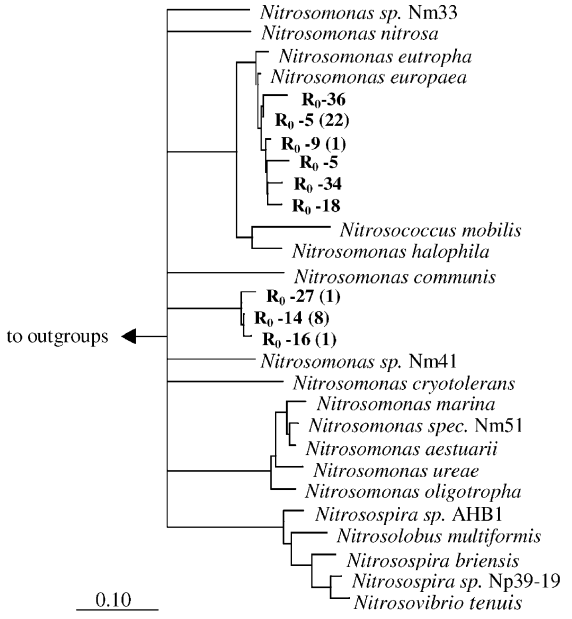

Fig. 5. Phylogenetic FITCH tree reflecting the relationships of the ammonia-oxidizers in reactor R_0 based on *amoA* sequences. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length. Numbers in brackets indicate the number of clones with almost identical sequences (>99% amino acid sequence similarity) which were retrieved from the reactor.

operation modes (N and C+N operation) the major difference is that during pure nitrification all beta-subclass *Proteobacteria* were ammonia-oxidizers, while during acetate dosing an additional presumably heterotrophic beta-subclass population developed. With respect to the different hydraulic retention times the major difference is a presumably heterotrophic alpha-subclass *Proteobacteria* population that occurred during pure nitrification only in reactor R_2 biofilm and disappeared after introduction of acetate.

3.4. Population dynamics in biofilm and suspended biomass

Values for each probe's targeted-bacteria are depicted in Table 3 for biofilm and in Table 4 for suspended biomass. The relative biovolumes of the different microbial populations (determined by FISH) were normalized by taking into account the differences in biomass (measured as dry weight) in the different reactors and samples. The FISH biovolume percentages for the sample with the highest biomass per reactor volume (sample 6) were kept unchanged and the FISH percentages of the other samples were reduced according to the biomass differences.

The fraction of the heterotrophic beta-*Proteobacteria* population (HET) present in the biofilm of reactors R_1 and R_2 can be determined as follows:

$$(\mathrm{Area}_{\mathrm{BET42a}})_{\mathrm{HET}} = \mathrm{Area}_{\mathrm{BET42a}} - \mathrm{Area}_{\mathrm{Nso1225}}. \quad (1)$$

Simultaneous hybridization of biofilm samples with probes ALF1b and Ntspa662 designed for specific detection of the alpha-subclass of *Proteobacteria* and the genus *Nitrospira*, respectively, demonstrated that members of the genus *Nitrospira* are non-specifically targeted by probe ALF1b. Consistent with this finding, a recent data base inspection demonstrated that *Nitrospira*-like 16S rRNA-sequences retrieved from wastewater treatment plants possess the full match target site of probe ALF1b. Consequently, *Nitrospiras* have to be included in the list of non-alpha-subclass *Proteobacteria* targeted by probe ALF1b [8]. The relative biovolume labeled with probe ALF1b was higher than the one labeled with probe Ntspa662 in biofilm samples taken from reactor R_2, except for the last biofilm sample during phase III (Table 3). This demonstrates that an ALF1b-positive population not related to *Nitrospira* appeared in the biofilm when the hydraulic retention time changed from 0.8 to 5.0 h, corresponding to the transition from reactor R_0 to reactor R_2 (phase II—N operation mode), and disappeared after the operation of reactor R_2 with acetate for 50 days (phase III—C+N operation mode).

The fraction of the alpha-*Proteobacteria* population (HET) developed in the biofilm from reactor R_2 during pure nitrification (phase II) can be estimated as follows:

$$(\mathrm{Area}_{\mathrm{ALF1b}})_{\mathrm{HET}} = \mathrm{Area}_{\mathrm{ALF1b}} - \mathrm{Area}_{\mathrm{Ntspa662}}. \quad (2)$$

The fraction of biofilm and suspended biomass bacteria identified with specific gene probes (F) in relation to all bacteria (EUB338 probe set) was calculated as listed below:

Biofilm samples

$$\mathrm{Area}_{\mathrm{ALF1b}} \approx \mathrm{Area}_{\mathrm{Ntspa662}} \Rightarrow \mathrm{F} = \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{Ntspa662}}, \quad (3)$$

$$\mathrm{Area}_{\mathrm{ALF1b}} > \mathrm{Area}_{\mathrm{Ntspa662}} \Rightarrow \mathrm{F} = \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{ALF1b}}. \quad (4)$$

Suspended biomass samples

$$\mathrm{F} = \mathrm{Area}_{\mathrm{ALF1b}} + \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{GAM42a}} + \mathrm{Area}_{\mathrm{CF319a}}. \quad (5)$$

The population dynamics of nitrifiers and heterotrophs in the biofilm and suspended biomass from reactors R_0, R_1 and R_2 during the different phases of operation is depicted in Fig. 6. For most biofilm and suspension samples, more than 80% of the bacteria detectable by the EUB338 probe set could simulta-

Table 3
Microbial community composition of reactors R_0, R_1 and R_2 biofilm during the different phases of operation[a]

Sample/reactor	NF	Oligonucleotide probes						F
		ALF1b	BET42a	Nso1225	NEU	Ntspa662	NIT3	
Phase I—N removal								
1 R_0	0.74		**39**	**39**	**25**	**38**	**0**	105
		—	(53±4)	(53±4)	(34±3)	(52±4)	(0)	
2 R_0		—	—	—	(56±5)	(30±5)	0	—
3 R_0	0.74	**20**	**41**	**41**	**19**	**20**	**0**	82
		(27±3)	(55±4)	(55±4)	(26±3)	(27±3)	(0)	
Phase II—N removal								
4 R_1	0.68	**21**	**30**	**30**	**16**	**23**	**0**	78
		(31±3)	(45±6)	(45±6)	(24±6)	(33±5)	(0)	
R_2	0.67	**35**	**21**	**21**	**14**	**27**	**0**	83
		(52±3)	(31±5)	(31±5)	(21±3)	(41±2)	(0)	
Phase III—C + N removal								
5 R_1	0.68	**12**	**26**	**19**	**12**	**14**	**0**	59
		(18±3)	(39±5)	(27±3)	(17±3)	(21±2)	(0)	
R_2	0.75	**30**	**29**	**21**	**19**	**23**	**0**	80
		(40±7)	(39±5)	(28±7)	(25±5)	(30±6)	(0)	
6 R_1	0.70	**24**	**34**	**21**	**5**	**22**	**0**	81
		(34±6)	(49±8)	(30±4)	(7±2)	(32±5)	(0)	
R_2	1.00	**29**	**60**	**0**	**0**	**31**	**3**	94
		(29±6)	(60±4)	(0)	(0)	(31±6)	(3±1)	

[a] The relative biovolumes of probe-defined bacterial populations (values in brackets) and the respective normalized values in regard to the biomass content of the samples (bold values) are given in columns 3–8. No normalized values are given for sample 2 since the biomass content was not determined for this sample. The fraction of bacteria detectable with the bacterial probe set, which were identified with specific oligonucleotide probes, is given in column 9. Values listed in the table are the average percentage ±95% confidence interval. For all biofilm samples no signals were observed with probes specific for the *Cytophaga-Flavobacterium*-cluster (CF319a) and the gamma-subclass of *Proteobacteria* (GAM42a), respectively. NF = normalization factor; — = not determined.

Table 4
Microbial community composition of reactors R_1 and R_2 suspended biomass during combined nitrification and organic carbon removal (columns 3–6)[a]

Sample/reactor	NF	Oligonucleotide probes				F
		ALF1b	BET42a	GAM42a	CF319a	
Phase III—C + N removal						
5 R_1	0.18	**2.2**	**11.5**	**0**	**0.2**	76
		(12±1)	(64±2)	(0)	(1±0)	
R_2	0.17	**1.6**	**14**	**0.5**	**0.9**	99
		(10±1)	(81±1)	(3±0)	(5±1)	
6 R_1	0.18	**3.1**	**14.6**	**0.2**	**0.2**	100
		(17±3)	(81±2)	(1±0)	(1±0)	
R_2	0.17	**1.2**	**13**	**1.9**	**1.5**	104
		(7±1)	(77±4)	(11±1)	(9±2)	

[a] Column 7 displays the fraction of bacteria detectable with the bacterial probe set which were identified with specific oligonucleotide probes. Values listed in the table are the average percentage ±95% confidence interval (values in brackets). NF = normalization factor.

neously be classified by at least one of the more specific probes applied (Tables 3 and 4; Eqs. 3–5). However, this does not hold true for the biofilm and suspended biomass samples taken from reactor R_1 during the accidental overloading period with ammonium (sample 5). In this sample 41% of the biofilm bacteria and 24%

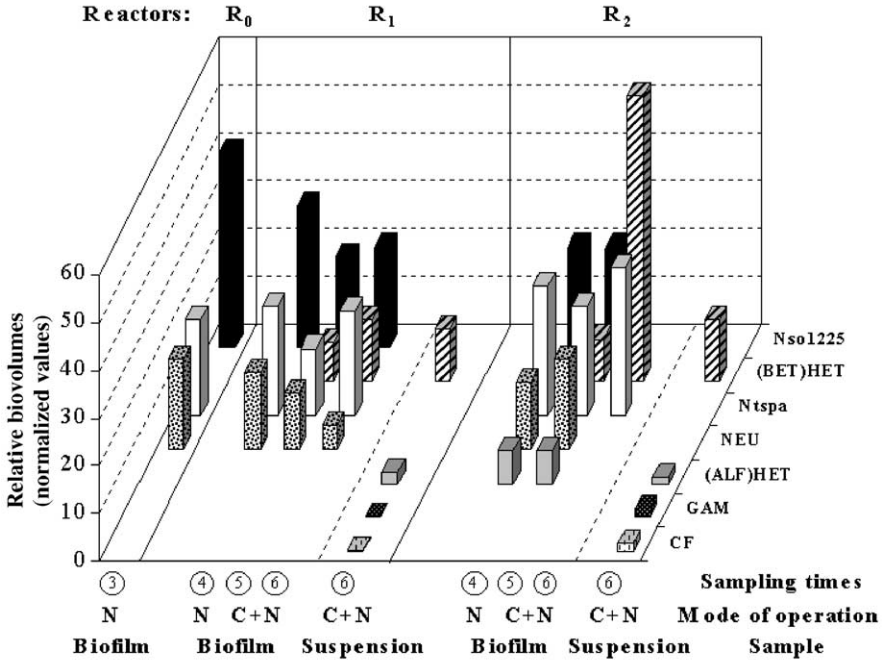

Fig. 6. Population dynamics of ammonia-oxidizers, nitrite-oxidizers and heterotrophs in the biofilm during process operation of reactors R_1 and R_2 with pure nitrification [phase I (sampling point 3) and phase II (sampling point 4)] and combined nitrification and organic carbon removal (phase III—sampling points 5 and 6). In addition, the microbial community composition of the suspended biomass is displayed for both reactors for sampling point 6.

of the suspended bacteria which were detectable by FISH could not further be classified with the more specific probes. Whether these bacteria appeared in the reactor due to the acetate addition or the elevated ammonia concentrations could not be clarified.

