**Lehrstuhl für Mikrobiologie**

# Structure and function of microbial aggregates in wastewater treatment: Floc formation and aerobic ammonia/anaerobic ammonium oxidation

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**My parents**

**Meinen Eltern**

# A Introduction

Since the first humans began to settle, they started to produce waste and wastewater in an excessive way. Their uncontrolled disposal within the cities caused outbreaks of serious diseases like typhus or cholera, which forced many lives. With the beginning of the industrialization in the 19$^{th}$ century and the exponential increase of produced waste, strategies to clarify sewage compounds were developed (e.g. Kröhnke, 1900). During the last century, various changes and improvements were applied to these techniques. Today for treatment of large amounts of municipal wastewater most commonly the activated sludge system (see below) is used. Nevertheless, this method is often hampered by serious problems, like sludge bulking caused by activated sludge flocs with poor settling ability or by complete breakdowns of nitrification. Since nitrification, which is the oxidation of ammonia –one of the most toxic nitrogen compounds in sewage–, over nitrite to nitrate, is a central process in nutrient removal via wastewater treatment, nitrification failure results in environmental pollution.

Though wastewater treatment systems are vital to our environment, they are often poorly understood. The general introduction of this thesis will give a brief summary of the current knowledge in wastewater treatment with special regard to (i) attached and suspended biofilms like activated sludge flocs, and (ii) ammonium/ammonia oxidation. In addition, it will summarize problems of common microbiological techniques for the examination of wastewater treatment systems.

## A1 Wastewater treatment systems

This section provides a short introduction of commonly used and recently introduced wastewater treatment systems, which were investigated in this study.

### A1.1 The activated sludge process

The most common technique in biological wastewater treatment is the activated sludge process (Tchobanoglous and Burton, 1991). It consists of an aerated suspension of a mixed microbial culture, which spontaneously forms flocs. The aeration is applied by compressed air, by pure oxygen or by a mechanical setup and fulfills two essential functions. It provides (i) a steady upflow or turbulence with a rate exactly balancing the settling velocity of the activated sludge (Figure 1) and (ii) the oxygen required for the microbial oxidation of waste water compounds like ammonia. After a certain dwell of the sewage in the aerated basin, it is transferred to a secondary clarifier, where the flocs are left settling. During this time anaerobic processes like denitrification can be observed. The cleared water is removed and the remaining sludge is partly recycled to the activated sludge basin to keep a high biomass concentration in the aeration tank and to provide a continuous reinoculation. This results in an extended retention time of the sludge and an improved adaptation of microorganisms to their environment. The not recycled part of the floc biomass matching the amount of biomass produced in the activated sludge basin is transferred as excess sludge to the sludge dewatering.

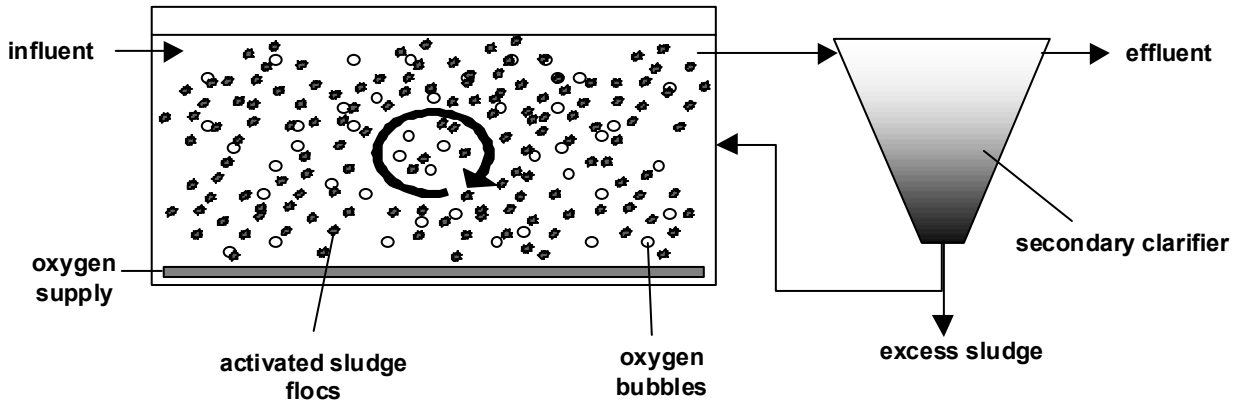



Fig. 1: Schematic drawing of an activated sludge basin

## A1.2 The trickling filter

The trickling filter is the most widely distributed biofilm reactor. As fill material for the filter tower lava stones or alternatively plastics are in use. The wastewater is poured over the top of the biofilm covered fill material and is cleaned by trickling down to the bottom, where it is removed. Aeration is accomplished through the bottom of the filter tower by a steady air flow only driven by differences in temperature from the filter material to the waste water and the surrounding air (Figure 2; Henze et al., 1997).

The major problem in the conventional trickling filter is clogging. Small plants with a low load and unhindered growth of biofilm often show local clogging. The biofilm grows too thick and the circulation of air is no longer possible. Consequently, the biofilm is starting to foul and to degrade until air is able to circulate again. Another factor influencing these plants is the grazing of the biofilm by worms, snails and larvae. Nevertheless, is the load increased in such plants the clogging runs out of control till a certain point, when the shearing effect of the water trickling through the filter becomes this high, that biofilm is continuously washed out of the system.

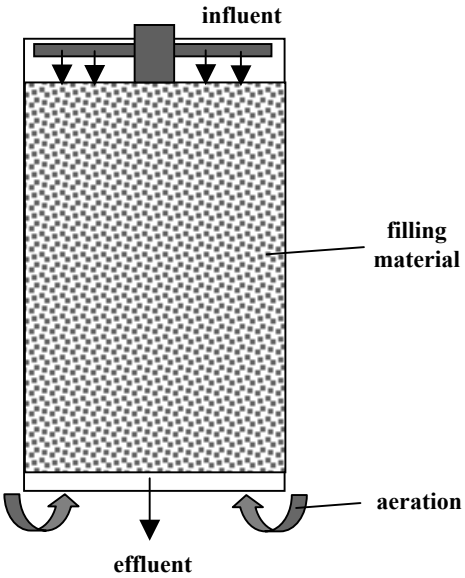



Fig. 2: Schematic drawing of a trickling filter

## A1.3 The sequencing batch reactor

The sequencing batch reactor (SBR) was introduced by Irvine *et al.* in 1977. This reactor type uses a process were populations of organisms are discontinuously provided with medium or waste water (Figure 3; Rubio *et al*., 1988; Wilderer, 1992).

The reactor sequence starts with the addition of nutrient during a defined filling phase. By choosing the filling velocity and the volume transfer rate a control of the occurrence and frequency of substrate concentration increase is possible. While slow filling and therefore continuously low substrate concentration within the reactor will select for organisms with high substrate affinity (low $k_s$ value), a fast filling and temporarily increase of substrate concentration lowers the selective pressure on organisms with high $k_s$ value.

With adjusting the biomass retention time (remaining sludge volume in the reactor/removed sludge volume per day) the medium growth rate limit for organisms to stay in the reactor can be set. A high sludge age provides an opportunity for slow growing organisms to hold their ground in the reactor.

The continuous change from high to low substrate concentration also stimulates the production of extracellular polymeric substances (EPS) during starvation periods. This could lead to a better aggregation and lower heterogeneity of the flocs resulting in a decrease of the sludge volume index (SVI).

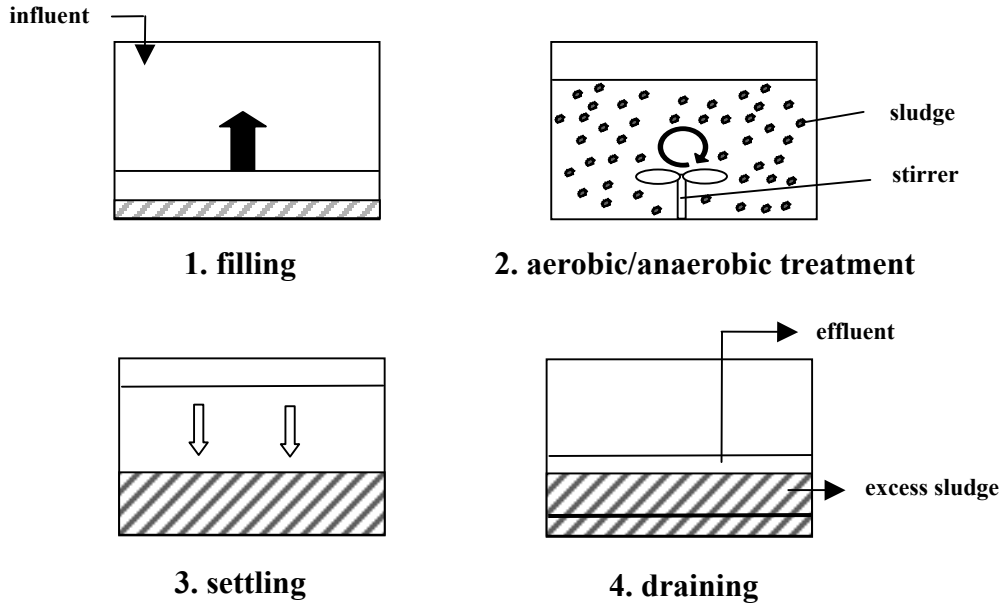



Fig 3: Schematic of a SBR cycle. The aerobic/anaerobic treatment (step 2) could be also applied in separated steps.

The major advantage of the SBR is its flexibility in providing different growth conditions in well-defined phases during the batch sequence. For example, the reactor could be driven aerated continuously, alternatingly aerated and not aerated to support nitrification and denitrification in one reactor, and also completely not aerated to force anaerobic growth (Strous et al., 1998).

## A1.4 The rotating biological contactor

The rotating biological contactor consists of discs in a row situated on a turning axe (Figure 4A). Normal plants possess 3 to 4 turning rows of discs ordered in sequence (Figure 4B), which are partially drowned in a basin filled with wastewater (Figure 4A). Since the biofilm keeps growing on the smooth surface of the discs, biomass is permanently sheared off into the basin. When the biofilm gets in contact with the air during a turn its surface will become oxygen saturated and oxygen dependent physiological processes are performed. After drowning of the biofilm into the water the oxygen diffusion turns into the wastewater and supplies the suspended biomass with oxygen. Another effect of the turning of the row of discs is the creation of turbulences, which keep the suspended biomass floating and causes a permanent wash out of biomass according to the construction of the discs and the basin into a secondary clarifier.

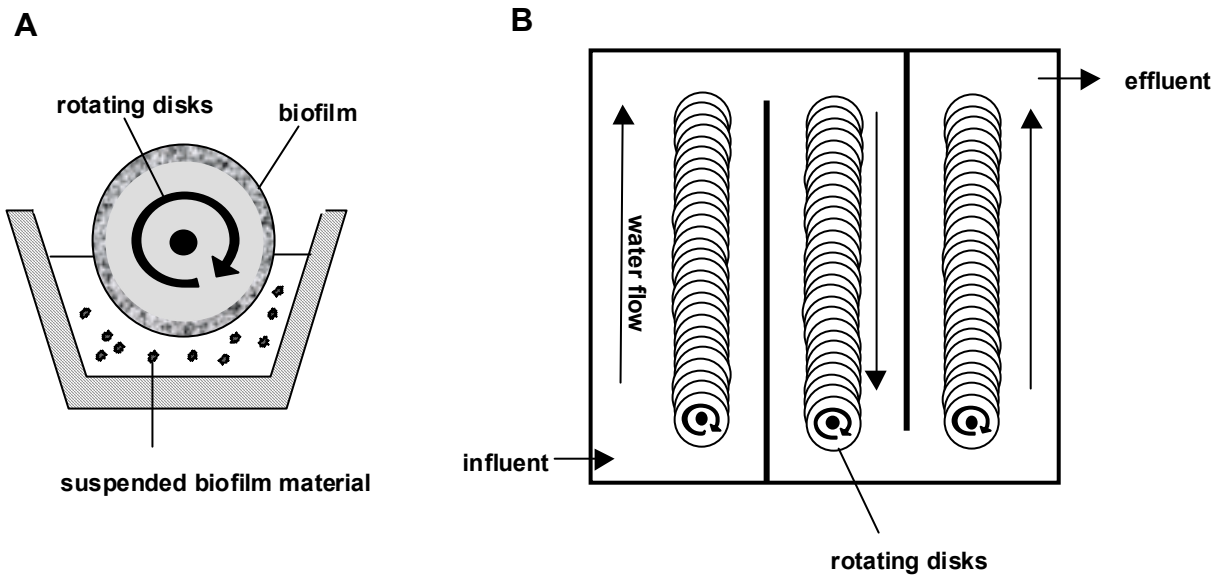



Fig 4: **A** Cross section of a rotating disk biological contactor. **B** Schematic of the layout of a rotating disk biological contactor.

## A1.5 The moving bed reactor

Similar to a fixed bed reactor the biofilm in a moving bed reactor grows on a small cylindric plastic material (Figure 5A). These carriers are not tightly packed, but floating in the waste water like activated sludge on a steady upflow or turbulence with a rate exactly balancing their settling velocity. Since this upflow or turbulence can be generated by either influx of gas (e.g. oxygen, Figure 5B) or stirring (Figure 5C), an aerobic or anaerobic operation of the moving bed reactor is possible (Odegaard *et al.,* 1994). This technique provides a higher settling rate, because of the bigger, more compact, biofilm covered plastic beads. This results in a better biomass retention as in the normal activated sludge process. Adversely, the reactor is more difficult to operate and more energy is necessary for the suspension of the carrier.

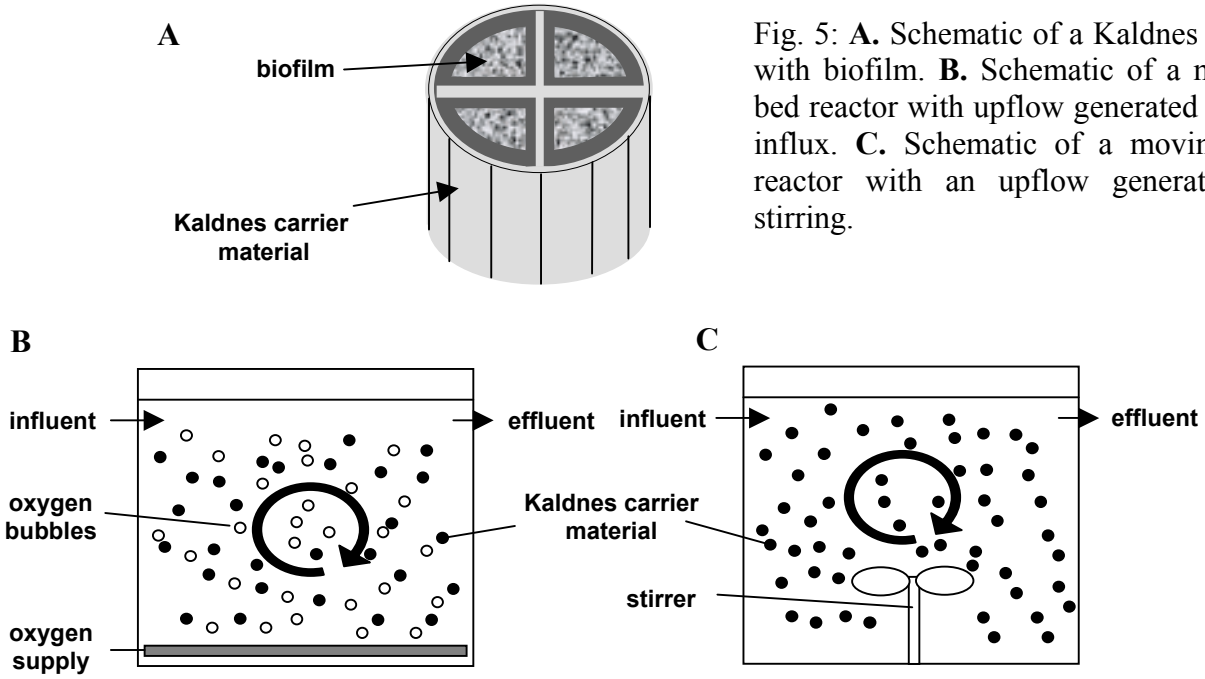



Fig. 5: **A.** Schematic of a Kaldnes carrier with biofilm. **B.** Schematic of a moving bed reactor with upflow generated by gas influx. **C.** Schematic of a moving bed reactor with an upflow generated by stirring.

## A2. Biofilms in waste water treatment

All the above mentioned techniques use biofilms in different forms for the conversion of nutrients and toxic inorganic or organic compounds of wastewater. But not only attached biofilms (biofilm is originally defined as conglomerates of microorganisms and extracellular polymeric substances attached to a surface; Characklis and Wilderer, 1989; see below) are

applied, but also in an extended version of the biofilm definition suspended forms like flocs or granules. Regarding full-scale wastewater treatment, the activated sludge floc rather compromises the most common biofilm.

## A2.1 Biofilm structure and function

Although the overall biofilm structure is dependent on growth rate (Tijhuis *et al.*, 1996), substrate concentration (Wimpenny and Colasanti, 1997), shear stress (van Loosdrecht *et al.,* 1997), grazing and cell surface properties (deBeer and Kühl, 1999), the most prominent structure of biofilms consist of more or less dense bacterial cell clusters surrounded by EPS with interstitial voids (Figure 6, Figure 7). A steep gradient within the biofilm leads to chemical conditions providing via substrate limitation different ecological niches in close neighborhood. For example, ammonium is converted on the oxygenated surface of the biofilm to nitrate (nitrification), which diffuses to deeper anaerobic parts of the biofilm. There nitrate is used as electron acceptor and thus reduced by classical denitrification to dinitrogen gas (Schramm *et al.,* 1996).

Many of the beneficial properties for bacteria within a biofilm are mediated by the EPS, which includes a variety of substances (mainly polysaccharides, but also proteins and free DNA; Sutherland, 1985). Most bacteria are capable of producing EPS, either as capsules attached to the cell or as secretion in the extracellular environment (e.g. Omar *et al.,* 1983; Uhlinger and White, 1983; Unz, 1989; Dugan, 1992). The role of EPS ranges from influences in cellular processes that relate to microbial competitiveness and reproductive success, such as the formation of microcolonies (Allison and Sutherland, 1987), attachment to surfaces (Marshall *et al.,* 1989) and increased resistance to antimicrobial substances (Uhlinger and White, 1983), to concentration of nutrients (Decho, 1990).

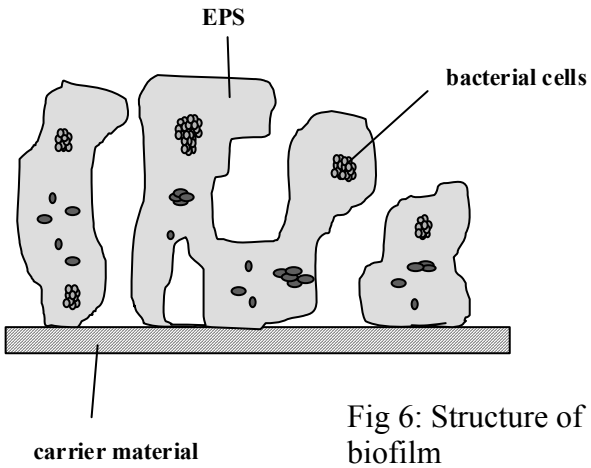



Fig 6: Structure of a common attached biofilm

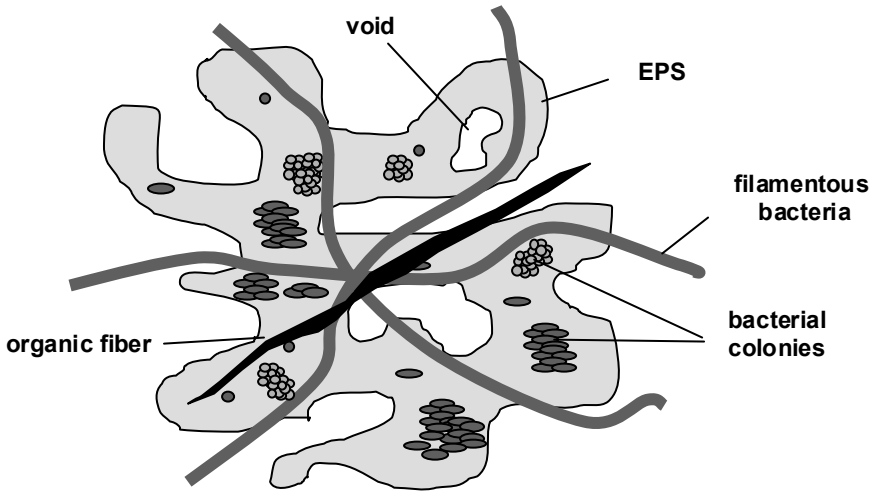



Fig 7: Common structure of an activated sludge floc (Wilén, 1999; modified)

## A2.2 Activated sludge flocs

The activated sludge floc, though not attached, also comprises the features of a biofilm. Clusters of cells and filamentous bacteria as backbone (Szegin *et al*., 1978) are imbedded in EPS occasionally with voids and a diffusion boundary layer. With regard to a better settling the ideal form of an activated sludge floc is large and compact (Figure 7). But this structure is, for example, not always suitable for an efficient nutrient transfer from the floc surface to the center resulting in decreased metabolic rate in the floc. In reality, the perfect floc morphology is difficult to achieve in wastewater treatment plants and thus various types of problems are directly related to the floc structure.

The most prominent malfunction is sludge bulking which is caused by extensive growth of filamentous bacteria leading to a less compact sludge with a reduced settling ability (e.g. Wanner and Grau, 1988; Jenkins, 1993; Novak *et al*., 1994; Wanner, 1994). Another undesired effect is the occurrence of pinpoint flocs. These are small flocs and dispersed bacteria, which do not settle by gravity. This causes a poor clarification and a low dewaterability (Pipes, 1979; Karr and Keinath, 1978). At last, there is the phenomenon of scumming or foaming of the sludge which is triggered by non-degradable surfactants or by the presence of certain bacteria like *Nocardia* sp., *Gordonia sp., Rhodococcus sp.* or "*Microthrix parvicella"* (e.g. Blackall, 1994; Goodfellow *et al.,* 1996; Davenport *et al.,* 2000). Thereby the sludge is building thick foam on the surface of the settler (Kappler and Gujer, 1994).

The quality of activated sludge flocs is currently monitored by the measuring of the sludge volume index (SVI) which is the volume occupied by 1g sludge after settling of 30 minutes in a cylinder. Values lower 100mg/l indicate normally a good settling sludge, while sludges with

values over 150mg/l are considered as bulking. In general, the SVI is well correlated with the functionality of the plant, but it provides no detailed information about the reason for the malfunction. As demonstrated the SVI is affected by the abundance of filamentous bacteria (e.g. Jenkins 1993; Wanner, 1994), floc size and heterogeneity (Barber and Veenstra, 1986; Sadalgekar *et al*., 1988; Andreadakis, 1993), and the chemical composition of the EPS (Urbain *et al*., 1993). Therefore, it is not possible to maintain a stable activated sludge process just on the basis of the SVI. A more comprehensive knowledge on the links between microbial community composition and floc structure could be achieved by the application of molecular methods like fluorescence in situ hybridization for the detection of bacteria responsible for bulking (e.g. Wagner *et al*., 1994a; Wagner *et al*., 1994b; Davenport et al., 2000) and further modern chemical and microscopic tools for the determination of EPS composition and floc architecture (e.g. Zartarian *et al*., 1997).

## A2.3 Granular sludge

Granular sludge was first described in upflow anaerobic sludge blanket reactors (UASBR; Lettinga, *et al*., 1980). These anaerobic granules comprise a common and very complex structure with two major zones, referred to as the cortex and the medulla, providing several ecological niches (Figure 8; MacLeod *et al*., 1990; Macario *et al*., 1991). It was possible to shift the bacterial populations by applying different substrates (Grotenhuis *et al.,* 1991) or to introduce a certain de novo degradative pathways into the anaerobic granular sludge by inoculation with a pure culture (Ahring *et al.,* 1992) without alteration of the granule architecture.

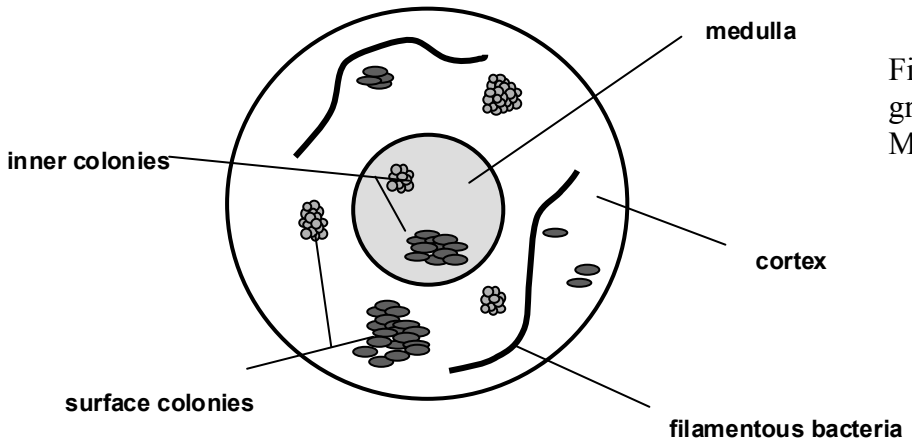



Fig. 8: Structure of anaerobic granular sludge according to Macario *et al.* (1991)

Keeping in mind the problems occurring in conventional sewage treatment regarding sludge bulking and foaming in activated sludge systems or the often fruitless application of bioaugmentation (Bouchez *et al*., 2000), the process stability, high settling ability and grazing resistance offered by granular sludge could provide important advantages. However, aerobic processes like nitrification and phosphorous removal could not be applied to anaerobic granular sludge. Therefore, the development and characterization of aerobic granules (van Loosdrecht *et al,* 1997; Etterer *et al,* 2001), which were also characterized as part of this thesis, is a prerequisite to establish this technique in conventional aerobic sewage treatment.

## A3 Ammonia/ammonium oxidation in microbial biofilms

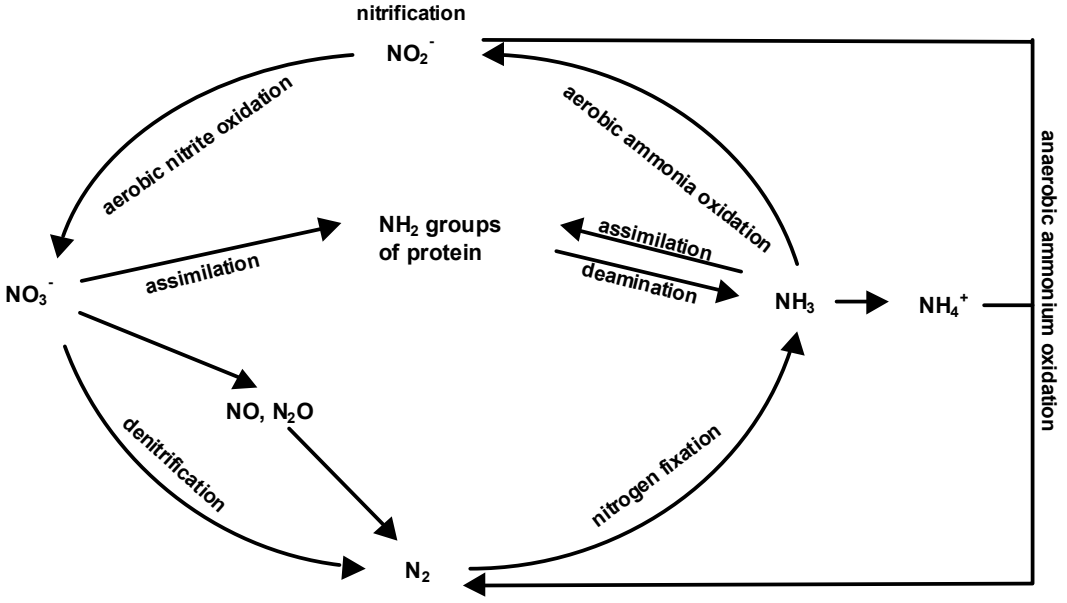



Fig. 9: Extended nitrogen cycle

The oxidation of ammonia or its ionized form ammonium to nitrate as a prerequisite for its further conversion to dinitrogen gas is one of the key processes in the global nitrogen cycle (Figure 9, Prosser, 1989). Due to the toxicity of ammonium and other higher oxidized nitrogen compounds to aquatic life (Painter, 1986, Arthur *et al*., 1987) and the risk of eutrophication of aquatic systems the N-removal represents an integral part of the secondary treatment in wastewater treatment plants. Two pathways for the biologically mediated oxidation of ammonium in the environment are known so far.

## A3.1 The aerobic ammonia oxidation

In conventional wastewater treatment plants ammonia is first converted by ammonia oxidizing bacteria to nitrite. Complete N-removal requires a further oxidation the nitrite to nitrate and its subsequent reduction in an additional heterotrophic step (denitrification) to dinitrogen gas. Since the whole process of N-removal consists of different stages, which need also different prerequisites to keep functional like, for example, the provision of enough carbon source to maintain denitrification, it is time consuming and difficult to control.

The aerobic ammonia oxidation is a two step reaction (eq. 1). The copper containing membrane-bound protein ammoniummonooxygenase (AMO) catalyzes the initial oxidation of ammonia to the intermediate hydroxylamine (Hollocher *et al*., 1981; Wood, 1986; Bedard and Knowles, 1989). Subsequently, the periplasmatic, tetrameric heme protein hydroxylamine oxidoreductase (HAO) oxidizes the hydroxylamine to nitrite (Sayavedra-Soto *et al*., 1994).

eq. 1: $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NO_2^- + 5H^+ + 4e^-$

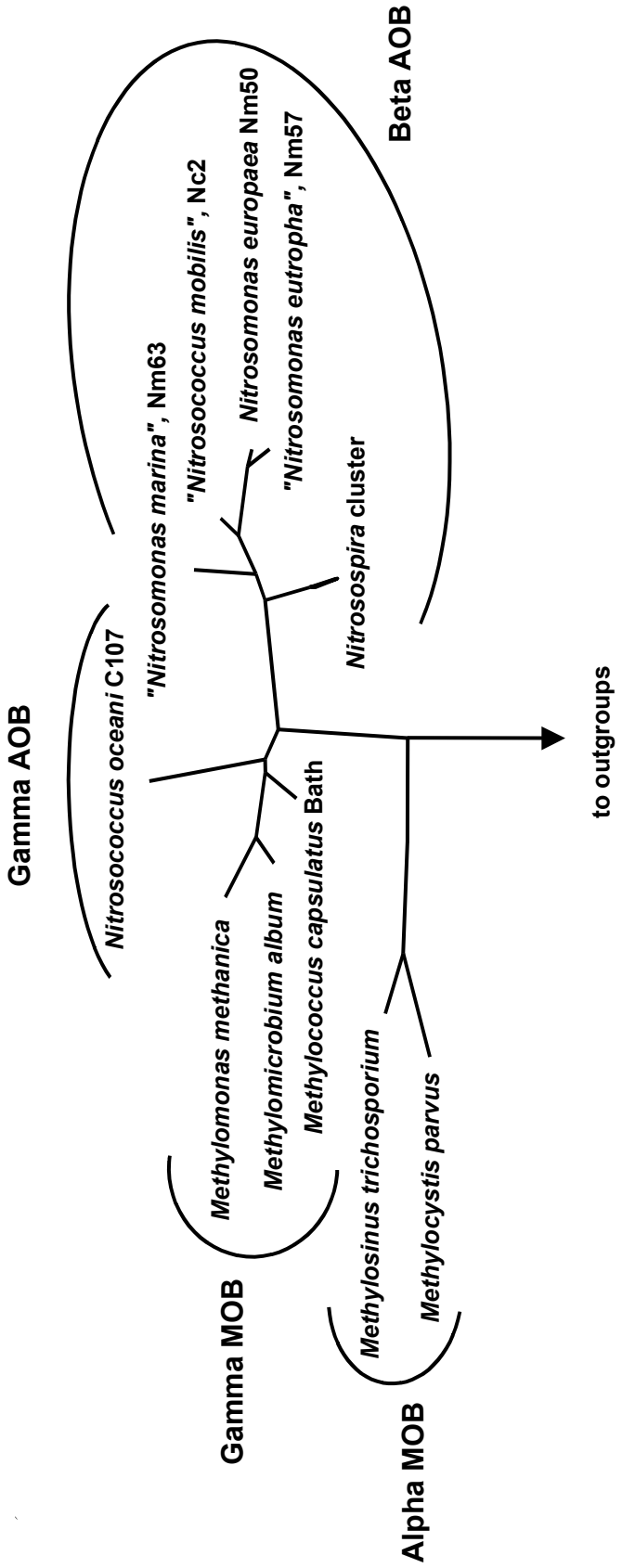
Since the redox potential of the reaction sequence is too low for a direct NADH regeneration, a reverse electron transport is necessary for gaining redox-equivalents (Bock *et al*., 1986).

Previous studies based on comparative 16S rRNA gene sequence analysis showed that the so far cultured and described species of autotrophic aerobic ammonia oxidizers (e.g. Koops *et al*., 1976, Watson *et al*., 1989; Koops *et al*., 1991) form two different monophyletic clusters within the *Proteobacteria* (e.g. Head *et al*., 1993; Stehr *et al*., 1995; Pommerening-Röser *et al*., 1996). The species *Nitrosococcus oceani* and *Nitrosococcus halophilus* belong to the *Gammaproteobacteria*, whereas the genera *Nitrosomonas* (including the species *Nitrosococcus mobilis*)*, Nitrosospira, Nitrosolobus* and *Nitrosovibrio* belong to the *Betaproteobacteria* (Figure 10). The later three genera are very closely related on the 16S rRNA gene level and it was therefore suggested to lump them together into the single genus *Nitrosospira* (Head *et al*., 1993).