3.5. Population dynamics during phase I

Biofilm samples were taken before and after the storage period preceding operation of reactor R_0 (samples 1 and 2) and after 112 days of operation (sample 3), in order to evaluate the effects of the storage period on the microbial community composition.

The samples were hybridized in situ with probes Nso1225, NEU and Ntspa662, as well as the EUB338 probe set. After the storage period (sample 2) the fraction of ammonia-oxidizers was larger than during normal operation (samples 1 and 3) while the relative abundance of nitrite-oxidizers decreased (data not shown), suggesting that the nitrite-oxidizers decayed faster than the ammonia-oxidizers under the selected storage conditions. The characterization of the biofilm from reactor R_0 during operation (sample 3) showed that the ammonia-oxidizing population detected with probe Nso1225 was able to recover a comparable relative abundance as before the storage period (sample 1), while the nitrite-oxidizing population decreased considerably from 38% (sample 1) to 20% (sample 3). Furthermore, the morphology of the nitrite-oxidizing clusters changed from big clusters present before the storage period (sample 1), to small ones mixed with net-like structures afterwards (sample 3). Despite the decrease in the relative abundance of nitrite-oxidizers, no nitrite accumulation was observed during phase I. This suggests that the nitrite-oxidizers present in net-like structures in sample 3 are more active (possibly due to a better accessibility to substrates) than those occurring in big clusters in sample 1.

3.6. Population dynamics during phase II

In the transition from phases I to II the amount of biofilm particles from reactor R_0 was split up between reactors R_1 and R_2, while the NH_4^+-N load per reactor was reduced from 1.24 to 0.48–0.61 $kg\,m^{-3}\,d^{-1}$. Despite the lower amount of biofilm particles in R_1 and R_2 than in R_0, the biomass concentration per reactor volume was similar in both reactors during phases I and II (Table 2). This can be explained by a lower biofilm detachment rate in reactors R_1 and R_2 caused by a decrease in the

collision frequency between biofilm particles. Consequently, the NH_4^+-N load to biomass ratio was higher in R_0 ($0.46\,kg\,kg^{-1}\,d^{-1}$) than in R_1 ($0.25\,kg\,kg^{-1}\,d^{-1}$) and R_2 ($0.24\,kg\,kg^{-1}\,d^{-1}$). As a result the effluent NH_4^+-N concentration of the reactors dropped from $1.8\,mg\,l^{-1}$ (R_0) to $0.6\,mg\,l^{-1}$ (R_1) and $0.2\,mg\,l^{-1}$ (R_2), respectively. The normalized abundance of ammonia-oxidizers in the biofilm decreased from reactor R_0 (sample 3) to reactor R_1 (sample 4) and reactor R_2 (sample 4), respectively (Fig. 6; Table 3). The NH_4^+-N concentration in reactors R_1 and R_2 was lower than the assumed saturation constant of $0.7\,mg\,l^{-1}$ [18], and thus most likely limited the amount of ammonia-oxidizers in the biofilm. Unlike the ammonia-oxidizing population the relative abundance of nitrite-oxidizers increased in R_2 and remained approximately constant in R_1 during phase II (N operation). According to Eq. (2), in reactor R_2 a heterotrophic population (8%) detectable with probe ALF1b developed.

3.7. Population dynamics during phase III

The newly introduced supply of acetate during phase III induced the growth of heterotrophic microorganisms both in suspension and in the biofilm in both reactors. Biofilm samples collected 12 days after acetate addition (sample 5) showed the presence of a thin layer of heterotrophic beta-*Proteobacteria* distributed discontinuously on top of the nitrifying biofilm, accounting for 7–8% of the bacterial biofilm population in both reactors. Like during phase II, a heterotrophic population detectable with probe ALF1b (7%) was exclusively present in reactor R_2, 12 days after addition of acetate. During the first 12 days of phase III the abundance of nitrite-oxidizers decreased in the biofilm of both reactors, while the ammonia-oxidizers decreased only in R_1. The latter observation corresponds with the higher loss of ammonium removal observed in reactor R_1, (28% loss) compared to reactor R_2 (19% loss).

The ammonium removal in reactor R_2 continued to decrease during the subsequent days of acetate addition until a complete breakdown of the nitrification process happened. After 50 days of acetate addition (sample 6), no ammonia-oxidizing bacteria could be detected in reactor R_2 while the relative in situ abundance of *Nitrospira*-like nitrite-oxidizers was similar to the one during phase II (N operation). At that time reactor R_2 biofilm was dominated by heterotrophic beta-*Proteobacteria* that amounted to 60% of the total biovolume labeled with the bacterial probe set. In contrast to reactor R_2, the extended addition of acetate did not cause a complete breakdown of nitrification in reactor R_1. In this reactor, the ammonium removal efficiency stabilized at 69% and the heterotrophic beta population in the biofilm amounted to a maximum biovolume fraction of only 13% (sample 6). As in reactor R_2, the nitrite-oxidizing population in reactor R_1 did recover its initial relative abundance during prolonged acetate dosage.

4. Discussion

In this study the microbial community composition and dynamics in two nitrifying biofilm reactors (differing in hydraulic retention time) was monitored using molecular tools during a shift in process operation from pure nitrification to combined nitrification and organic carbon removal. In general, the dynamics of the microbial communities correlated well with the performance of the respective reactors. In the following sections several interesting findings are discussed in more detail.

4.1. Composition and dynamics of bacterial populations in the reactors

In both reactors at least two populations of beta-subclass ammonia-oxidizers were present. As demonstrated by oligonucleotide probing and comparative AmoA sequence analysis, one of these populations was closely related to the model organism *Nitrosomonas europaea*, while the other population surprisingly showed no close relationship with recognized ammonia-oxidizers. Nitrite oxidation was catalyzed in both reactors mainly by *Nitrospira*-like bacteria confirming the recently recognized importance of these bacteria for nitrite oxidation in several environments (e.g. [13,14,19]). In both reactors Nitrospiras occurred in previously not observed net-like structures in the biofilm. In the present work, *Nitrobacter* could only be detected in small numbers in the biofilm of reactor R_2 during simultaneous addition of acetate and ammonium (phase III) and a concurrent increase of the NO_2^--N concentration from $0.02\,mg\,l^{-1}$ (phase II) to $0.39\,mg\,l^{-1}$ (phase III). This result is consistent with the recently published hypothesis that Nitrospiras are k-strategists (and thus thrive at low nitrite concentrations) while *Nitrobacter* as r-strategist can compete successfully only in environments with relatively high nitrite concentrations [19]. The absence of detectable Nitrobacters in reactor R_1 during phase III might have been caused by the lower NO_2^--N accumulation ($0.22\,mg\,l^{-1}$) in this reactor compared to reactor R_2. In addition, *Nitrobacter* might have benefited in phase III in R_2 from its capability to grow mixotrophically with acetate while Nitrospiras might not possess this capability [14]. On the other hand we yet have no explanation for the observed increase of *Nitrospira*-like nitrite oxidizers during phase III in both reactors despite the decreasing or even failing nitrification during this period.

Despite the lack of organic carbon dosing during phase II a presumably heterotrophic population affiliated with the alpha-subclass *Proteobacteria* was present in the biofilm of reactor R_2 (5.0 h retention time), but disappeared during reactor operation with acetate (end of phase III). Most likely this population was growing on soluble microbial products (SMP) formed by active nitrifiers in the biofilm [20] since SMP concentration should be higher in reactor R_2 than in reactor R_1 due to the difference in retention time. In phase III the supply of acetate provoked the breakdown of the nitrification process in reactor R_2, consequently SMP were no longer produced and the alpha-proteobacterial population disappeared from the biofilm.

During combined nitrification and organic carbon removal in phase III a higher group-level diversity of heterotrophic microorganisms was found in suspension than in the biofilm. This result might be explained by the fact that the heterotrophic biofilm community is exposed to a higher shear stress compared to the suspended consortia. Thus, only fast growing heterotrophs can maintain themselves in the outer layer of the biofilm, a selection pressure which might reduce diversity.

4.2. Population dynamics versus reactor performance

During phase II (pure nitrification) the biofilm was, as expected, dominated by ammonia- and nitrite-oxidizing bacteria in both reactors. After acetate addition (phase III), the formation of a thicker layer of heterotrophic bacteria in reactor R_2 (with long retention time) on the surface of the nitrifying biofilm led to increased oxygen mass transfer resistance from bulk liquid to the nitrifiers. This coincided with a drastic reduction in the ammonia-oxidizing population and a subsequent breakdown of the nitrification process. In contrast, reactor R_1 operated with short retention time displayed a smaller increase of heterotrophic biofilm bacteria on the surface which corresponded to the less pronounced reduction in nitrification performance. An alternative hypotheses for the breakdown of the nitrification in reactor R_2 during phase III would be that nitrifiers but not the heterotrophs were lost from the biofilm due to detachment and finally washed out of the reactor. This would be consistent with the absence of in situ detectable ammonia-oxidizers in sample 6 of reactor R_2. However, there are two lines of evidence that oxygen limitation and not selective washout caused the nitrification breakdown. Firstly, the even slower growing nitrite-oxidizers still were detected in significant amounts (31%) within the biofilm of reactor R_2 at the end of phase III. Secondly, the re-establishment of 90% of the nitrification capacity within 14 days after stop of acetate addition (Fig. 4; phase IV) in reactor R_2 contradicts the possibility of a previous complete depletion of ammonia-oxidizers. The mass accumulation rate for nitrifying biofilm of $0.03\,kg\,m^{-3}\,d^{-1}$, [21] excludes the chance of such a fast recovery by re-growth. Keeping in mind previously published data [2] our findings were unexpected since we assumed that reactor R_2 will, due to its longer retention time, favor suspended growth of the fast reproducing heterotrophs and thus allow for a higher biofilm-mediated nitrification during the presence of acetate.

What remains to be discussed is (i) why a thicker heterotrophic biofilm is formed on top of the nitrifying biofilm in reactor R_2 compared to reactor R_1 and (ii) why heterotrophic biofilm formation and subsequent loss of nitrifying capacity was not observed in the airlift reactor of [2] after addition of organic carbon. Heterotrophic biofilm formation in reactor R_2 could be explained by increased liquid phase viscosity in this reactor due to the presence of extracellular biopolymers which accumulated compared to reactor R_1 due to the increased liquid retention time in reactor R_2. Increased liquid viscosity will lead to a stronger air bubble coalescence, thus decreasing the volume occupied by the gas phase, reducing the driving force for the circulation and ultimately the shear stress for the biofilm ([22,23]). Consistent with this argumentation, surface protuberances were observed by us microscopically in the biofilm of R_2 (reflecting the lower shear stress) while the biofilm of reactor R_1 was characterized by a much smoother biofilm surface (indicative for a higher shear stress [23]). Differences in liquid phase viscosity or other factors influencing turbulence and thus shear stress and substrate availability (e.g. reactor geometry, friction, structure of the biofilm support particles etc.) could also be responsible for the inconsistent results between this study and the work of van Benthum and co-workers. Independent from the actual reason(s) causing these inconsistent results of both studies, it is important to note that extension of hydraulic retention time caused dramatically different effects in similar biofilm reactors.

5. Conclusions

The following main conclusions can be drawn from the present study:

1. No major effect of the hydraulic retention time on the diversity of nitrifying bacteria in the biofilm was observed. A group of ammonia-oxidizers not closely related to any described reference strain was identified in the biofilm from both reactors showing that even in systems working with defined conditions, the bacterial diversity is not completely described.
2. Combined nitrification and carbon removal under oxygen limiting conditions could be accomplished in the biofilm reactor with low hydraulic retention time but failed in the reactor with high hydraulic retention

time. This unexpected finding was caused by the formation of a thick heterotrophic layer on top of the nitrifying biofilm in the latter reactor that limited the nitrifiers' oxygen supply. Thus, extension of the hydraulic retention time is not always sufficient to improve combined nitrification and organic carbon removal in biofilm reactors.

3. Today, the battery of molecular tools allows to precisely determine ecological key parameters of complex microbial communities present in engineered systems. In addition to species richness and evenness also the in situ activity of probe identified bacteria can be analyzed [24]. Future interdisciplinary research at the interface between molecular microbial ecology and civil engineering will almost certainly allow for a detailed understanding of the links between microbial diversity, process efficiency and process stability. For example, it should be possible by the use of molecular methods to define operational parameters which selectively increase the diversity within important functional groups of bacteria (e.g. nitrifiers) and thus render the microbial community more resistant against perturbations. Armed with such knowledge innovative strategies for process control and design as well as for bioaugmentation can be developed.

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ANHANG IV

Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm

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Community Structure and Activity Dynamics of Nitrifying Bacteria in a Phosphate-Removing Biofilm

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The microbial community structure and activity dynamics of a phosphate-removing biofilm from a sequencing batch biofilm reactor were investigated with special focus on the nitrifying community. O_2, NO_2^-, and NO_3^- profiles in the biofilm were measured with microsensors at various times during the nonaerated-aerated reactor cycle. In the aeration period, nitrification was oxygen limited and restricted to the first 200 μm at the biofilm surface. Additionally, a delayed onset of nitrification after the start of the aeration was observed. Nitrate accumulating in the biofilm in this period was denitrified during the nonaeration period of the next reactor cycle. Fluorescence in situ hybridization (FISH) revealed three distinct ammonia-oxidizing populations, related to the *Nitrosomonas europaea, Nitrosomonas oligotropha,* and *Nitrosomonas communis* lineages. This was confirmed by analysis of the genes coding for 16S rRNA and for ammonia monooxygenase (*amoA*). Based upon these results, a new 16S rRNA-targeted oligonucleotide probe specific for the *Nitrosomonas oligotropha* lineage was designed. FISH analysis revealed that the first 100 μm at the biofilm surface was dominated by members of the *N. europaea* and the *N. oligotropha* lineages, with a minor fraction related to *N. communis*. In deeper biofilm layers, exclusively members of the *N. oligotropha* lineage were found. This separation in space and a potential separation of activities in time are suggested as mechanisms that allow coexistence of the different ammonia-oxidizing populations. Nitrite-oxidizing bacteria belonged exclusively to the genus *Nitrospira* and could be assigned to a 16S rRNA sequence cluster also found in other sequencing batch systems.

Modern biological treatment of wastewater involves not only C removal, but also elimination of the nutrients P and N (5, 20). This requires the combined or sequential actions of various groups of microorganisms, such as heterotrophic bacteria, phosphate-accumulating organisms (PAO), and nitrifying and denitrifying bacteria. Consequently, purification plants and processes have become increasingly complex to satisfy the needs of the different microorganisms, usually in several reactor stages (5, 27). The integration of different functions in a single reactor would save reaction space and time and therefore is desirable from an economical point of view. However, difficulties often arise in establishing stable nitrification in such complex systems. Nitrifying bacteria (i.e., ammonia-oxidizing bacteria [AOB] and nitrite-oxidizing bacteria [NOB]) usually show low maximum growth rates, relatively low substrate affinities, and high sensitivity to toxic shocks or sudden pH changes (17, 25, 41). In the presence of organic matter, they can be easily outcompeted by heterotrophs for oxygen (56) and ammonia (19). Other problems to be solved are the inhibition of denitrification by the presence of oxygen (5) and the need for cyclic changes of oxic and anoxic (i.e., free of oxygen and nitrate) conditions for biological phosphate removal (34). Biofilm systems are an obvious option for such multifunctional reactors. Slow-growing organisms remain in the reactor by their attached growth; the biofilm matrix might protect bacteria from grazing, harmful substances, or sudden pH shifts; and biofilms can be stratified and therefore provide oxic and anoxic reaction zones (11). During the last 5 years, several studies have addressed nitrifying biofilms through a combination of microsensor measurements and 16S rRNA-based methods, such as fluorescence in situ hybridization (FISH) (39, 48, 50). This approach revealed, e.g., the identity and spatial arrangement of AOB and NOB in various nitrifying systems (39, 48–50) and provided a first estimate of their in situ reaction rates and substrate affinities (48). However, very little is known about how and which nitrifying bacteria are adapted to competition with heterotrophs in more complex systems and how they interact with other processes.

Recently, a biofilm system was proposed that integrates enhanced biological phosphate removal (EBPR) and nitrification and denitrification in a single reactor (3, 18). The biofilm is subjected to a sequencing batch mode, in which an anoxic treatment period is followed by an oxic period to allow for net accumulation of polyphosphate in the biomass, which is removed from the system by backwashing at regular intervals. Substrate balances revealed that the removal of organic carbon and EBPR were successfully combined with nitrogen removal via nitrification and denitrification (3).

In the present study, the microbial ecology of this combined nitrification-EBPR biofilm process was investigated by using microsensor analysis and various molecular techniques, i.e., FISH and analysis of 16S ribosomal DNA (rDNA) and *amoA* gene sequences. The objectives were to reveal which populations contribute to which part of the process, which AOB and NOB persist under these highly competitive and transient conditions, and how nitrifying activity overlaps, in time and space, with heterotrophic activity, especially EBPR.

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MATERIALS AND METHODS

Process description. A 20-liter sequencing batch biofilm reactor (SBBR) was established as described previously (18). The artificial wastewater was composed of $Na(CH_3COO) \times 3H_2O$ (103 mg liter^{-1}), peptone (200 mg liter^{-1}), $(NH_4)_2SO_4$ (63 mg liter^{-1}), KH_2PO_4 (44 mg liter^{-1}), KCl (14 mg liter^{-1}), and yeast extract (3 mg liter^{-1}), leading to influent concentrations of 12 mg liter^{-1} for P and 38 mg liter^{-1} for N and a chemical oxygen demand (COD) of about 270 mg liter^{-1}. Oxygen and phosphate concentrations in the bulk water were regularly monitored by online measurements with an oxygen electrode (Oxy 196; WTW, Weilheim, Germany) and with a P analyzer (Phosphax Inter; Dr. Lange, Düsseldorf, Germany). Ammonium, nitrate, and COD were determined photometrically with standard test kits (LCK 303, 339, and 314; digital photometer ISIS 6000; Dr. Lange, Düsseldorf, Germany). The lengths of the operation periods were as follows: 20 min of filling (min 0 to 20), 160 min of nonaerated recirculation (min 20 to 180), 260 min of recirculation with aeration (min 180 to 440), and 40 min of draining (min 440 to 480). The process temperature was kept at 20°C. Biofilm was grown on substratum, Kaldnes elements (Purac, Merseburg, Germany): i.e., plastic rings (diameter, 8 mm, height, 8 mm) designed so biofilm could adhere to both the outer surface and the central spaces within the ring. To remove biofilm material with incorporated polyphosphate, the system was backwashed once a week with pressurized air and water. Removal of biomass from the central spaces of the Kaldnes elements was not efficient, leading to complete clogging of these spaces. The preceding FISH analysis of the biofilm structure as described below revealed no visual difference in the composition and spatial organization of the main microbial populations in the upper parts of biofilms originating from the external substratum surface and from the biofilm of clogged central spaces. Therefore, elements filled completely with biogenic material were chosen for microsensor measurements and FISH.

Microsensor measurements. To allow measurements during reactor operation, Kaldnes elements with biofilm were transferred during the initial filling period from the reactor to a separate 750-cm^3 flow chamber coupled to the recirculation of the reactor. Microsensors were inserted through small holes in the top lid that were closed with stoppers during the nonaeration period. Vertical concentration microprofiles in the biofilm were measured for oxygen with Clark-type microsensors (45) and for ammonium, nitrite, and nitrate with potentiometric ion-selective microelectrodes of the LIX type (15) as described previously. At least 20 profiles were measured for each parameter at different times covering the whole course of a treatment cycle. Measurements were distributed over four cycles to check for the similarity of conditions on different days of operation.

Rate calculations. Due to the periodic changes in the process conditions, the microprofiles measured in situ do not represent a steady-state situation. Therefore, in the case of nitrate, profiles were corrected to allow the application of a one-dimensional diffusion-reaction model for calculation of specific volumetric rates of net consumption or production. In each nitrate profile, the first value measured in the bulk water was set to $t = 0$. Then for each depth, the concentration change over time was calculated from two subsequent profiles. The resulting slope was used to correct the time-dependent change of concentration points at $t > 0$ in each profile by linear interpolation. In that way, every profile was corrected for the dynamics of concentration changes leading to the elimination of transience. The correction procedure just described influenced the absolute values of concentration in the deeper biofilm, but did not change the shapes of the profiles. The correction-of-profile data were not applied to oxygen profiles, because their appearance and the stability of bulk values throughout the oxic period of the process indicated a clear steady-state situation with respect to oxygen.