Since isolation of ammonia oxidizing bacteria from environmental systems is difficult and time consuming, the 16S rRNA approach has frequently been applied to investigate the natural diversity of ammonia oxidizing bacteria (e.g. Stephens *et al.,* 1996; Kowalchuk *et al.,* 1997). Furthermore, quantitative dot blot hybridization as well as fluorencence in situ hybridization, was used to quantify the abundance of AOB in environmental systems (e.g. Wagner *et al*., 1995;

Mobarry *et al*., 1996; Schramm *et al.,* 1996; Juretschko *et al*., 1998; Okabe *et al.,* 1999). But with very high 16S rRNA gene sequence similarities within the closely related aerobic ammonia oxidizing bacteria of the *Betaproteobacteria* the 16S rRNA gene as phylogenetic marker was not longer sufficient to provide a fine scale differentiation of these organisms. After initial studies (Holmes *et al*., 1995, Rotthauwe *et al*., 1995) Rotthauwe and coworkers proposed in 1997 the less conserved *amo*A-gene coding for the active site carrying AmoA subunit of AMO as additional phylogenetic marker for fine scale differentiation of ammonia oxidizing bacteria (Figure 11). The evolution of the *amo*-homologous genes in those ammonia-oxidizers investigated to date has occurred orthologously rather than by horizontal gene transfer (Klotz and Norton, 1998). The genes of the three subunits of AMO (*amoA*, *amoB, amoC*) are located in 1 to 3 operon copies (McTavish *et al*., 1993; Klotz and Norton, 1995; Norton *et al*., 1996; Sayavedra-Soto *et al*., 1998), but the high similarity of these multiple operons indicate paralogous evolution (Klotz and Norton, 1998). Therefore, *amoA* fulfills the criteria to be used as phylogenetic marker (Ludwig *et al*., 1998) and thus enables a comparison of *amoA*/AmoA phylogeny with 16S rRNA gene phylogeny. Keeping in mind that nitrification in wastewater treatment is often affected by serious breakdowns, it is necessary to learn more about the links between nitrifier community composition and process stability. The 16S rRNA and *amoA* approach are valuable tools for high-resolution composition studies of ammonia oxidizer populations in complex systems, but these approaches suffer from incomplete coverage of the recognized ammonia-oxidizer species in the respective data sets. Furthermore, the available sequences are mostly partial and of minor quality. In environmental studies, phylogenetic analyses with this rudimentary data set often indicated “new species“ of ammonia oxidizers (e.g. Stephen *et al.,* 1996; Ward 1996; McCaig *et al.,* 1999). However, a definite interpretation of the obtained phylogenetic data is only possible if all recognized species of ammonia oxidizers are reliably (full sequence data with few ambiguities) represented in the data set.

Fig. 10 (next page): Phylogenetic neighbor-joining tree based on 16S rRNA reflecting the relationship between ammonia oxidizing bacteria (AOB) of the *Beta*- and *Gammaproteobacteria* and methane oxidizing bacteria (MOB) of the gamma and *Alphaproteobacteria*. MOBs were included, because their particulate methane monooxygenase (PMO) is highly homologous to the ammonium monooxygenase (AMO) of AOBs (see Figure 11). Only sequences with more than 1000 bases were taken into account (status end 1999). The bar indicates 10% estimated sequence divergence. It should be noted, that for some of the species shown complete recently published 16S rRNA sequence data (Purkhold *et al.* (2000)) were used to reconstruct this tree.


Gamma AOB
Nitrosococcus oceani C107
"Nitrosomonas marina", Nm63
"Nitrosococcus mobilis", Nc2
Nitrosomonas europaea Nm50
"Nitrosomonas eutropha", Nm57
Nitrosospira cluster
Beta AOB
Methylomonas methanica
Methylomicrobium album
Methylococcus capsulatus Bath
Gamma MOB
Methylosinus trichosporium
Methylocystis parvus
Alpha MOB
to outgroups
0.10

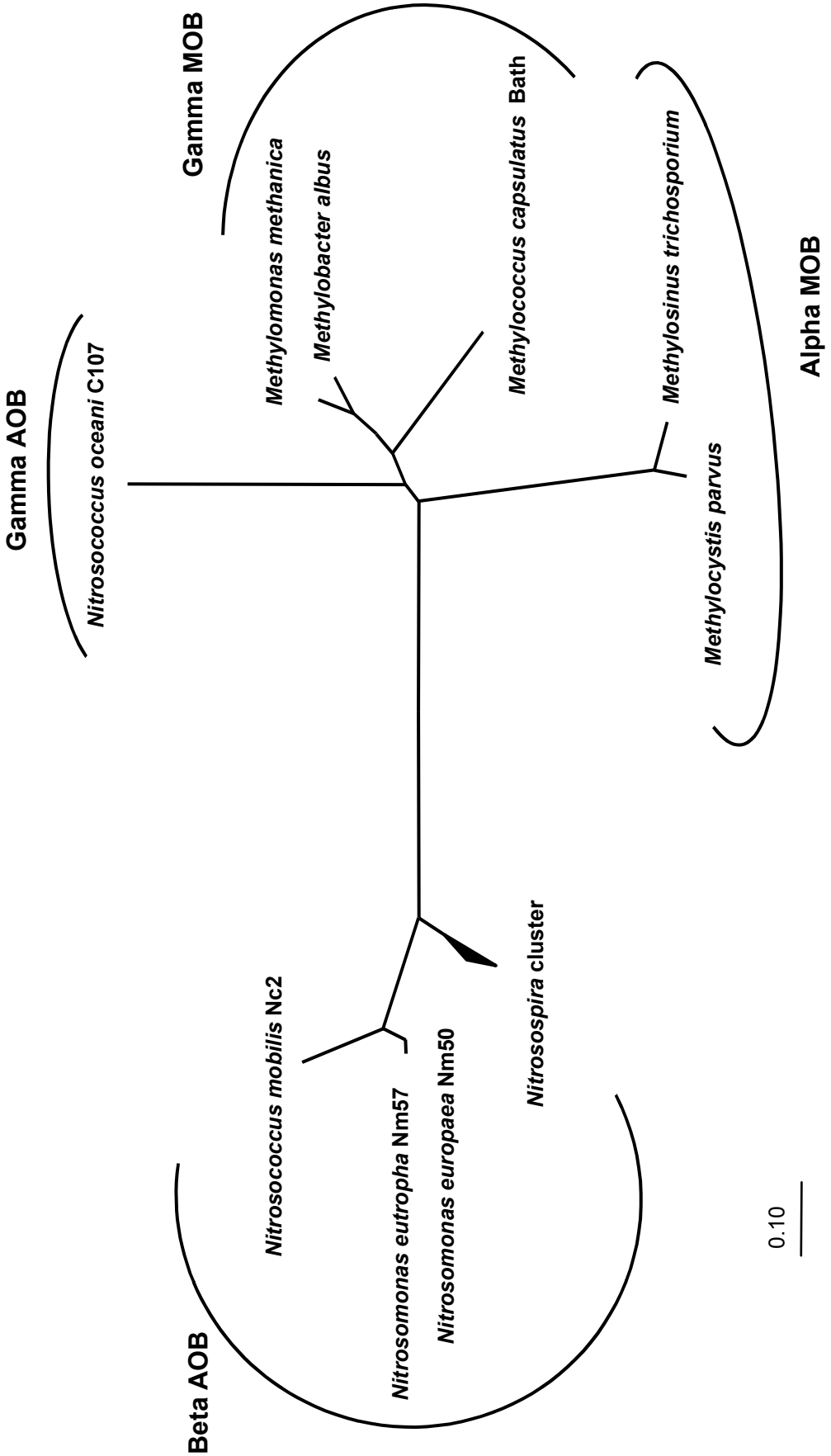

Gamma MOB
Gamma AOB
Alpha MOB
Beta AOB
Nitrosococcus oceani C107
Methylomonas methanica
Methylobacter albus
Methylococcus capsulatus Bath
Methylosinus trichosporium
Methylocystis parvus
Nitrosococcus mobilis Nc2
Nitrosomonas eutropha Nm57
Nitrosomonas europaea Nm50
Nitrosospira cluster
0.10

Fig. 11 (previous page): Phylogenetic Fitch-Margoliash tree based on deduced AmoA and PmoA sequences reflecting the relationship between ammonia oxidizing bacteria (AOB) of the *Beta-* and *Gammaproteobacteria* and methane oxidizing bacteria (MOB) of the *Gamma-* and *Alphaproteobacteria* (status 1999). MOBs were included, because their particulate methane monooxygenase (PMO) is highly homologous to the AMO of AOBs. The bar indicates 10% estimated sequence divergence.

## A3.2 The anaerobic ammonia oxidation

The existence of anaerobic ammonium oxidation as the reaction of ammonium as electron donor with nitrite as electron acceptor was first postulated by Broda in 1977. He considered the amount of free energy gained in this reaction as sufficient for autotrophic growth. The process itself was discovered 18 years later in a fluidized bed reactor (Mulder *et al*., 1995). The first yet not isolated organism, which catalyzed the anaerobic ammonium oxidation, was provisionally named *Candidatus* "Brocadia anammoxidans" (Strous, 2000).

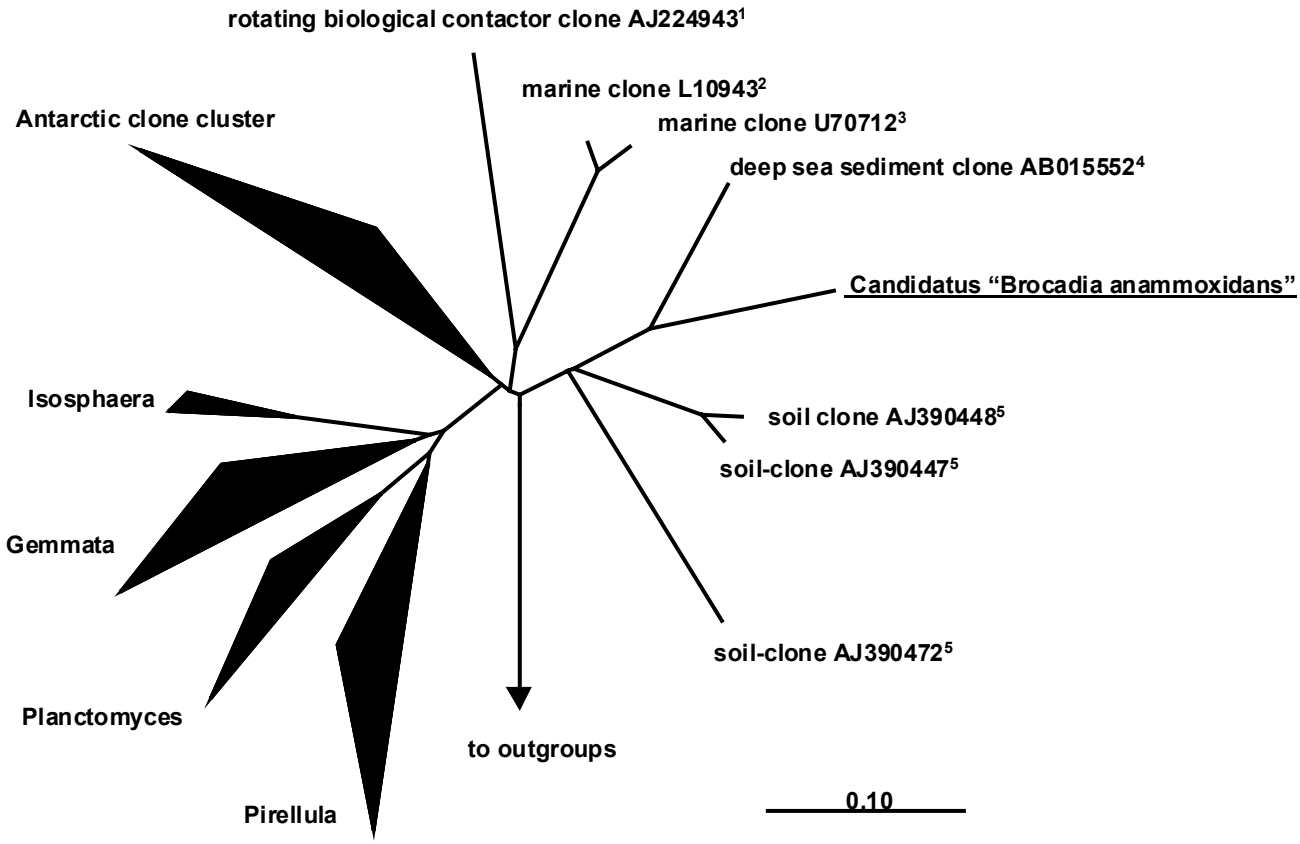


Fig. 12 (previous page): Phylogenetic tree based on 16S rRNA reflecting the relationships of *Candidatus* "Brocadia anammoxidans", environmentally derived 16S rRNA sequences of organisms with unknown physiology affiliated to the order *Planctomycetales,* the "classical" planctomycetes represented by isolates (genera *Pirellula, Planctomyces, Gemmata, Isosphaera*), and other reference organisms. The triangles indicate phylogenetic groups. The tree is based on results of maximum likelihood analyses on different data sets. The bar represents 10% estimated sequence divergence. The Antarctic clone cluster comprise mainly Antarctic clones, but also a methanogenic sludge clone and a sequencing batch reactor clone (for details please refer to Schmid *et al*., 2001). References: [1] Van der Meer *et al*., 1999; [2] DeLong *et al*., 1993; [3] Rappe *et al*., 1997; [4] Li *et al*., 1999; [5] Derakshani *et al*., 2001.

Three enzymes are proposed to be involved in the anaerobic ammonium oxidation. In an initial reaction ammonium is converted by a putative enzyme hydrazine hydrolase with hydroxylamin to hydrazine (eq. 2; Schalk *et al*., 1998). Subsequently, the hydrazine disintegrates mediated by a hydrazine oxidizing enzyme to dinitrogen gas (eq. 3; Strous 2000).The hydroxylamine for the initial reaction has to be recovered by the reduction of nitrite with a nitrite reducing enzyme (eq. 4).

eq. 2: $NH_4^+ + NH_2OH \rightarrow N_2H_4 + H_2O + H^+$

eq. 3: $N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$

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

Interestingly *Candidatus* " Brocadia anammoxidans could be identified as member of a deep branching lineage within the order *Planctomycetales* (Figure 12; Strous *et al*., 1999; Strous, 2000). The order *Planctomycetales* was first described in 1986 by Schlessner and Stackebrandt and includes four genera (*Planctomyces, Pirellula, Gemmata* and *Isosphaera*). Up to now, within this order only 7 species are validly described and represented by pure cultures isolated from various aquatic systems (Schlesner, 1994, Fuerst 1995; Ward *et al*., 1995; Griepenburg *et al.,* 1999). As indicated by numerous 16S rRNA gene sequences retrieved from different environmental systems (e.g. Liesack and Stackebrandt, 1992, De Long *et al*., 1993; Ward *et al.,* 1995; Griepenburg *et al.,* 1999; Derakshani *et al*., 2001) the diversity of yet uncultured planctomycetes is very high. Despite their almost ubiquitous abundance, only few properties are known of the members of the order *Planctomycetales*. They are all peptidoglycane less, budding

bacteria largely differing in morphology (Fuerst, 1995) within a fast evolving lineage indicated by large sequence distances (Woese, 1987). Since all so far isolated planctomycetes are considered heterotrophic, the anaerobic ammonium oxidation as autotrophic process comprises a distinct physiology within the planctomycetes.

The potential application of anaerobic ammonium oxidation in wastewater treatment is obvious, when it is kept in mind that the process is autotrophic and leads to dinitrogen gas. This is ideal for the treatment of ammonium rich wastewater with low organic carbon content, which does not maintain heterotrophic denitrification. The recently developed "Completely autotrophic N-removal over nitrite" (CANON) process (Strous, 2000), which could be realized so far only in lab reactors, represents the most interesting application of the process. It comprises a partial ammonium oxidation to nitrite by classical ammonia oxidizing bacteria prior to an anaerobic ammonium oxidation (Jetten *et al*., 1999) to dinitrogen gas. Since this process could be located in one reactor, great enhancements in speed and process control are at hand. However, the anaerobic ammonium oxidation by planctomycetes has not yet been detected in real wastewater treating systems. Nitrogen losses not explainable by classical denitrification were reported for some oxygen limited full scale plants, but also the classical ammonia oxidizing bacteria *Nitrosomonas europaea* and *Nitrosomonas eutropha* are capable to oxidize ammonium or hydroxylamine with nitrite or nitrogen dioxide as electron acceptor (Bock et al., 1995; De Bruijn et al., 1995; Schmidt and Bock, 1997). Nevertheless, the conversion rates are very low in this process and hence these bacteria are unlikely candidates causing the above described nitrogen losses.

For the fast and reliable investigation of the causative agents of the observed nitrogen losses FISH would a suitable method. However, the available probe set for anaerobic ammonium oxidizing bacteria was constructed on the basis of one 16S rRNA sequence from *Candidatus* "Brocadia anammoxidans" (Strous *et al.,* 1999; Strous *et al.,* 2000). Since it is unlikely that this organism is the only anaerobic ammonium oxidizing bacterium and the phylogenetic distances within the planctomycetes are large, it is almost certain that FISH probes constructed specifically for *Candidatus* "Brocadia anammoxidans" are not able to detect the full diversity of anaerobic ammonium oxidizing bacteria. Therefore, an elaborated detection system could discover not only new species of anaerobic ammonium oxidizing bacteria in the environment with, for example, better oxygen tolerance or higher growth rate than *Candidatus* "Brocadia anammoxidans". Also

the identification of the species diversity and activity in the samples could provide information about process stability and enhanced plant control.

## A4 Scope of the thesis

Basically, the gain of knowledge accomplished by this thesis could be separated in two parts, both focusing on problems during waste water treatment.

The first part is focusing on activated sludge settling. The methods, which are currently in use for describing activated sludge floc properties indicative for the quality of sludge settling are only covering certain aspects of this problem like floc size or heterogeneity and are often time consuming. Therefore, molecular methods mainly based on the CLSM were accustomed to a quick and high accurate determination of different physical, chemical and microbial population parameters, which might contribute to floc settling. With these utilities, it was possible to investigate the characteristics of flocs during their enrichment for poor settlers. Data derived from these studies may contribute to a better understanding of the sludge settling and help to identify and prevent beginning sludge malfunctions.
By partly using these methods aerobic granules could also be characterized. However, further investigations must show if their application in full-scale wastewater treatment plants or as a tool for an enhanced bioaugmention is worthwhile.

The second part of the thesis focused on the identification and detection of bacteria with the physiotype of ammonium/ammonia oxidation as key processes in the wastewater treatment. The 16S rRNA and *amoA* approaches would be most suitable for this. However, there was the problem that the information obtained with them was based on incomplete data sets and therefore difficult to interpret. With the now elaborated tools we investigated different environments and could obtain a more accurate detection of the diversity of aerobic ammonia oxidizing bacteria. Since a higher diversity might positively influence the process stability of ammonia oxidation, a fast determination of population structure will direct the plant control to a more stable population.
So far, the only possibility to detect anaerobic ammonium oxidizing bacteria is by FISH probes specific for *Candidatus* “Brocadia anammoxidans”. This seems hardly enough to detect the full

diversity of anaerobic ammonium oxidizing bacteria. In this thesis, wastewater treatment systems with unexpected nitrogen losses were investigated for the presence of additional species and genera of anaerobic ammonium oxidizing bacteria. Therefore, a PCR for the specific amplification of the planctomycete 16S rRNA gene was applied. Phylogenetic analyses of the obtained sequences led to the assignment of a new genus (represented by the species *Candidatus* "Kuenenia stuttgatiensis") affiliated to *Candidatus* "Brocadia anammoxidans". However, only one probe of the probe set designed for *Candidatus* "Brocadia anammoxidans" targeted *Candidatus* "Kuenenia stuttgatiensis". Consequently, new FISH probes were designed for the detection of all recognized anaerobic ammonium oxidizing bacteria and a specific differentiation of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgatiensis". As detected by FISH *Candidatus* "Kuenenia stuttgatiensis" was the dominant organisms in the investigated anaerobic ammonium oxidizing system and hence *Candidatus* "Kuenenia stuttgatiensis" was postulated as a new anaerobic ammonium oxidizing bacterium. The combination of the specific amplification of the planctomycete 16S rRNA gene to identify new potential anaerobic ammonium oxidizing bacteria in environmental samples and FISH applied with a comprehensive probe set is a prerequisite for investigating species richness and evenness of anaerobic ammonium oxidizing bacteria in the environment.

In addition to diversity analysis, information about the *in situ* activity of the detected anaerobic ammonium oxidizers would be required to better understand their ecophysiology. It has been postulated in the past that the signal intensities from FISH targeting rRNA, which are dependent on the ribosome content of the target cell, can be used to infer activity and even growth rates (DeLong *et al.,* 1989; Poulsen *et al.,* 1993). Although the link between FISH signal intensity and activity probably holds true for most fast growing heterotrophic microorganisms, past research and data from this thesis show that slow growing organisms including anaerobic ammonium oxidizing bacteria keep a high ribosome content during inhibition and starvation (Wagner *et al.,* 1995; Morgenroth *et al.,* 2000). For in situ activity analysis of anaerobic ammonium oxidizers we therefore developed and applied a set of fluorenscently labeled oligonucleotide probes specific for the intergenic spacer region between the 16S and 23S rRNA genes of anaerobic ammonium oxidizers. The cellular content of spacer region transcripts is dependent on the ribosome production (the non-coding parts of the intergenic spacer region are rapidly degraded after cleavage of the precursor RNA). Assuming that ribosomes are only produced in non-inhibited and non-starved cells the deduction of cell activity with this method is more reliable.

This fast detection of cell activities of populations could provide relevant information to shorten the startup of anaerobic ammonium oxidizing plants and allows a sophisticated control of the process.

# B Materials and Methods

## B1. Sampled environments

Environments studied in this thesis are listed and briefly described in Table 1.

**Table 1.** Description of environments analyzed in this study

| | |
|---|---|
| **Anammox enrichment lab scale reactor, Delft, The Netherlands** | Sequencing batch reactor containing a 80-90% enriched culture of *Candidatus* "Brocadia anammoxidans" |
| **Granular sludge lab scale reactor, Garching** | Sequencing batch reactor containing aerobically grown granules; the medium contained 27-29 mg/l ammonium and 7-8 mg/l phosphate. |
| **Semitechnical plant, Großlappen** | Experimental activated sludge plant fed with municipal waste water |
| **Semitechnical plant, Stuttgart** | System consisting of two trickling filters with biofilm grown on plastic filling material; the aerobic trickling filter 1 performs a partial oxidation of ammonium to nitrite; the anaerobic trickling filter 2 performs an anaerobic ammonium oxidation; the plant is fed with synthetic waste water |
| **WWTP Mechernich** | Rotating biological contactor treating ammonium rich landfill leachate |
| **WWTP Kraftisried** | Waste water treatment plant treating the effluent of an animal rendering plant, population equivalent 6000 |
| **WWTP Dietersheim high load stage** | High load stage of the municipal waste water treatment plant Munich 2, population equivalent 2100000 |
| **WWTP Großlappen high load stage** | High load stage of the municipal waste water treatment plant Munich 1, population equivalent 2000000 |
| **Mongolian soda lakes** | Soda lakes in Mongolia with high pH and high salinity |

## B2. Isolation of high molecular weight DNA

### B2.1 Extraction of DNA from pure cultures

The isolation of pure culture DNA was performed according to a simplified protocol. For the isolation of DNA from pure bacterial cultures, the cells were suspended in TE buffer containing 10mM Tris/HCl and 10mM sodium EDTA. Cell lysis was performed by addition of sodium dodecyl sulfate (SDS) to 2% final concentration and incubation at 65°C for 1h. Subsequently, the DNA was separated from cell debris by a phenol chloroform (phenol:chloroform:isoamylalcohol 25:24:1) extraction. After centrifugation the aqueous phase was transferred to a new tube and the extraction was repeated with chloroform:isoamylalcohol (24:1) to remove residual Phenol. The

DNA, which remained in the aqueous phase, was precipitated by adding NaCl to a final concentration of 1M and 0.6 volume of 2-propanol for 1 h at room temperature. The DNA was washed with 70% ethanol, air-dried and dissolved in 10mM Tris/HCl (pH 8). The amount and quality of DNA were determined by spectrophotometric analysis of the ratio of optical densities at 260 and 280 nm (Sambrook *et al.,* 1989)

### B2.2 Extraction of DNA from Biofilm samples

Biofilm samples had to be pretreated prior to DNA extraction, because most organisms in biofilms are tightly embedded in a hull of extracellular polymeric substances (EPS). Pretreatments used in this thesis were enzymatic digestion and mechanical disruption (Klein *et al.,* 2001) of EPS and cell walls.

#### B2.2.1 Mechanical EPS and cell wall disruption

DNA isolations with the bead beating technique were performed with the Fastprep Bead-beater (BIO 101, Vista) and the FastDNA kit (BIO 101, Vista) according to the manual of the manufacturer. Large yields of high molecular DNA were obtained with the bead beater setting: 3x 15s at a speed of 4.5.

#### B2.2.2 Enzymatic digestion of EPS

Enzymatic digestion was combined with an altered version of the DNA isolation protocol published by Zhou *et al.* (1996). A 0.25-g activated sludge pellet was resuspended in a 2-ml tube with a screw-on plastic cap with 675 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 [CTAB]) and treated for 30 s with a blender (Ultraturrax; Janke and Kunkel, Freiburg, Germany). After addition of 50 µl of enzyme mixture I (each at 10 mg/liter; Table 2), the mixture was incubated for 30 min at 37°C. Subsequently, 50 µl of enzyme mixture II (each at 10 mg/liter; Table 2) was added and the mixture was incubated for another 30 min at 37°C.

**Table 2.** Content of enzyme mixes I and II with provider and enzyme activities

| | Enzyme | Provider | Target |
|---|---|---|---|
| **Enzyme Mix I** | lysozyme | Fluka, Buchs, Switzerland | murein |
| | lipase type 7 | Sigma, Deisenhofen, Germany | tri-, di-, monoglycerides |
| | pectinase (from A. niger, contains also cellulases and hemicellulases) | Roth, Karlsruhe, Germany | pectin, cellulose |
| **Enzyme Mix II** | proteinase K | Boehringer, Mannheim, Germany | proteins, peptides, glycoproteins |
| | protease type 9 | Sigma, Deisenhofen, Germany | proteines, peptides |
| | pronase E | Serva, Heidelberg, Germany | all proteins |

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 centrifugation the aqueous phase was carefully transferred to a fresh tube. Residual phenol was removed by a chloroform:isoamyalcohol (24:1), extraction. Nucleic acids were precipitated from the aqueous phase by incubation with 0.6 volume of 2-propanol for 1 h at room temperature. Precipitated DNA was treated further as described in section B2.1.

A prominent problem of DNA preparations from environmental samples is their high content of humic substances, which bind to DNA and can reduce efficiency of DNA amplification or hybridization (Alm *et al.,* 2000). It is difficult to remove these substances from DNA, but compounds like cetyltrimethylammonium bromide (CTAB), polyphenyl polypyrolidone (PVPP) or Sephadex have been used to significantly lower the content of humic substances in DNA preparations (e.g. Tsai and Olson, 1992; Young et al, 1993; Zhou *et al,* 1996; Jackson *et al.,* 1997; Kowalchuk *et al.,* 1997). Although both DNA extraction protocols for biofilms described here used agents to remove humic acids, a brown coloring of the preparations indicated residual humic compounds. However, these substances did apparently not hamper polymerase chain reactions (PCR) performed with these samples.

## B3. Polymerase chain reaction

Amplifications of the *amoA* gene or rRNA genes were performed in a 96 micro well plate (Biorad, München, Germany) with a gradient cycler (Eppendorf, Hamburg, Germany). The standard reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). A general PCR program is given in Table 3.

**Table 3.** General program for the polymerase chain reaction

| | | | |
|---|---|---|---|
| initial denaturing | 94°C | 30-240sec | |
| denaturing | 94°C | 15-45sec | 25-30 cycles |
| annealing | 48-60°C | 20-50sec | |
| elongation | 72°C | 40-180sec | |
| final elongation | 72°C | 60-600sec | |

**Table 4**. References, sequences, annealing temperatures and target sites for primers used for PCR

| Trivial name | Sequence 5'-3'[e] | Annealing temperatures | Target site |
|---|---|---|---|
| 616V | AGAGTTTGATYMTGGCTCAG | 48-60°C | 16S rRNA gene, 8-27[a] |
| Pla46F | GGATTAGGCATGCAAGTC | 58°C | 16S rRNA gene, 46-63[a] |
| 1390R | GACGGGCGGTGTGTACAA | 58°C | 16S rRNA gene, 1390-1407[a] |
| 630R | CAKAAAGGAGGTGATCC | 48-60°C | 16S rRNA gene, 1529-1545[a] |
| 1037R | CGACAAGGAATTTCGCTAC | 58°C | 23S rRNA gene, 1930-1948[b] |
| amoA-1F | GGGGTTTCTACTGGTGGT | 55°C | *amo*A, 332-349[c] |
| amoA-2R | CCCCTCKGSAAAGCCTTCTTC | 55°C | *amo*A, 802-822[c] |
| amoA-3F | GGTGAGTGGGYTAACMG | 48°C | *amo*A, 295-310[c] |
| amoB-4R | GCTAGCCACTTTCTGG | 48°C | *amo*B, 30-44[d] |

[a] 16S rRNA position, *E.coli* numbering (Brosius *et al.*, 1978), [b] 23S rRNA position, *E.coli* numbering (Brosius *et al.,* 1980), [c] *amo*A gene positions of *Nitrosomonas europaea*, [d] *amo*B gene positions of *Nitrosococcus oceani* C-107, [e] IUB code: K=G/T, M=A/C, S=C/G, Y=C/T

Duration of the different steps was set according to the length of the expected PCR fragments. Annealing temperatures were applied specific for each primer pair (Table 4). The optimal annealing temperature for the primer set Pla46F and 1390R was determined by using the thermal gradient function of the gradient cycler. 12 different annealing temperatures between 44-64°C were tested. Since the amplification efficiency for different templates in one sample can deviate, it is possible that the ratios of obtained amplification products does not match the ratio of applied templates. This bias increases with every amplification cycle (Suzuki *et al.,* 1996). Thus the amount of PCR cycles was kept as low as possible. The presence and size of amplification products were determined by agarose (0.8% for large and 1.7% for small DNA fragments) gel electrophoresis of 5 µl aliquots of the PCR products.

## B4. Gel retardation *amo*A amplificates

The gel retardation of amplified *amo*A fragments was performed in a Hoefer HE33 submarine gel electrophoresis unit (Pharmacia Biotech, Freiburg, Germany) with cooled base using a modification of the protocol published by Wawer *et al.* (1995). The 2% agarose gel consisted of 35 ml 0.5 x TBE (0.0445 M Tris/HCl, 0.0445 M boric acid, 0.001 M EDTA, pH 8.3) and 0.7 g Nusieve 3:1 agarose. After cooling down the agarose mixture to 70°C, 35 µl of the DNA ligand bisbenzimide, to which long chains of polyethylene glycol 6000 are covalently coupled (Hanse Analytik Yellow; Hanse Analytik, Bremen, Germany), was added to the gel. The dye bisbenzimide binds preferentially to A + T rich sequence motifs in the DNA and retards them compared to DNA sequences with low A+T content. After poring, the gel was solidified in the dark. The electrophoresis was performed applying a voltage of 100V for 80 min with 0.5 x TBE as running buffer. After the run, the gel was stained with SYBR Green I (FMC BioProducts, Rockwell, US) nucleic acid stain (5 µl in 50 ml 0.5 x TBE) for 1 h in the dark. The bands on the gel were visualized using UV illumination (364nm) and cut out with a capillary. The gel fragments were resuspended in sterile $H_2O$ and reamplified.

## B5. Cloning of PCR products

### B5.1 Transformation of PCR products

The biofilm-derived rDNA PCR products were cloned by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). The major advantage of the TOPO TA cloning kit is that the duration of the ligation step mediated by vector-bound DNA-topoisomerases I can be reduced to 5 minutes. Furthermore, even with relatively large PCR products (5 kb) very high ligation and transformation efficiencies could be achieved. Other features of the pCR®2.1-TOPO vector (included in the kit) are the *lacZ* operon with the multiple cloning site for an easy blue/white screening of clones and resistance genes for kanamycine and ampicilin. Plasmid-DNA was isolated with the Quiaprep spin miniprep kit (Quiagen, Hilden, Germany).

### B5.2 Screening of plasmids by restriction digestion

After cloning of PCR fragments, plasmids with an insert of the expected size were identified by EcoRI digestion and subsequent agarose (0.8%) gel electrophoresis . All plasmids were additionally digested with "sure cutting" restriction enzymes (Mbo I, Alu 1) to obtain fingerprints of the cloned PCR fragments. To harvest a maximum diversity within the sequenced clones of one clone library, at least one plasmid of each unique restriction pattern was sequenced. All restriction enzymes used in this thesis were purchased at MBI Fermentas (St. Leon Roth, Germany). For restriction sites of the enzymes see Table 5.

**Table 5**. Restriction enzymes and respective restriction sites

| Restriction enzyme | Restriction sites |
|---|---|
| Eco RI | G↓AATTC |
| Alu I | AG↓CT |
| Mbo I | ↓GATC |

## B6. Determination of nucleotide sequences

All sequencing in this thesis were performed according to the dideoxy method, also called chain termination method (Sanger et al., 1977), with the Thermo Sequenase Cycle Sequencing Kit (Amersham, Little Chalfont, United Kingdom) and an infrared automated DNA sequencer (model LiCor Longreadir DNA 4200, MWG - Biotech, Ebersberg, Germany). This sequencer uses the primer-labeling technique, where the primer added to the sequencing reactions is fluorescently labeled (IRD 800, MWG - Biotech, Ebersberg, Germany). For each sample four separated sequencing reaction each with one of the ddNTP terminators must be performed. The sequencing reactions are transferred on four lanes, for each base one, on a sequencing poly acryl amide (PAA) gel. The resulting pattern is read out by a computer program. Compared to the dye termination method, the main disadvantages of this technique are the low number of simultaneously applicable samples (12 with the configuration of the sequencers used in this thesis) and the gel run duration of 12 to 16h. However, the reading length of up to 1200 bases with high accuracy is most suitable for sequencing long DNA fragments like rRNA genes.