Volumetric nitrification rates were calculated by applying a one-dimensional diffusion-reaction model to the corrected data. Each profile results from a combination of production (P), consumption (C), and diffusive transport, as described by Fick's second law of one-dimensional diffusion, which under steady-state conditions [$\delta c(z, t)\delta t = 0$)] can be written as $P - C = -D_s \cdot \delta^2 c(z,t)/\delta z^2$, where D_S is the effective diffusion coefficient, c is the solute concentration, z is depth, and t is time. Assuming a zero order reaction, volumetric net production was subsequently calculated by quadratic regression for each depth (38).

DNA extraction. DNA was extracted from native biofilm samples stored at −70°C with the FastDNA-Extraction kit for soil (Bio 101, Carlsbad, Calif.), as described in the manufacturer's instructions. The quality of DNA was checked by agarose (1% [wt/vol]) gel electrophoresis.

16S rDNA analysis. A 1-kb fragment of the 16S rDNA gene was amplified with the complement of probe NOLI191 (43) as the forward primer and the unlabeled probe Nso1225 (35) as the reverse primer. The following reaction mixture was used: 50 pmol of each primer, 2.5 μmol of each deoxynucleoside triphosphate (dNTP), 1× PCR buffer, 1 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom), and 50 to 100 ng of template DNA. The mixture was adjusted to 100 μl with sterile water. PCR was performed with an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with 35 cycles with hot start. The annealing temperature of 56°C for the primer set was determined in previous PCRs run at different temperatures. After checking an aliquot of the PCR product by agarose gel electrophoresis, the DNA was directly ligated into the pGEM-T vector (Promega, Mannheim, Germany) according to the manufacturer's instructions and subsequently transformed to competent high-efficiency *Escherichia coli* cells (strain JM109; Promega, Mannheim, Germany). White and blue screening was used to screen for recombinant transformants. Inserts of positive clones were analyzed for redundancy by amplified rDNA restriction analysis (ARDRA) (44). One representative clone was sequenced for each distinct fragmentation pattern by *Taq* Cycle sequencing with the PCR primers or primers M13uni and M13rev on a model ABI 377 sequencer (PE Corporation, Norwalk, Conn.). The sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (32). Sequences were aligned and analyzed by use of the ARB software package (Technical University Munich, Munich, Germany) according to the last release of the 16S rDNA sequence database of December 1998 as well as individually added sequences of recent publications. Trees were calculated for the clones and selected related sequences following the suggestions of Ludwig et al. (31). For tree calculation, maximum-parsimony, distance matrix, and maximum-likelihood methods were used, and the results were combined in a consensus tree.

TABLE 1. Organisms used for determination of T_d, their sequence at the target region, and hybridization results with probe Nmo218

Organism or probe	Sequence	FISH result with probe Nmo218
Probe sequence Nmo218 (3′→5′)	TACGAAAACCTCGCCGGC	
Target sequence (5′→3′)	AUGCUUUUGGAGCGGCCG	
Nitrosomonas ureae Nm10[a]	.....	+
Nitrosomonas cryotolerans Nm55[a]	G.....	+
Nitrosomonas aestuarii Nm36[a]	G.....	+
Pseudomonas lemoignei DSM 7445[b]	.....A.....	−
Nitrosomonas communis Nm2[a]	G.......A.....	−
Nitrosococcus mobilis Nc2[a]	GC.....	−

[a] Donated as fixed pure cultures from G. Timmermann, University of Hamburg, Hamburg, Germany.
[b] Active culture received from the German Culture Collection (DSMZ) and fixed as described in the text.

Probe design. Based on the newly retrieved and published sequences of the *Nitrosomonas oligotropha* lineage (40), a probe was designed by using the PROBE_DESIGN tool of ARB. The dissociation temperature of the probe was determined by hybridization with a pure culture of *Nitrosomonas ureae* isolate Nm10 at formamide concentrations ranging from 0 to 60%. Analysis was done by confocal laser-scanning microscopy (CLSM) and image analysis as described elsewhere (13). The specificity of the probe was checked against the ARB database and experimentally tested under the optimal hybridization conditions with the strains listed in Table 1.

FISH. Complete substratum elements with adhering biofilm were fixed with fresh 4% paraformaldehyde solution and alternatively with ethanol, washed with phosphate-buffered saline (PBS), and stored in PBS-ethanol (1:1) at −20°C until further processing (1, 33). After freezing and removal of the plastic material, radial biofilm sections with a thickness of 14 μm were prepared at −18°C, immobilized on gelatin-coated microscope slides, and dehydrated in an ethanol series (50). In situ hybridizations of cells in the biofilm were performed with fluorescently labeled, rRNA-targeted oligonucleotide probes according to the method of Manz et al. (33). The probes and conditions used are listed in Table 2. Probes labeled with the sulfoindocyanine dyes Cy3 and Cy5 were obtained from Interactiva (Ulm, Germany) and Biometra (Göttingen, Germany). In cases in which stringency conditions did not allow simultaneous hybridization with several probes, multiple probe hybridization was performed in subsequent steps by first hybridizing with the probe of higher stringency (58). The biofilm was additionally stained with 4′, 6′-diamidino-2-phenylindole (DAPI) after the hybridization step with a solution of 1 mg liter^{-1} for 10 min or 100 mg liter^{-1} for 10 s for the purpose of polyphosphate staining (23). Samples were analyzed by standard epifluorescence microscopy on a Zeiss Axioplan II microscope and by CLSM on a Zeiss LSM 510 microscope (Carl Zeiss, Jena, Germany).

Quantification of AOB and NOB. Total AOB abundance was quantified by microscopical counting of cells hybridized with probes Nso1225 and Nso190 in

TABLE 2. Oligonucleotide probes and hybridization conditions applied in this study

Probe	Probe sequence (5′→3′)	Target site[a]	Target organism(s)	% of formamide[b]	NaCl concn (mM)[c]	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S (338–355)	Domain *Bacteria*	20	225	1
NON338	ACTCCTACGGGAGGCAGC	16S (338–355)	Complementary to EUB338	20	225	33
ALF968	GGTAAGGTTCTGCGCGTT	16S (968–985)	Most α-proteobacteria and other bacteria	35	80	37
ALF1b	CGTTCGYTCTGAGCCAG	16S (19–35)	Most α-proteobacteria and other bacteria	20	225	33
ALF4-1322	TCCGCCTTCATGCTCTCG	16S (1322–1339)	Subgroup 4 of α-proteobacteria	40	56	37
RRP1088	CGTTGCCGGACTTAACC	16S (1088–1104)	Genera *Rhodobacter*, *Rhodovulum*, *Roseobacter*, and *Paracoccus* and other bacteria	0	900	37
BET42a[d]	GCCTTCCCACTTCGTTT	23S (1027–1043)	β-Proteobacteria	35	80	33
HGC69a	TATAGTTACCACCGCCGT	23S (1901–1918)	Gram-positive bacteria with high GC content	20	225	46
HGC1351	CAGCGTTGCTGATCTGCG	16S (1351–1368)	Gram-positive bacteria with high GC content	30	112	16
GAM42a[d]	GCCTTCCCACATCGTTT	23S (1027–1043)	γ-Proteobacteria	35	80	33
Nso1225	CGCCATTGTATTACGTGTGA	16S (1225–1244)	Ammonia-oxidizing β-proteobacteria	35	80	35
Nso190	CGATCCCCTGCTTTTCTCC	16S (190–208)	Ammonia-oxidizing β-proteobacteria	55	20	35
Nsm156	TATTAGCACATCTTTCGAT	16S (156–174)	Various *Nitrosomonas* spp.	5	636	35
Nsv443	CCGTGACCGTTTCGTTCCG	16S (444–462)	*Nitrosospira* spp.	30	112	35
NmII	TTAAGACACGTTCCGATGTA	16S (120–139)	*Nitrosomonas communis* lineage	25	159	40
NmIV	TCTCACCTCTCAGCGAGCT	16S (1004–1023)	*Nitrosomonas cryotolerans* lineage	35	80	40
NEU[d]	CCCCTCTGCTGCACTCTA	16S (653–670)	Halophilic and halotolerant members of the genus *Nitrosomonas*	40	56	60
Nse1472[e]	ACCCCAGTCATGACCCCC	16S (1472–1489)	*N. europaea*	50	28	22
NmV	TCCTCAGAGACTACGCGG	16S (174–191)	*Nitrosococcus mobilis* lineage	35	80	40
NOLI191	CGATCCCCCACTTTCCTC	16S (191–208)	Various members of the *Nitrosomonas oligotropha* lineage	30	112	43
cNOLI[f]	CGATCCCCCACTTTCCCC	16S (191–208)				This study
Nmo218	CGGCCGCTCCAAAAGCAT	16S (218–235)	*Nitrosomonas oligotropha* lineage	35	80	This study
NIT3[d]	CCTGTGCTCCATGCTCCG	16S (1035–1048)	*Nitrobacter* spp.	40	56	61
NSR826	GTAACCCGCCGACACTTA	16S (826–843)	Various *Nitrospira* spp.	20	225	49
NSR1156	CCCGTTCTCCTGGGCAGT	16S (1156–1173)	Various *Nitrospira* spp.[g]	30	112	49
Ntspa712[d,h]	CGCCTTCGCCACCGGCCTTCC	16S (712–732)	Phylum *Nitrospira*	35	80	14
Ntspa662[d,h]	GGAATTCCGCGCTCCTCT	16S (662–679)	Genus *Nitrospira*	35	80	14

[a] rRNA position according to *E. coli* numbering (9).
[b] Percentage of formamide in the hybridization buffer.
[c] Concentration of sodium chloride in the washing buffer.
[d] Used together with an equimolar amount of unlabeled competitor oligonucleotides as indicated in the reference.
[e] Referred to as S-*-Nse-1472-a-A-18 in the reference.
[f] Unlabeled competitor oligonucleotide applied in equimolar amount to discriminate against weak mismatches in *Thiobacillus thioparus* and two sequences of *Azoarcus* spp.
[g] Excluding clones described by Burrell et al. (10).
[h] Referred to as S-*-Ntspa-0712-a-A-21 and S-G-Ntspa-0662-a-A-18, respectively, in the reference.

several fields of view along a 50-μm-thick horizontal layer up to a biofilm depth of 600 μm. Distinct populations of AOB were counted after hybridization with probes NEU and NOLI191. Quantification of NOB was done by CLSM as previously described (48); i.e., optical sections with a defined thickness of 0.6 μm were scanned, and by determining the signal area and subsequent multiplication with a volume-specific cell abundance, the cell number for a given volume was calculated. Thorough calibration for NOB quantification was performed by counting DAPI-stained cells in various scanned fields of view ($n = 30$), leading to volumetric indices with a 95% confidence interval of ±7%. Results with all probes used for quantification were corrected for nonspecific binding according to results with probe NON338 as a negative control.

For quantitative population analysis with FISH, means and medians were calculated to describe the distribution of the AOB and NOB populations.

Analysis of *amoA* sequences. To supplement results from FISH and the specific 16S rDNA library, comparative sequence analysis of biofilm-derived 491-bp fragments of the ammonia monooxygenase gene (*amoA*) was performed as previously described (42). Amplificates of *amoA* were separated according to their GC content by agarose gel retardation as described by Schmid et al. (47).

Nucleotide sequence accession number. The 16S rDNA partial sequences obtained in this study are available from the EMBL nucleotide sequence database under accession no. AJ297415 to AJ297419. The *amoA* partial sequences appear under accession no. AF293065 to AF293075 and AY007575.

RESULTS

Functional analysis. Concentrations of the solutes measured by microsensors in the bulk liquid of the measuring setup closely reflected the bulk liquid composition in the reactor as determined independently by online monitoring (data not shown).

During the nonaeration period, oxygen could be detected in neither the bulk water nor the biofilm at any time. During the aeration period of the process, the biofilm was supplied with oxygen at 227.8 ± 5.4 μM (mean ± standard deviation [SD], $n = 16$), corresponding to 80.2% ± 1.9% air saturation. Oxygen penetration into the biofilm was limited to a depth of 200 μm (Fig. 1), leaving substantial parts of the biofilm anoxic during the whole treatment. The average areal oxygen uptake remained stable throughout the oxic period at 0.84 ± 0.05 μmol cm^{-2} h^{-1} (mean ± SD, $n = 16$). Neither the penetration depth nor the slope through the diffusive boundary layer was significantly altered during the oxic period (Fig. 1).

With ammonium, nitrate, and nitrite, absolute concentrations showed a certain variability, but the shapes and time courses of profiles between different cycles were similar. A strong initial decrease of ammonium directly after filling from 2,300 μM ($t = 25$ min) to 950 μM ($t = 60$ min) could be observed in the biofilm, analogous to the decline within the bulk water in the first 60 min (profiles not shown). Later, the concentration of ammonium decreased less but continuously up to the end of the treatment cycle to 800 μM. The concentration in the biofilm followed this pattern. Because no gradients of ammonium were observed above or within the biofilm, ammonium uptake of the whole biofilm or of any specific layer could not be quantified.

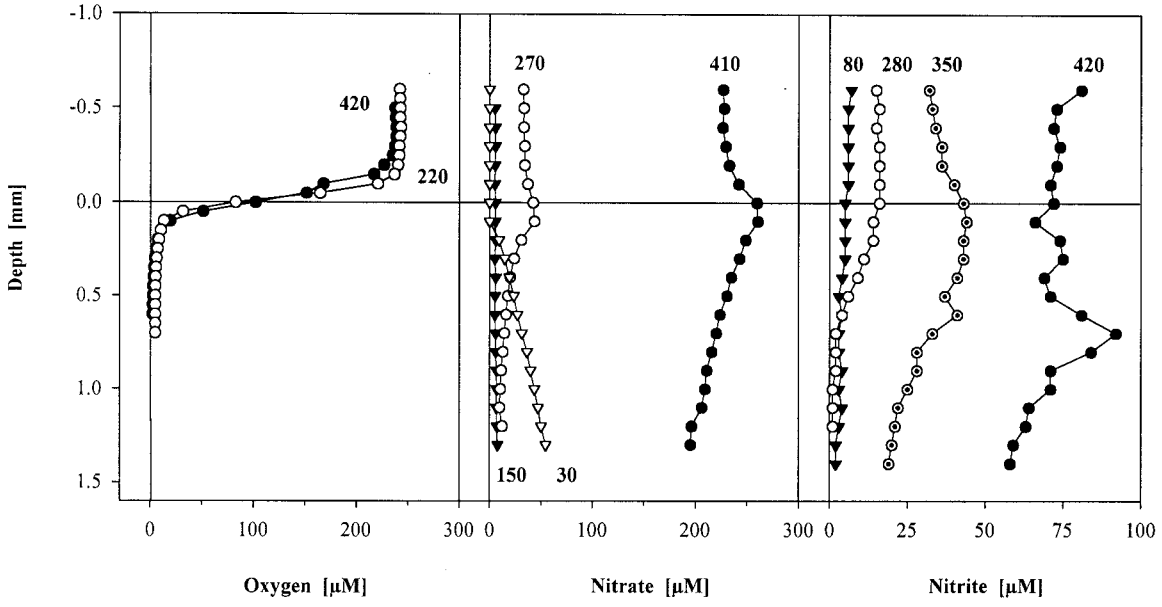


FIG. 1. Representative examples of vertical concentration microprofiles of oxygen, nitrate, and nitrite measured in biofilm at different times of the reactor cycle. Numbers refer to the time in minutes after start of the treatment cycle (start of aeration, t = 180 min).

Production of nitrate during oxic conditions was restricted to a narrow surface layer of about 200 μm, causing an accumulation of nitrate in the bulk water of up to 230 μM in the final period of the treatment (Fig. 1). The highest concentration of nitrate measured in the productive layer was 260 μM. During the following nonaeration period (after draining and refilling the system), the remaining nitrate was detected up to a concentration of 200 μM in the deeper biofilm zones, but it continuously decreased during the anoxic period. At the beginning of the aeration (t = 180 min), no nitrate from the previous treatment cycle was left in the biofilm (Fig. 1). The shape of nitrate profiles supported a denitrifying activity in the deeper biofilm layer. Nitrite accumulated during aeration up to 75 μM. In the second half of the aeration treatment period, an even stronger local production of nitrite occurred in the deeper biofilm layers at a depth of 600 to 700 μm, which was probably due to denitrification, with concentrations of more than 90 μM (Fig. 1).

During the aeration period, there was a conspicuous delay in the first occurrence of both nitrite and nitrate compared to the onset of aeration. Detectable amounts of both solutes (>1 μM) were measured first at t = 270 min, i.e., 90 min after onset of aeration.

A nitrogen balance based on nitrification products (300 μM), remaining ammonium (800 μM), and an assumed stripping and incorporation into bacterial biomass of 25% (61) indicates an unresolved N loss of about one-third.

Calculation of nitrate production. Volumetric nitrate production rates were separately estimated for the productive surface (depth of 0 to 200 μm) and the deeper biofilm (300 to 600 μm) from profile data measured in four different batch runs. The deeper layer showed some denitrifying activity up to about 1.0 μmol cm^{-3} h^{-1} at t = 360 min, evolving together with nitrifying activity in the upper layer. The nitrate production in the surface layer during aeration reached maximum estimated rates of 1.7 μmol cm^{-3} h^{-1} (corresponding to 0.03 μmol cm^{-2} h^{-1}) and thus accounted for about 7% of the oxygen uptake at the end of the process (Fig. 2).

Broad-scale community structure. Among all phylogenetic groups tested, four groups dominated the biofilm community, as shown by FISH: (i) members of the gram-positive bacteria with high DNA G+C content (GPBHGC), which were mainly coccoid cells forming loose aggregates; (ii) members of the β-proteobacteria, forming dense layers at the very surface and dense globular aggregates mostly located within the upper 200 μm; (iii) a population morphologically similar to the

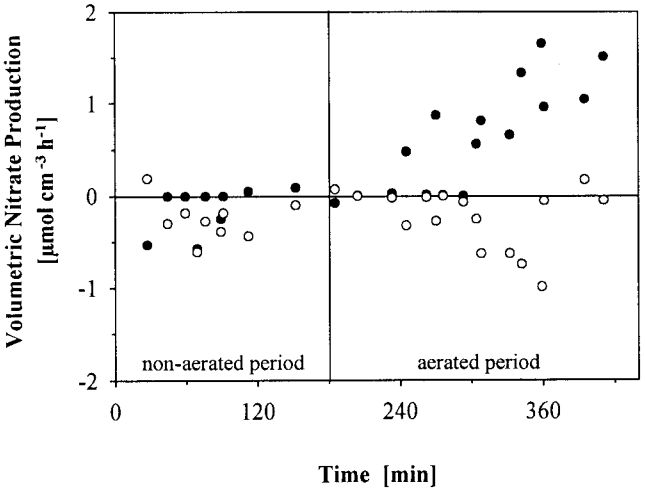


FIG. 2. Volumetric net production and consumption of nitrate at the biofilm surface (0 to 200 μm [●]) and in the deeper biofilm (300 to 600 μm [○]) during the reactor cycle. Data points originate from measurements of nitrate microprofiles of four different batch runs. For details of calculations, see text.

GPBHGC, with cells typically arranged in tetrads, that hybridized with probes ALF968, ALF1b, and GAM42a, but not with probes for subgroups of the α-proteobacteria (Fig. 3A), leaving their phylogenetic affiliation as yet unresolved; and (iv) members of the phylum *Nitrospira* (see below). Other phylogenetic groups together did not account for more than 20% of the microbial community in the biofilm (data not shown). The vast majority of all cells were located in the upper 300 μm of the biofilm, whereas in the deeper, permanently anoxic layers, only a few cell aggregates could be detected occasionally.

Additional staining with DAPI in high concentrations, indicating polyanionic inclusions by yellow fluorescence (23), was exclusively colocalized with hybridization signals for GPBHGC. However, only a fraction of the GPBHGC population was stained yellow with DAPI.