**Table 6**. Standard amplification program used for sequencing

| | | | |
|---|---|---|---|
| initial denaturing | 94°C | 300sec | |
| denaturing | 94°C | 30sec | 25-30 cycles |
| annealing | 45/60°C | 30sec | |
| final elongation | 72°C | 300sec | |

Biofilm-derived rDNA, and *amoA* PCR products were cloned before sequencing as described in B5. PCR fragments amplified from pure culture were sequenced directly (without intermediate cloning). Sequencing reactions were amplified with a PTC 100 Thermocycler (MJ Research, Watertown, USA) used a standard amplification program listed in Table 6. For information about the applied sequencing primers see Table 7.

**Table 7.** Sequencing primers, their respective target sites and annealing temperatures

| Trivial name | Direction | Sequence 5'-3'[a] | Annealing temperatures | Target site |
|---|---|---|---|---|
| 609V | forward | TTAGATACCCCDGTAGT | 45°C | 16S rRNA gene, 785-806 |
| 699R | reverse | AGGGTTGCGCTCGTTGC | 45°C | 16S rRNA gene, 1099-1114 |
| 630R | reverse | CAKAAAGGAGGTGATCC | 45°C | 16S rRNA gene, 1529-1545[b] |
| 1035R | reverse | TTCGCTCGCCRCTAC | 45°C | 23S rRNA gene, 242-256[c] |
| 1274V | forward | GCGTRCCTTTTGTAKAATG | 45°C | 23S rRNA gene, 559-577[c] |
| 1020R | reverse | TCTGGGYTGTTYCCCT | 45°C | 23S rRNA gene, 975-990[c] |
| M13F (-20) | forward | GTAAAACGACGGCCAGT | 60°C | vector, 391-406 |
| M13R | reverse | CAGGAAACAGCTATGAC | 60°C | vector, 205-221 |

[a] IUB code: D=A/G/T, K=G/T, R=A/G, Y=C/T

## B7. Phylogenetic inference

The rRNA as part of ribosomes is ubiquitous in all cellular live forms and displays functional stability. It consists of regions with different level of conservation which allows to determine phylogenetic relationships from distantly related species as well as from highly related ones. These features make rRNA a very powerful phylogenetic marker (Woese, 1987; Ludwig *et al*., 1998). However, rRNA represents not the only molecule suitable for phylogeny inference. For phylogenetic analysis of prokaryotes the elongation factor TU, *recA*, ribosomal proteine genes and the ATPase have successfully been analyzed (Liao and Dennis, 1994; Eisen, 1995; Brendel *et al.,* 1997; Ludwig *et al.,* 1998) For guilds of microorganisms genes encoding key enzymes of the respective physiotype (so-called functional genes) can be used for deducing evolutionary relationships. For example, the *amoA* gene and the *dsrAB* genes have been used to investigate the evolutionary history of ammonia oxidizers (Rotthauwe *et. al.,* 1997) and sulfate-reducing prokaryotes (Wagner *et al.,* 1998b; Klein *et al.,* 2001), respectively.

For phylogenetic analyses many computer programs and internet services are available, but the ARB program package (used in this thesis) developed at the Department of Microbiology at the Technical University Munich is one of the most powerful software packages (Strunk and Ludwig, 1997). Its sequence databases currently encompass about 22000 16S rRNA, and about 1500 23S rRNA (all available in public data bases) entries.

**Table 8.** Basic description of treeing methods (from Ludwig *et al.,* 1998)

| | |
|---|---|
| **Distance matrix (neighbor joining)** | The basis of this method is the measuring of distances between sequences. These values are usually transferred into phylogenetic distances by a correction factor (various algorithms are available) The phylogenetic distances contain the phylogenetic and branch length information |
| **Maximum parsimony** | This method uses real sequence information. A tree is the most parsimoniest, if the minimum number of differences between the sequences is sufficient in account to put the species into a phylogenetic order. Branch length information is not available |
| **Maximum likelihood** | This most sophisticated of all treeing methods takes the whole sequence information into account. The applied models in this method use parameters like transition/tranversion ratios, back mutation etc. to calculate branch lengths. During tree reconstruction first basic trees with few species will be calculated. The one which is the most likely (shortest branch length between species) will be taken to add the next species. The new species will be put into all possible positions within the tree and again the most likely will be taken to go on with adding species. Trees are very reliable, but the high calculation efforts allow only limited amounts of species |

## B7.1 Phylogenetic analysis of rRNA sequences

New rRNA sequences were added to the respective 16S or 23S rRNA sequence data set of the ARB program package. While ISR sequences were not aligned, 16S and 23S rRNA sequences were aligned automatically using the respective tool of the ARB package (O. Strunk and W. Ludwig, http://www.arb-home.de). Subsequently, the alignments were corrected by visual inspection. Additionally, a combined 16S and 23S rRNA sequence database was generated including all members of the *Planctomycetales* for which 16S and 23S rRNA sequence information is available (Ward *et al.,* 2000). Phylogenetic analyses of rRNA sequences were performed by applying neighbor-joining, ARB parsimony, and maximum likelihood analyses (Table 8) 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 (Maidak *et al.,* 1996). 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. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling).

## B7.2 Phylogenetic analysis of *amoA* sequences

The *amoA* sequences were added to an ARB *amoA* sequence database which contains all publicly available *amoA* sequences. Deduced amino acid sequences were manually 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. For facilitation of protein alignment more recent versions of the ARB software packages have the CLUSTALW alignment tool included (Thompson *et al.,* 1994). 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 (Table 8) using the tools described above. Bootstrap analysis for protein-level (AmoA) and nucleotide-level (*amoA*,) phylogenetic analyses were performed for parsimony using the tool in the PHYLIP program package. For each calculation, 100 bootstrap resamplings were analyzed.

## B8. Probe design

Probes targeting rRNA were designed with the probe design tool of the ARB program package. Probe sequences as well as positions and qualities of mismatches of the suggested probes on the probe design output list to non target organisms were checked by the probe match tool of the ARB program package and manually in the ARB sequence editor. In some cases probes had to be adjusted by increasing their length or shifting their position on the alignment for few bases to increase their specificity.

Probes targeting the intergenic spacer region between the 16S and 23S rDNA (ISR) of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgatiensis", respectively, were designed manually in the ARB editor according to the following criteria: (i) probes must not have overlapping target regions; (ii) GC content and length of the probeswere adjusted such that they possess comparable theoretical melting temperatures Tm (defined as temperature, where 50% of the probe have melted of the target region) to allow their simultaneous application; (iii) tRNA coding regions must not be used for probe design. All ISR targeting probes were checked against the ARB 16S and 23S rRNA datasets as well as against all nucleotide databases via the BLAST similarity search tool (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al.*, 1990) to exclude unexpected false positive results.

## B9. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes was first introduced for identification of microorganisms 1989 (De Long *et al*., 1989) and subsequently found widespread application in environmental microbiology (e.g. Amann *et al*., 1990; Amann *et al.,* 1995; Manz *et al*., 1992; Wagner *et al*., 1994a; Wagner *et al*., 1998a). The oligonucleotides used in this method usually have a length between 18 and 22 nucleotides and target regions on the rRNA with different evolutionary conservation. Therefore, the specificity of the probes can be adjusted ranging from the domain to the species level (high conservation of the target site – broad specificity and vice versa). For adjusting the stringency in FISH experiments formamide is applied to the hybridization buffer, which destabilizes the DNA(probe)-RNA(target) hybrid

(1,7% formamide in the hybridization buffer has the same effect as increasing the hybridization temperature by 1°C).

Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from ThermoHybaid/Interactiva division (Ulm, Germany). Hybridizations were performed as described by Amann (1995). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (Wagner *et al.,* 1994a). Optimal hybridization conditions for newly designed probes were determined by using the hybridization and wash buffers described by Manz *et al.* (1992) The *in situ* probe dissociation temperatures were evaluated by measuring the relative fluorescence intensity of bacteria after hybridization at different stringencies as described by Daims *et al.* (1999). For information about previously published probes used in this study, please refer to Table 9.

**Table 9**. Previously published oligonucleotide probes used in this thesis with probe sequences, specificity, target sites, formamide concentrations in the hybridisation buffer as well as NaCl concentrations in the washing buffer required for specific in situ hybridisation

| Trivial name (if available); (reference) | OPD[a] designation | Specificity | Sequence 5'-3' | % Formamide/ mM [NaCl] [b] | Target site[c] |
|---|---|---|---|---|---|
| ACA 23a (Wagner *et al.,* 1994b) | S-P-Acin-0652-a-A-18 | *Acinetobacter sp.* | ATCCTCTCCCATACTCTA | 35/80 | 16S, 652-669 |
| Act 1458 (Rabus *et al.,* 1999) | S-P-AzTh-0338-a-A-21 | *Azoarcus/Thauera* | GAATCTCACCGTGGTAAGCGC | 50/28 | 16S, 1458-1479 |
| Alf968 (Neef, 1997) | S-P-Alf-0968-a-A-18 | *Alphaproteobacteria* | GGTAAGGTTCTGCGCGTT | 20/225 | 16S, 968-985 |
| Bet42a (Manz *et al.,* 1992) | L-P-Bet-1027-a-A-17 | *Betaproteobacteria* | GCCTTCCCACTTCGTTT | 35/80 | 23S, 1027-1043 |
| CF319a (Wagner *et al.,* 1994a) | S-P-CF-0319-a-A-18 | *Cytophaga/ Flavobacterium* | TGGTCCGTGTCTCAGTAC | 35/80 | 16S, 319-336 |
| CNIT3 (Wagner *et al.,* 1996) | S-G-Nbac-1035-b-A-18 | Competitor to NIT3 | CCTGTGCTCCAGGCTCCG | 35/80 | 16S, 1035-1052 |
| Cte[d)] (Schleifer *et al.,* 1992) | S-G-Cte-659-a-A-18 | *Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.* | TTCCATCCCCCTCTGCCG | 20/225 | 16S, 659-676 |
| Eub338 (Amann *et al.,* 1990) | S-D-Bact-0338-a-A-18 | Bacteria | GCTGCCTCCCGTAGGAGT | 0/900 | 16S, 338-355 |
| Eub338II (Daims *et al.,* 1999) | S-D-Bact-0338-a-A-18 | Bacterial lineages not covered by probe EUB338 | GCAGCCACCCGTAGGTGT | 0/900 | 16S, 338-355 |
| Eub338III (Daims *et al.* 1999) | S-D-Bact-0338-a-A-18 | Bacterial lineages not covered by probe EUB338 and EUB338II | GCTGCCACCCGTAGGTGT | 0/900 | 16S, 338-355 |

| | | | | | |
|---|---|---|---|---|---|
| Euk 516 (Amann *et al.,*1995) | S-D-Euk-0516-a-A-16 | *Eucarya* | ACCAGACTTGCCCTCC | 0/900 | 18S, 516-531 |
| Gam42a (Manz *et al.,* 1992) | L-P-Gam-1027-a-A-17 | *Gammaproteobacteria* | GCCTTCCCACATCGTTT | 35/80 | 23S, 1027-1043 |
| Hgc69a (Roller *et al.,* 1994) | L-P-HGC-1901-a-A-18 | *Actinobacteria* | TATAGTTACCACCGCCGT | 25/159 | 23S, 1901-1918 |
| Neu (Wagner *et al.,* 1995) | S-*-Neu-0653-a-A-18 | Halophilic and halotolerant *Nitrosomonas* sp. | CCCCTCTGCTGCACTCTA | 40/56 | 16S, 653-670 |
| NIT 3 (Wagner *et al.,* 1996) | S-G-Nbac-1035-a-A-18 | genus *Nitrobacter sp.* | CCTGTGCTCCATGCTCCG | 35/80 | 16S, 1035-1052 |
| NmV (Juretschko et al., 1998) | S-S-Ncmob-0174-a-A-18 | *Nitrosococcus mobilis* | TCCTCAGAGACTACGCGG | 35/80 | 16S, 174-191 |
| Nsm156 (Mobarry *et al.,* 1996) | S-G-Nsm-0155-a-A-19 | genus *Nitrosomonas* | TATTAGCACATCTTTCGAT | 5/636 | 16S, 156–174 |
| Nso1225 (Mobarry *et al.,* 1996) | S-P-Betao-1225-a-A-20 | ammonia oxidizers of the *Betaproteobacteria* | CGCCATTGTATTACGTGTGA | 35/80 | 16S, 1225-1244 |
| Nso 190 (Mobarry *et al.,* 1996) | S-P-Betao-0190-a-A-19 | ammonia oxidizers of the *Betaproteobacteria* | CGATCCCCTGCTTTTCTCC | 55/20 | 16S, 190-208 |
| Nsv 443 (Mobarry *et al.,* 1996) | S-F-Nsp-0444-a-A-19 | *Nitrosospira*-cluster | CCGTGACCGTTTCGTTCCG | 30/112 | 16S, 444-462 |
| - (Daims *et al.,* 2001) | S-G-Ntspa-0662-a-A-18 | genus *Nitrospira* sp. | GGAATTCCGCGCTCCTCT | 35/80 | 16S, 662-679 |
| Lgca (Meier *et al.,* 1999) | S-P-LGC-0354-a-A-18 | *Firmicutes* | TGGAAGATTCCCTACTGC | 20/225 | 16S, 354-371 |
| Lgcb (Meier *et al.,* 1999) | S-P-LGC-0354-b-A-18 | *Firmicutes* | CGGAAGATTCCCTACTGC | 20/225 | 16S, 354-371 |
| Lgcc (Meier *et al.,* 1999) | S-P-LGC-0354-c-A-18 | *Firmicutes* | CCGAAGATTCCCTACTGC | 20/225 | 16S, 354-371 |
| PAO651 (Crocetti *et al.,* 2000) | S-*-PAO-0651-a-A-18 | Phosphate accumulating bacteria affiliated to *Rodocyclus* | CCCTCTGCCAAACTCCAG | 35/80 | 16S, 651-668 |
| Pla46 (Neef *et al.,* 1998) | S-P-Planc-0046-a-A-18 | *Planctomycetales* | GACTTGCATGCCTAATCC | 25/159 | 16S, 46-63 |
| Sna 23a (Wagner *et al.,* 1994a) | S-S-Sna-0656-a-A-18 | *Sphaerotilus natans* and relatives | CATCCCCCTCTACCGTAC | 45/40 | 16S, 656-673 |
| Zra 23a (Rosello-Mora *et al.,* 1995) | S-S-Zora-0647-a-A-18 | *Zoogloea ramigera*$^{T}$ | CTGCCGTACTCTAGTTAT | 35/80 | 16S, 647-664 |
| - (Strous, 2000) | S-*-Amx-0156-a-A-18 | *Candidatus.* "B. anammoxidans" | CGGTAGCCCCAATTGCTT | 40/56 | 16S, 156-173 |
| - (Strous, 2000) | S-*-Amx-0223-a-A-18 | *Candidatus.* "B. anammoxidans" | GACATTGACCCCTCTCTG | 40/56 | 16S, 223-240 |
| - (Strous, 2000) | S-*-Amx-0432-a-A-18 | *Candidatus.* "B. anammoxidans" | CTTAACTCCCGACAGTGG | 40/56 | 16S, 432-449 |
| - (Strous, 2000) | S-*-Amx-0613-a-A-22 | *Candidatus.* "B. anammoxidans" | CCGCCATTCTTCCGTTAAGCGG | 40/56 | 16S, 613-634 |
| - (Strous, 2000) | S-*-Amx-0820-a-A-22 | *Candidatus.* "B. anammoxidans" and *Candidatus.* "K. stuttgartiensis" | AAAACCCCTCTACTTAGTGCCC | 40/56 | 16S, 820-841 |
| - (Strous, 2000) | S-*-Amx-0997-a-A-21 | *Candidatus.* "B. anammoxidans" | TTTCAGGTTTCTACTTCTACC | 20/225 | 16S, 997-1017 |
| - (Strous, 2000) | S-*-Amx-1015-a-A-18 | *Candidatus.* "B. anammoxidans" | GATACCGTTCGTCGCCCT | 60/14 | 16S, 1015-1032 |
| - (Strous, 2000) | S-*-Amx-1154-a-A-18 | *Candidatus.* "B. anammoxidans" | TCTTGACGACAGCAGTCT | 20/225 | 16S, 1154-1171 |

| | | | | | |
|---|---|---|---|---|---|
| - (Strous, 2000) | S-*-Amx-1240-a-A-23 | *Candidatus.* "B. anammoxidans" | TTTAGCATCCCTTTGTACCAACC | 60/14 | 16S, 1240-1262 |
| - (Juretschko *et al.,* 2002) | S-*-Zora-1414-a-A-20 | *Zoogloea ramigera*[T] and affiliated molecular isolates | TTCTGGTAAACCCCACTCCC | 25/159 | 16S, 1414-1433 |

[a)]Oligonucleotide probe database (Alm *et al.,* 1996), [b)] Formamide in the hybridization buffer and [NaCl] in the washing buffer, respectively. [c)] rRNA positions, *E. coli* numbering (Brosius *et al.,* 1981), [d)] Probe also used together with NEU as unlabeled competitor, [e)] Probe used as unlabeled competitor together with NIT 3.

## B10. Confocal laser scanning microscopy

Compared to conventional epifluorescence microscopy the major advantage of confocal laser scanning microscopy (CLSM) is that light of a single focal plane can be acquired. Since the intensity of the emitted light of one focal plane is normally very low the fluorescently labeled sample has to be exited by monochromatic laser light. The focal plane of the sample is scanned in two dimensions. The emitted light from each spot is projected through a pinhole onto a detector and enhanced by a photo multiplier The pinhole eliminates scattered light and light emitted from above or below the focal plane. The thickness of the focal plane can be adjusted by changing the diameter of the pinhole. By moving the object desk up and down it is possible to scan different optical planes of the sample. These optical planes (or slices) can be combined by image analysis software to a 3D presentation.

During this thesis image acquisitions were performed with a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Jena, Germany) together with the standard software package delivered with the instrument (version 2.1). The CLSM was equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) as well as long pass and band pass filters for the emission wavelengths of the dyes used in this thesis.

## B11. Image analysis

For image analysis several computer programs were developed in C, Microsoft Excel and for the Carl Zeiss Vision Kontron KS400 (version 3.1) and applied (Table 10). All programs are available on request.

**Table 10**. Description of programs used in this thesis.

| Program name | Reference | Program type | Description |
|---|---|---|---|
| **3DVASD** | this thesis | Kontron KS 400 macro | **3DVASD - 3 Dimensional Volume and Surface Determination**<br><br>3D image stacks acquired with the CLSM are processed to a complete 3D reconstruction. After setting a threshold (pixels with intensities[a] below this value are not considered for further calculations) the gaps between the optical slices were extrapolated by enlargement of all pixels of each single slice by half of the slice distance in both directions of the z-axis. These 3D pixels are so-called voxels. Non-stained enclosures within the aggregate structure are filled out by the software. All voxels which form a connected structure are counted and multiplied by the volume of one voxel (dimension of a pixels in x- and y-direction and the slice distance in µm). The overall surface of an aggregate is determined by adding voxel surfaces, which do not touch other voxels.<br><br>**Purpose:** 3D volume and surface measurement of aggregates<br><br>**Output:** Volume and surface area of aggregates |
| **DfDist** | This thesis | C program | For each measurement 20 random confocal optical biofilm section images (thickness 1 µm) are processed with pixel intensity threshold values (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250[a], see 3DVASD).For each threshold value the distances of all pixels to each other are measured. However, this process requires immense calculation power. Therefore, images exceeding 50.000 pixels after setting the threshold are not considered.<br><br>**Purpose:** Calculation of the fractal dimension Df of aggregates (first part)<br>**Output:** Text files for each threshold value with distances |
| **DfCal** | This thesis | MS Excel macro | This program reads the output files of DfDist and correlates the distance values for each threshold (Thill *et al.,* 1998). The size range of objects which are considered for the calculation of the fractal dimension Df can be set interactively choosing a range of distances (e.g. 10-250 µm for microbial aggregates). The Df for each threshold is calculated.<br><br>**Purpose:** Calculation of the fractal dimension Df of aggregates (second part)<br>**Output:** Df for each threshold value |

| | | | |
|---|---|---|---|
| **RAM** | Schmid et al. 2000; Daims et al., 2001 | Kontron KS 400 macro | **RAM – Relative Area Measurement**<br><br>The program determines for optical sections the ratio of the area of cells stained with a specific probe to the area of cells labeled with a general probe (e.g. the bacterial probe set EUB338, EUB338-II, EUB338-III) or DNA stains like SYBR Green I(FMC Bioproducts, Rockland) or 4,6-diamidino-2-phenylindole (DAPI). For each measurement, analysis of 20 randomly recorded optical sections (magnification of 400) of slides completely covered with biofilm material is generally sufficient to obtain statistically significant data (Bouchez et al., 2000).<br><br>**Purpose:** Quantitative in situ analysis of the composition of microbial communities<br><br>**Output:** Ratio of the area of cells stained with a specific probe to the area of cells stained with a general probe or a DNA-staining dye |
| **SIM** | Daims *et al.,* 1999 | Kontron KS 400 macro | **SIM – Signal Intensity Measurement**<br><br>This program measures intensities of object on images. The size range of these objects is set interactively on screen by choosing the largest and smallest object which should be considered. Objects identified as artifacts can be removed on screen and will not be included in the computation.<br><br>**Purpose:** Determination of optimal probe stringency by measuring of probe intensities in a formamide row.<br><br>**Output:** Mean intensity of all objects and single object intensities for each image |
| **SIIM** | This thesis | Kontron KS 400 macro | **SIIM Split Image Intensity Measurement**<br><br>A high resolution image (2048x2048 pixels) is split into squares of 256x256 pixels. Subsequently, the ratio of all pixels (no threshold applied) to the total amount of pixels is calculated.<br><br>**Purpose:** Determination of a distribution profile of organisms on image<br><br>**Output:** List of ratios of stained to total amount of pixels of the squares |
| **SIAM** | This thesis | Kontron KS 400 macro | **SIAM Split Image Area Measurement**<br><br>A high resolution images (2048x2048 pixels) is split into squares of 256x256 pixels. Subsequently, the mean intensity of all pixels in each square is measured.<br><br>**Purpose:** Determination of an activity profile of organisms on a digital image<br><br>**Output:** List of mean intensities of the squares |

[a)] Pixels from 8 bit images can have grey (intensity) values from 0 to 255

# C Results

## C1. Characterization of activated sludge flocs

(published in Schmid *et al.,* submitted)

During this thesis a new approach was developed and evaluated to characterize biovolume, heterogeneity and the microbial community composition of activated sludge flocs by using confocal laser scanning microscopy and digital image analysis. To identify floc parameters which differ between flocs with good and bad settling ability, bad settling activated sludge flocs were enriched from activated sludge of three different waste water treatment plants (wwtp). Subsequently, floc architecture as well as the microbial community composition of the flocs were determined and compared to the respective original activated sludge.

### C1.1 Evaluation of the 3D volume measurement

For 3D volume and surface measurement of biological aggregates by confocal laser scanning microscope a well-suited staining procedure for activated sludge flocs was developed. Furthermore, the available set of tools implemented in the software package Kontron KS400 (Carl-Zeiss Vision; version 3.1) was complemented by the newly developed macro 3DVASD (for specifications see Table 10). This macro measures the volume and surface area of distinct particles on 3D image stacks.

The time required for sample treatment (staining with fluoresceine isothiocyanide FITC and wash steps) and image acquisition (without data analysis) was approx. 7 hours for a single sample. Transport and storage of activated sludge samples might influence the number, shape, and volume of the activated sludge flocs. Thus, the influence of sample storage was investigated by keeping an activated sludge sample for 6 hours, 1, 2, 3 and 5 days at 4°C, respectively. An aliquot was stained and observed immediately after sampling. No pronounced changes in measured floc parameters could be observed during storage.

To determine the number of flocs which need to be analyzed to get a reliable statistical floc volume distribution, the volumes of 600, 3000, 6000, and 15000 randomly selected flocs originating from the same activated sludge sample (Dietersheim wwtp) were measured. While floc volume distributions deviated significantly for less than 6000 acquired flocs, they were

almost identical for 6000 and 15000 acquired flocs. 6000 flocs correspond to about 75 confocal images.

In the next step, the largest distance between two confocal optical sections which can be applied without significant decrease of the accuracy of the analysis, was defined by taking 20 CLSM stack images of an activated sludge sample using 1 µm distance intervals between the optical sections (distances <1 µm were not analyzed since they are not practicable due to bleaching effects caused by the extended excitation time). After data acquisition, individual sections were removed from each stack image to obtain modified data sets with distances between the sections of 2, 4, and 8 µm. The use of section distances of 1, 2, and 4 µm resulted in only marginal variations in the determined floc volumes and distributions. The total volumes of all measured flocs using 1, 2, and 4 µm section distance, respectively, differed only by 3.5%. However, increase of section distance to 8 µm led to significant alterations of floc volumes and distributions.

## C1.2 Physical floc properties

The physical floc properties were determined for the semitechnical plant in Großlappen and the Dietersheim wwtp. Generally, the small flocs were most abundant [below 256 $\mu m^3$ (diameter of about 9 µm)], while the large ones comprised almost the complete volume [above 131073 $\mu m^3$ (diameter above about 140 µm)]. The poor settling flocs remaining in the supernatant after 20 minutes of settling in Dietersheim were slightly more voluminous and the abundance of small flocs decreased. In contrast, the poor settling flocs in Großlappen showed a shift to smaller volumes in the larger volume classes [above 131073$\mu m^3$ (diameter above about 140 µm)]. A significant increase in the number of small flocs was not visible.

In both plants, the fractal dimension ($D_f$) of the poor settling flocs had mean values (1.9 for Großlappen and 1.8 for Dietersheim) lower than the $D_f$ of the original sludge flocs (2.2 for Großlappen and 2.1 for Dietersheim), indicating that the poor settling flocs were more heterogeneous. This finding was also supported by measurement of the ratio of the floc surface to the surface of a sphere with an identical volume ($S_{floc}/S_{sphere}$), which showed a marked increase for poor settling flocs in both plants.

## C1.3 Chemical composition of activated sludge

The chemical composition of the solids was determined in the activated sludge samples from the wwtps Großlappen (high load stage), Dietersheim (high load stage) and Poing (Table 1 section B). In all plants the relative carbohydrate content decreased while the DNA content increased when the poor settling flocs were enriched. In contrast, the change in the content of humic substances and proteins did not show a general pattern as it increased, decreased or remained constant in the wwtps investigated.

## C1.4 Microbial population structure of activated sludge flocs

Group-specific probes listed in Table 9 were applied to investigate the microbial population structure of the activated sludge of the wwtps Großlappen (high load stage), Dietersheim (high load stage) and Poing by FISH. Activated sludge from all three plants was dominated by *Betaproteobacteria* and *Actinobacteria.* In Poing also *Firmicutes* were numerous. In medium amounts, members of the *Alpha*- and *Gammaproteobacteria* could be detected in Dietersheim and Poing. *Bacteroidetes* and *Planctomycetes* played only a minor role in these two sludges. The Großlappen sludge contained the most heterogeneous bacterial population and a relatively high proportion of the bacteria could not be identified by any of the probes applied (18%).

After enrichment for poor settling sludge two major shifts in the population could be observed in the Dietersheim sludge. The *Betaproteobacteria* decreased from about 62% to 40%, and the percentage of *Alphaproteobacteria* was only about half of the original (about 15% to 7%).

In poor settling sludge from Großlappen all bacteria could be detected with the applied group-specific oligonucleotide probes and the relative abundance of the *Alpha*, *Beta*- *Gammaproteobacteria* and *Firmicutes* raised accordingly. This increase was most significantly for *Gammaproteobacteria* (from about 6% to 12%).

In the poor settling flocs from Poing the abundance of *Actinobacteria* dropped significantly (from 35% to 10% of all bacteria detected with the EUB probe mixture) after enrichment for poor-settling flocs. A large fraction (about 25%) of the bacterial population in the poor settling flocs could be detected only with the EUB probe mixture and must thus be affiliated with bacterial lingeages for which no specific probes were applied.

Additionally, a general shift in the filament index to higher values (from 2 to 4 for Dietersheim and Großlappen, and from 3 to 5 for Poing, respectively) could be observed for all wwtps after enrichment for poor settling flocs.

## C2. Characterization of the of the population structure and distribution in aerobic granular sludge

Since aerobic granular sludge shows an excellent settling ability, they could be an alternative to conventional activated sludge systems. The aim of this study was to investigate for the first time the microbial community composition of aerobic granular sludge and to link the obtained data with physiological properties of the granules. A sequencing batch reactor (SRB) with alternating anaerobic/aerobic conditions was used to produce the granules. The reactor was operated in sequencing batch mode with alternating aerobic/anaerobic phases and was fed with synthetic wastewater, containing glucose as major carbon source and peptone as the organic nitrogen source.

### C2.1. Physiological properties of the aerobic granular sludge

In the reactor ammonia was usually degraded up to 100 %. During the cycle nitrite and nitrate were detectable only in very low concentrations (0.23 and 0.02 mg/l, respectively). 30-35 % of the COD reduction occurred in the anaerobic phase and a removal efficiency between 88 and 96% could be observed usually. The phosphate removal ranged from 24 % (in the first weeks after start-up) to 78%.

### C2.2 Microbial population composition of the aerobic granular sludge

#### C2.2.1 Investigation of the microbial population structure by FISH

Initially, granules were analyzed using a previously published set of rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH, Table 9). Almost all (>99%) of the bacteria in the granules were detectable by FISH as demonstrated by simultaneous staining

with the EUB338 probe set and the nucleic acid stain SYBRGreen. The aerobic granular sludge was dominated by *Betaproteobacteria* (88,8% +/- 1,6%). In addition isolated microcolonies of *Gammaproteobacteria* (5,7% +/- 3,3%) were observed. However, other group specific probes for the detection of *Alphaproteobacteria, Firmicutes, Actinobacteria, Cytophaga/Flavobacterium* and, *Planctomycetales* as well as the probe specific for *Eucarya* did not hybridize significantly (all below 1% of all detectable organisms). In an attempt to refine this analysis many genus or species specific probes were applied in addition (Table 9), but none of these probes yielded a detectable signal in the aerobic granular sludge.

### C2.2.2 Determination of the microbial population structure by the full cycle 16S rRNA approach

For high resolution analysis of the microbial population structure aerobic granular sludge derived DNA was utilized as template for PCR-mediated amplification of almost full-length 16S rRNA gene fragments. Obtained amplificates were cloned and from the resulting clone library 32 clones were sequenced. These clones were classified into operational taxonomic units (OTUs) which each encompass clones which share 97% or more sequence similarity with each other. Applying this criterion, the 32 molecular isolates from the granules could be assigned to 10 OTUs. 6 out of 10 OTUs consisted of only one clone. Consequently, the 32 clones analyzed were estimated to represent 81% of the OTUs deposited within the library according to the following formula (Giovannoni *et al.,* 1995; Singleton *et al.,* 2001):

$$\mathbf{C_{coverage} = [1-(n1/N)] \times 100\% = [1-(6/32] \times 100\% = 81\%}$$

n1 = number of OTUs represented by only a single clone
N = total number of clones analyzed

14 of the 32 clones were phylogenetically affiliated to the type strain of *Zoogloea ramigera*$^T$ (ATCC 19544; Unz, 1971, Figure 13) However, no positive hybridization signal within the granules was observed after application of the oligonucleotide probe Zra 23a (Table 9) described to be specific for *Zoogloea ramigera*$^T$ (Rossello-Mora et al., 1995) Analysis of the *Zoogloea ramigera*$^T$ related clone sequences revealed, that none of them possessed a fully complementary

target site of probe Zra 23a. Thus the recently designed oligonucleotide probe S-*-Zora-1414-a-A-20 (Juretschko *et al.,* 2002; Table 9) specific for *Zoogloea ramigera*$^{T}$ , which also targeted the clones obtained from the aerobic granules without a mismatch, was applied. FISH with probe S-*-Zora-1414-a-A-20 revealed that *Zoogloea ramigera*$^{T}$-like organisms represented the majority of the bacterial population of the aerobic granules (88,4% +/- 4,1%, Figure 14 A).

**Table 11.** Oligonucleotide probes developed and applied in this thesis**.**

| OPD[a] designation | Specificity | Sequence 5'-3' | Target site | % Formamide/ mM NaCl[e] |
|---|---|---|---|---|
| I-*-Ban-0071(*B.anam.*)-a-A-18 | *Candidatus* "Brocadia anammoxidans" | CCCTACCACAAACCTCGT | ISR, 71-88[b] | 10/450 |
| I-*-Ban-0108(*B.anam.*)-a-A-18 | *Candidatus* "Brocadia anammoxidans" | TTTGGGCCCGCAATCTCA | ISR, 108-125[b] | 10/450 |
| I-*-Ban-0222(*B.anam.*)-a-A-19 | *Candidatus* "Brocadia anammoxidans" | GCTTAGAATCTTCTGAGGG | ISR, 222-240[b] | 10/450 |
| I-*-Ban-0389(*B.anam.*)-a-A-18 | *Candidatus* "Brocadia anammoxidans" | GGATCAAATTGCTACCCG | ISR, 389-406[b] | 10/450 |
| I-*-Kst-0031(*K.stutt.*)-a-A-18 | *Candidatus* "Kuenenia stuttgartiensis" | ATAGAAGCCTTTTGCGCG | ISR, 31-48[b] | 10/450 |
| I-*-Kst-0077(*K.stutt.*)-a-A-18 | *Candidatus* "Kuenenia stuttgartiensis" | TTTGGGCCACACTCTGTT | ISR, 77-94[b] | 10/450 |
| I-*-Kst-0193(*K.stutt.*)-a-A-19 | *Candidatus* "Kuenenia stuttgartiensis" | CAGACCGGACGTATAAAAG | ISR, 193-211[b] | 10/450 |
| I-*-Kst-0288(*K.stutt.*)-a-A-20 | *Candidatus* "Kuenenia stuttgartiensis" | GCGCAAAGAAATCAAACTGG | ISR, 288-297[b] | 10/450 |
| S-S-Ban-0162(*B.anam.*)-a-A-18[f] | *Candidatus* "Brocadia anammoxidans" | CGGTAGCCCCAATTGCTT | 16S rRNA insertion | 40/56 |
| L-*-Amx-1900-a-A-21 | Both anaerobic ammonium oxidizing bacteria | CATCTCCGGCTTGAACAA | 23S rRNA, 1900-1483[c] | 30/112 |
| S-S-Kst-0158-a-A-18 | *Candidatus* "Kuenenia stuttgartiensis" | GTTCCGATTGCTCGAAAC | 16S rRNA insertion | 25/159 |
| S-*-Kst-1275-a-A-20 | *Candidatus* "Kuenenia stuttgartiensis" | TCGGCTTTATAGGTTTCGCA | 16S rRNA, 1275-1294 | 25/159 |
| S-*-Clta-0467-a-A-18 | Aerobic granules derived clones affiliated to *Tolumonas auensis* | ACGTCAATGTTGATGCGT | 16S rRNA, 467-484 | 15/318 |

[a] Oligonucleotide probe database (Alm *et al*., 1996),[b] Position of intergenic spacer region (ISR) with regard to the respective target organism, [c] 23S rRNA position, *E.coli* numbering (Brosius *et al.,* 1980), [d]16S rRNA position, *E.coli* numbering (Brosius *et al*., 1978), [e] % Formamid in the hybridization buffer and mM NaCl in the washing buffer, respectively, required for specific *in situ* hybridization.