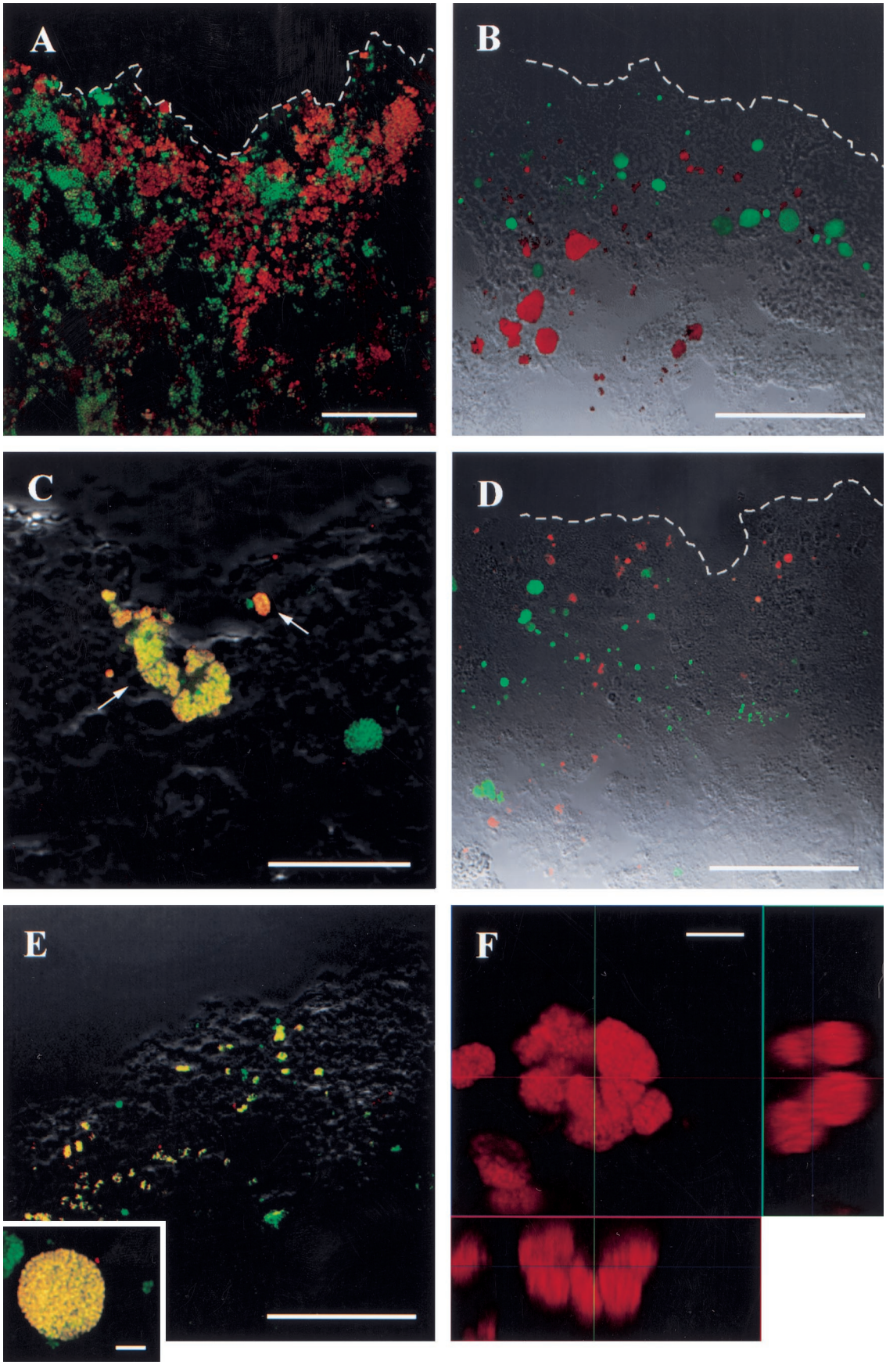
AOB community structure. A common problem for the quantification of nitrifying bacteria is the formation of dense aggregates resulting in a typical patchy distribution of AOB and NOB (Fig. 3). Therefore, neither normal distribution of values nor homogeneity of variances is given throughout the biofilm. For that reason, the median may be a better representative of cell densities than the mean and will be given in the following sections. To allow comparison with other studies, however, both means and medians are displayed in Fig. 4.

The globular aggregates hybridizing with probe BET42a were shown to belong to the AOB of β-proteobacteria by FISH with probes Nso1225 and Nso190 (Fig. 3B). The abundance of AOB was highest at the biofilm surface (Nso1225, 2.9×10^9 cm^{-3}; Nso190, 2.0×10^9 cm^{-3}) and declined below 1×10^8 cm^{-3} within the first 200 μm. A cumulative mean of 95% of all AOB could be detected within the first 200 μm (Fig. 4A). A few single aggregates of β-ammonia oxidizers occurred in the deeper biofilm as well, but the abundance in these layers on average was very low. Hybridization with probes Nsm156 and Nsv443 revealed that the complete AOB community consisted of members of the genus *Nitrosomonas.* Within this genus, three different subgroups of AOB were detected. The smallest fraction (which was not further quantified) belonged to the *Nitrosomonas communis* lineage (40) of β-subclass AOB, as identified by hybridization with probes Nso1225 and NmII (Fig. 3C). They formed small aggregates and were restricted to the upper 100 μm. The two dominant subpopulations showed distinct distribution patterns. One population belonged to the *Nitrosomonas europaea* lineage of β-subclass AOB (40), as identified by hybridization with probes Nso1225, Nsm156, and NEU (Fig. 3D). Hybridization with Nse1472 or NmV resulted in no signals, indicating that the population is not identical to *N. europaea* or *Nitrosococcus mobilis*. The second population hybridized with Nso1225 and NOLI191 (Fig. 3D and E), a probe that had been designed for the *N. oligotropha* lineage (40) based solely on the sequence of *N. ureae* (43). Both groups together accounted for from 55 to 100% of β-subclass AOB hybridizing with probe Nso1225 in the upper 200 μm of the biofilm. At the surface, 22 and 33% of Nso1225-positive cells hybridized with probes NEU and NOLI191, respectively. At a depth of 200 μm, about 90% of Nso1225-positive cells hybridized with probe NOLI191, whereas the abundance of the *N. europaea* lineage declined to less than 10% (Fig. 4B). No signals were detected after hybridizations with probe NmIV specific for *Nitrosomonas cryotolerans*.

AOB-specific PCR. Because hybridization with a single probe is sometimes not sufficient to prove the identity of a given cell (2), a specific PCR and cloning strategy was applied to support the occurrence of populations affiliated with the *N. oligotropha* lineage within the biofilm. From the 26 clones analyzed by ARDRA, 10 different restriction patterns were obtained. Three of the patterns, representing a total of 13 clones, were indicative of sequences most similar to *Nitrosomonas* isolate JL21 (55), a member of the *N. oligotropha* lineage (Fig. 5). Two patterns, representing a total of eight clones, belonged to sequences most similar to *Nitrosomonas communis* Nm2. The remaining five clones with different ARDRA patterns represented single sequences not related to the genus *Nitrosomonas*. The amplification of these sequences and of the ones similar to *Nitrosomonas communis* was due to insufficient primer discrimination under the conditions chosen.

Design and application of probe Nmo218. The sequence of probe NOLI191 differs from the target sites of most of the recently reported 16S rRNA sequences of the *N. oligotropha* lineage (42, 52, 55). Furthermore, the probe hybridizes to organisms (e.g., *Pseudomonas lemoignei*) not belonging to the intended target group. Therefore, we designed probe Nmo218 encompassing the whole *N. oligotropha* lineage (Fig. 5). The probe sequence and binding sites of target and nontarget organisms are displayed in Table 1. The dissociation temperature of the probe was 40.1 ± 1.7°C, and the optimal formamide concentration in the hybridization buffer was 35%. When applied under these conditions, probe Nmo218 did not hybridize with any negative controls, except for *Nitrosomonas cryotolerans* and *Nitrosomonas aestuarii* (Table 1). (The sequences of five clones with identical binding sites to *N. cryotolerans* or *N. aestuarii* might also not be discriminated.) The occurrence of *N. cryotolerans* can, however, be ruled out by parallel use of probe NmIV. Hybridization of probe Nmo218 to the biofilm resulted in a picture similar to that with probe NOLI191 (Fig. 3D and E). However, simultaneous hybridization with both probes revealed a certain number of organisms only hybridizing with either of the two probes. Cells of *N. aestuarii* hybridized with probe Nmo218 (Table 1), but not with probe NOLI191. Consequently, cells showing this hybridization pattern might be related to *N. aestuarii*.

AOB diversity assessed by comparative *amoA* sequence analysis. Specific amplification of the *amoA* gene fragments from extracted biofilm DNA and subsequent separation by gel retardation resulted in three clearly visible *amoA* bands. All bands were excised and separately cloned and sequenced. A total of 12 *amoA* clones (4 from each band) were analyzed, which represented five phylogenetically distinct β-subclass AOB (Fig. 6). Two clusters (representing two different bands), each containing four *amoA* sequences, were found within the *Nitrosomonas marina-N. oligotropha* lineages (which cannot clearly be distinguished by using *amoA* sequences) (42). The third band contained a higher diversity of *amoA* sequences. One *amoA* clone was closely related to *Nitrosomonas communis.* Another clone was affiliated with *Nitrosococcus mobilis,* which could not be detected in the biofilm by FISH. The two remaining *amoA* clones obtained from the third band clustered together and formed an independent lineage not closely related to any described AOB.


A
B
C
D
E
F

NOB community structure. The only NOB detected in the system were of the genus *Nitrospira,* as identified by hybridization with probes Ntspa712, Ntspa662, and NSR826 (Fig. 3B and F). Low numbers of cells also hybridized with probe NSR1156. The abundance of *Nitrospira* spp. in the upper 100 μm of the biofilm was 1.1×10^{11} cm^{-3} and therefore was about 30 times higher than the abundance of β-subclass AOB, as quantified with probe Nso1225. In comparison to the AOB, the vertical distribution was broadened towards depth. Within the section investigated, a 95% limit in cumulative (mean) abundance was reached, although not before a depth of 400 μm, and the numbers were high even in deeper layers of the biofilm (Fig. 4C).

Cell-specific nitrification rates. The average cell density of NOB in the upper 200 μm of the biofilm where nitrification was mainly performed was 6.6×10^{10} cm^{-3}. Because nitrate production in this layer during aeration on average was 0.7 μmol cm^{-3} h^{-1}, conversion rates were about 0.01 fmol of nitrite cell^{-1} h^{-1} for the final aeration period. Maximum rates were 0.025 fmol of nitrite cell^{-1} h^{-1}. Cell-specific ammonia oxidation rates can only be roughly estimated based on nitrate production rates. With the average abundance of AOB of 1.09×10^{9} cm^{-3} in the upper 200 μm, mean conversion rates were at least 0.65 fmol of ammonium cell^{-1} h^{-1} (maximum of 1.5 fmol of ammonium cell^{-1} h^{-1}). However, because of the accumulation of nitrite within the nitrification zone, this number is clearly an underestimate.

DISCUSSION

Biofilm activity and community structure. In the biofilm studied, biological phosphorus removal is combined with nitrogen removal via nitrification and denitrification. Nitrification is typically performed by distinct phylogenetic groups and will be discussed later. For biological P removal, involvement of various groups has been suggested: e.g., GPBHGC (12, 59), members of the genus *Rhodocyclus* (7, 8, 21), or a mixture of multiple species (34). The high abundance of GPBHGC and the detection of DAPI-stainable inclusions indicative of polyphosphate (23) in part of this population strongly promote the first hypothesis for our system. However, the fixation procedure used might have led to the disappearance of polyphosphate granules (21), and therefore PAO, especially among gram-negative bacteria, might have been overlooked.

Denitrification cannot at all be assigned to a certain microbial population, nor is it restricted to a certain zone of the biofilm. During the anoxic period, the surface layers denitrify nitrate that has been accumulated during the oxic period of the preceding cycle (Fig. 1 and 2) and by that contribute about 20% to the total N loss. During the oxic period, the deeper parts of the biofilm remain anoxic and are provided with nitrate via nitrification (Fig. 1). The contribution of denitrification in the 300- to 600-μm-deep biofilm during the oxic period to total N loss is about 15%. Because there are reports of denitrifying PAO (28, 57) and because GPBHCG as potential PAO were found in close proximity to nitrifying bacteria, the source of nitrate, involvement of PAO in denitrification appears to be possible. However, this cannot be proven based upon our data.

Competition for oxygen. Both nitrification and phosphate uptake require oxygen. Therefore, competition for oxygen between the respective microbial populations during the aeration period is to be expected. Microsensor data indicate that the nitrifying bacteria were oxygen limited. Oxygen penetration was low, whereas ammonium was present in excess during the whole process. The distribution of AOB and NOB in general corresponds to the oxygen penetration depth, supporting the findings of earlier studies (39, 50). The delayed onset of nitrification after the start of the aeration and the accumulation of nitrite despite the high abundance of NOB very likely reflect the limited supply of oxygen. Due to their high K_m for oxygen, AOB and NOB are poor competitors compared to heterotrophic bacteria (17, 41, 56). Thus, during the initial aeration period, oxygen is taken up preferentially by heterotrophs as the PAO. As the phosphate uptake rate declines with the ongoing aeration period (data not shown), the stoichiometric oxygen demand of PAO metabolism decreases as well (51). Consequently, during the course of the aeration period, phosphate accumulation is most likely replaced by nitrification in terms of oxygen consumption.

Nitrification rates. The handling of concentration data to estimate volumetric nitrate production rates (see Materials and Methods) is based on two assumptions: (i) low lateral heterogeneity of the concentration in the biofilm at a certain time and (ii) slow dynamics of the concentration changes due to activity compared to those due to diffusion (i.e., a pseudo-steady-state situation). Structural analysis showed a certain lateral inhomogeneity based mostly on the clustering of nitrifiers (Fig. 3B), but inhomogeneity is low in the horizontal direction, and the resolution of the measurements was not in the range of the cluster size. Therefore, the first assumption is not violated. For a similar system (36), it was shown that the time required to reach equilibrium was 1.8 min in a biofilm with a thickness of 500 μm. This is quite short compared to the overall cycle dynamics observed here. Nevertheless, the second assumption may introduce some error, especially in highly dynamic layers. The rates determined here should therefore be regarded as best estimates.

The rates of both estimated cell-specific in situ ammonia oxidation and nitrate production are on the order of magni-

FIG. 3. Confocal laser-scanning micrographs of vertical thin sections of biofilm as hybridized with different fluorescent oligonucleotide probes. (A) Overview of the biofilm surface hybridized with ALF968 and HGC1351 (in green and red, respectively). (B) Aggregates of β-subclass AOB as hybridized with Nso1225 (green) and NOB of the genus *Nitrospira* (Ntspa662, red). (C) Clustered aggregates (arrows) of members of the *N. communis* lineage (NmII, red) among the population β-subclass AOB (Nso1225, green). Colocalization of the two probes results in a yellow color. (D) Aggregates of members of the *N. europaea* lineage as hybridized with probe NEU (green) and of the *N. oligotropha* lineage as hybridized with probe NOLI191 (red). (E) Colocalization of signals after hybridization with probes specific for the β-subclass AOB (Nso1225, green) and the *N. oligotropha* lineage (Nmo218, red); the insert shows a big aggregate as hybridized with probes Nso1225 (green) and NOLI191 (red). (F) Orthogonal representation of a dense assemblage of *Nitrospira* sp. as hybridized with probe Ntspa662. Scale bars are 50 μm (A, B, D, and E), 25 μm (C), and 5 μm (insert in panels E and F), respectively. Dashed lines indicate the surface of the biofilm exposed to the wastewater.

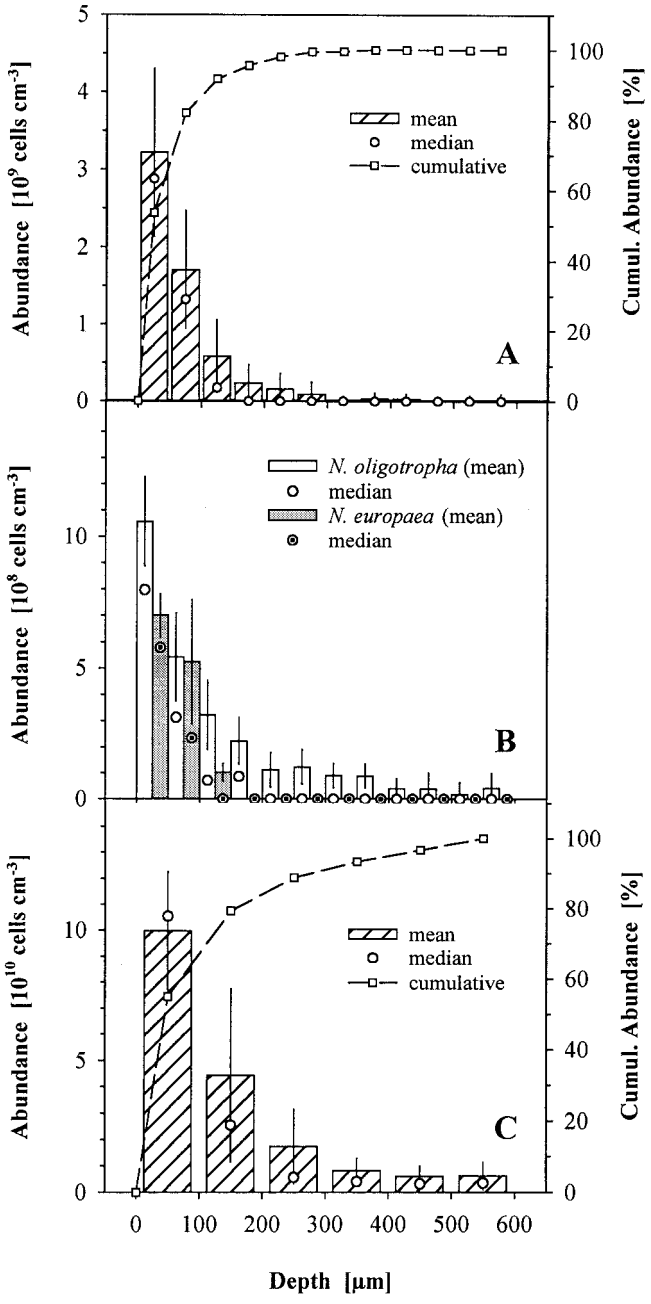


FIG. 4. Depth distribution of β-subclass AOB (A), of populations affiliated with different lineages of the genus *Nitrosomonas* (B), and of the NOB *Nitrospira* spp. (C) in the biofilm investigated. Numbers are given as volume-specific abundances. Cumul, cumulative. Note the different scales of the ordinates.

tude of those calculated for aggregates of a nitrifying fluidized bed reactor based on microsensor measurements (48) and the ammonia oxidation rate estimated by a process mass balance of a sewage treatment plant (60). Unfortunately, the resolution of our measurements was too low to distinguish between the ammonia oxidation rate of the surface layer (with the mixed nitrifying community) and that of the deeper layer (with the *N. oligotropha*-like population). Also, the different activities of each of the individual AOB populations in the surface layer or of individual cells within a monospecies cluster cannot be resolved. Therefore, it has to be noted that the cell-specific ammonia oxidation rate calculated here represents an average value for a diverse assemblage of AOB consisting of at least three different populations.

Community structure of AOB. The combined approach of FISH, 16S rDNA, and *amoA* analyses led to the unexpected but consistent finding that there were several different AOB populations occurring in close spatial vicinity. In contrast, previous studies in biofilms and activated sludge usually reported a single population dominating the system, e.g., populations related to *N. europaea* (39, 50), *Nitrosococcus mobilis* (22), or members of the genus *Nitrosospira* (39, 49). This raises the question of what mechanisms allow for the coexistence of the different AOB populations in the biofilm studied here.

N. communis-like AOB were found only within the first 100 μm at the biofilm surface and only in low abundance. Isolates of this lineage originally were obtained from soils (24) and were recently also detected in activated sludge and biofilm systems (42). Unfortunately, the lack of ecophysiological data about *N. communis* and its exclusive occurrence in the same zone with both members of the *N. europaea*- and *N. oligotropha*-like AOB leave the specific adaptation of *N. communis*-like AOB to our system unresolved.

The second population was identified as members of the *N. europaea* lineage, although these bacteria were not identical to *N. europaea* itself. The low number of *amoA* sequences analyzed allows for some hidden diversity, because several clones might be represented by one band in the gel retardation. The *N. europaea*-like population detected by FISH thus might be unrepresented by any *amoA* clone. Members of this phylogenetic cluster, like, e.g., *N. europaea* and *N. eutropha,* are typically isolated from activated sludge systems (24), and the maximum substrate conversion rates for *N. europaea* are high compared to those of other strains of AOB (41). Pure culture and chemostat experiments revealed low substrate affinities for *N. europaea* with K_m (NH_4^+) values in the range of 0.4 to 7 mM (41) and 0.88 to 1.96 mM (29), respectively. The same is true for oxygen affinity, with K_m (O_2) values between 6.9 and 17.4 μM (29). While ammonium concentrations in the biofilm exceeded most of the reported K_m values, oxygen limitation for *N. europaea*-like AOB is obvious. Between a depth of 100 and 200 μm, the oxygen concentration decreases from 33.8 ± 5.45 μM to 7.0 ± 1.01 μM (mean ± SD, $n = 15$). Consequently, *N. europaea*-like AOB virtually disappear within these layers (Fig. 4B). Here, the biofilm is dominated by *N. oligotropha*-like AOB with an abundance of 1×10^8 to 2×10^8 cells cm^{-3} down to a depth of 400 μm. Based on 16S rDNA sequence analysis, members of the *N. oligotropha* lineage (also referred to as *Nitrosomonas* cluster 6a [26, 54]) have recently been detected in freshwater and brackish environments (52, 53), terrestrial habitats (26, 54), and activated sludge (42, 55). This suggests high physiological versatility and ecological importance. Isolates of this lineage are sensitive to ammonium concentrations exceeding 10 to 60 mM (24, 53, 55), show low K_m (NH_4^+ plus NH_3) values, and possess urease activity (53). These features support adaptation of the *N. oligotropha* lineage to low substrate concentrations. Although no kinetic data with respect to oxygen are available, this might also imply high affinity towards oxygen. Lower K_m (O_2) values of the *N. oligotropha*-like AOB