On basis of several 16S rRNA gene sequences related to the *Gammaproteobacterium Tolumonas auensis* probe S-*-Clta-0467-a-A-18 was designed (Table 11). Probe S-*-Clta-0467-a-A-18 has one mismatch to *Tolumonas auensis* and at least three mismatches to all other 16S rRNA gene sequences in the database. The optimal stringency for probe S-*-Clta-0467-a-A-18 was determined by recording an in situ probe dissociation curve by varying the formamide and salt concentrations in the hybridization and wash buffer as described by Manz *et al.,* 1992 and Daims *et al.,* 1999. Applying stringent hybridization conditions (15% formamide in the hybridization

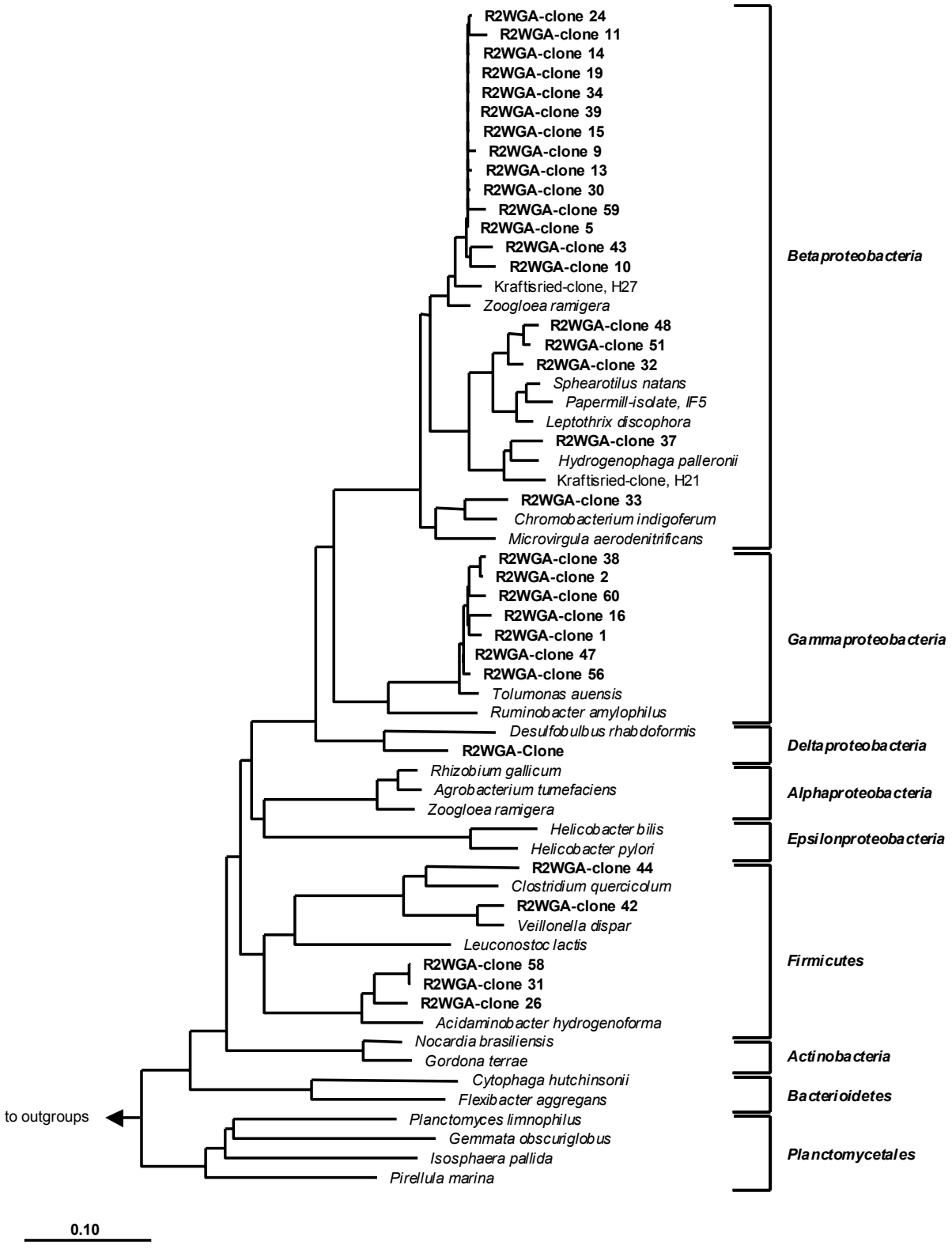



Fig. 13 Phylogenetic 16S rRNA gene tree reflecting the relationship of 32 aerobic granule clones (labeled R2WGA) and reference organisms belonging to different phylogenetic groups (indicated by brackets) within the *Bacteria*. The tree was constructed by creating a scaffolding tree of full sequences by the maximum-likelihood method and a subsequent adding of shorter sequences. The bar represents 10% sequence divergence.

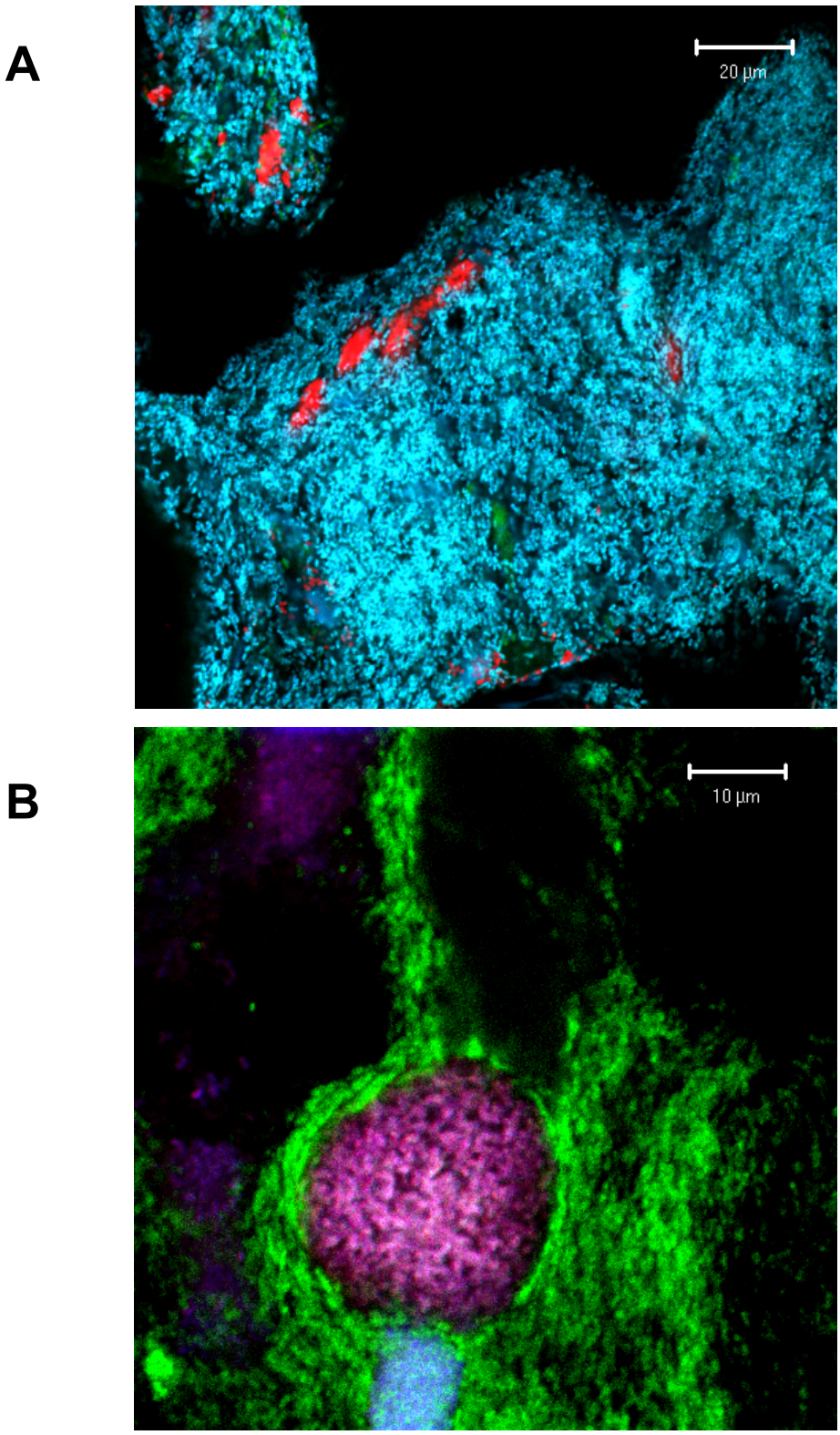



Fig. 14**. A.** In situ identification of *Zoogloea ramigera*-like organisms (probe S-*-Zora-1414-a-A-20 labeled with Cy5, Table 9), *Betaproteobacteria* (Bet 42a labeled with Fluos) and *Gammaproteobacteria* (Gam 42a labeled with Cy3, Table 9) in a cryosection of an aerobic granule. *Zoogloea ramigera*-like organisms appear turquoise because of overlapping labels. **B:** In situ identification of *Tolumonas auensis*-like organisms in a cryosection of an aerobic granule by simultaneous hybridization with probe S-*-Clta-0467-a-A-18 (labeled with Cy3; Table 11), Bet 42a (Table 9) specific for *Betaproteobacteria* (labeled with Fluos) and Gam 42a (Table 9) specific for *Gammaproteobacteria* (labeled with Cy5). *Tolumonas auensis*-like organisms appear purple due to overlapping labels.

buffer and 318 mM NaCl in the washing buffer, respectively), probe S-*-Clta-0467-a-A-18 stained about 5% of the bacteria detectable by FISH and demonstrated that *Tolumonas auensis*-like organisms were forming the cell clusters of *Gammaproteobacteria* within the aerobic granules (Figure 14 B).

Surprisingly, ammonia oxidizing bacteria were not detected by FISH with probes Nso1225 and Nso190 both specific for betaproteobacterial ammonia oxidizers. Also sequences of them were not present in the clone library. However, the aerobic granular sludge showed ammonia oxidizing activity. Thus inhibition experiments using 2,4 dinitrophenol, which inhibits all organisms, were performed. A decrease of ammonia oxidation activity indicated its biological origin. Further tests with allyl-thio-urea, which specifically inhibits ammonia oxidizing bacteria, will reveal whether the aerobic ammonia oxidation in the granules is autotrophic or heterotrophic.

### C2.2.3 Spatial distribution of “active” microorganisms within the granule

It could be demonstrated by using the SIIM macro (Table 10) that the cells immobilized at the surface of the granules had the highest ribosome content possibly indicating maximum microbial activity at the surface of the granules. Deeper inside the granules the bacteria possessed a significantly reduced ribosome content possibly caused by limited substrate availability. In accordance with a high surface activity maximum cell densities (SIAM macro, Table 10) were recorded at the surface region of the granules.

## C3. Phylogenetic analysis and fluorescence in situ hybridization of aerobic ammonia oxidizing bacteria

### C3.1 The ammonia oxidizing population of the wastewater treatment plant in Kraftisried (published in Juretschko *et al.,* 1998)

The industrial waste water treatment plant in Kraftisried (Allgäu, Germany) receives sewage from an animal waste processing plant. The decay of protein-rich material causes a high ammonium load in the influent, which can reach up to 5000 mg/l. The sewage is treated in one

intermittently aerated basin. This method creates oxic and anoxic zones to support nitrification as well as denitrification. Due to this plant design, the removal N compound reached up to 90 % during the time this experiments were performed..

### C3.1.1 In situ characterization of the population structure of ammonia oxidizing bacteria

The composition of the nitrifying consortium in the activated sludge of the Kraftisried waste water treatment plant was analyzed by fluorescence in situ hybridization with a set of previously published probes by S. Juretschko (Juretschko *et al.,* 1998). 16 to 20% of the total number of cells (determined by DAPI staining) were assigned to the genus *Nitrosomonas* of betaproteobacterial ammonia oxidizers by simultaneous hybridization with probes Nso190, Nsm156, and NEU (Table 9). 99% of the ammonia oxidizers detectable with probe NEU hybridized also with the *N. mobilis*-specific probe NmV. A minor amount of bacteria hybridized exclusively with either probe NEU or probe NmV.

### C3.1.2 Comparative *amoA* sequence analysis

Two ammonia oxidizer strains (Nm93 and Nm103) were isolated by G. Timmermann from the Kraftisried wwtp. Strains Nm 93 and Nm 103 were identified by comparative 16S rRNA sequence analysis (performed by S. Juretschko) as *Nitrosococcus mobilis* and *Nitrosomonas europaea*, respectively. A 665-bp fragment of the *amoA* gene of both ammonia oxidizer isolates was PCR-amplified with the primer set described to be able to amplify all betaproteobacterial ammonia oxidizers by Sinigalliano *et al.* (1995). Nucleic acid sequence analysis of both *amoA* fragments revealed that the sequence of isolate Nm103 was identical to that of the corresponding *amoA* fragment of *N. europaea* Nm50 and unexpectedly displayed only a single base substitution compared to the sequence of isolate Nm93 (99.8% sequence similarity). Initially, the primer set published by Sinigalliano *et al.,* (1995) was also applied for direct analysis of AOB diversity in the Kraftisried waste water treatment plant. However, no amplification product was obtained in these experiments. To enhance the sensitivity of the *amoA* PCR, an additional inner primer pair for nested amplification of *amoA* from *N. europaea* was designed (expected fragment size of 625 bp). The nested PCR products retrieved from the activated sludge were used to

generate an *amoA* gene library. All 13 randomly selected clones showed high similarities to *N. europaea* Nm50 (>99%).

After revaluating the primer pair published by Sinigalliano *et al.* (1995) with a refined data set for the *amoA* gene (Purkhold et al., 2000), it became obvious that these primers only amplified the *amoA* of Nitrosomonas europaea. Thus the *amoA* gene from isolates Nm93 and Nm103 as well as Kraftisried activated sludge was also amplified with a primer set published by Rotthauwe *et al.* (1997). This primer set was also on the basis of new *amoA* data specific for all betaproteobacterial ammonia oxidizers (Purkhold *et al.,* 2000). Nm103 *amoA* and 2 clones derived from the activated sludge showed high similarities with *N. europaea.* However, Nm93 *amoA* and 7 activated sludge clones were almost identical with the *amoA* gene of the type strain of *Nitrosococcus mobilis* Nc2.

## C3.2 *AmoA* based phylogenetic analysis of the ammonia oxidizer population in waste water treatment plants

(pulished in Purkhold et al., 2000)

*AmoA* PCR products (using the primers described by Rotthauwe et al., 1997) were obtained of 4 nitrifying waste water treatment samples (Kraftisried waste water treatment plant, Stuttgart trickling filter 1 and 2 and Mechernich biological contactor; Table 1). Phylogenetic analysis was performed with 35 randomly selected clones. In all plants *amoA* sequences were affiliated to the *Nitrosomonas europaea/Nitrosococcus mobilis* cluster While *N. europaea* and *N. eutropha* related sequences could be found in all of the four plants, only the trickling filter 2 in Stuttgart and the wastewater treatment plant in Kraftisried harbored ammonia oxidizer species, which grouped with *Nc. mobilis*. Remarkably, all four nitrifying waste water treatment samples contained at least two different species of ammonia oxidizing bacteria. As indicated at Purkhold *et al.* (2000) there are also plants, which harbored up to 5 different ammonia oxidizer (e.g. BIOFOR 2 biofilm)

### C3.3 Phylogenetic analysis of alkaliphilic ammonia oxidizing isolates from Mongolian soda lakes

(published in Sorokin *et al.,* 2001)

In the course of this thesis D. Sorokin approached us to perform phylogenetic analyses of ammonia oxidizing strains, which he isolated from Mongolian soda lakes. These lakes represent a unique ecosystem with two extreme properties. High concentrations of alkaline salts create a high saline and high pH environment.

All five isolates (AN1-5), which we obtained from D. Sorokin, possessed 16S rRNA genes with identical sequence. Comparative 16S rRNA sequence analysis demonstrated that the isolates are affiliated with the halophilic ammonia oxidizer *Nitrosomonas halophila*. The low sequence divergence of the 16S rRNA gene (below 1%) with that species indicated that the new alkali-tolerant isolates might belong to the species *N. halophila.* Consistent with these results the *amoA* sequence of the isolate ANs1 showed the highest sequence similarity (95.6% on the nucleic acid level and 98.6% on the amino acid level) with the respective gene of *N. halophila.* This was confirmed by DNA-DNA hybridization studies performed by others.

## C4 Phylogeny and in situ detection of anaerobic ammonia oxidizing bacteria

### C4.1 Discovery of a new anaerobic ammonium oxidizing bacterium

(published in Schmid *et al.,* 2000)

A two-stage semi-technical trickling filter reactor system for the treatment of effluent from anaerobic sludge digestion (typically containing between 500-2500 mg $NH_4^+$-N $l^{-1}$) was maintained at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management at the University of Stuttgart. A partial aerobic nitrification was realized in trickling filter 1 by regulating the ammonium conversion rate via the influent load of ammonium. In that stage 60 % of the ammonium were oxidized to nitrite. Most likely due to ammonia-inhibition no nitrite oxidation occurred in trickling filter 1. Subsequently, the effluent of trickling filter 1 was used as influent for the anoxic trickling filter 2 designed for anaerobic ammonium-oxidation. Actually, in the experimental period the second trickling filter of the semi technical plant in Stuttgart

exhibited anaerobic ammonium oxidation. Trickling filter 2 removed more than 88% of the ammonium and 96% of the nitrite present in the influent. Furthermore, an average production of 64 mg $l^{-1}$ of nitrate (11.3% formation) was observed. Thus, the average total nitrogen removal in trickling filter 2 was 81.2%.

### C4.1.1 In situ detection of ammonia oxidizing bacteria in trickling filter 2 with previously published oligonucleotide probes

Nine 16S-rRNA targeted oligonucleotide probes designed for specific *in situ* detection of the previously recognized anaerobic ammonium-oxidizer Candidatus "Brocadia anammoxidans" (Table 9) were used for *in situ* hybridization of biofilm samples of trickling filter 2. Only probe S-*-Amx-0820-a-A-22 (Table 9) showed bright hybridization signals. Simultaneous application of the *Planctomycetales*-specific probe Pla46 (Table 9) demonstrated that bacteria detectable with probe S-*-Amx-0820-a-A-22 made up more than 99% of the area of those cells stained with probe Pla46.

### C4.1.2 *Planctomycetales* specific 16S rRNA gene sequence analysis of the biofilm in trickling filter 2

A phylogenetic inventory of members of the *Planctomycetales* present in trickling filter 2 was established by using probe Pla46 as forward primer in combination with the universal reverse primer 1390R for PCR 16S rRNA gene amplification. After cloning the PCR product, nine sequences (1363-1365 bp in length) were highly similar to each other (more than 98.8% sequence similarity) but only distantly related to all other sequences presently represented in public 16S rRNA databases. Among those the highest similarity values were calculated for the previously recognized anaerobic ammonium-oxidizer *Candidatus* "Brocadia anammoxidans" (90% - 91%). Other members of the *Planctomycetales* and all other organisms displayed very low sequence similarities below 81%. Consistent results were obtained after application of different treeing methods for phylogenetic analysis of the retrieved *Planctomycetales*-related sequences. Neighbor-joining, maximum-likelihood and maximum parsimony analysis agreed that these sequences form a grouping with the deep-branching anaerobic ammonium-oxidizer within the *Planctomycetales*. Bootstrap support for the clustering of the biofilm-retrieved sequences

with the anaerobic ammonium-oxidizer and for the monophyly of the *Planctomycetales* and the anaerobic ammonium-oxidizer related sequences is highly significant. The newly discovered anaerobic ammonium oxidizer was provisionally named *Candidatus* "Kuenenia stuttgartiensis"

### C4.1.3 Probe design specific for the newly discovered anaerobic ammonium oxidizer

The oligonucleotide probe S-*-Kst-1275-a-A-20 (Table 11) was designed complementary to a specific target region shared between the nine (anaerobic ammonium-oxidizer related) 16S rRNA gene biofilm sequences. Probe S-*-Kst-1275-a-A-20 had at least three mismatches with respect to all other available 16S rRNA sequences. An *in situ* probe dissociation curve was recorded for probe S-*-Kst-1275-a-A-20 with fixed biofilm samples using increasingly stringent conditions. Probe S-*-Kst-1275-a-A-20 yielded strong signals up to 25% (v/v) formamide in the hybridization buffer followed by a decline at 30% (v/v) formamide. Considering strength and position of the mismatches in other known non-target organisms a formamide concentration of 25% (v/v) was chosen as optimal stringency for probe S-*-Kst-1275-a-A-20. After simultaneous hybridization of biofilm material with probe S-*-Kst-1275-a-A-20, and probe S-*-Amx-0820-a-A-22 (labeled with different dyes) exclusively double-labeled cells occurring in dense aggregates were observed.

### C4.1.4 Quantification of the anaerobic ammonium oxidizer population in trickling filter 2

Digital image analysis of confocal biofilm sections simultaneously hybridized with the Cy3-labeled EUB338 probe mixture (Daims *et al.,* 1999; Table 9) and the Cy5-labeled probe S-*-Kst-1275-a-A-20 demonstrated that bacteria related to the anaerobic ammonium-oxidizer occupied 49% (+/-12; 95% confidence limit) of the area of those bacterial cells detectable by *in situ* hybridization. In a control experiment, the relative area of the novel biofilm *Planctomycetales* after hybridization with probe S-*-Amx-0820-a-A-22 was determined to be 50% (+/-8; 95% confidence limit)**.** Since about 90% of the total bacterial cells within the biofilm stained with SYBR Green I were simultaneously detectable with the bacterial probe mixture, the bacteria related to anaerobic ammonium-oxidizer constitute almost half of the bacterial biomass in trickling filter 2.

### C4.1.5 Aerobic ammonia oxidizers in the biofilm of trickling filter 2

Biofilm of trickling filter 2 was investigated by fluorescence *in situ* hybridization with a set of 16S rRNA-targeted oligonucleotide probes specific for aerobic ammonia oxidizing bacteria of the *Betaproteobacteria* (Table 9). In confocal biofilm sections 27% (+/-8; 95% confidence limit) of the area of those cells detectable with the bacterial probe set was occupied by ammonia-oxidizers identified as halophilic or halotolerant members of the genus *Nitrosomonas* by simultaneous hybridization with probes Nso 1225, Nso190 and NEU (Table 9). In addition, no nitrite-oxidizing bacteria of the genera *Nitrobacter* and *Nitrospira* could be visualized by FISH.
For 16S rRNA-independent high resolution diversity analysis of betaproteobacteria*l* ammonia-oxidizers of trickling filter 2 the *amoA* approach was performed (Rotthauwe *et al.,* 1997). Gel retardation was performed with *amoA* gene fragments amplified from trickling filter 2 biofilm derived DNA. Three distinct bands were observed, cloned and sequenced. Phylogenetic analysis of the deduced amino acid sequences of the molecular isolates representing the respective bands demonstrated their grouping with *Nitrosococcus mobilis* (band 1; 3 clones, identity values between 95.7 and 97.2% on the amino acid level to the respective AmoA fragment of *N. mobilis), Nitrosomonas eutropha* (band 2, one clone, identity value of 95.0 % on the amino acid level to the respective AmoA fragment of *N. eutropha*), and *Nitrosomonas europaea* (band 3, 2 clones, identity values of 98.6 - 99.3% on the amino acid level to the respective AmoA fragment of *N. europaea)*.

## C4.2 Analysis of rRNA operon of anaerobic ammonium oxidizing bacteria

(published in Schmid *et al.,* 2001)

### C4.2.1 Helix 9 of the 16S rRNA of anaerobic ammonium oxidizing bacteria contains an insertion

16S rRNA gene sequences of the anaerobic ammonium oxidizing bacteria *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" contain an insertion with 20 nucleotides in length located within helix 9 (beginning at *E. coli* position 158). A pronounced secondary structure can be predicted for this insertion leading to the proposal of the two new

subhelices 9a and 9b. With the exception of the *Planctomycetales*-affiliated environmental 16S rRNA clone AF280847 (LaPara *et al*., 2000) which contains 14 inserted nucleotides at this site, this insertion is absent from all other 16S rRNA gene sequences in the ARB data base. The presence of the insertion in the mature 16S rRNA of the anaerobic ammonium oxidizing bacteria was demonstrated using the insertion-targeted oligonucleotide probes S-*-Amx-0156-a-A-18 (specific for *Candidatus* "Brocadia anammoxidans", Table 9) and the newly designed probe S-S-Kst-158-a-A-18 (specific for *Candidatus* "Kuenenia stuttgartiensis", Table 11) for FISH. Probe S-S-Kst-158-a-A-18 has at least four mismatches with all other available 16S rRNA sequences including *Candidatus* "Brocadia anammoxidans". Optimal stringency for probe S-S-Kst-158-a-A-18 was determined by a probe dissociation curve and accordingly set to 25% (v/v) formamide. Probes S-*-Amx-0156-a-A-18 andS-S-Kst-158-a-A-18 hybridized specifically to their respective target organisms with an intensity comparable to the 16S rRNA-targeted probe S-*-Amx-0820-a-A-22.

### C4.2.2 Phylogenetic analysis of the rRNA operon of anaerobic ammonium oxidizing bacteria

In contrast to most other recognized members of the *Planctomycetales* (Liesack and Stackebrandt, 1989; Menke *et al*., 1991; Ward *et al*., 2000) the 23S rRNA gene sequence of both investigated anaerobic ammonium oxidizing bacteria is directly linked by an ISR (approx. 450 bp) to the 16S rRNA gene. *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" possess 90.5% and 87.8% sequence similarity on the 16S and 23S rRNA level, respectively. 16S and 23S rRNA sequence similarities between the anaerobic ammonium oxidizing bacteria and all other planctomycetes are below 78% and 72%, respectively. Phylogenetic analysis were performed independently for 16S rRNA and 23S rRNA as well as for a concatenated 16S and 23S rRNA data set. Independent from the data set analysed and the treeing method used the anaerobic ammonium oxidizing bacteria form a stable, deep branching line of descent within the order *Planctomycetales.*

### C4.2.3 Design of an oligonucleotide probe targeting the 23S rRNA of all anaerobic ammonium oxidizing bacteria

The obtained 23S rRNA gene sequences were used for design of the oligonucleotide probe L-*-Amx-1900-a-A-21 specific for both anaerobic ammonia-oxidizers (Table 11). Probe L-*-Amx-1900-a-A-21 has at least three mismatches with respect to all other available 23S rRNA sequences. The optimal hybridization stringency for probe L-*-Amx-1900-a-A-21 was determined by recording an *in situ* probe dissociation curve with an enrichment culture of *Candidatus* "Brocadia anammoxidans" and biofilm material containing *Candidatus* "Kuenenia stuttgartiensis". 30% (v/v) formamide was chosen as optimal stringency for probe L-*-Amx-1900-a-A-21.

## C4.3 *In situ* detection of anaerobic ammonium oxidizing bacteria using intergenic spacer region (ISR) targeted oligonucleotide probes

The ISRs between the 16S and 23S rRNA genes of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis is about 450 bp long and contains coding regions for the tRNAs tAlanine and tIsoleucine. Four probes were designed for each of the anaerobic ammonium oxidizer targeting the ISR (tRNA genes excluded) and applied in FISH. Each probe of both sets had at least four mismatches with all 16S and 23S rRNA sequences and all other sequences at GenBank (www.ncbi.nlm.nih.gov). For optimal signal intensities the four ISR probes for each species (labeled with the same dye) were applied together. This resulted in strong, specific hybridization signals for *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis". No addition of formamide to the hybridization buffer was required to obtain specific signals. The specificity of the ISR probes was confirmed by simultaneous application of 16S and 23S rRNA-targeted probes (S-*-Amx-820-a-A-22 and L-*-Amx-1900-a-A-21, labeled with different dyes, Table 9 and 11, respectively; Figure 15) specific for the ammonium oxidizing bacteria. The suitability of the ISR probes to measure activity changes of ammonium oxidizing bacteria was evaluated with cells of *Candidatus* "Brocadia anammoxidans" which were inhibited by exposure to oxygen for different time periods. Active cells (maintained at anoxic conditions) are detectable by both the rRNA and the ISR-targeted probes. However,

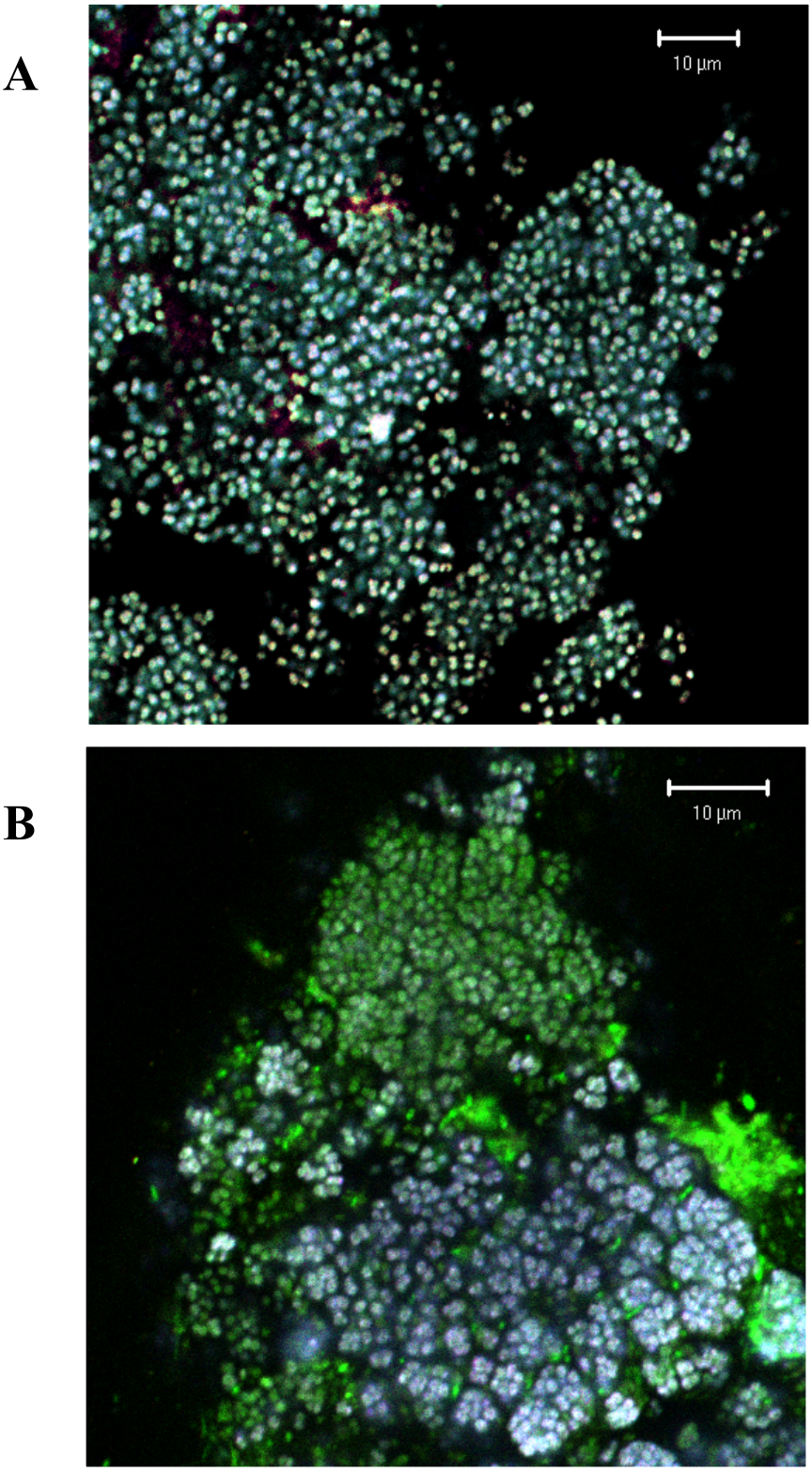



Fig. 15**. A**. Enrichment culture of *Candidatus* "Brocadia anammoxidans" hybridized with the *Candidatus* "Brocadia anammoxidans" specific ISR targeting probes (labeled with Cy3), probe S-*-Amx-820-a-A-22 (labeled in Cy5) and the EUBmix (labeled in Fluos). *Candidatus* "Brocadia anammoxidans" cells appear white, because of simultaneous hybridization of all three probes. **B**. Biofilm of trickling filter 2 of the semitechnical plant in Stuttgart hybridized with the *Candidatus* "Kuenenia stuttgartiensis" specific ISR targeting probes (labeled with Cy3), probe S-*-Amx-820-a-A-22 (labeled in Cy5) and the EUBmix (labeled in Fluos). *Candidatus* " Kuenenia stuttgartiensis" cells appear white, because of simultaneous hybridization of all three probes.

after 1 h of oxygen exposure only a minor percentage (8 ± 1 %) of the anaerobic ammonium oxidizer cells showed ISR probe conferred fluorescence while cells could still be detected with specific 16S and 23S rRNA-targeted probes. After 4 h of oxygen incubation no ISR probe signals were detectable while the mean 16S and 23S rRNA-probe conferred fluorescence was only reduced to 42 ± 1 % (16S rRNA-targeted probe S-*-Amx-0156-a-A-18) and 72 ± 1 % (23S rRNA-targeted probe L-*-Amx-1900-a-A-21), respectively. Even after one day of complete physiological inhibition by oxygen exposure, the AAOB cells were still detectable with 16S and 23S rRNA probes while again no ISR probe signal was recorded.

# D Discussion

## D1. Characterization of activated sludge flocs

The activated sludge process is the most common technique in biological wastewater treatment but it is often hampered by plant malfunctions like sludge bulking and foaming. The settling quality of activated sludge flocs is routinely monitored by measuring the sludge volume index (SVI). The SVI is influenced by many parameters like the excessive growth of filamentous bacteria (e.g. Jenkins, 1993; Wanner, 1994), floc size and heterogeneity (e.g. Andreadakis, 1993), and chemical composition of the flocs (Urbain *et al*., 1993). Therefore the SVI gives no specific information on the causes of sludge bulking. This lack of fundamental data makes proper plant management not possible.

During the last thirty years two different approaches for determining more specific properties of activated sludge flocs have been used. Light microscopic surveys provided floc diameter distributions (e.g. Parker et al, 1971; Li and Ganczarczyk, 1991; Jorand et al, 1995) and provisional information on the abundance (Jenkins, 1993) and identity (Eikelboom, 1971) of filamentous bacteria present in the sludge. The introduction of epifluorencence microscopy and fluorescence in situ hybridization (FISH) greatly improved the identification of filaments (e.g. Wagner *et al.,* 1994; de los Reyes *et al.,* 1997; Blackall *et al.,* 2000; Davenport *et al.,* 2000; Kanagawa *et al.,* 2000). But without adequate techniques for image analysis all microscopic methods were time consuming and not completely objective.

A second approach was the measurement of physical sludge properties like floc diameters and the fractal dimension $D_f$ of the flocs by light scattering. The structure of biological aggregates was deduced from the intensities of the scattered light measured as a function of scattering angles (e.g. Guan *et al.,* 1998). Despite simplicity of the measurement this technique has the disadvantages that no data about the microbial population structure of the aggregate can be obtained.

This thesis describes the introduction of confocal laser scanning microscopy (CLSM) and image analysis to floc characterization (Schmid *et al.,* submitted). This combination leads to a comprehensive description of the floc structure and the composition of the microbial population forming the floc. A protocol was developed which allows via CLSM image acquisition and

subsequent image analysis to determine actual biovolumes from three-dimensional images of unspecifically stained flocs (Schmid *et al.*, submitted). Since CLSM is an optical based method, results can always be controlled by visualization of the processed flocs. Additionally, heterogeneity factors like the fractal dimension (e.g. Thill *et al.,* 1998) and the ratio of real surface of a floc and the surface of a sphere with the same volume as the floc ($S_{floc}/S_{sphere}$; Schmid *et al.*, in preparation) can be calculated from the original images taken for biovolume measurements. CLSM in combination with fluorescence in situ hybridization as applied in this thesis is also the most suitable tool for qualitative and quantitative microbial population analysis (e.g. Wagner *et al.*, 1994; Wagner, *et al.*, 1998; Daims *et al.,* 2001). The image acquisition can be done with an automated stage and a suitable macro for the image acquisition software. For the calculation of physical parameters of the flocs and quantification of bacterial population macros for the Carl Zeiss Vision software package Kontron KS400 3.0 are already available (Schmid *et al.,* submitted; Schmid *et al.,* 2000, Daims et al., 2001). Due to the high flexibility of this approach, it can be applied fully or in parts to all forms of microbial aggregates as indicated in previous studies (e.g. Lawrence *et al.*, 1991) and also in this thesis (Etterer *et al.,* in preparation). However, this approach holds also some minor problems. Though the measuring of real biovolumes is more comprehensive than the determination of diameters, a transfer of volume values to diameters is necessary for comparison of the obtained results with those of other studies (e.g. Parker et al, 1971; Li and Ganczarczyk, 1991; Jorand et al, 1995). Since diameters could not directly deduced from the obtained volume values, diameters had to be calculated from biovolumes by using the respective $S_{floc}/S_{sphere}$ heterogeneity value. Additionally, the available FISH probe set does not target specifically all different bacterial species present in an activated sludge. It is likely, that both efficient floc formers and bacterial species, which hamper floc settling, are detected with the same for example group-specific oligonucleotide probe. This may lead to inconsistent results during comparison of flocs of different origin and settlling ability. A construction of new specific probes for activated sludge relevant organisms will help to solve this problem. There is also no efficient way to analyze the chemical composition of biological aggregates in situ. So it has to be determined classically by isolation of the components (e.g. Nielsen et al. 1996). Nevertheless with this set of mainly CLSM based methods it was possible for the first time to observe significant shifts in activated sludge floc architecture, chemical composition and population structure occurring during enrichment for flocs with bad settling properties (Schmid *et al.*, submitted). In an experiment activated sludge was left settling in a

cylinder for a period of time. Poorly settling flocs were enriched in the water phase and sampled at certain time points. In the different plants investigated in this study only slight shifts in the biovolume distribution were visible. Parameters like Df and $S_{floc}/S_{sphere}$ generally indicated a higher heterogeneity for sludge enriched for poorly settling flocs. For all plants the carbohydrate content decreased if flocs with bad settleability were enriched. Changes in the content of humic substances, proteins and DNA during enrichment of flocs with bad settleability differed between different plants and did not obey general rules. Shifts in the population structure of each plant could be observed, but since group specific probes were used in this study, it is not possible to tell whether poor settling flocs doe contain more filamentous bacteria or not.
Nevertheless, a further technical improvement, which reduces costs in terms of equipment and manpower needed for the surveys could render the application of the set of tools described in this study to a widely used approach for monitoring the settle ability of activated sludge.

## D2. Development and characterization of aerobic granules

The ability to detect structural and microbial changes of sludge flocs is a prerequisite for the determination of causes for settling problems in wwtps, but there is still the problem of finding an adequate response to stop those undesired developments. Currently, one option is the supply of chemical settling enhancers. However, addition of these chemicals is cost intensive and burdens the environment. Another imaginable approach is bioaugmentation (van Limbergen *et al.*, 1998) with recognized floc-forming bacteria. However, this method is often hampered by the inflexibility of the applied cultures and sludge of other plants to adapt to the new environment and by a massive grazing of *Protozoa* (Bouchez *et al.*, 2000). In principle, granules formed by microbial communities could be used as efficient vectors to introduce allochthonous bacteria into an activated sludge community For anaerobic granules it is known that they provide (i) a stable structure even under different growth conditions (Grotenhuis *et al.*, 1991) and (ii) the possibility to introduce new degradative pathways by inoculation with bacteria possessing the wanted metabolism (Ahring *et al.*, 1992). However, for the application in aerobic wastewater treatment it is necessary to produce aerobic granules with similar and evaluated abilities. This thesis reports for the first time the determination of the microbial community of these aerobic granules (Etterer *et al.*, in preparation). Application of the full-cycle rRNA approach revealed that *Zoogloea ramigera* neotype strain (ATCC 19544; Unz, 1971) related organisms mainly form the matrix of

the aerobic granules. Image analysis showed that this matrix contains at least two compartments. At the surface of the granule the density and ribosome content of the cells were very high, which might indicate a growing zone. The *Zoogloe ramigera* cells in the core region of the granule are less dense and contain less ribosomes. This could be an effect of nutrient or oxygen depletion. In fact experiments with microsensors performed by T. Etterer showed that oxygen could not penetrate the granule completely. The biological phosphorous removal (BioP) of the aerobic granules is a strong indication that the partitioning of aerobic granules in different ecological niches could provide habitats for establishing different physiotypes like comprehensive N-removal.

Since the aerobic granules exhibited also a measurable ammonia oxidation activity, it was surprising that ammonia oxidizing bacteria were not detected by FISH. A chemical binding of the ammonium to substances in the reactor could not be supported, because inhibition of the ammonia oxidation with 2,4 dinitrophenol showed its biological origin. The ammonia oxidizing activity of the aerobic granular sludge might be caused by heterotrophic nitrifying organisms. However, this has to be proven by further inhibition experiments with allyl-thio-urea, which specifically inhibits autotrophic ammonia oxidizing bacteria (Bedard and Knowles, 1989).

Aerobic granules have the potential to harbor different physiological traits, which can be introduced by stimulating the growth (e.g. by altering media composition) or by inoculation of certain bacterial strains. Further they are resistant to grazing, because of their size and compactness. These features make aerobic granules a promising tool for future, more reliable bioaugmentation strategies.

## D3. Detection of aerobic ammonium oxidation by a 16S rRNA and *amoA* gene based approach

Nitrification is one of the key processes in wastewater treatment, but still the ecology and phylogeny of the responsible organisms is poorly understood. For ammonia oxidizers (on which this part of the thesis will be focused) two detection and identification systems are commonly in use. One of these is based on the 16S rRNA, and includes fingerprinting techniques (e.g. Kowalchuk *et al.,* 1997; Aakra *et al.,* 1999), phylogenetic analysis (e.g. McCaig *et al.,* 1999; Mendum *et al.,* 1999; Stephens *et al.,* 1996) and FISH for the detection of these organisms in the environment (e.g. Wagner *et al.*, 1995; Mobarry *et al.*, 1996; Juretschko *et al.*, 1998). However,

especially finger printing techniques based on amplification of rRNA of ammonia oxidizers are hampered by a big shortcoming. The 16S rRNA of ammonia oxidizing bacteria does not provide primer target sites suited for a specific amplification of all betaproteobacterial ammonia oxidizing bacteria. Thus a complete detection of all relevant ammonia oxidizers by applying one 16S rRNA gene targeting primer pair is not possible. Otherwise *amoA* (coding for the active site of the ammonium monooxygenase) contains these betaproteobacterial ammonia oxidizer specific primer target sides and therefore the *amoA* approach is more sophisticated and provides comparable results (e.g. Rotthauwe et al., 1995; Rotthauwe *et al.*, 1997). But the 16S rRNA as well as the *amoA* approach were handicapped by incomplete sequence data sets. Short and badly determined sequences of even characterized species of ammonia oxidizing bacteria made an assignment of environmental sequences to their correct phylogenetic positions and a specific probe and primer design hardly possible (Purkhold *et al.*, 2000).

The completion of 16S rRNA and the *amoA* sequence data sets now including full sequences of all previously described aerobic ammonia oxidizing bacteria (Purkhold *et al.*, 2000) showed that many of the "new" ammonia oxidizer sequences proposed in different studies (e.g. Stephens *et al.,* 1996; Ward 1996; McCaig *et al.,* 1999) were in truth affiliated to already recognized species (Purkhold *et al.*, 2000). A revaluation of the specificity of published primers and oligonucleotide probes based on the 16S rRNA for beta-subclass ammonia oxidizers revealed that none of them showed 100% of their proposed sensitivity or specificity (Purkhold *et al.*, 2000). This most likely caused biases in previous environmental studies by use of, for example, primers with underestimated specificity (e.g. Kowalchuk *et al.*, 1997; Ward *et al.,* 1997; Ward *et al.,* 2000).

Also investigations in this thesis were hampered by such inconsistencies (Juretschko *et al.,* 1998). In samples of the wastewater treatment plant connected to the animal rendering plant in Kraftisried/Allgäu a FISH survey indicated a large amount of *Nitrosococcus mobilis* as the dominant ammonia oxidizer. Only isolation of this organism succeeded, while both 16S rRNA and *amoA* clone libraries did not contain this species. The non-appearance of 16S rRNA clones of *Nitrosococcus mobilis* could be explained by DNA isolation and PCR biases (e.g. Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Tanner *et al*., 1998). However, the lack of *amoA* clones remained uncertain until it turned out that the used primer set for this investigation (Sinigallioano *et al.,* 1995), which was described as specific for all beta ammonia oxidizers, based on newly obtained data could only detect *Nitrosomonas europaea* (Purkhold *et al.,* 2000). Using a primer set with a much better coverage of aerobic ammonia oxidizer species (Rotthauwe

*et al.,* 1997) for *amoA* PCR, *amoA* of *Nitrosococcus mobilis* could be detected in the Kraftisried plant (Purkhold *et al.,* 2000). Therefore, as a general guideline, the *amoA* and the 16S rRNA approach should always be applied in parallel for environmental diversity surveys of aerobic ammonia oxidizers. Also results obtained from PCR dependent fingerprinting techniques should subsequently be controlled by sequencing or by hybridization with suitable probes of resulting gel bands (Purkhold *et al.*, 2000).

All the environmental systems investigated in this thesis (Juretschko *et al*, 1998; Helmer *et al*, 1999, Schmid *et al.*, 2000) showed a consortium of ammonia oxidizing bacteria with a considerable diversity. However, the Kraftisried plant is exceptional in this context, because the population of the at least 3 ammonia oxidizing species as detected by FISH was by far dominated by *Nitrosococcus mobilis*. This ammonia oxidizing species was previously only recognized in brackish water (Koops *et al.*, 1976). Adapted to salty environments this species seems to have a selective advantage in the salt rich habitat of an animal rendering waste water treatment plant. If the living conditions change; other ammonia oxidizers present, which are better adapted to the new conditions might take over the ammonia oxidation. This would prevent an intoxication of the system, which could lead to a breakdown of the plant. Consequently the diversity of ammonia oxidizing bacteria might be an indicator for the process stability of the ammonia oxidation. Therefore, this thesis contributed to the elaborated tool set (Juretschko *et al*, 1998; Purkhold *et al.*, 2000) necessary for the direct detection of ammonia oxidizing bacteria in the environment, which should enable us to learn more about systems with different stability.

## D4. Detection of anaerobic ammonium oxidation in the environment

1998 an unexpected nitrogen loss in the rotating biological contactor in Mechernich treating landfill leachate was reported (Helmer *et al.*, 1999). Since this plant contained very high amounts of aerobic ammonia oxidizers, as demonstrated by quantitative FISH (Helmer *et al.*, 1999) this phenomenon was initially attributed to the capability of these bacteria to perform a limited anaerobic oxidation of ammonia (e.g. Bock *et al.*, 1995; Jetten *et al.*, 1999). However, one year later a similar nitrogen loss was observed in a semitechnical trickling filter system in Stuttgart. This plant was designed for anaerobic ammonium oxidation in accordance to the findings of a group of scientists in Delft, The Netherlands (e.g. van der Graaf *et al.,* 1996; Strous *et al.,* 1999; Strous, 2000). At the same time, these researches reported on the successful enrichment of a new

organism affiliated to the order *Planctomycetales* and provisionally named *Candidatus* "Brocadia anammoxidans" from a Dutch waste water treatment plant. This bacterium is capable to oxidize ammonium anaerobically with rates which significantly exceed those of the aerobic ammonia oxidizers (Strous *et al.,* 1999). Consequently, efforts were made in this thesis to detect this novel organism in Stuttgart. Surprisingly, it turned out that the anaerobic trickling filter in Stuttgart indeed contained a highly abundant organism affiliated to *Candidatus* "B. anammoxidans", but only with a relatively low 16S rRNA gene sequence similarity indicating genus level diversity within the anaerobic ammonium oxidizing bacteria. Consequently, this novel annaerobic ammonia oxidizer was named *Candidatus* "Kuenenia stuttgartiensis" (Schmid *et al.,* 2000). The trickling filter from Stuttgart was the first environmental system were anaerobic ammonia oxidizing bacteria could be detected and identified in situ. Consistent with the findings in the Stuttgart trickling filter, *Candandidatus* "Kuenenia stuttgartiensis" could subsequently be found in very high abundance also in the Mechernich plant (Helmer *et al.*, 2002), indicating that this species might be more abundant than *Candidatus "*Brocadia. anammoxidans". in environmental systems (see also Egli *et al.,* 2001).

Apparently, the process of anaerobic ammonium oxidation has a very high commercial value, because it enables a cost effective treatment of wastes with high ammonium load and low organic carbon content (Strous, 2000). Thus a sophisticated system based on phylogenetic analysis and FISH (Schmid *et al.,* 2000; Schmid *et al.,* 2001) is needed for the detection of new species of anaerobic ammonia oxidizing bacteria in the environment, to determine the composition of anaerobic ammonium oxidizing population and to monitor shifts in these populations. This data is a prerequisite to learn more about the ecology of anaerobic ammonium oxidizing bacteria and it will help to gain information about the process stability in newly developed full-scale anaerobic ammonium oxidizing plants.
However, for the startup and for the further management of a full-scale anaerobic ammonium oxidizing plant it will be a prerequisite to monitor changes of the ammonium oxidizing activity. A deduction of the cell activity from signal intensities of FISH targeting the rRNA as suggested by Poulsen et al. (1993) for fast growing heterotrophs was not applicable. For ammonia oxidizers as slow growing chemolithoautothrophic organisms it was shown that they keep their ribosome content stable even in longer periods of inhibition (Wagner *et al.,* 1995) or starvation (Morita, 1993; Morgenroth *et al.,* 2000). In the course of inhibition experiments for this thesis, this was

also proven for anaerobic ammonium oxidizing bacteria (Schmid *et al.*, 2001). An alternative is the concept is based on observations by Cangelosi and Brabant (1997). The availability of intergenic spacer region RNA in the cell is dependent on the ratio of its degradation and production. Only in the increased transcription of the rRNA operon in active cells leads to an accumulation of intergenic spacer RNA in cell to detectable levels by FISH (Oerther *et al*. 20009 Therefore, fluorescently labeled oligonucleotide probes targeting the intergenic spacer region between the 16S and 23S rRNA genes of *Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans" were used in this thesis (Schmid *et al.,* 2001) to monitor activity changes during inhibition with oxygen of anaerobic ammonium oxidizing bacteria. The continuous monitoring of cell activity during the startup and operation of anaerobic ammonium oxidizing plants could help to shorten the time period to obtain a stable population and allows a sophisticated control of the process.

The detection of the anaerobic ammonium oxidizing bacteria in the environment is just the beginning, because virtually nothing is known about the genome or the biochemical composition of anaerobic ammonium oxidizing bacteria. Approaches like environmental genomics (e.g. Stein *et al.,* 1996; Schleper *et al.,* 1997; Béjà *et al.,* 2000; Rondon *et al.,* 2000) cloning and sequencing of large fragments of environmental DNA) could give further insight in the possibilities of these bacteria. With genomic sequence information the analyses of interesting genes and their products will become possible. These and other methods will contribute in future studies to get a full picture of the anaerobic ammonium oxidation.

# E Summary

Microbial aggregates are complex structures formed by a variety of organisms and are found in many environments. Due to their complexity, they can integrate different physiological properties and degradation pathways. Waste water treatment takes advantage of these properties, but has also to deal with the uncertainties arising from exploiting living organisms. This thesis addresses some fundamental aspects of microbial aggregates in waste water treatment systems by investigating the architecture, biodiversity and physiology.

In the first part of this thesis, methods to determine the biovolume and heterogeneity factors of activated sludge flocs were developed by applying confocal laser scanning microscopy in combination with various digital image analysis tools. These techniques were complemented by chemical analysis of extra-cellular polymeric substances and by quantitative fluorescence in situ hybridization for determining the bacterial community composition of activated sludge. This set of methods was applied on activated sludge flocs with good as well as poor settling properties from three wastewater treatment plants. All measured parameters differed significantly between the different floc types Interestingly, the fractal dimension of flocs with poor settling ability was always small compared to good settling flocs. Furthermore, pronounced shifts in microbial community composition between flocs with good and bad settling properties could be observed.

In addition, the microbial community composition of an aerobic granular sludge, which allowed to combine excellent settling ability with nitrification and efficient phosphorous removal, was investigated for the first time. The application of the full-cycle rRNA approach revealed that a population affiliated with *Zoogloea ramigera*$^{T}$ formed the matrix of these granules and constituted about 90% of the total population. Other bacteria mainly affiliated with the *Gammaproteobacteria* were imbedded in this matrix. Further experiments have to show if it is possible to introduce allochthonous microbial strains with different physiological properties into these granules without changing their physical structure. This would allow to use such granules as inoculum for biaugmentation approaches.

The second part of the thesis focused on the ammonium removal in wastewater treatment plants. In order to prevent eutrophication of receiving waters the oxidation of ammonia to nitrite is one of the key processes in waste water treatment. In this thesis the diversity of aerobic ammonia oxidizing in different nitrifying reactors was monitored by methods based on refined data sets for

the 16S rRNA and *amoA* genes. These efforts led to the identification of the numerically dominant ammonia-oxidizers in various waste water treatment plants. Future research will show whether the diversity of ammonia-oxidizing bacteria is linked with process stability.

In order to learn more about diversity and habitats of the recently discovered but yet not culturable anaerobic ammonium oxidizing bacteria, which oxidize ammonium with nitrite under anaerobic conditions to dinitrogen gas, a rRNA gene based system for detecting anaerobic ammonium oxidizing bacteria was developed and applied to environmental systems. These experiments revealed an unexpected genus-level diversity of anaerobic ammonium oxidizing bacteria within the order *Planctomycetales* and led to the identification of a previously unknown anaerobic ammonium oxidizing bacterium, *Candidatus* “Kuenenia stuttgartiensis”. For obtaining more information on the in situ physiology of anaerobic ammonium oxidizing bacteria, oligonucleotide probes targeting the intergenic spacer region (ISR) between the 16S and 23S rRNA gene were developed and successfully applied for fluorescence in situ hybridization. The ISR is degraded relatively fast and accumulates only in active cells. By using the ISR approach the inhibitory effect of oxygen on anaerobic ammonium oxidizing bacteria was demonstrated. In future experiments these tools will be helpful to monitor the start-up phase of waste water treatment reactors with anaerobic ammonium oxidizing activity.

# E Zusammenfassung

Mikrobielle Aggregate sind komplexe Strukturen, die aus einer grossen Anzahl von Organismen bestehen und in vielen Lebensräumen angetroffen werden. Aufgrund ihrer Komplexität können sie verschiedene metabolische Eigenschaften aufweisen. Die Abwassereinung zieht Vorteile aus diesen Eigenschaften, muss aber auch mit Unwägbarkeiten, die mit dem Ausbeuten von lebenden Organismen einhergehen, fertig werden. Diese Arbeit beschäftigt sich mit einigen fundamentalen Aspekten von mikrobiellen Aggregaten in der Abwassereinigung wie ihre Architektur, Physiologie und die Diversität der enthaltenen Mikroorganismen.

Im ersten Teil dieser Arbeit wurden Methoden zur Bestimmung von Biovolumina und Heterogenitätsfaktoren von Belebschlammflocken entwickelt. Verwendet wurde zu diesem Zweck eine Kombination von Konfokaler Laser Scanning Mikroskopie und digitaler Bildanalyse. Chemische Analysen von extrazellulären polymeren Substanzen und einer Quantifizierung der mikrobiellen Population mittels Fluoreszenz in situ Hybridisierung komplettierten diese Techniken. Anhand dieser Methoden wurden Belebschlammflocken mit unterschiedlichen Absetzeigenschaften von drei Abwassereinigungsanlagen untersucht. Die Messergebnisse zwischen den verschiedenen Flockenarten variierten deutlich. Auffallend war, dass die fraktale Dimension von Flocken mit schlechtem Absetzverhalten stets geringer war als von denen mit gutem Absetzverhalten. Des weiteren konnten klare Veränderungen in der mikrobiellen Populationszusammensetzung der unterschiedlichen Flockenarten festgestellt werden.

Zum ersten Mal wurde die Populationszusammensetzung von aeroben granulärem Schlamm bestimmt. Dieser zeichnet sich durch sehr gute Absetzbarkeit in Kombination mit Nitrifikation und effizienter Phosphateliminierung aus. Die Anwendung des rRNA Ansatzes zeigte, dass Bakterien, die nahe mit *Zoogloea ramigera*$^{T}$ verwandt sind, die Matrix dieser Granula bilden und ca. 90% der Gesamtpopulation darstellen. Andere Bakterien, überwiegend *Gammaproteobacteria*, waren in deutlich geringerer Zahl in diese Matrix eingebettet. Weitere Experimente müssen zeigen, ob es möglich ist allochthone Stämme von Mikroorganismen mit verschiedenen physiologischen Eigenschaften in diesen Granula anzusiedeln. Das würde die Verwendung dieser Granula als inoculum für Bioaugmentationsanwendungen erlauben.

Der zweite Teil dieser Arbeit beschäftigte sich mit der Eliminierung von Ammonium in Abwassereinigungsanlagen. Um die Eutrophierung von Gewässern zu vermeiden, ist die

Oxidation von Ammonium zu Nitrit einer der vordringlichsten Prozesse in der Abwassereinigung. In dieser Arbeit wurde mit Methoden, die auf komplettierten 16S rRNA and *amoA* Gen Datensätzen beruhten, die Diversität von aeroben Ammoniak Oxidierern in verschiedenen nitrifizierenden Reaktoren beobachtet. Dadurch konnten dominante ammoniakoxidierende Bakterien in verschiedenen Abwassereinigungsanlagen identifiziert werden. Weitere Analysen werden zeigen, ob die Diversität von Ammoniak Oxidierern einen Einfluss auf die Prozessstabilität hat.

Um mehr über die Diversität und Lebensräume von den kürzlich entdeckten, aber nicht kultivierbaren, anaeroben ammoniumoxidierenden Bakterien, die Ammonium mit Nitrit unter anaeroben Bedingungen zu Luftstickstoff oxidieren, zu erfahren, wurde eine auf dem rRNA Gen basierende Methode zum Nachweis von anaeroben ammoniumoxidierenden Bakterien entwickelt und auf Umweltproben angewandt. Dabei zeigte sich eine unerwartete Diversität anaerober ammoniumoxidierender Bakterien auf Gattungsebene in der Ordnung der *Planctomycetales*. Zudem konnte das vorher noch unbekannte anaerobe ammoniumoxidierende Bakterium *Candidatus* "Kuenenia stuttgartiensis" beschrieben werden. Um mehr Information über die in situ Physiologie der anaeroben ammoniumoxidierenden Bakterien zu erhalten, wurden Oligonukleotidsonden, die die Spacerregion zwischen dem 16S und 23S rRNA Gen (ISR) erfassen, konstruiert und erfolgreich bei Fluoreszenz in situ Hybridisierung angewendet Die ISR wird relativ schnell abgebaut und ist nur in aktiven Zellen detektierbar. Mit dem ISR Ansatz konnte der hemmende Effekt von Sauerstoff auf die anaeroben ammoniumoxidierenden Bakterien demonstriert werden. In zukünftigen Experimenten werden diese Methoden hilfreich sein, um Abwassereinigungsreaktoren mit anaerober Ammonium Oxidationsaktivität während der Startphase zu beobachten.

# F References

(This section only refers to the Introduction, Materials and Methods, Results and Discussion parts of this thesis)

**Aakra, Å., J. B. Utaker, and I. F. Nes.** 1999. RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int. J. Syst. Bacteriol. **49:**123–130.

**Ahring, B.K., Christiansen, N., Mathrani, I., Hendriksen, H.V., Macario, A.J.L., and E.C. Macario.** 1992. Introduction of a novo bioremediation ability, acryl reductive dechlorination into anaerobic granular sludge by inoculation of sludge with *Desulfomonile tiedjei*. Appl. Environ. Microbiol. **58**: 3677-3682.

**Allison, D. G. and I. W. Sutherland.** 1987. The role of exopolysaccharides in adhesion of fresh water bacteria. J. Gen. Microbiol. **133:** 1319-1327.

**Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., and L. Raskin.** 1996. The oligonucleotide probe database. Appl Environ Microbiol **62:** 3557-3559.

**Alm, E.W., Zheng, D., and L. Raskin.** 2000. The presence of humic substances and DNA in RNA extracts affects hybridization results. Appl. Environ. Microbiol. 66: 4547-4554.

**Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and D.J. Lipman.** 1990. Basic local alignment search tool (BLAST). J. Mol. Biol. **215:** 290-296.

**Amann, R.I., Krumholz, L., and D.A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172**:762-770.

**Amann, R. I.** 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p. 1-15. In A.D.C. Akkerman, J.D. van Elsas and F.J. de Bruijn (ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dortrecht, Netherlands.

**Amann, R.I., Ludwig, W., and K.-H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143-169.

**Anderson, I.C., Poth, M., Homstead, J., and D. Burdige**. 1993. A comparison of NO and $N_2O$ production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrphic nitrifier *Alcaligenes faecalis*. Appl. Environ. Microbiol. **59:**3525-3533.

**Andreakis, A. D.** 1993. Physical and chemical properties of activated sludge floc. Wat. Res. **27:** 1707-1714.

**Arthur, J.W., C.W. West, K.N. Allen, and S.F. Hedke.** 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bull. Environ. Contam. Toxicol. **38**:324-331.

**Barber, J.** B. and J. N. Veenstra. 1986. Evaluation of biological sludge properties influencing volume reduction. J. Wat. Poll. Cont. Fed. **58:** 149-156.

**Bedard, C. and R. Knowles.** 1989. Physiology, biochemistry, and specific inhibitors of $CH_4$, $NH_4^+$, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. **53:** 68-84.

**Béjà, O., Suzuki, M. T., Koonin, E. V., Aravind, L., Hadd, A., Nguyen, L. P., Villacorta, R., Amjadi, M., Garrigues, C., Jovanovich, S. B., Feldman, R. A. and E. F. DeLong.** 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environ. Microbiol. **2:** 516-529.

**Blackall, L.L., Seviour, E.M., Cunningham, M.A., Seviour, R.J., and P. Hugenholtz.** "*Microthrix parvicella*" is a novel, deep branching member of the Actinomycetes subgroup. 1994. Syst. Appl. Microbiol. **17:**513-518.

**Blackall, L.L., Seviour, E.M., Bradford, D, Rossetti, S, Tandoi, V, and R.J. Seviour.** 2000. 'Candidatus Nostocoida limicola', a filamentous bacterium from activated sludge. Int J Syst Evol Microbiol **50**:703-709.

**Bock, E., H.-P. Koops, and H. Harms.** 1986. Cell biology of nitrifying bacteria. *In*: Nitrification. Prosser,J.I.(ed.), Special publications of the society for general microbiology, Vol. **20**, Oxford, IRL press.

**Bock, E., Schmidt, I., Stüven, R., and D. Zart.** 1995. Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. **163:** 16-20.

**Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., Moletta, R. and M. Wagner.** 2000. Ecological study of a bioaugmentation failure. Environmental Microbiology **2:** 179-190.

**Brendel, V., Brocchieri, L., Sandler, S. J., Clark, A. J., and S. Karlin.** 1997. Evolutionary comparisons of RecA-like proteins across all major kingdoms of living organisms. J. Mol. Evol. **44:** 528-541.

**Broda, E.** 1977. Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. **17:** 491-493.

**Brosius, J., Palmer, M. L., Kennedy, P. J., and H. F. Noller.** 1978. Complete sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:** 4801-4805.

**Brosius, J., Dull, T. J., H. F. Noller.** 1980. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:** 201-204.

**Brosius, J., Dull, T. J., Sleeter, D. D. and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal operon from *Escherichia coli*. J. Mol. Biol. **148:** 107–127.

**Cangelosi, G.A and W.H. Brabant.** 1997. Depletion of pre-16S rRNA in starved *Escherichia coli* cells. J. Bacteriol. **179:** 4457-4463.

**Charackalis, W. G. and P. A. Wilderer.** 1989. Structure and function of biofilms. John Wiley and Sons Ltd., Chichester.

**Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D., and L. L. Blackall.** 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation Appl. Envir. Microbiol. **66:** 1175-1182.

**Daims H., Brühl, A., Amann, R., Schleifer, K.-H., and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434-444.

**Daims, H., Ramsing, N.B., Schleifer K.-H., and M. Wagner.** 2001. Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence *in situ* hybridization. Appl. Environ. Microbiol. **67**:5810-5818.

**Davenport, R.J., Curtis, T.P., Goodfellow, M., Stainsby, F.M., and M. Bingley.** 2000. Quantitative use of fluorescent in situ hybridization to examine relationships between mycolic acid-containing Actinomycetes and foaming in Activated sludge plants. Appl. Environ. Microbiol. **66:**1158–1166

**De Beer, D**. **and M. Kühl.** 1999. Interfacial processes, gradients and metabolic activities in microbial mats and biofilms. Chapter15 in B. P. Boudeau and B. B. Joergensen (ed.). The benthic boundary layer. Oxford University Press, Oxford.

**Decho, A**. **W.** 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanogr. Mar. Biol. Annu. Rev. **28:** 73-153.

**De Bruijn, P., Van de Graaf, A. A., Jetten, M. S. M., Robertson, L. A., and J. G. Kuenen.** 1995. Growth of *Nitrosomonas europaea* on hydroxylamine. FEMS Microbiol. Lett. **125:** 179-184.

**DeLong, E. F., Wickham G. S., and N. R. Pace.** 1989. Phylogenetic stains: ribosomal rRNA-based probes for the identification of single microbial cells. Science **243:**1360-1363.

**DeLong, E. F., Franks, D. G. and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38(5):** 924-934.

**De los Reyes, F. L., W. Ritter, and L. Raskin.** 1997. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. **63:**1107–1117.

**Derakshani, M., Lukow, T. and W. Liesack.** 2001. Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. Appl. Environ. Microbiol. **67:** 623-631.

**Dugan, P. R., Stoner, D. L., and H. M. Pickrum.** 1992. The genus *Zoogloea*. pp. 3952 - 3964 In Balows A., Tüper H. G., Dworkin M., Harder W., Schleifer K.-H. (eds). The Prokaryotes. 2nd ed. Springer Verlag, New York.

**Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., Van der Meer, J. R. and A. J. B. Zehnder.** 2001. Enrichment and characterization of a new Anammox bacterium from a rotating biological contactor treating an ammonium-rich leachate. Arch. Microbiol. **175:** 198-207.

**Eisen J. A.** 1995. The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. J. Mol. Evol. **41:** 1105-1023.

**Etterer, T. and P.A. Wilderer.** 2001. Generation and properties of aerobic granular sludge. Wat. Sci. Tech., **43:** 19-26. **Etterer, T., Schmid, M. C., Zhu, J., Wilderer, P. A. and M. Wagner.** Aerobic granular sludge formation: Towards structure and function. In preparation for the Journal of Biotechnology.

**Fuerst, J. A.** 1995. The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology **141:** 1493-1506.

**Giovannoni, S. J., Mullins, T. D., and K. G. Field.** 1995. Microbial diversity in oceanic systems: rRNA approaches to the study of unculturable microbes,In: Molecular Ecology of Aquatic Microbes (J. Joint, ed.) Springer-Verlag, Berlin/Heidelberg, Germany.

**Goodfellow, M., R. Davenport, F. M. Stainsby, and T. P. Curtis.** 1996. Actinomycete diversity associated with foaming in activated sludge plants. J. Ind. Microbiol. **17:**268–280.

**Grotenhuis, J. T., Smit, M., Plugge, C. M., Xu, Y. S., van Lammeren, A. A., Stams, A. J., and A. J. Zehnder.** 1991. Bacteriological composition and structure of granular sludge adapted to different substrates. Appl. Environ. Microbiol. **57:** 1942-1949.

**Griepenburg, U., Ward-Rainey, N., Mohamed, S., Schlesner, H., Marxen, H., Rainey, F. A., Stackebrandt, E. and G. Auling.** 1999. Phylogenetic diversity, polyamine pattern and DNA base composition of members of the order *Planctomycetales*. Int. J. Syst. Bacteriol. **49:** 689-696.

**Guan J, Waite T. D., and R. Amal** 1998. Rapid structure characterization of bacterial aggregates. Environ. Sci. Technol. **32**: 3735-3742.

**Head, I. M., W. Hiorns, T. Embley, A. McCarthy, and J. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **13:**1147-1153.

**Helmer, C., Kunst, S., Juretschko, S., Schmid, M. C., Schleifer, K.-H. and M. Wagner.** 1999. Nitrogen loss in a nitrifying biofilm system. Wat. Sci. Tech. **39(7):** 13-21

**Helmer-Madhok, C., Schmid, M., Filipov, E., Gaul, T., Hippen, A., Rosenwinkel, K.-H., Seyfried, C.F., Wagner M. and S. Kunst.** 2002. Deammonification in biofilm systems: population structure and function. Wat. Sci. Tech. 46(1-2): 223-231.

**Henze, M., Harremoes, P., Jansen, J. C. and E. Arvin.** 1997. Wastewater treatment. 2. Edition. Springer-Verlag, Heidelberg, Germany.

**Hollocher, T. C., Tate, M. E. and D. J. D. Nicholas.** 1981. Oxidation of ammonia by *Nitrosomonas europaea*: definitive 18O - tracer evidence that hydroxylamine involves a monooxygenase. J. Biol. Chem. **256:** 10834-10836.

**Holmes, A. J., Costello, A., Lidstrom, M. E. and J. C. Murrell.** 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett. **132:** 203-208.

**Irvine, R. L., Fox, T. P. and R. O. Richter.** 1977. Investigation of fill and batch periods of sequencing batch biological reactors. Water Res. **11:** 713-715.

**Jackson, C.R., Harper, J.P., Willoughby, D., Roden E.E, and P.F. Churchill.** 1997. A simple, efficient method for the separation of humic substances and DNA from environmental samples. Appl. Environ. Microbiol. **63:** 4993-4995.

**Jenkins, D., Richard, M. G. and G. J. Daigger.** 1993. Manual on the causes and control of activated sludge bulking and foaming; 2. Edition. Lewis Publishers Michigan 48118 U.S.A.

**Jetten, M. S. M., Strous, M., van de Pas-Schoonen, K. T., Schalk, J., van Dongen, U. G. J. M., van de Graaf, A., Logemann, S., Muyzer, G., van Loosdrecht, M. C. M. and J. G. Kuenen.** 1999. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. **22:** 421-437.

**Jorand, F., Zartarian F., Thomas F., Block J.C., Bottero J.Y., Villemin G., Urbain, V. and J. Manem.** 1995. Chemical and structural (2D) linkage between bacteria within activated sludge flocs. Wat. Res. **29**:1639-1647.

**Juretschko, S., Timmermann, G., Schmid, M.C., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64**:3042-3051.

**Juretschko, S., Loy, A., Lehner, A. and M., Wagner.**2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. **25:** 84-99.

**Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M., and M. Wagner.** 2000. Phylogenetic relationships of filamentous bacteria of the Eikelboom Type 021N isolated from bulking activated sludge and development of an encompassing set of 021N-specific oligonucleotide probes. Appl. Environ. Microbiol. **66:** 5043-5052.

**Kappeler, J. and W. Gujer.** 1994. Influence of waste water composition and operation conditions on activated sludge bulking and scumming. Wat. Sci. Tech. **30:** 181-189.

**Karr, P. R. and T. M. Keinath.** 1978. Influence on particle size on sludge dewaterability. J. Wat. Poll. Cont. Fed. **50:** 1911-1928.

**Klein, M., Friedrich, M., Roger, A.J., Hugenholtz, P., Fishbain, S., Abicht, H., Blackall, L.L., Stahl, D.A., and M. Wagner.** 2001. Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. J. Bacteriol., **183**: 6028-6035.

**Klotz, M. G. and J. M. Norton.** 1995. Sequence of an ammonia monooxygenase subunit A-encoding gene from *Nitrosospira* sp. NpAV. Gene **163**: 159-160**.**

**Klotz, M. G. and J. M. Norton.** 1998. Multiple copies of ammonia monooxygenase (amo) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. FEMS Microbiol Lett. **168:** 303-311.

**Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosocccus mobilis* nov. sp. Arch. Microbiol. **10:**277-282.

**Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus.* Arch. Microbiol. **15:**244-248.

**Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. J. Gen. Microbiol. **13:**1689-1699.

**Kowalchuk, G. A., J. Stephen, W. De Boer, J. Prosser, T. Embley, and J. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **6:**1489-1497.

**Kröhnke, O.** 1900. Die Reinigung des Wassers für häusliche und gewerbliche Zwecke. Verlag von Ferdinand Enke, Stuttgart, Germany.

**Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W., and D.E. Caldwell.** 1991. Optical sectioning of microbial biofilms. J. Bacteriol. **173:**6558-6567.

**Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw W. and A. Klapwijk.** 1980. Use of the UASBR concept for biological WWT, especially for anaerobic treatment. Biotech. Bioeng. 22, 699-734.

**Li, D.-H. and J. J. Ganczarczyk.** 1991. Size distribution of activated sludge flocs. Research J. Wat. Poll. Cont. Fed. **63**:806-814.

**Li, L., Kato, C., and K. Horikoshi.** 1999. Bacterial diversity in deep-sea sediments from different depths. Biodivers. Conserv. **8:** 659-677.

**Liao, D., and P. P. Dennis.** 1994. Molecular phylogenies based on ribosomal protein L11, L1, L10, and L12 sequences. J. Mol. Evol. **38:** 405-419.

**Liesack, W. and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an australian terrestrial environment. J. Bacteriol. **174:** 5072-5078.

**Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. and K.-H. Schleifer**. 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis. **19:** 554-568.

**Macario, A. J. L., Visser, F. A., van Lier, J. B. and E. Conway de Macario.** 1991. Topography of methanogenic subpopulations in a microbial consortium adapting to thermophilic conditions. J. Gen. Microbiol. **137:** 2179-2189.

**MacDonald, R. M.** 1986. Nitrification in soil: an introductory history p. 1-16. *In* J. I. Prosser (ed), Nitrification, vol. 20. IRL Press, Oxfor, United Kingdom.

**MacLeod, F. A., Guiot, S. R. and J. W. Costerton.** 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl Environ. Microbiol. **56:** 1598-1607.

**Maidak, B. L., Olsen, G. J., Larsen, N., Overbeck, R., Mc Caughey, M. J., and C. R. Woese.** 1996. The ribosomal database project (RDP). Nucleic Acid Res **24:** 82-85.

**Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. System. Appl. Microbiol. **15:**593-600.

**Marshall, P. A., Loeb, G. I., Cowan, M. M. and M. Fletcher.** 1989. Response of microbial adhesives and biofilm matrix polymers to chemical treatments as determined by interference

reflection microscopy and light section microscopy. Appl. Environ. Microbiol. **55:** 2827-2831.

**Mendum, T. A., Sockett, R. E., and P. R. Hirsch**. 1999. Use of Molecular and Isotopic Techniques To Monitor the Response of Autotrophic Ammonia-Oxidizing Populations of the Subdivision of the Class Proteobacteria in Arable Soils to Nitrogen Fertilizer. Appl. Environ. Microbiol. **65:** 4155-4162.

**McCaig, A. E., Phillips, C. J., Stephen, R. S., Kowalchuk, G. A:, Harvey, S. M., Herbert, R. A. , Embley, T. M. and J. I. Prosser.** 1999. Nitrogen cycling and community structure of proteobacterial β-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. Appl. Environ. Microbiol. **65:**213-220.

**McTavish, H., J. A. Fuchs, and A. B. Hooper.** 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436-2444.

**Meier, H., Amann, R., Ludwig, W., and K.H. Schleifer.** 1999. Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. **22:** 186-196.

**Meincke, M., Krieg, E. and E. Bock.** 1989. *Nitrosovibrio* spp. The dominant ammonia-oxidizing bacteria in building sandstone. Appl. Environ. Microbiol. **55:** 2108-2110.

**Mobarry, B. K., M. Wagner, V. Urbain, B. Rittmann, and D. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **6:**2156-2162.

**Morgenroth, E., Obermayer, A., Arnold, E., Brühl, A., Wagner, M., and P. A. Wilderer.** 2000. Effect of long term idle periods on the performance of sequencing batch reactors. Wat. Sci. Technol. **41(1):**105-113.

**Morita, R. Y.** 1993. Bioavailability of energy and starvation state. *In* S. Kjelleberg (ed.) Starvation in bacteria. Plenum Press, New York.

**Mulder, A., Van de Graaf, A. A., Robertson, L. A. and J. G. Kuenen.** 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. **16:** 177-183.

**Neef, A.** 1997. Anwendung der in situ Einzelzell-Identifizierung von Bakterien in komplexen mikrobiellen Biozönosen. PhD-theses at TU München.

**Neef, A., Amann, R. I., Schlesner, H., and K.-H. Schleifer.** 1998. Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiology **144:** 3257-3266.

**Nielsen, P.H., Frølund, B., and K. Keiding.** 1996.Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. Appl. Microbiol. Biotechnol. **44:**823-830.

**Norton, J. M., J. M. Low, and M. G. Klotz.** 1996. The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol. Lett. **139:**181-188.

**Novak, L., Larrea, L., Wanner, J. and J. L. Garcia-Heras.** 1994. Non-filamentous activated sludge bulking caused by *Zoogloea*. Wat. Sci. Tech. **29:** 301-304.

**Oerther, D.B., Pernthaler, J., Schramm, A., Amann, R., and L. Raskin.** 2000. Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Appl Environ Microbiol **66:** 2154-2165.

**Odegaard, H., Rusten B. and T, Westrum**. 1994. A new moving bed biofilm reactor – applications and results. Wat. Sci. Tech. **29(10-11):** 157-165.

**Okabe, S., Satoh, H., and Y., Watanabe.** 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **65:**3182-3191.

**Omar, A. S., Weckesser, J. and H. Mayer.** 1983. Different polysaccharides in the external layers (capsule and slime) of the cell envelope of *Rhodopseudomonas capsulata* Sp11. Arch. Microbiol. **136:** 291-296.

**Painter, H. A.** 1986. Nitrification in the treatment of sewage and wastewaters, p. 185-211. *In* J. I. Prosser(ed), Nitrification, vol. 20. IRL Press, Oxfor, United Kingdom.

**Parker, D.S., Kaufmann, W.J. and D. Jenkins.** 1971. Physical conditioning of activated sludge flocs. J. Wat. Poll. Cont. Fed. **43,** 1817-1833.

**Pipes, W**. O. 1979. Bulking, deflocculation and pin point floc. J. Wat. Poll. Cont. Fed. **51:** 62-70.

**Polz, M. F. and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:** 3724-3730.

**Pommerening-Röser, A., G. Rath, and H.-P. Koops.** 1996. Phylogenetic diversity within the genus *Nitrosomonas*. System. Appl. Microbiol. **19:**344-351.

**Poulsen, L.K., Ballard, G., and D.A., Stahl.** 1993. Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:** 1354-1360.

**Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microbiol. Physiol. **30:**125-181.

**Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H.-P. 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:** 5368-5382.

**Rabus, R., Wilkens, H., Schramm, A., Harms, G., Behrends, A., Amann, R., and F. Widdel.** 1999. Anaerobic utilization of alkylbenzenes and n-alkanes from crude oil in an enrichement culture of denitrifying bacteria affiliating with the β-subclass of *Proteobacteria*. Environmental Microbiology **1:** 145-157.

**Rappé, M. S., Kemp, P. F. and S. J. Giovannoni.** 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. Limnol. Oceanogr. **42(5):** 811-826.

**Roller, C., Wagner, M., Amann, R., Ludwig, W., and K.-H. Schleifer.** 1994. In situ probing of Gram positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotide probes. Microbiology **140:** 2849-2858.

**Rondon, M., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M., Loiacono, R. K. A., Lynch, B. A., MacNeil, I. A., Minor, C., Tiong, C., Gilman, L.M., Osburne, M. S., Clardy, J., Handelsman, J. and R. M. Goodman.** 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol**.** 66: 2541-2547.

**Rossello – Mora, R. A., Wagner, M., Amann, R., and K.-H. Schleifer.** 1995. The abundance of *Zoogloea ramigera* in sewage treatment plants. Appl. Environ. Microbiol. **61:** 702 - 707.

**Rotthauwe, J. H., de Boer, W. and W. Liesack.** 1995. Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira* sp. AHB1 and *Nitrosolobus multiformis* C-71. FEMS Microbiol Lett. **133:** 131-135.

**Rotthauwe, J.-H., K.-P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704-4712.

**Rubio, M. A., Görg, S. and P. A. Wilderer.** 1988. The SBR Technique: Control of species composition of mixed cultures. Forum Mikrobiologie 5: 169-175.

**Sadelgekar, V. V., Mahajan, B. A. and A. M. Shaligram.** 1988. Evaluation of sludge settleability by floc characteristics. J. Wat. Poll. Cont. Fed. **60:** 1862-1863.

**Sambrook, J., Fritsch, E. F. and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, ColdSpring Harbor, N.Y.

**Sanger, F., Nicklen, S., and A.R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463-5467

**Sayavedra-Soto, L.A., Hommes, N.G., and D.J. Arp.** 1994. Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. J. Bacteriol. **176:**504-510.

**Schalk, J., Oustad, H., Kuenen, J. G. and M. S. Jetten.** 1998. The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. FEMS Microbiol Lett. **158:** 61-67.

**Schalk, J., de Vries, S., Kuenen, J. G. and M. S. Jetten.** 2000. Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry **39:** 5405-5412.

**Schleifer K. H., Ludwig, W. and R. Amann.** 1992. Gene probes and their use in microbiology. Naturwissenschaften **79:** 213-219.

**Schleper, C., Swanson, R. V., Mathur, E. J. and E. F. DeLong.** 1997. Characterization of a DNA polymerase from the uncultivated psychrophilic archaeon *Cenarchaeum symbiosum*. J Bacteriol. **179:**7803-11.

**Schlesner, H. and E. Stackebrandt.** 1986. Assignment of the genera *Planctomyces* and *Pirella* to a new family *Planctomycetaceae* fam. nov. and description of the order *Planctomycetales* ord. nov. Syst. Appl. Microbiol. **8:** 174-176.

**Schlesner, H.** 1994. The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. Syst. Appl. Microbiol. **17:** 135-145.

**Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.-H. and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:** 93-106.

**Schmid, M., Schmitz-Esser, S. Jetten, M. and M. Wagner.** 2001. 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium oxidizing bacteria: implications for phylogeny and *in situ* detection. Environ. Microbiol. **3:** 450-459.

**Schmid, M., Thill, A., Purkhold, U., Walcher, M., Bottero, J. Y., Ginestet, P., Nielsen, P. H., Wuertz, S. and M. Wagner.** Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis. Submitted to Water Research.

**Schmidt, I. and E. Bock.** 1997. Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. **167:** 106-111.

**Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K.-H. Schleifer.** 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **6:** 4641-4647.

**Schramm, A., de Beer, D., Wagner, M. and R. Amann.** 1998. Identification and activities *in situ* of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Envir. Microbiol. **64:** 3480-3485.

**Singleton, D. R., Furlong, M. A., Rathbun, S. L., and W. B. Whitman.** 2001. Quantitative comparison of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. **67:** 4374-4376.

**Sinigalliano, C. D., D. N. Kuhn, and R. D. Jones.** 1995. Amplification of the *amoA* gene from diverse species of ammonia-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl. Environ. Microbiol. **61:** 2702-2706.

**Smalla, K., Cresswell, N., Mendonca-Hagler, L.C., Wolters, A., and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. of Appl. Bacteriol. **74:**78-85.

**Sorokin, D., Tourova, T., Schmid, M. C., Wagner, M., Koops, H.-P., Kuenen, J. G. and M. Jetten**. 2001. Isolation and properties of obligately chemolithoautotrophic and extremely alkalitolerant ammonia-oxidizing bacteria from Mongolian soda lakes. Arch. Microbiol. 176: 170-177.

**Stehr, G., Zörner, S., Böttcher, B., and H.-P. Koops.** 1995a. Exopolymers: An ecological characteristic of a floc-attached, ammonia-oxidizing bacterium.Microb. Ecol. **30:**115-126.

**Stein, J. L., Marsh, T. L., Wu., K. Y., Shizuya, H. and E DeLong.** 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planctonic marine Archaeon. J. Bacteriol **178**: 591-599.

**Stephen, J.R., McCaig, A.E., Smith, Z. , Prosser, J.I., and T.M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147-4154.

**Strous, M., Heijnen, J. J., Kuenen, J. G., and M. S. M. Jetten.** 1998.The sequencing batch reactor as a powerful tool to study very slowly growing micro-organisms. Appl. Microbiol. Biotechnol. **50:** 589-596.

**Strous, M., Fuerst, J. A., Kramer, E. H. M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R. I., Kuenen, J. G. and M. S. M. Jetten.** 1999. Missing lithotroph identified as new planctomycete. Nature **400:** 446-449.

**Strous, M.** 2000. Microbiology of anaerobic ammonium oxidation. PhD thesis. Delft University Press, Delft The Netherlands, ISBN 90-9013621-5.

**Strunk, O., and W. Ludwig**. 1997. ARB software program package; http://www.biol.chemie.tu-muenchen.de/pub/ARB/

**Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. **39:** 243-270.

**Suzuki,M. T. and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62: 625-630.

**Szegin, M., Jenkins D. and D. S. Parker.** 1978. A unified theory of filamentous sludge bulking. J. Wat. Poll. Cont. Fed. **50:** 362-381.

**Tanner, M. A., Goebel, B. M., Dojka, M. A. and N. R. Pace.** 1998. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. Appl. Environ. Microbiol. **64:**3110-3113.

**Tchobanoglous, G**. **and F. L. Burton.** 1991. Wastewater engineering; treatment, disposal and reuse. McGraw-Hill Inc., New York.

**Thill, A., Veerapaneni, S., Simon, B., Wiesner, M., Bottero, J.Y. and Snidaro D.** (1998) Determination of Structure of Aggregates by Confocal Scanning Laser Microscopy. J. Colloid Interface Sci. **204**: 357-362.

**Tijhuis, L., Hijman, B., van Loosdrecht, M. C. M. and J. J. Heijnen.** 1996. Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. Appl. Microbiol. Biotech. **45:** 7-17.

**Tortoso, A. C. and G. L. Hutchinson.** 1990. Contribution of autotrophic and heterotrophic nitrifiers to soil NO and $N_2O$ emission. Appl. Environ. Microbiol. **56:** 1799-1805.

**Tsai, Y.L. and B.H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292-2295.

**Uhlinger, D. J. and D. C. White.** 1983. Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. Appl. Environ. Microbiol. **45:** 64-70.

**Unz, R. F.** 1971. Neotype strain of *Zoogloea ramigera* Itzigsohn. Request for an opinion. Int. J. Syst. Bacteriol. **21:** 91-99.

**Unz, R. F.** 1989. *Zoogloea*. pp. 214-219 In Krieg, N. R., and J. G. Holt (eds). Bergey´s Manual of Systematic Bacteriology (Volume 1). Wiliams and Wilkins, Baltimore/London.

**Urbain, V**., Block, J. C. and J. Manem. 1993. Bioflocculation in activated sludge: an analytical approach. Wat. Res. **27:** 829-838.

**Van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., and J.G. Kuenen.** 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro- organisms in a fluidized bed reactor. Microbiology **142**:2187–2196.

**Van der Meer, J. R., Werlen, C. and H. Siegrist.** 1999. Genbank direct submission. Accession number: AJ224943

**Van Limbergen, H., Top, E.M. and W. Verstraete.** 1998. Bioaugmentation in activated sludge. Current features and future perspectives. Appl. Environ. Microbiol. **50:** 16-23.

**Van Loosdrecht, M. C. M., Picioreanu C. and J. J. Heijnen.** 1997a. A more unifying hypothesis for biofilm structures. FEMS Microbiol. Ecol. **24:** 181-183

**Wagner, M., R. Amann, P. Kämpfer, B. Aßmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer.** 1994a. Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. System. Appl. Microbiol. **17:**405-417.

**Wagner, M., Erhart, R.,Manz, W., Amann, R., Lemmer, H., Wedi, D., and K.-H. Schleifer.** 1994b. Development of a rRNA-targeted oligonucleotide probe for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. **60:**792-800.

**Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. System. Appl. Microbiol. **18:**251-264.

**Wagner, M., Rath, G., Koops, H.-P., Flood, J. and R. Amann.** 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. Wat. Sci. Tech. **34:** 237-244.

**Wagner, M., Hutzler, P., and R. Amann.** 1998a. 3-D analysis of complex microbial communities by combining confocal laser scanning microscopy and fluorescence in situ hybridization (FISH).1. In: Digital Image Analysis of Microbes (Wilkinson, M.H.F.; Schut, F., eds.): 467-486. John Wiley & Sons, Chichester, England.

**Wagner, M., Flax, J. L., Brusseau, G. A., and D. A. Stahl.** 1998b. Phylogeny of dissimilatory sulfit reductases supports an early origin of sulfate respiration. J. Bacteriol. **180:** 2975-2982.

**Wanner, J**. **and P. Grau.** 1988. Filamentous bulking in nutrient removal activated sludge systems. Wat. Sci. Tech. **20:** 1-8.

**Wanner, J.** 1994. Activated sludge bulking and foaming control. Technomic Publishing Company, Lancaster U.S.A.

**Ward, B. B.** 1996. Nitrification and denitrification: probing the nitrogen cycle in aquatic environments. Microb. Ecol. **32:**247–261.

**Ward, B.B., M.A. Voytek, and K.-P. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers by specific PCR amplification. Microb. Ecol. **33:**87-96.

**Ward, B.B., Martino, D.P., Diaz, M.C., and S.B. Joye.** 2000. Analysis of ammonia-oxidizing bacteria from hypersaline Mono Lake, California, on the basis of 16S rRNA sequences. Appl. Environ. Microbiol. **66**:2873-81

**Ward, N., Rainey, F. A., Stackebrandt, E. and H. Schlesner.** 1995. Unraveling the extent of diversity within the order *Planctomycetales*. Appl. Environ. Microbiol. **61:** 2270-2275.

**Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Ammonia-oxidizing bacteria. *In*: Bergey`s Manual of Systematic Bacteriology, Vol. III Staley, J. T., M. P. Bryant, N. Pfennig, J. G. Holt, Eds., pp.1818-1834: Williams & Wilkins, Baltimore, USA.

**Wawer, C., Rüggeberg, H., Meyer, G. and G. Muyzer.** 1995. A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified DNA fragments. Nucleic Acid Res. **23:** 4928-4929.

**Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

**Wood, P. M.** 1986. Nitrification as a bacterial energy source. *In* J. I. Prosser(ed), Nitrification, vol. 20. IRL Press, Oxford, United Kingdom.

**Wimpenny, J. W.T. and R. Colasanti.** 1997. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. FEMS Microbiol. Ecol. 22: 1-16

**Wilderer, P. A.** 1992. Sequencing batch biofilm reactor technology. In: Harnessing biotechnology for the 21st century, Ladisch, M. R. and Bose, A. (eds), American Chemical Society, pp. 474-479.

**Young, C.C.**, **Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R., and S.M. Hinton.** 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl. Envir. Microbiol. **59:** 1972-1974.

**Zartarian, F., Mustin, C., Villemin, G., Ait-Ettager, T., Thill, A., Bottero, J. Y., Mallet, J. L. and D. Snidaro.** 1997. Three-dimensional modeling of an activated sludge floc. Langmuir **13**: 35-40.

**Zhou, J., Bruns, M.A., and J.M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316-322.

## List of Publications

1. Contribution to manuscripts presented in this thesis

**Juretschko, S., Timmermann, G., Schmid, M.C., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64**:3042-3051.

Sequencing and phylogenetic analysis of *amoA,* development of the DNA isolation protocol and editorial help by M. S.

**Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.-H. and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:** 93-106.

Concept by M.W., M.K., M.S.. Sequencing and phylogenetic analysis of *amoA* and 16S rRNA gene, probe design and fluorescence in situ hybridization by M.S.. Gel retardation by M.K. and M.S.. Writing by M.W. and M.S.

**Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H.-P. 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:** 5368-5382.

Supply of 35 *amoA* sequences from environmental samples and editorial help by M.S..

**Sorokin, D., Tourova, T., Schmid, M. C., Wagner, M., Koops, H.-P., Kuenen, J. G. and M. Jetten**. 2001. Isolation and properties of obligately chemolithoautotrophic and extremely alkalitolerant ammonia-oxidizing bacteria from Mongolian soda lakes. Arch. Microbiol. 176: 170-177.

Sequencing and phylogenetic analysis of 16S rRNA gene and *amoA* by M. S.. Writing of the phylogeny part of the manuscript by M.W. and M.S.

**Schmid, M., Schmitz-Esser, S. Jetten, M. and M. Wagner.** 2001. 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium oxidizing bacteria: implications for phylogeny and *in situ* detection. Environ. Microbiol. **3:** 450-459.

Concept by M.W. and M.S.. Sequencing by S.S. and M.S. and phylogenetic analysis of rRNA genes and ISR, probe design and fluorescence in situ hybridization by M.S.. Writing by M.W. and M.S.

**Schmid, M., Thill, A., Purkhold, U., Walcher, M., Bottero, J. Y., Ginestet, P., Nielsen, P. H., Wuertz, S. and M. Wagner.** Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis. Submitted to Water Research.

Concept by M.W., P.G. and M.S.. Development of a staining technique for activated sludge flocs, image acquisition, development and application of the 3DVASD macro for volume and surface determination by M.S.. Development of programs for Df calculation and application by A.T. and M.S.. Writing by P.N., M.W. and M.S.

## 2. Contribution to manuscripts prepared, but not explicitly included, in the course of this thesis

**Helmer, C., Kunst, S., Juretschko, S., Schmid, M. C., Schleifer, K.-H. and M. Wagner.** 1999. Nitrogen loss in a nitrifying biofilm system. Wat. Sci. Tech. **39(7):** 13-21

Sequencing and phylogenetic analysis of *amoA* by M. S.

**Helmer-Madhok, C., Schmid, M., Filipov, E., Gaul, T., Hippen, A., Rosenwinkel, K.-H., Seyfried, C.F., Wagner M. and S. Kunst.** 2002. Deammonification in biofilm systems: population structure and function. Wat. Sci. Tech. 46(1-2): 223-231.

Sequencing, phylogenetic analysis and FISH by M.S.

**Etterer, T., Schmid, M. C., Zhu, J., Wilderer, P. A. and M. Wagner.** Aerobic granular sludge formation: Towards structure and function. In preparation for the Journal of Biotechnology.

Sequencing, phylogenetic analysis, probe design and microbial population analysis by M.S.. FISH by T.E. and M.S. Writing by T.E., M.W. and M.S..

## 3. Further Publications

**Steidle, A., Sigl, K., Schuhegger, R., Ihring, A., Schmid, M., Gantner, S., Stoffels, M., Riedel, K., Givskov, M., Hartmann, T., Langebartels, C. and L. Eberl.** Visualization of N-acylhomoserine lactone (AHL)-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl. Environ. Microbiol. **67:**5761-5770.

**Schmid, M., Schmitz-Esser, S., Jetten, M., and M. Wagner.** 2001. The rDNA operon of anaerobic ammonium oxidizing bacteria : Implications for phylogeny and in situ detection. In "Der Stickstoff im Wasser/Abwasser" (ed. Metzger J. W.). ISBN 3-486-26528-8. Oldenbourg Industrieverlag GmbH, München.

**Jetten, M.S.M., Schmid, M., Schmidt, I., Wubben, M., van Dongen, L., Abma, W., Sliekers, O.A., Revsbech, N.P., Beaumont, B., Ottosen, L.M., Volcke, E., Laanbroek, H.J., Campos-Gomez, J.L., Cole, J.A., van Loosdrecht, M.C.M., Mulder, J.W., Fuerst, J.A., Richardson, D., van de Pas, K.T., Mendez-Pampin, R., Third, K., Cirpus, I.Y., van Spanning, R.J.M., Nielsen, L.P., Op den Camp, H.J.M., Schultz, C., Gundersen, J.K., Vanrolleghem, P., Strous, M., Wagner, M., and J.G. Kuenen.** 2002**.** Implementation of EU guidelines for Nitrogen removal by improved control and application of new nitrogen-cycle bacteria. Re/views in Environ. Sci. technol. **1:**51-63.

**Schmidt, I., Sliekers, O., Schmid, M., Cirpus, I., Strous, M., Bock, E., Kuenen, J.G., and M.S.M. Jetten.** 2002. Aerobic and anaerobic ammonia oxidizing bacteria – competitors or natural partners? FEMS Microbiol. Ecol. **39:**175-181.

# Appendix A

Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: Nitrosococcus mobilis and Nitrospira-Like Bacteria as Dominant Populations

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# Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: *Nitrosococcus mobilis* and *Nitrospira*-Like Bacteria as Dominant Populations

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**The ammonia-oxidizing and nitrite-oxidizing bacterial populations occurring in the nitrifying activated sludge of an industrial wastewater treatment plant receiving sewage with high ammonia concentrations were studied by use of a polyphasic approach. In situ hybridization with a set of hierarchical 16S rRNA-targeted probes for ammonia-oxidizing bacteria revealed the dominance of *Nitrosococcus mobilis*-like bacteria. The phylogenetic affiliation suggested by fluorescent in situ hybridization (FISH) was confirmed by isolation of *N. mobilis* as the numerically dominant ammonia oxidizer and subsequent comparative 16S rRNA gene (rDNA) sequence and DNA-DNA hybridization analyses. For molecular fine-scale analysis of the ammonia-oxidizing population, a partial stretch of the gene encoding the active-site polypeptide of ammonia monooxygenase (*amoA*) was amplified from total DNA extracted from ammonia oxidizer isolates and from activated sludge. However, comparative sequence analysis of 13 *amoA* clone sequences from activated sludge demonstrated that these sequences were highly similar to each other and to the corresponding *amoA* gene fragments of *Nitrosomonas europaea* Nm50 and the *N. mobilis* isolate. The unexpected high sequence similarity between the *amoA* gene fragments of the *N. mobilis* isolate and *N. europaea* indicates a possible lateral gene transfer event. Although a *Nitrobacter* strain was isolated, members of the nitrite-oxidizing genus *Nitrobacter* were not detectable in the activated sludge by in situ hybridization. Therefore, we used the rRNA approach to investigate the abundance of other well-known nitrite-oxidizing bacterial genera. Three different methods were used for DNA extraction from the activated sludge. For each DNA preparation, almost full-length genes encoding small-subunit rRNA were separately amplified and used to generate three 16S rDNA libraries. By comparative sequence analysis, 2 of 60 randomly selected clones could be assigned to the nitrite-oxidizing bacteria of the genus *Nitrospira*. Based on these clone sequences, a specific 16S rRNA-targeted probe was developed. FISH of the activated sludge with this probe demonstrated that *Nitrospira*-like bacteria were present in significant numbers (9% of the total bacterial counts) and frequently occurred in coaggregated microcolonies with *N. mobilis*.**

Nitrification, the bacterially catalyzed oxidation of ammonia to nitrate is a key process in the global cycling of nitrogen (39) and an integral component of modern wastewater treatment plants. Reduction of the ammonia content of sewage is important, as ammonia is toxic to aquatic life (e.g., reference 3) and creates a large oxygen demand in receiving waters. Furthermore, nitrification is a prerequisite for total N removal from sewage via subsequent denitrification. Two distinct, physiologically defined groups of bacteria catalyze the two separate steps involved in nitrification (7, 27). First, chemolithoautotrophic ammonia-oxidizing bacteria convert ammonia to nitrite, which is subsequently transformed to nitrate by nitrite-oxidizing bacteria. Sixteen species of lithoautotrophic ammonia-oxidizing bacteria have been isolated and validly described (21, 23, 25, 26, 60). Based on comparative 16S rRNA gene (rDNA) sequence analysis, cultured ammonia-oxidizing bacteria comprise two monophyletic groups within the *Proteobacteria*. *Nitrosococcus oceanus* and *N. halophilus* belong to the gamma subclass of the class *Proteobacteria* (63), while the members of the genera *Nitrosomonas* and *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* (the latter three being closely related to each other [16]), as well as *Nitrosococcus mobilis* (actually a member of the genus *Nitrosomonas*) constitute a closely related assemblage within the beta subclass of *Proteobacteria* (16, 38, 47, 50, 51, 62). Based on ultrastructural properties, cultivated nitrite-oxidizing bacteria have been assigned to the four recognized genera *Nitrobacter*, *Nitrospina*, *Nitrococcus*, and *Nitrospira*. Comparative 16S rDNA sequence analyses revealed that one of these genera, *Nitrobacter* (61), with its three species (5, 6), is a member of the alpha subclass of *Proteobacteria* (37, 50). The genera *Nitrospina* (58) and *Nitrococcus* (58), with one species each, belong to the delta and gamma subclass of *Proteobacteria*, respectively (50). The remaining genus, *Nitrospira* (59), encompassing the species *Nitrospira moscoviensis* (14) and *N. marina* (59), is a member of the *Nitrospira* phylum of the domain *Bacteria* (14).