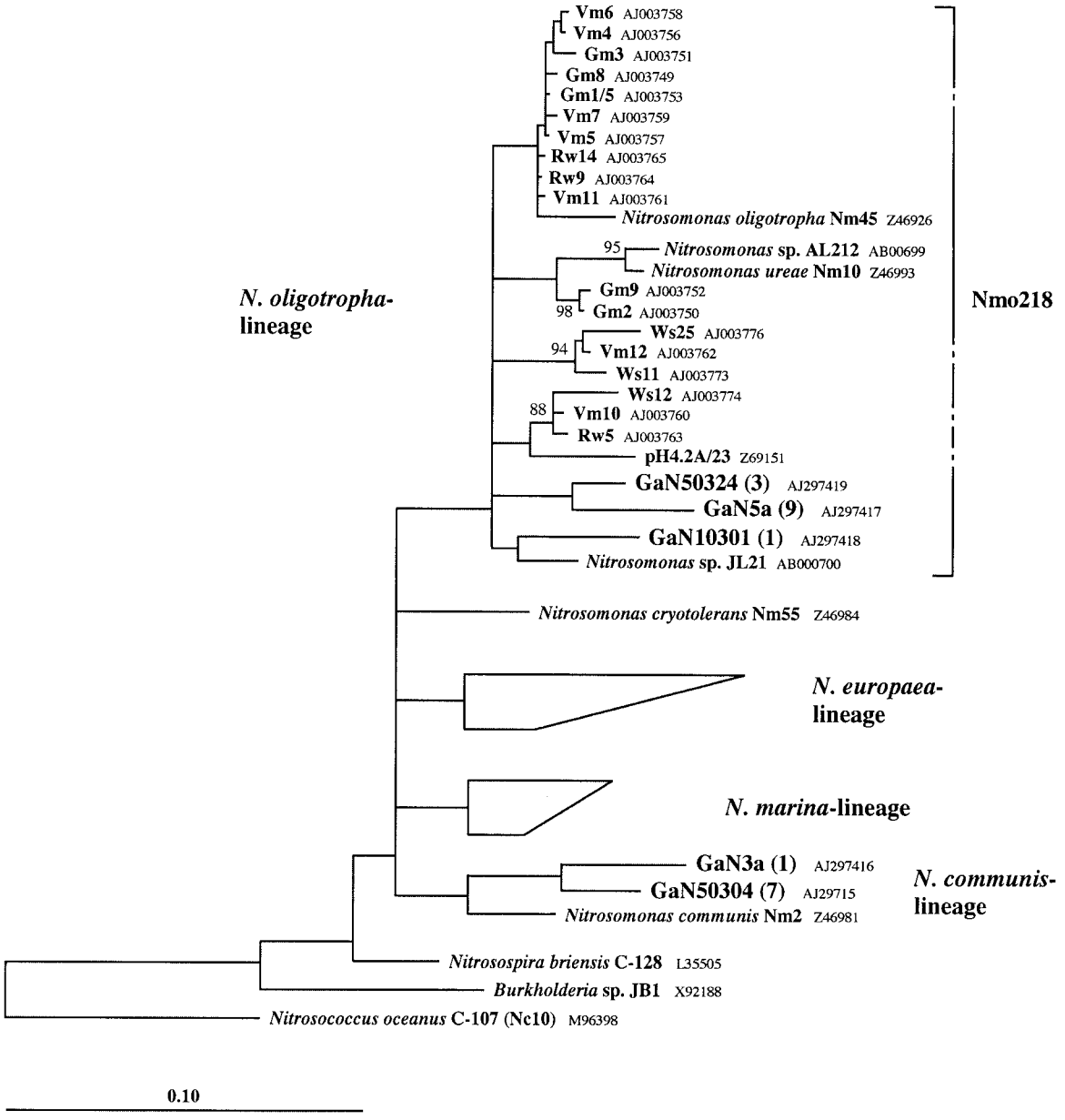


FIG. 5. Phylogenetic tree of the genus *Nitrosomonas* inferred from comparative analysis of 16S rDNA sequence data. The accession numbers of the published sequences used are given in the tree. Sequences with accession no. AB000701, AB000702, AJ005546, M96399, M96402, M96403, and Z46987 represent the *N. europaea* lineage, and sequences with accession no. AJ003777, M96400, Z46990, Z69091, and Z69097 represent the *N. marina* lineage. The numbers of clones with identical ARDRA patterns for each sequence are given in parentheses. Phylogenetic reconstruction is based on a maximum-likelihood tree calculated from 950 informative positions with a genus-specific 50% positional variability filter. Tree topology was tested by distance matrix and maximum-parsimony methods, and a consensus tree was drawn. Multifurcations connect branches for which a relative order could not unambiguously be determined by the different treeing methods used. Bootstrap values (100 cycles) refer to the maximum-parsimony tree. Values smaller than 80% were omitted. The sequence of *N. oligotropha* isolate Nm45 was added to the consensus tree by the ARB maximum-parsimony method without changing the tree topology. The bar represents 10% estimated sequence divergence.

than the values reported for *N. europaea*-related AOB could be responsible for the outcompetition of the latter at the oxic-anoxic transition zone in the biofilm. At the biofilm surface, however, both populations were found to coexist in almost equal abundance. Assuming higher maximum substrate conversion rates for *N. europaea*-like AOB (41), they should be able to outcompete other AOB in this zone. For explanation of the co-occurrence, the dynamics of the system have to be taken into account (i.e., the metabolic activity of the populations might be separated in time). In the initial aeration period, *N. oligotropha*-like AOB in particular might be active, because, as suggested above, their higher oxygen affinity allows competition for oxygen with the highly active heterotrophic PAO. In the late oxic period, when oxygen uptake of the heterotrophic PAO decreases, *N. europaea*-like AOB could become more active. Their relatively high maximum substrate conversion

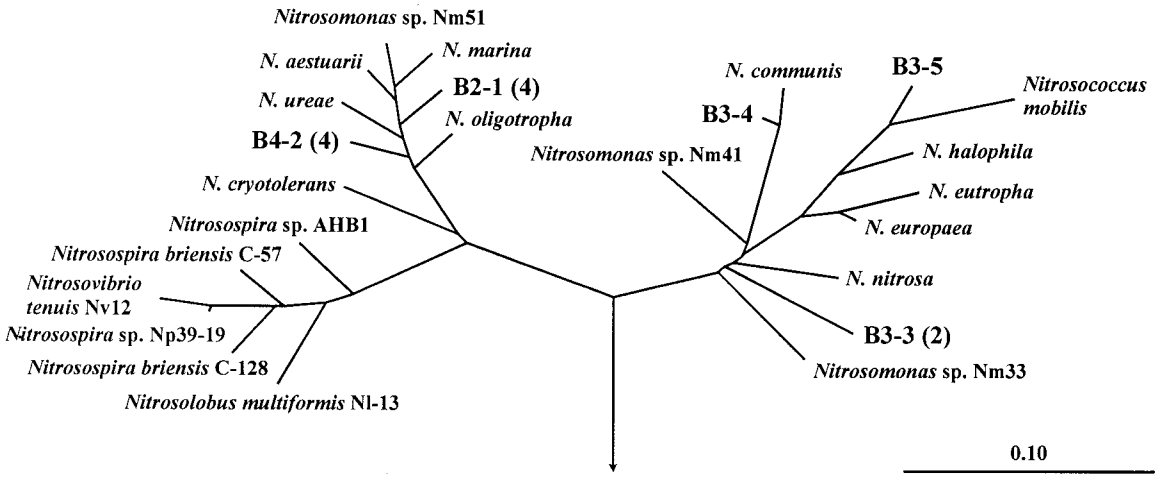


FIG. 6. Phylogenetic FITCH-Margoliash *amoA* tree (using global rearrangement and randomized input order [7 jumbles]) showing the position of the 12 recovered biofilm sequences in relation to described β-subclass AOB (42). The bar indicates 10% estimated sequence divergence. The root was determined with the *amoA* sequences of the γ-subclass ammonia-oxidizing bacteria (42). Cloned *amoA* sequences with amino acid similarities >99% are represented by a single clone: the number in parentheses indicates the number of clones for each representative. Clones labeled with the prefixes B2, B3, and B4 were obtained from different gel retardation bands.

rate might compensate for their time-limited activity and contribute to the successful establishment of this population in the biofilm. It has been shown for biofilm populations of AOB that the abundance is likely not to be affected by short starvation periods (i.e., a few days). Furthermore, recovery for both growth and activity of AOB from starvation is very rapid. This effect is likely to be coupled to high densities of AOB, as found in biofilms, and is probably due to cell-cell signaling (4). The high cell density and occurrence in dense clusters we found are in agreement with this hypothesis. Based on this strategy, it can be argued that even merely short-term activity would allow AOB to persist in the biofilm.

In addition, there are reports about an anoxic type of metabolism in *N. europaea* and *N. eutropha* when electron acceptors other than oxygen are used (6). The ability to survive or even thrive during anoxic conditions is, however, likely to differ among the three AOB populations. Therefore, the nonaeration period of the reactor cycle might be another factor to support a mixed community of AOB. However, alternative hypotheses explaining the coexistence are possible. Detection of AOB populations in situ does not prove their recent activity (60). It can be speculated that the ability to maintain the ribosome content during inactive periods might be stronger in the *N. oligotropha* lineage than in the *N. europaea* lineage. This would cause *N. europaea*-like AOB to become more rapidly undetectable by FISH in deeper layers of the biofilm compared to the results with *N. oligotropha*. Currently, we have no evidence in favor of this hypothesis, but future studies including the detection of local activity (e.g., by use of microautoradiography) (30) might help to support or reject this hypothesis. By *amoA* analysis, three more clones could be identified, related to *Nitrosococcus mobilis* (B3–5) and not closely affiliated with any isolated *Nitrosomonas* strain (B3–3). Because neither population could be identified by FISH, we assume the populations were either low in numbers or were in a dormant state.

NOB population. Using different probes for NOB, the existence of a certain population of *Nitrospira* spp. could be proven, whereas *Nitrobacter* spp. were not detected. This is in agreement with several other culture-independent studies of engineered systems (10, 22, 39, 48, 49, 61). Analysis of the probe match pattern in the current data set by the software package ARB (Technical University Munich, Munich, Germany) showed that it fits a cluster of closely related sequences in the genus *Nitrospira* (strains with accession no. Y14636 to Y14643) retrieved from a nitrite-oxidizing sequencing batch reactor by Burrell et al. (10). The NOB population in our system is thus likely to be affiliated with this particular cluster of the genus *Nitrospira*. It might be speculated whether this cluster possesses common functional features leading to a competitive advantage under the periodically changing conditions typical for sequencing batch reactors. The cell densities of *Nitrospira* spp. found in this study are on the order of magnitude of those reported from a purely nitrifying fluidized bed reactor (48). However, the abundance is 1 order of magnitude higher than that of the AOB. The question remains of how such a high abundance is supported, when the substrate turnover is speculated to be low (48).

Conclusions. Through the combination of microsensor measurements and molecular methods, it was possible to resolve the structure and activity of the nitrifying community in a complex, P-removing biofilm. Some first insights were obtained into the mechanism that allows for the coexistence of different populations of AOB in the same biofilm, i.e., separation of their distributions in space and separation of their activities in time. The design of probe Nmo218 specific for the *N. oligotropha* lineage will enable in situ detection and quantification of *N. oligotropha*-like AOB in future studies to collect more information about their natural abundance and ecology.

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1. **Purkhold, U., A. Pommering-Röser, S. Juretschko, M. C. Schmid, H.-P. Koops, and M. Wagner.** (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66**(12): 5368-5382.
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