Investigation of the diversity and ecology of nitrifying bac-

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TABLE 1. Probe sequences, target sites, and formamide concentrations in the hybridization buffer required for specific in situ hybridization

| Probe | Sequence (5′–3′) | Target site[a] (16S rRNA positions) | % Formamide |
|---|---|---|---|
| Nso190 | CGATCCCCTGCTTTTCTCC | 190–208 | 55 |
| Nso1225 | CGCCATTGTATTACGTGTGA | 1225–1244 | 35 |
| Nsv443 | CCGTGACCGTTTCGTTCCG | 444–462 | 30 |
| Nsm156 | TATTAGCACATCTTTCGAT | 156–174 | 5 |
| NEU | CCCCTCTGCTGCACTCTA | 653–670 | 40 |
| CTE | TTCCATCCCCCTCTGCCG | 659–676 | —[b] |
| S-*-Nse-1472-a-A-18 | ACCCCAGTCATGACCCCC | 1472–1489 | 50 |
| NmV | TCCTCAGAGACTACGCGG | 174–191 | 35 |
| S-*-Ntspa-1026-a-A-18 | AGCACGCTGGTATTGCTA | 1026–1043 | 20 |
| NIT3 | CCTGTGCTCCATGCTCCG | 1035–1048 | 40 |
| CNIT3 | CCTGTGCTCCAGGCTCCG | 1035–1048 | —[c] |

[a] *E. coli* numbering (10).
[b] Used as unlabeled competitor together with probe NEU.
[c] Used as unlabeled competitor together with probe NIT3.

teria in natural and engineered systems by traditional cultivation techniques has been hampered by their slow growth rates and by the biases inherent in all culture-based studies (e.g., reference 4; 53, 60). Most studies on nitrification were performed with *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, as they represent ammonia- and nitrite-oxidizing bacteria, which are easy to obtain from international bacterial culture collections but might not represent those nitrifying bacteria dominant in the environments analyzed (39, 60). This appears particularly likely in light of recent molecular studies exploiting 16S rDNA sequence information which demonstrated a sequence diversity in the monophyletic line of the beta subclass ammonia oxidizers which significantly exceeded the species diversity recognized in cultured ammonia oxidizers (28, 31, 48). Unfortunately, the limited degree of sequence diversity within some of the environmental ammonia oxidizer sequence clusters makes it difficult to interpret how many of the sequences represent new, as yet uncultured, species (46). In addition, quantitative dot blot (19) and in situ hybridization (56) studies using 16S rRNA-targeted probes specific for the nitrite-oxidizing bacteria of the genus *Nitrobacter* indicated low *Nitrobacter* numbers in a variety of nitrifying environments and consequently highlighted the importance of non-*Nitrobacter* nitrite-oxidizing bacteria for the nitrification process.

Recently, the battery of molecular tools used to infer the presence of ammonia-oxidizing bacteria in the environment in a cultivation-independent way has been supplemented by sets of specific or semispecific PCR primers for amplification of 16S rDNA (12, 18, 38, 52, 57) or the ammonia monooxygenase structural gene *amoA* (15, 40, 44). While such PCR-based methods have provided exciting new insights into the sequence diversity and environmental distribution of ammonia oxidizers, they do not permit accurate quantification of cell numbers. For direct enumeration and simultaneous in situ analysis of the spatial distribution of environmental populations of nitrifying bacteria, we and others have used in situ hybridization with fluorescent oligonucleotide probes (33, 43, 55, 56). Since the presence of ammonia oxidizers can be correlated with their characteristic activity, in situ probe counts can be compared with total nitrification rates to calculate the specific in situ activity per cell (55). However, isolation of dominant ammonia- and nitrite-oxidizing bacteria identified by molecular methods is still required to obtain a more comprehensive picture of their physiology. The inevitable bias induced by standard cultivation can be partly compensated for by the monitoring of enrichment and isolation using hybridization with oligonucleotide probes designed from environmentally derived 16S rRNA sequences (22).

The present study was undertaken to identify the most important species of the nitrifying bacterial population present in activated sludge with a high nitrifying capacity that originated at an industrial wastewater treatment plant. The diversity of ammonia-oxidizing bacteria was studied by (i) fluorescent in situ hybridization techniques on activated sludge samples using previously published phylogenetic probes (33, 38, 55), (ii) comparative sequence analysis of environmentally derived *amoA* gene sequences, and (iii) isolation and subsequent characterization (using fluorescent in situ hybridization, 16S rDNA sequencing, and DNA-DNA hybridization) of the numerically dominant ammonia oxidizer population. Since we failed in a previous study to detect nitrite-oxidizing bacteria of the genus *Nitrobacter* in the activated sludge we analyzed (56), we used comparative analysis of 16S rDNA sequences to test for the presence of other nitrite-oxidizing genera. Confocal laser scanning microscopy and fluorescent in situ hybridization using probes designed from environmentally derived sequences affiliated with the genus *Nitrospira* were used to monitor their abundance and spatial distribution in activated sludge.

(A preliminary part of this work has been presented at the Second International Conference on Microorganisms in Activated Sludge and Biofilm Processes, 21 to 23 July 1997, Berkeley, Calif.)

## MATERIALS AND METHODS

**Organisms, culture conditions, and cell fixation.** The nitrifying bacteria investigated in this study were cultured as described previously (6, 26). For in situ hybridization, cells were fixed with paraformaldehyde (2) from cultures which had oxidized 70 to 80% of the ammonia and nitrite, respectively, originally present in the cultivation medium.

**Sampling.** Grab samples were collected in October 1996 and September 1997 from the intermittently aerated nitrification-denitrification basin of an industrial wastewater treatment plant receiving sewage from an animal waste processing facility (Tierkörperbeseitigungsanstalt Kraftisried, Kraftisried, Germany; 6,000 population equivalents [PE] [1 PE = 60 g of biological oxygen demand $day^{-1}$). For DNA isolation, aliquots of the samples were pelleted by centrifugation (10,000 × *g* for 2 min), immediately frozen on dry ice, and stored at −80°C after their arrival at our laboratory. For in situ hybridization, a subsample of activated sludge was fixed for 3 h with 4% paraformaldehyde as described by Amann (2).

**Oligonucleotide probes.** The following 16S rRNA-targeted oligonucleotide probes were used: (i) NEU, complementary to a signature region of most halophilic and halotolerant ammonia oxidizers (55); (ii) Nso190, specific for the ammonia oxidizers in the beta subclass of *Proteobacteria* (33); (iii) Nso1225, specific for the ammonia oxidizers in the beta subclass of *Proteobacteria* (33); (iv) Nsm156, specific for the *Nitrosomonas* cluster (33); (v) Nsv443, specific for the *Nitrosospira* cluster (33); (vi) NmV, specific for the *N. mobilis* lineage (38); (vii) S-*-Nse-1472-a-A-18, targeted against ammonia oxidizer isolate Nm103 and all

other members of the *N. europaea* lineage; (viii) NIT3, complementary to a region of all previously sequenced *Nitrobacter* species (56); and (ix) S-*-Ntspa-1026-a-A-18, specific for *N. moscoviensis* and activated-sludge-derived clone sequences A-4 and A-11. The sequences and target sites of all of the probes are listed in Table 1. The probes developed in this study were named in accordance with the standard proposed by Alm et al. (1). The names of previously published probes were left unchanged to avoid confusion. Oligonucleotides were synthesized with a C6-trifluoracetylamino amino linker at the 5′ end (Interactiva, Ulm, Germany). Labeling with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) and with the monofunctional, hydrophilic sulfoindocyanine dyes Cy3 and Cy5 (Amersham, Buckinghamshire, United Kingdom) and purification of the oligonucleotide-dye conjugates were performed as described by Amann (2). For labeling with the Cy dyes, the fluorochrome was suspended in a 1:1 mixture of 200 mM sodium carbonate buffer (pH 9.0) and dimethyl formamide.

**In situ hybridization and probe-specific cell counts.** Optimal hybridization conditions were determined for probes NmV, S-*-Nse-1472-a-A-18, and S-*-Ntspa-1026-a-A-18 by using the hybridization and wash buffers described by Manz et al. (30). Optimal hybridization stringency required the addition of formamide to final concentrations of 20% for probe S-*-Ntspa-1026-a-A-18, 35% for probe NmV, and 50% for probe S-*-Nse-1472-a-A-18. All hybridizations were performed at a temperature of 46°C. Subsequently, a stringent wash step was performed for 10 min at 48°C. Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (54). Probes NEU and NIT3 were applied together with the competitor oligonucleotides CTE and CNIT3, respectively (55, 56; Table 1). Dual staining of cells with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent oligonucleotides was modified from the method of Hicks et al. (17) so that cells were stained after in situ hybridization with DAPI (0.5 μg $ml^{-1}$) for 10 min on ice. Probe-specific cell counts were determined by enumerating at least 5,000 cells stained with DAPI.

**Microscopy.** A Zeiss LSM 510 scanning confocal microscope equipped with a UV laser (351 and 364 nm), an Ar ion laser (450 to 514 nm), and two HeNe lasers (543 and 633 nm) was used to record optical sections. Image processing was performed with the standard software package delivered with the instrument (version 1.5). Reconstructed and processed images were printed by using the software package Microsoft Power Point (version 7.0) in combination with a Kodak 8650 PS printer.

**Enrichment and isolation of ammonia oxidizers.** Enrichments were performed on a mineral salt medium containing 10 or 100 mM $NH_4Cl$, 0.4 mM $KH_2PO_4$, 1 mM KCl, 0.2 mM $MgSO_4$, 10 or 200 mM NaCl, 5-g $liter^{-1}$ $CaCO_3$, 1-m $liter^{-1}$ 0.05% (wt/vol) cresol red solution, and 1-ml $liter^{-1}$ trace element solution [0.2 mM $MnSO_4$, 0.8 mM $H_3BO_3$, 0.15 mM $ZnSO_4$, 0.03 mM $(NH_4)_6Mo_7O_{24}$, 2.5 mM $FeSO_4$, and 0.1 mM $CuSO_4$ in 0.01 N HCl]. Isolations were carried out by plating the enrichments on mineral medium solidified by addition of 10 g of agar per liter and picking single colonies after 2 months of incubation at 30°C. Purity of the cultures was checked by microscopic examinations and by inoculation of an organic medium containing 0.5-g $liter^{-1}$ (each) yeast extract, beef extract, and peptone (pH 7.4).

**Enrichment and isolation of nitrite oxidizers.** A nitrite-oxidizing bacterium was enriched and isolated from the activated sludge as described by Bock et al. (6). Purity of the nitrite oxidizer isolate was checked by (i) inoculation of an organic culture medium (containing 0.5 g of yeast extract, 0.5 g of peptone, 0.5 g of beef extract, and 0.584 g of NaCl per liter; pH 7.4) and (ii) fluorescent in situ hybridization with probe NIT3 (56).

**PCR amplification of 16S rDNA.** DNA was extracted from a 0.25-g (wet weight) activated sludge pellet by using three different methods (11, 45, 64). For PCR amplification of the 16S rDNA of the ammonia and nitrite oxidizer isolates, high-molecular-weight DNA was isolated by the method of Chan and Goodwin (11). Oligonucleotide primers targeting the 16S rDNAs of all bacteria were used for PCR with a thermal capillary cycler (Idaho Technology, Idaho Falls) to obtain almost-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the primers were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*Escherichia coli* 16S rDNA positions 8 to 27 [10]) and 5′-CAKAAAGGAGGTGATCC-3′ (*E. coli* 16S rDNA positions 1529 to 1545). Reaction mixtures were prepared in accordance with the manufacturer's recommendations in a total volume of 50 μl by using 20 mM $MgCl_2$ reaction buffer. Thermal cycling was carried out with an initial denaturation step of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 51°C for 20 s, and elongation at 72°C for 30 s; cycling was completed by a final elongation step of 72°C for 1 min. Positive controls containing purified DNA from *E. coli* were included in all sets of amplifications along with negative controls (no DNA added). The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of the reaction product.

**PCR amplification of the *amoA* gene fragment.** For PCR amplification of the *amoA* gene fragment of the ammonia oxidizer isolates, high-molecular-weight DNA was isolated by the method of Chan and Goodwin (11). A 665-bp fragment of the *amoA* gene was amplified from 100 ng of DNA by primers AMO-F and AMO-R (44) for PCR with a thermal capillary cycler (Idaho Technology). Reaction mixtures with each primer at 15 pM 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 of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 48°C for 40 s, and elongation at 72°C for 50 s; cycling was completed by a final elongation step of 72°C for 1 min. For *amoA* gene fragment amplification from the bacterial population present in the activated sludge, total genomic DNA was extracted by the following protocol. A 0.25-g activated sludge pellet was resuspended in a 2-ml polypropylene tube with a screw-on plastic cap with 675 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) and treated for 30 s with a blender (Ultraturrax; Janke and Kunkel, Freiburg, Germany). After addition of 50 μl of enzyme mixture I (lysozyme [Fluka, Buchs, Switzerland], lipase Typ7 [Sigma, Deisenhofen, Germany], pectinase [Roth, Karlsruhe, Germany], and β-glucuronidase [Sigma], each at 10 mg $liter^{-1}$), the mixture was incubated for 30 min at 37°C. Subsequently, 50 μl of enzyme mixture II (proteinase K [Boehringer Mannheim], protease Typ9 [Sigma], and pronase P [Serva, Heidelberg, Germany], each at 10 mg $liter^{-1}$) was added and the mixture was incubated for another 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 phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged again for 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 volume 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 50 μl of double-distilled $H_2O$. The amount and quality of DNA were determined by spectrophotometric analysis of the ratio of optical densities at 260 and 280 nm (41). Amplification of *amoA* gene fragments was initially performed with 25, 50, and 100 ng of DNA and primers AMO-F and AMO-R as described above. To increase the specificity of *amoA* amplification from activated sludge, a nested PCR protocol was developed. For this purpose, two additional primers, AMO-F2 (5′-AAGATGCCGCCGGAAGC-3′) and AMO-R2 (5′-GCTGCAATAACTGTGGTA-3′), comprising the inner primer set, were designed from the *amoA* sequences of *N. europaea* Nm50, isolate Nm93, and isolate Nm103. AMO-F2 and AMO-R2 hybridize to nucleotide positions 288 to 305 and 895 to 913, respectively, of the published *N. europaea* sequence (32). For nested PCR, 25, 50, and 100 ng of genomic DNA isolated from activated sludge was amplified by using primers AMO-F and AMO-R and the conditions described above. A 1-μl volume of the reaction product was subjected to a further round of PCR amplification with 15 pM (each) AMO-F2 and AMO-R2 as a second primer set. Reaction mixtures were prepared as described above. Thermal cycling was carried out by an initial denaturation step of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 40 s, and elongation at 72°C for 50 s; cycling was completed by a final elongation step of 72°C for 1 min. Positive controls containing purified DNA from *N. europaea* Nm50 were included in all of the amplification sets along with negative controls (no DNA added). The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of the reaction product. Ethidium bromide-stained bands were digitally recorded with a Cybertech video documentation system (Cybertech, Hamburg, Germany).

**Cloning, sequencing, and phylogeny inference.** 16S rDNA and *amoA* PCR products were excised from the agarose gel, purified with an agarose gel extraction kit (Boehringer Mannheim), and subsequently ligated, in accordance with the manufacturer's recommendations, into the cloning vector (pCRII) supplied with the Original TA cloning kit (Invitrogen Corp., San Diego, Calif.). One *amoA* gene library and three 16S rDNA clone libraries, reflecting the three methods used for DNA extraction, were generated. Nucleotide sequences were determined by the dideoxynucleotide method (42) by cycle sequencing of purified plasmid preparations (Qiagen, Hilden, Germany) with a Thermo Sequenase Cycle sequencing kit (Amersham) and an infrared automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. Dye-labeled sequencing primers (Li-Cor) were used. The 16S rDNA sequences were added to the 16S rRNA sequence database of the Technische Universität München by use of the ARB program package (49). The ARB_EDIT tool was used for sequence alignment. Alignments were refined by visual inspection. Deduced amino acid sequences for *amoA* were aligned manually by pooling the amino acids into six groups with the GDE 2.2 sequence editor implemented in the ARB software package. Nucleic acid sequences of the *amoA* gene fragments were then aligned in accordance with the amino acid alignment. Nucleic acid similarities were computed by using the appropriate tool in the ARB program package. Phylogenetic analyses based on 16S rDNA were performed by applying the ARB parsimony tool and maximum-likelihood analysis (fast DNAml; 29) to different data sets. Checks for chimeric sequences were conducted by independently subjecting the first 513 5′ base positions, the middle 513 base positions, or the last 513 3′ base positions of the insert sequence to phylogenetic analysis. Phylogenetic trees based on comparative analysis of the *amoA* gene fragments were computed by performing maximum-likelihood analysis on *amoA* nucleic acid alignments with the appropriate tool in the ARB software package.

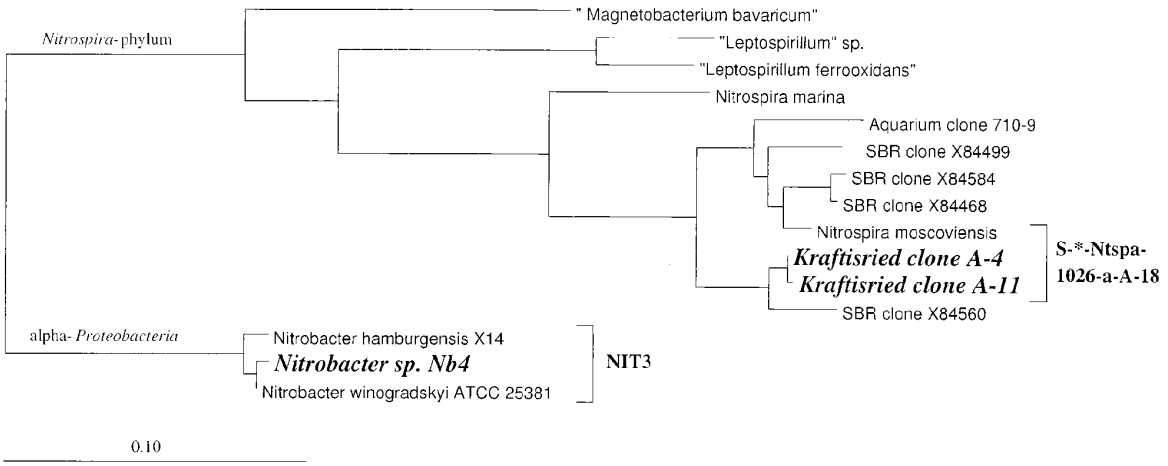


FIG. 1. Phylogenetic tree showing the relationships of the *Nitrospira*-like organisms represented by 16S rDNA clones A-4 and A-11 from activated sludge, *Nitrobacter* isolate Nb4, and their closest relatives. The tree is based on the results of maximum-likelihood analysis. Together with a 16S rDNA clone retrieved from an SBR (GenBank accession no. X84560; 8), both clone sequences form a separate lineage within the nitrite-oxidizing bacteria of the *Nitrospira* group, which is also supported by maximum-parsimony and neighbor-joining analyses. Target organisms for probes S-*-Ntspa-1026-a-A-18 and NIT3 are indicated by brackets. Due to partial sequencing of the SBR clones (8), no information about their sequence in the target region of probe S-*-Ntspa-1026-a-A-18 is available. The bar represents 10% estimated sequence divergence.

**DNA-DNA hybridization.** DNA similarities were estimated by photometric determination of thermal renaturation rates (13) as described by Koops and Harms (24).

**Nucleotide sequence accession numbers.** The sequences obtained in this study are available in GenBank under accession no. AF037105 (Nm93; 16S rRNA), AF037106 (Nm103, 16S rRNA), AF037107 (Nm103; *amoA*), AF037108 (Nm93; *amoA*), and AF043707 to AF043719 (*amoA* activated sludge clones 1 to 13).

## RESULTS

The Kraftisried wastewater treatment plant receives sewage with exceptionally high $NH_4^+$ concentrations (up to 5,000 mg liter$^{-1}$) which stem from the decay of protein-rich material handled by the adjoining animal waste processing facility. Due to intermittent aeration and the high nitrifying and denitrifying capacity of its activated sludge, the Kraftisried plant was able to remove more than 90% of the N compounds from the sewage during the sampling period (October 1996 to October 1997).

**Activated-sludge-derived 16S rDNA sequences affiliated with the genus *Nitrospira*.** Three different methods were used for DNA extraction of activated sludge sampled in October 1996. For each DNA preparation, 16S rDNA products were separately amplified and used for the generation of 16S rDNA libraries. A total of 60 clones were randomly selected from the libraries for comparative sequence analysis. Interestingly, none of the 16S rDNA sequences analyzed grouped within the monophyletic clade of the beta subclass of ammonia oxidizers (data not shown). However, 2 of the 60 sequences analyzed were unambiguously affiliated with nitrite-oxidizing bacteria of the genus *Nitrospira* (Fig. 1). Both sequences were found in the 16S rDNA library generated after DNA extraction by the method of Chan and Goodwin (11). Within the genus *Nitrospira*, these almost identical clone sequences showed about 94 and 88% 16S rRNA sequence similarity to *N. moscoviensis* and *N. marina*, respectively (Table 2). Based on these two activated-sludge-derived 16S rDNA sequences, oligonucleotide probe S-*-Ntspa-1026-a-A-18 was designed (Table 1).

**In situ characterization of the population structure of nitrifying bacteria.** The composition of the nitrifying consortium in the activated sludge was analyzed by fluorescent in situ hybridization with a set of previously published probes (33, 38, 55, 56). By searching for ammonia oxidizers in both samples, no hybridization signals could be obtained after application of *Nitrosospira* cluster-specific probe Nsv443, but 16 (October 1996) to 20% (September 1997) of the total number of cells stained with the intercalating dye DAPI could be assigned to the *Nitrosomonas* cluster of the beta subclass of ammonia oxidizers by

TABLE 2. Similarity ranking of clones A-4 and A-11 retrieved from activated sludge and members of the *Nitrospira* phylum

| rDNA source | % Similarity to rDNA of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A-4 | A-11 | 710-9 | SBR clone X84560 | *N. moscoviensis* | *N. marina* | *Leptospirillum* sp. | *L. ferrooxidans* |
| A-4 sequence | | | | | | | | |
| A-11 sequence | 99.8 | | | | | | | |
| 710-9 sequence | 92.2 | 92.0 | | | | | | |
| SBR clone X84560 | 97.0 | 96.5 | 92.1 | | | | | |
| *N. moscoviensis* | 94.0 | 93.8 | 94.7 | 91.6 | | | | |
| *N. marina* | 88.2 | 88.0 | 87.2 | 83.0 | 88.8 | | | |
| *Leptospirillum* sp. | 80.9 | 80.7 | 79.9 | 76.5 | 80.0 | 81.3 | | |
| *L. ferrooxidans* | 81.6 | 81.4 | 79.2 | 77.3 | 80.8 | 81.4 | 93.0 | |
| *M. bavaricum* | 80.3 | 80.2 | 78.5 | 73.9 | 79.9 | 80.8 | 79.7 | 81.4 |

simultaneous binding of probes Nso190, Nso1225, Nsm156, and NEU. In an additional hybridization experiment performed on both samples, more than 99% of the ammonia oxidizers detectable with probe NEU could simultaneously be visualized with *N. mobilis*-specific probe NmV. However, a few bacterial microcolonies did not show simultaneous binding of probes NEU and NmV but, instead, hybridized exclusively with either probe NEU or probe NmV. Looking for nitrite-oxidizing bacteria within the activated sludge, we were not able to detect any *Nitrobacter* cells by in situ hybridization with probe NIT3. However, in situ hybridization with probe S-*-Ntspa-1026-a-A-18, under stringent hybridization conditions (20% formamide in the hybridization buffer), revealed significant numbers (in both samples, approximately 9% of the total number of cells stained by DAPI) of *Nitrospira*-like cells to be present in the samples analyzed. Hybridization of the two activated sludge samples with probes NmV and S-*-Ntspa-1026-a-A-18 demonstrated that *Nitrospira*-like cells occurred as small microcolonies in close proximity to *N. mobilis* (Fig. 2).

**Isolation of nitrifying bacteria.** To obtain cultures of the numerically dominant nitrifying bacterial species, enrichments were initiated by inoculating small amounts of activated sludge into a series of different enrichment media. After plating of the enrichments on isolation agar and picking of colonies about 8 weeks later, eight ammonia oxidizer isolates and one nitrite oxidizer isolate were obtained. Using whole-cell hybridization of the ammonia oxidizer isolates with the respective probe set, we could distinguish between two types of ammonia oxidizers. Four of the isolates (Nm94, Nm100, Nm103, and Nm106) showed positive hybridization signals with probes Nso190, Nso 1225, Nsm156, and NEU but were not detectable with probe NmV. The remaining isolates (Nm93, Nm99, Nm104, and Nm107) showed a hybridization pattern identical to that observed for the *N. mobilis*-like cells present in the activated sludge. Nearly complete 16S rDNA sequences were determined for Nm93 and Nm103, representing the two types of ammonia oxidizers isolated. The 16S rRNA primary structures of isolates Nm93 and Nm103 shared overall similarities of more than 98% with *N. mobilis* Nc2 and *N. europaea* Nm50, respectively (Table 3; Fig. 3). Since the 16S rRNA similarities of both isolates to validly described species were greater than 97%, we performed DNA-DNA reassociation studies to clarify their species affiliations (46). The DNA similarity value measured for isolate Nm 93 and *N. mobilis* Nc2 was 82.7%, while the respective value for isolate Nm103 and *N. europaea* Nm50 was 84.8%. Based on the 16S rRNA sequence of isolate Nm103, oligonucleotide probe S-*-Nse-1472-a-A-18 was developed. Whole-cell hybridization experiments demonstrated that this probe also hybridized to *N. europaea* Nm50, *N. halophila* Nm1, and *N. eutropha* Nm 57 under stringent conditions (Table 4). Simultaneous in situ hybridization of the activated sludge with probes NEU, NmV, and S-*-Nse-1472-a-A-18, each labeled with a different fluorescent dye, revealed that all NEU-positive, NmV-negative cells were stained with probe S-*-Nse-1472-a-A-18.

The only nitrite-oxidizing isolate obtained (Nb4) could be assigned to the genus *Nitrobacter* by successful whole-cell hybridization with probe NIT3 (56). Consistent with the probing result, phylogenetic analysis of a partial 16S rRNA sequence (676 bp) of Nb4 revealed high similarity (99.8%) to the 16S rRNA of *N. winogradskyi*.

**Comparative *amoA* sequence analysis.** For isolates Nm93 and Nm103, a 665-bp fragment of the *amoA* gene was amplified with the primer set described by Sinigalliano et al. (44). Nucleic acid sequence analysis of both *amoA* fragments revealed that the sequence of isolate Nm103 was identical to that of the corresponding *amoA* fragment of *N. europaea* Nm50 (32) and displayed only a single base substitution compared to the sequence of isolate Nm93 (99.8% sequence similarity). For fine-scale diversity analysis of environmental populations of *N. europaea* and *N. mobilis*, a fragment of the *amoA* gene was amplified from DNA extracted from activated sludge for comparative sequence analysis. Initial *amoA* amplification experiments were performed with the primer set published by Sinigalliano et al. (44). While this primer pair amplified the expected 665-bp fragment from *N. europaea* Nm50, Nm93, and Nm103, no such product was observed for the Kraftisried activated sludge when 25 or 50 ng of DNA was used for amplification (Fig. 4). After increasing the amount of DNA used for *amoA* PCR to 100 ng, small amounts of a 665-bp amplification product, as well as larger and smaller products, were observed (Fig. 4). We excised and purified the 665-bp product from the agarose gel for subsequent cloning. However, sequence analysis of 20 randomly selected clones revealed that none of them had an *amoA*-related insert (data not shown). To enhance the specificity and sensitivity of the *amoA* PCR, we designed an additional inner primer pair for nested amplification of *amoA* from *N. europaea* and *N. mobilis*. By applying the nested PCR assay, we obtained a single amplification product with the expected length of 625 bp from both *N. europaea* Nm50 and Kraftisried activated sludge (Fig. 4). The nested PCR products retrieved from the activated sludge were used to generate an *amoA* gene library. A total of 13 clones were randomly selected for comparative sequence analysis. All of the nucleic acid sequences obtained were highly similar to each other (>99.8% sequence similarity) and to the partial *amoA* sequence of clone SP3 derived from activated sludge by Rotthauwe et al. (40; >99.7% sequence similarity). Six of the *amoA* clone nucleic acid sequences were identical to the *amoA* genes of *N. europaea* Nm50 and Nm103 and had a single base substitution in comparison with the *amoA* gene of the *N. mobilis* isolate Nm93. The remaining seven *amoA* clones had one to four base substitutions compared to the *amoA* sequences of *N. europaea* Nm50 (32), Nm103, and Nm 93. Consequently, the Kraftisried *amoA* sequences were lumped together with *N. europaea*, *N. mobilis*, and clone sequence SP3 in a phylogenetic tree for the *amoA* sequences reconstructed by the maximum-likelihood method (Fig. 5).

## DISCUSSION

Recent studies of nitrifying bacteria in activated sludge systems by fluorescent in situ hybridization with rRNA-targeted oligonucleotides indicated that part of the numerically important ammonia oxidizer assemblage has not yet been characterized (33) and that the importance of nitrite oxidizers other than *Nitrobacter* spp. for sewage treatment might have been underestimated (56). To learn more about the nitrifying bacteria involved in sewage treatment, the nitrifying consortium present in the nitrifying-denitrifying activated sludge from an industrial sewage treatment plant was characterized by a combination of molecular and cultivation-based techniques. In situ diversity analysis with multiple probes demonstrated that at least three different types of ammonia-oxidizing bacteria were present in activated sludge from the Kraftisried plant. It was surprising to find that *N. mobilis*-like cells were of the numerically dominant ammonia oxidizer type, as this species was originally isolated from brackish water (23) and had not been reported to contribute to nitrification in wastewater treatment. A second, small population of cells showed an unexpected hybridization pattern indicative of a novel, as-yet-uncultured (or if cultured, then previously not characterized on the 16S rRNA

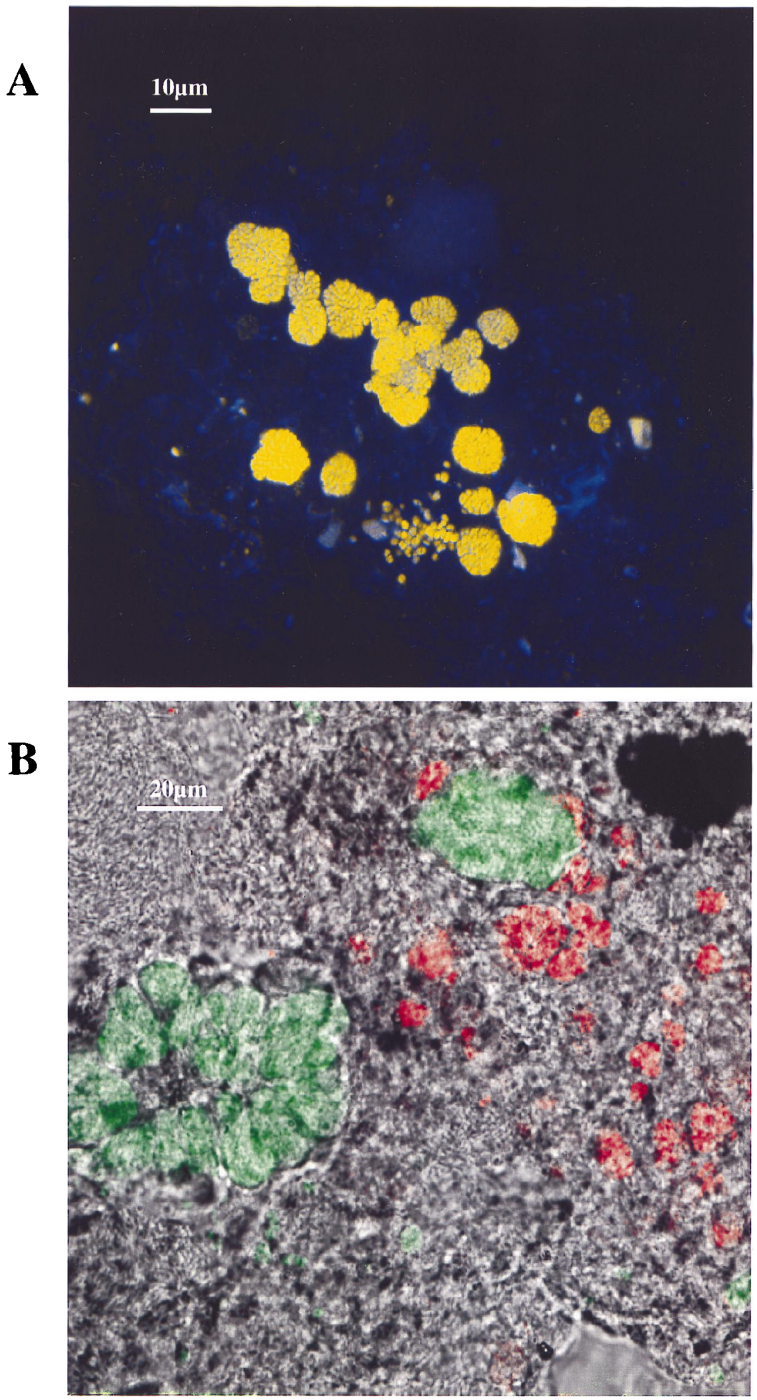


FIG. 2. In situ identification of nitrifying bacteria in activated sludge from the Kraftisried plant. (A) Simultaneous in situ hybridization with Cy3-labeled probe NmV and FLUOS-labeled probe NEU. *N. mobilis* cells appear yellow because of the overlapping labels. For visualization of the activated sludge floc, its autofluorescence was recorded with a 633-nm laser and colored blue by image analysis. (B) Simultaneous in situ identification of *N. mobilis* and *Nitrospira*-like bacteria after in situ hybridization with FLUOS-labeled probe NmV (green) and Cy3-labeled probe S-*-Ntspa-1026-a-A-18 (red). A phase-contrast image was superimposed for visualization of the floc material.

TABLE 3. 16S rRNA sequence similarities of ammonia-oxidizing bacteria Nm93 and Nm103 isolated from activated sludge and members of the *Nitrosomonas* cluster

| rDNA source | % Similarity to rDNA of: | | | | |
|---|---|---|---|---|---|
| | *N. mobilis* Nm93 | *N. europaea* Nm103 | *N. europaea* | *N. mobilis* | *N. eutropha* |
| *N. mobilis* Nm93 | | | | | |
| *N. europaea* Nm103 | 95.4 | | | | |
| *N. europaea* | 95.6 | 99.1 | | | |
| *N. mobilis* | 98.2 | 95.1 | 95.5 | | |
| *N. eutropha* | 94.9 | 97.7 | 98.1 | 94.4 | |
| *N. halophila* | 96.1 | 97.3 | 98.1 | 95.6 | 96.6 |

TABLE 4. Reference strains analyzed by whole-cell hybridization with oligonucleotides NEU and S-*-Nse-1472-a-A-18

| Organism | Strain[a] | Hybridization[b] with probe: | |
|---|---|---|---|
| | | S-*-Nse-1472-a-A-18 | NEU |
| *Nitrosomonas europaea* | Nm103 | + | + |
| *Nitrosococcus mobilis* | Nm93 | − | + |
| *Nitrosomonas europaea* | Nm50$^T$ | + | + |
| *Nitrosomonas halophila* | Nm1$^T$ | + | + |
| *Nitrosomonas eutropha* | Nm57$^T$ | + | + |
| *Nitrosomonas cryotolerans* | Nm55$^T$ | − | + |
| *Nitrosomonas aestuarii* | Nm36$^T$ | − | + |
| *Nitrosomonas marina* | Nm22$^T$ | − | + |
| *Nitrosomonas oligotropha* | Nm45$^T$ | − | − |
| *Nitrosomonas ureae* | Nm10$^T$ | − | − |

[a] T = type strain.
[b] +, hybridized; −, failed to hybridize.

level) ammonia oxidizer. This population hybridized with probes Nso190, Nso1225, and Nsm156 and *N. mobilis*-specific probe NmV but did not hybridize with probe NEU, which targets halophilic and halotolerant members of the genus *Nitrosomonas*, including *N. mobilis*. A third population of ammonia oxidizers with a low in situ abundance in the Kraftisried plant hybridized with probes Nso190, Nso1225, Nsm156, NEU, and S-*-Nse-1472-a-A-18—a hybridization pattern which is indicative of *N. halophila*, *N. europaea*, and *N. eutropha*. Despite the high in situ abundance of ammonia oxidizers in the activated sludge (10 to 20% of the total number of cells), none of the 60 sequences analyzed from the three 16S rDNA gene libraries obtained from the activated sludge was affiliated with ammonia oxidizers belonging to the beta subclass of *Proteobacteria*. The 16S rDNA amplification procedure did, however, work well when DNA from the ammonia oxidizer isolates was used as the template (see below). Plausible explanations for the absence, or at least significant underrepresentation, of ammonia oxidizer 16S rDNA sequences in the gene libraries are that (i) the three different DNA extraction techniques applied were not sufficiently rigorous to lyse the dense microcolonies of ammonia oxidizers in the activated sludge and/or that (ii) PCR or cloning biases occurred. Since microcolonies of ammonia oxidizers similar in architecture to those in the Kraftisried plant have been observed in situ in many nitrifying sewage disposal plants (55) and natural systems (56), results of DNA extraction and PCR-based molecular techniques alone for detection and diversity analysis of ammonia-oxidizing bacteria have to be carefully interpreted. Such an analysis might overlook numerically dominant populations in the environment.

Another goal of the present study was to obtain a pure culture isolate for each of the in situ-detected ammonia ox-

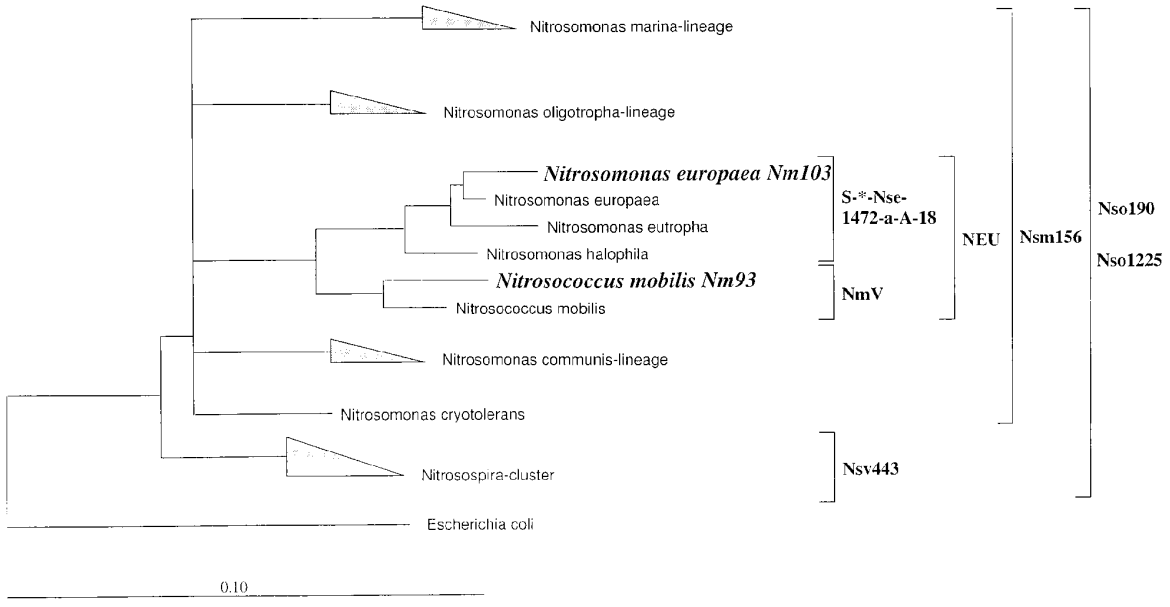


FIG. 3. Phylogenetic tree showing the relationships of ammonia oxidizer isolates *N. mobilis* Nm93 and *N. europaea* Nm103 and their closest relatives among the beta subclass of *Proteobacteria*. The tree is based on the results of maximum-likelihood analysis. Target organisms for probes Nso190, Nso1225, Nsm156, Nsv443, NEU, S-*-Nse-1472-a-A-18, and NmV are indicated by brackets. The bar represents 10% estimated sequence divergence.

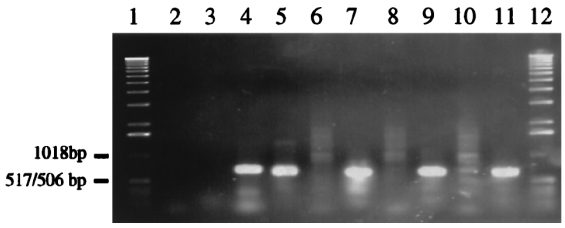


FIG. 4. Direct and nested PCR amplification of an *amoA* fragment from *N. europaea* Nm50 and from activated sludge (Kraftisried). Direct PCR amplification was performed with primers AMO-F and AMO-R (lanes 2, 4, 6, 8, and 10), and nested PCR amplification was performed with primers AMO-F and AMO-R, followed by primers AMO-F2 and AMO-R2 (lanes 3, 5, 7, 9, and 11). Lanes: 1 and 12, 1-kb DNA ladder; 2 and 3, water control; 4 and 5, 25 ng of *N. europaea* DNA; 6 and 7, 25 ng of activated sludge DNA; 8 and 9, 50 ng of activated sludge DNA; 10 and 11, 100 ng of activated sludge DNA.

idizer types. Screening with the ammonia-oxidizing bacterial probe set revealed that half of the ammonia-oxidizing isolates obtained by standard cultivation techniques were *N. mobilis* like, while the other half of the isolates could be assigned to the NEU-positive, NmV-negative *N. europaea* type. This distribution is in contrast to the abundance of both of the ammonia oxidizer populations found in the activated sludge and reflects the different salt requirements of the two ammonia oxidizer types. As most of our enrichments were performed with low-salt media (10 mM NaCl) composed for limnetic and terrestrial ammonia oxidizers, *N. mobilis*, which is characterized by an obligate salt requirement, was outnumbered in these enrichments by *N. europaea*. We failed to isolate the putative novel population of ammonia oxidizers observed in activated sludge by in situ hybridization. This cultivation failure may have been caused by low in situ abundance or by unique physiological properties not addressed by the enrichment strategies applied. It is also possible that the observed novel population could not be obtained by selective cultivation because it belongs to a hypothetical taxon closely related to the beta subclass ammonia oxidizer clade which does not oxidize ammonia. To date, no precedent for such a dramatic phenotype variation within the monophyletic beta-subclass ammonia oxidizers has been reported.

Two isolates representing the two types of ammonia-oxidizing bacteria obtained were identified as *N. mobilis* and *N. europaea* by comparative 16S rDNA sequence analysis (Fig. 3) and DNA-DNA hybridization, thereby confirming the specificity of the probes used for in situ analysis. As *N. mobilis* isolate Nm93 grows significantly slower in low-salt media than does *N. europaea* isolate Nm103, it appears likely that this species has been overlooked in activated sludge in previous laboratory culture studies.

Rotthauwe et al. (40) found the topology of an *amoA*-based phylogenetic tree to be in good agreement with the topology of the corresponding 16S rDNA tree. Nucleotide sequence similarities of closely related ammonia oxidizers were significantly lower for the *amoA* genes than for the corresponding 16S rRNA. For example, *N. europaea* and *N. eutropha* share 98.2% sequence similarity on the 16S rRNA level, whereas the corresponding values for the 665-bp *amoA* fragment analyzed in the present study is only 87.3%. Since *N. europaea* and *N. mobilis* share 95.6% sequence similarity on the 16S rRNA level, it was surprising that we could detect one single base substitution only between the 665-bp *amoA* fragments of *N. europaea* isolate Nm103 and *N. mobilis* isolate Nm93. This is in accordance with the results of Böttcher (9), which revealed unexpected high sequence similarities among 613-bp *amoA* fragments amplified from different genera of ammonia-oxidizing bacteria. These results could indicate lateral *amoA* gene transfer events between species of ammonia oxidizers of different genera. This assumption implies that the *amoA* genes we sequenced fulfill the criterion of orthology. Since some ammonia oxidizers possess multiple copies of the *amoA* gene (34–36), it is possible that the primers we used amplified not all of the *amoA* copies of *N. mobilis* Nm93 and that its orthologous ammonia monooxygenase has been overlooked. This appears to be unlikely, however, as sequence variation between the multiple *amoA* copies of a single species analyzed so far was shown to be very low (34–36). Additional experiments have been initiated to provide evidence for or against the hypothesis of horizontal transfer of the *amoA* gene and, as a result, for or against the suitability of the *amoA*-encoded protein to accurately reflect the phylogenetic relationships between ammonia oxidizers.

*amoA* clone sequences were retrieved from activated sludge after combining a modified DNA extraction protocol with a newly developed nested PCR technique. Keeping in mind the possible inadequacy of the three DNA extraction techniques used to generate 16S rDNA gene libraries for efficient cell lysis of *N. mobilis* microcolonies (see above), we implemented several additional enzymatic pretreatments in a modified DNA extraction protocol. Nevertheless, none of the 13 *amoA* acti-

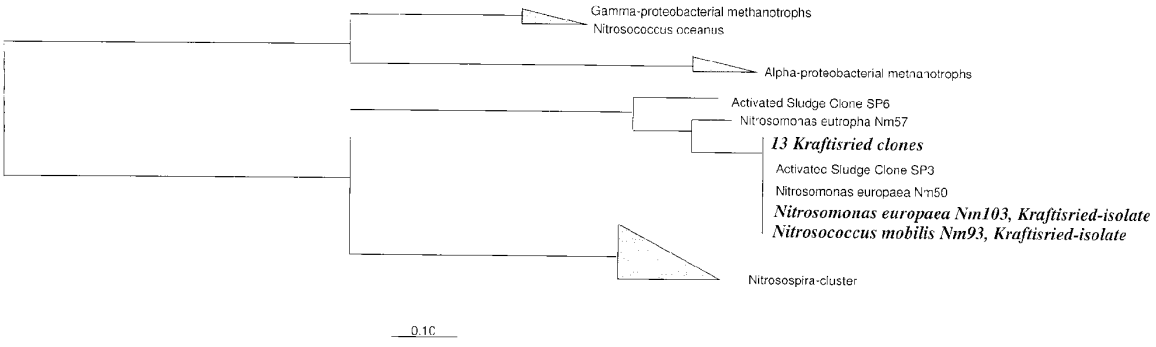


FIG. 5. Maximum-likelihood tree based on partial *amoA* gene sequences showing the phylogenetic positions of the *amoA* gene stretches from ammonia oxidizer isolates *N. mobilis* Nm93 and *N. europaea* Nm103 and the environmental sequences retrieved from activated sludge. The bar indicates 10% estimated sequence divergence.

vated sludge clones analyzed on the sequence level was identical to the *amoA* sequence of *N. mobilis* Nm93. Several *amoA* clones with sequence identity to the *amoA* sequence of *N. europaea* could be detected, however. This result, which is in apparent contrast to the in situ abundance of *N. mobilis* and *N. europaea*, and the fact that a nested PCR approach had to be used for successful *amoA* amplification from the activated sludge suggest that our modified DNA extraction protocol might still not be suitable for obtaining representative amounts of DNA from *N. mobilis* microcolonies. The development of more efficient DNA extraction protocols is a prerequisite for increasing the significance of DNA extraction-based molecular diversity analysis of ammonia oxidizers.

Results of the full-cycle rRNA approach presented here clearly show that *Nitrospira*-like bacteria, which previously had been found exclusively in marine environments (59), a heating system in Moscow (14), and freshwater aquaria (20), occurred as a dominant population in Kraftisried activated sludge, while *Nitrobacter* numbers were below the detection limit of the in situ hybridization technique ($10^3$ to $10^4$ cells $ml^{-1}$). In the activated sludge flocs, *Nitrospira*-like cells always were in the vicinity of *N. mobilis* microcolonies, which may reflect the syntrophic association between ammonia- and nitrite-oxidizing bacteria. Phylogenetic analysis of partial 16S rRNA sequences retrieved by Bond et al. (8) from a laboratory-scale sequencing batch reactor (SBR) in Australia demonstrated their association with the genus *Nitrospira* (Fig. 1), which indicates a widespread occurrence of *Nitrospira*-like cells in nitrifying activated sludge systems. Keeping in mind that low in situ *Nitrobacter* numbers have been reported for many nitrifying natural and engineered systems (56) and that the described species of *Nitrospira* grow significantly slower in pure culture than do *Nitrobacter* spp., it is tempting to speculate that *Nitrospira* spp. are responsible for nitrite oxidation in these environments but have previously been overlooked, as *Nitrobacter* outcompetes them during standard enrichment and isolation procedures. Consistent with this hypothesis is the fact that the only nitrite oxidizer isolate obtained from the Kraftisried plant could be assigned to the genus *Nitrobacter* by comparative 16S rRNA sequence analysis, while representatives of the in situ-dominant *Nitrospira*-like bacteria were missing among the isolates. It should be stressed, however, that comparative 16S rRNA analysis does not unambiguously prove that the molecular isolates of *Nitrospira*-like cells are indeed able to oxidize nitrite. For a detailed physiological characterization of *Nitrospira*-like organisms, representative isolates in pure culture will be required. Attempts to recover *Nitrospira*-like cells from activated sludge via probe-assisted isolation have been initiated. In the present report, we describe for the first time *N. mobilis* and *Nitrospira*-like bacteria as putative numerically dominant species of the nitrifying consortia in an industrial sewage treatment plant. The relevance of this finding lies in the importance of nitrifier activity for efficient nutrient removal in sewage treatment. Different species of ammonia- and nitrite-oxidizing bacteria most likely differ in their in situ growth kinetics, their ammonia and nitrite oxidation rates, their substrate and oxygen affinities, and their sensitivities to environmental perturbations. Probe-assisted isolation of numerically dominant nitrifying bacteria will allow a better understanding of the microbiology of the nitrification process and will help to improve the modeling, design, and operation of nitrifying wastewater treatment plants.

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## REFERENCES

1. **Alm, E., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557–3559.
2. **Amann, R. I.** 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p. 1–15. *In* A. D. C. Akkerman, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Arthur, J. W., C. W. West, K. N. Allen, and S. F. Hedke.** 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bull. Environ. Contam. Toxicol. **38:**324–331.
4. **Belser, L. W.** 1979. Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. **3:**309–333.
5. **Bock, E., H. Sundermayer-Klinger, and E. Stackebrandt.** 1983. New facultative lithotrophic nitrite-oxidizing bacteria. Arch. Microbiol. **136:**281–284.
6. **Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert.** 1990. A new facultative nitrite-oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch. Microbiol. **153:** 105–110.
7. **Bock, E., and H.-P. Koops.** 1992. The genus *Nitrobacter* and related genera, p. 2302–2309. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes 2nd ed. Springer-Verlag, New York, N.Y.
8. **Bond, P. L., P. Hugenholtz, J. Keller, and L. L. Blackall.** 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Appl. Environ. Microbiol. **61:**1910–1916.
9. **Böttcher, B.** 1996. Untersuchungen zur Phylogenie des ammoniakoxidierenden Systems nitrifizierender Bakterien. Ph.D. thesis. Universität Hamburg, Hamburg, Germany.
10. **Brosius, J., T. L. Dull., D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
11. **Chan, J. W. Y. F., and P. H. Goodwin.** 1995. Extraction of genomic DNA from extracellular polysaccharide synthesizing gram-negative bacteria. BioTechniques **1:**419–422.
12. **Chandler, D. P., R. W. Schreckhise, J. L. Smith, and H. Bolton, Jr.** 1997. Electroelution to remove humic compounds from soil DNA and RNA extracts. J. Microbiol. Methods **28:**11–19.
13. **DeLey, J., H. Cattoir, and A. Reynaerts.** 1970. The quantitative measurements of DNA hybridization from renaturation rates. Eur. J. Biochem. **12:** 133–142.
14. **Ehrich, S., B. Behrens, A. Lebedeva, W. Ludwig, and E. J. Bock.** 1995. A new obligately chemolithoautotrophic nitrite-oxidizing bacterium *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. Arch. Microbiol. **164:** 16–23.
15. **Hastings, R. C., M. T. Ceccherini, N. Miclaus, J. R. Saunders, M. Bazzicalupo, and A. J. McCarthy.** 1997. Direct molecular biological analysis of ammonia oxidizing bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol. Ecol. **23:**45–54.
16. **Head, I. M., W. Hiorns, T. Embley, A. McCarthy, and J. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **13:**1147–1153.
17. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
18. **Hiorns, W. D., R. Hastings, I. M. Head, A. McCarthy, J. Saunders, R. Pickup, and G. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonium-oxidizing bacteria. Microbiology **14:**2793–2800.
19. **Hovanec, T. A., and E. F. DeLong.** 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl. Environ. Microbiol. **62:**2888–2896.
20. **Hovanec, T. A., L. T. Taylor, A. Blakis, and E. DeLong.** 1998. *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. Appl. Environ. Microbiol. **64:**258–264.
21. **Jones, R. D., R. Morita, H.-P. Koops, and S. Watson.** 1988. A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. Can. J. Microbiol. **3:**1122–1128.
22. **Kane, M. D., L. K. Poulsen, and D. A. Stahl.** 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. **59:**682–686.
23. **Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov. sp. Arch. Microbiol. **10:**277–282.
24. **Koops, H.-P., and H. Harms.** 1985. Desoxyribonucleic acid homologies

among 96 strains of ammonia oxidizing bacteria. Arch. Microbiol. **141:**214–218.
25. **Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. **15:** 244–248.
26. **Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. J. Gen. Microbiol. **13:**1689–1699.
27. **Koops, H.-P., and U. C. Möller.** 1992. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2263. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes 2nd ed. Springer Verlag, New York, N.Y.
28. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **63:**1489–1497.
29. **Maidak, B. L., N. Larsen, M. McCaughey, R. Overbeek, G. Olsen, K. Fogel, J. Blandy, and C. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **2:**3485–3487.
30. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. **1:**593–600.
31. **McCaig, A. E., T. Embley, and J. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidizers. FEMS Microbiol. Lett. **12:**363–368.
32. **McTavish, H., J. A. Fuchs, and A. B. Hooper.** 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436–2444.
33. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
34. **Norton, J. M., J. M. Low, and M. G. Klotz.** 1996. The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol. Lett. **139:**181–188.
35. **Norton, J. M., and M. G. Klotz.** 1996. Nucleotide sequence of two gene copies encoding the subunit A of the ammonia monooxygenase from *Nitrosomonas eutropha* Nm57. GenBank Database, Bethesda, Md.
36. **Norton, J. M., J. Alzerreca, and M. G. Klotz.** 1997. Nucleotide sequence of two gene copies encoding the subunit A of the ammonia monooxygenase from *Nitrosolobus multiformis* C-71. GenBank Database, Bethesda, Md.
37. **Orso, S., M. Gouy, E. Navarro, and P. Normand.** 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. **44:**83–86.
38. **Pommerening-Röser, A., G. Rath, and H.-P. Koops.** 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Syst. Appl. Microbiol. **1:**344–351.
39. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
40. **Rotthauwe, J.-H., K.-P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704–4712.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **7:**5463–5467.
43. **Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K.-H. Schleifer.** 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **62:**4641–4647.
44. **Sinigalliano, C. D., D. N. Kuhn, and R. D. Jones.** 1995. Amplification of the *amoA* gene from diverse species of ammonia-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl. Environ. Microbiol. **61:**2702–2706.
45. **Smalla, K., N. Cresswell, L. C. Mendonca-Hagler, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74:**78–85.
46. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
47. **Stehr, G., B. Böttcher, P. Dittberner, G. Rath, and H.-P. Koops.** 1995. The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol. Ecol. **1:**177–186.
48. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147–4154.
49. **Strunk, O., and W. Ludwig.** 1997. ARB software program package; http://www.biol.chemie.tu-muenchen.de/pub/ARB/.
50. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
51. **Utåker, J. B., L. Bakken, Q. Jiang, and J. Nes.** 1995. Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. Syst. Appl. Microbiol. **1:**549–559.
52. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450.
53. **Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer.** 1993. Probing activated sludge with *Proteobacteria*-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
54. **Wagner, M., R. Amann, P. Kämpfer, B. Aßmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer.** 1994. Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. **1:**405–417.
55. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **1:** 251–264.
56. **Wagner, M., G. Rath, H.-P. Koops, J. Flood, and R. Amann.** 1996. *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Technol. **3:**237–244.
57. **Ward, B. B., M. A. Voytek, and K.-P. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers by specific PCR amplification. Microb. Ecol. **33:**87–96.
58. **Watson, S. W., and J. B. Waterbury.** 1971. Characteristics of two marine nitrite-oxidizing bacteria, *Nitrospina gracilis* nov. gen. nov. sp. and *Nitrococcus mobilis* nov. gen. nov. sp. Arch. Microbiol. **77:**203–230.
59. **Watson, S. W., E. Bock, F. W. Valois, J. B. Waterbury, and U. Schlosser.** 1986. *Nitrospira marina* nov. gen. nov. sp.: a chemolithotrophic nitrite-oxidizing bacterium. Arch. Microbiol. **144:**1–7.
60. **Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Ammonia-oxidizing bacteria, p. 1818–1834. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. III. The Williams & Wilkins Co., Baltimore, Md.
61. **Winogradsky, S.** 1892. Contributions à la morphologie des organismes de la nitrification. Arch. Sci. Biol. (St. Petersburg) **1:**88–137.
62. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of purple bacteria, the beta subdivision. Syst. Appl. Microbiol. **5:**327–336.
63. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of purple bacteria, the gamma subdivision. Syst. Appl. Microbiol. **6:**25–33.
64. **Zhou, J., M. A. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316–322.

# Appendix B

Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation

MARKUS SCHMID, ULF TWACHTMANN, MICHAEL KLEIN, MARC STROUS, STEFAN JURETSCHKO, MIKE JETTEN, JOERG W. METZGER, KARL-HEINZ SCHLEIFER, AND MICHAEL WAGNER

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SYSTEMATIC AND APPLIED MICROBIOLOGY

# Molecular Evidence for Genus Level Diversity of Bacteria Capable to Catalyze Anaerobic Ammonium-Oxidation

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## SUMMARY

Recently, a bacterium capable to oxidize ammonium anaerobically at a high rate was identified as novel member of the *Planctomycetales* (STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R. I., KUENEN, J. G., and JETTEN, M. S. M.. Nature 400, 446-449, 1999). Here we investigated the microbial community structure of a trickling filter biofilm with a high anaerobic ammonium oxidation activity. Fluorescence *in situ* hybridization (FISH) with a set of nine probes designed for specific identification of the recently described anaerobic ammonium oxidizer demonstrated that only one probe hybridized to bacteria within the biofilm. For phylogenetic characterization of putative biofilm anaerobic ammonium-oxidizers a full-cycle 16S rDNA approach was performed by using a *Planctomycetales*-specific forward primer for PCR amplification. Of the twenty-five 16S rDNA fragments (1364 bp in length) amplified from the biofilm, nine were affiliated to the *Planctomycetales*. Comparative analysis showed that these sequences were more than 98.9 % similar to each other but only distantly related to the previously recognized anaerobic ammonium-oxidizer (below 91% similarity) and all other organisms represented in public 16S rRNA databases (similarities of below 79 %). The retrieved sequences and the previously recognized anaerobic ammonium-oxidizer represent two well-separated groups of a deep-branching lineage within the *Planctomycetales*. Quantitative FISH analysis with a newly designed specific probe showed that the novel bacterium, provisionally classified as "*Candidatus* Kuenenia stuttgartiensis" constituted the dominant fraction of the biofilm bacteria. *In situ* probing revealed that ammonia-oxidizing bacteria of the beta-subclass of Proteobacteria were also present, albeit in significant smaller amounts, within the anoxic biofilm. Comparative sequence analysis of a stretch of the gene encoding ammonia-monooxygenase (*amoA*) demonstrated the occurrence of the DNA of at least three different populations of beta-subclass ammonia oxidizers within the biofilm.

**Key words:** Anaerobic ammonium-oxidation – diversity of *Planctomycetales* – beta-subclass ammonia oxidizers – trickling filter – nitrogen removal – biofilm – quantitative fluorescence in situ hybridization

## Introduction

Twenty-two years after Broda's remarkable theoretical consideration that lithotrophic microorganisms which exclusively gain energy by using ammonium as inorganic electron donor for denitrification should be able to exist in nature (BRODA, 1977), a novel member of the *Planctomycetales* capable of ammonium oxidation with nitrite as the electron acceptor under anoxic conditions was identified (STROUS et al., 1999). In addition to this autotrophic yet uncultured organism, anaerobic ammonium-oxidizing activity has been reported for some of the classical "aerobic" ammonia-oxidizers of the beta-subclass of *Proteobacteria* (BOCK et al., 1995; DE BRUIJN et al., 1995; KUAI and VERSTRAETE, 1998; POTH, 1986; POTH and FOCHT, 1995; SCHMIDT and BOCK, 1997). However, the anaerobic ammonium oxidation rate measured for enrichments of the novel planctomycete is more than twenty times higher than for pure cultures of the beta-subclass ammonia oxidizers (JETTEN et al., 1999).

Combined with recently developed nitrification systems which transform ammonium to nitrite with only

minor nitrate formation (ABELING and SEYFRIED, 1992; HELLINGA et al., 1998; LOGEMANN et al., 1998) the process of anaerobic ammonium oxidation allows for efficient denitrification in the absence of available organic carbon sources. Thus, implementation of this combined process appears to be a promising alternative for treatment of sewage with low C/N ratios in waste water treatment plants. Here we present data on the microbial community structure of an anoxic (anaerobic in the presence of nitrite) trickling filter biofilm showing high rates of anaerobic ammonium oxidation. A novel member of the *Planctomycetales* (FUERST, 1995) which is distantly related to the previously recognized anaerobic ammonium oxidizer (STROUS et al., 1999) was shown to dominate the biofilm by applying the full-cycle rRNA approach. Fluorescence in situ hybridization (FISH) with rRNA-targeted probes demonstrated that beta-subclass ammonia oxidizers represented 27% of the area of those bacteria detectable by FISH within this biofilm. *AmoA* gene fragments retrieved from the biofilm clustered together with the *Nitrosomonas europaea*-lineage and *Nitrosococcus mobilis*, respectively.

## Materials and methods

### Reactor operation and chemical analysis

A two-stage semi-technical trickling filter reactor system for the treatment of effluent from anaerobic sludge digestion is maintained at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management at the University of Stuttgart. A scheme of the reactor system and important parameters of the trickling filters are presented in Figure 1. The plant was designed for efficient nitrogen removal from the sludge liquor (typically containing between 500-2500 mg $NH_4^+$-N $l^{-1}$) via coupled partial aerobic nitrification (nitritation) and anaerobic ammonium oxidation. The ammonium conversion rate of trickling filter 1 is regulated via the influent load of ammonium so that 60 % of the ammonium is oxidized to nitrite. The resulting ratio of 1 : 1.3 of ammonium to nitrite in the effluent of trickling filter 1 is optimal for the subsequent anaerobic ammonium oxidation process in trickling filter 2 (STROUS et al., 1998; VAN DE GRAAF et al., 1996; VAN DE Graaf et al., 1997). Nitrite oxidation to nitrate by nitrite-oxidizers does not occur in trickling filter 1 most likely due to ammonia-inhibition. The effluent of trickling filter 1 containing a mixture of ammonium and nitrite is used as influent for the anoxic trickling filter 2 designed for anaerobic ammonium oxidation. The key parameters of trickling filter 2 are given in Table 1. The concentrations of ammonium, nitrite and nitrate in the influent and effluent of trickling filter 2 were determined photometrically (Merck, Spectroquant Darmstadt). Total nitrogen bounded (TNb; encompassing all inorganic and organic nitrogen compounds) was measured by a high temperature digestion process with subsequent chemiluminescence detection.

### Biofilm sampling

In December 1998 and April 1999, biofilm samples were removed together with their plastic support material (NOR-PAC, Norddeutsche Seekabelwerke GmbH, Nordenham, Germany) from trickling filter 2 of the semitechnical plant (Figure 1). Several pieces of the support material were immediately immersed in a fixative containing 4% (w/v) paraformaldehyde (4) and stored at 4°C for 8h. Subsequently, the biofilm was detached from its support material by gently mixing, washed with PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), transferred into 50% (v/v) PBS/EtOH and stored at –20 °C until use. Several other pieces of support material were transported on dry ice to the lab and stored at –20°C for subsequent nucleic acid extraction. After sampling trickling filter 2 was rinsed with dinitrogen gas to reestablish anoxic conditions.

### DNA extraction from biofilm

Biofilm material was removed from the plastic support by gently mixing in 10 ml 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% CTAB) (ZHOU et al., 1996). The plastic support was removed and the biofilm material was pelleted by centrifugation for 10 min at 5000 g (Hettich, Tuttlingen, Germany). Total genomic DNA was extracted as described previously for activated sludge by JURETSCHKO et al. (1998).

### PCR amplification of 16S rDNA

For preferential PCR amplification of 16S rDNA of members of the *Planctomycetales* the recently published phylum-specific probe Pla46 (*E. coli* positions 46-63; NEEF et al., 1998) was used as unlabeled derivative as forward primer in combination with the universal reverse primer 1390R (*E. coli* positions 1390-1407; ZHENG et al., 1996). PCR was performed in a 96 micro well plate (Biorad, München, Germany) with the gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 4 min at 94°C, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at different temperatures (see below) for 50 s, and elongation at 72 °C for 3 min. Cycling was completed by a final elongation step at 72° C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. Optimum annealing temperature for the used primer set in combination with the DNA retrieved from the biofilm was determined by using the annealing temperature gradient function of the thermal cycler. 12 different annealing temperatures between 44 and 64°C were tested. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products.

### PCR amplification of the amoA gene fragment

For PCR amplification of a stretch of the *amoA* gene the primer set amoA-1F and amoA-2R (ROTTHAUWE et al., 1997) was used. Amplification was performed in a total volume of

**Table 1.** Key parameters of trickling filter 2 during the sampling period

| $O_2$ [mg $l^{-1}$] | Temp. [°C] | pH | TOC (Infl. and Effl.) [mg $l^{-1}$] | $Q_{Influent}$ [l $d^{-1}$] | $Q_{Recycling}$ [l $d^{-1}$] |
|---|---|---|---|---|---|
| 0.00 – 0.08 | 23 - 26 | 7.7 - 8.4 | 5 - 10 | 42 - 55 | 200 - 370 |

50 µl with a thermal capillary cycler (Idaho Technology, Idaho Falls). Reactions contained 2 mM $MgCl_2$ (Idaho Technology), 1x BSA (Idaho Technology), 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega). The thermal cycling profile used for amplification was as follows: 30 s initial denaturation at 94°C; then 30 cycles consisting of 15 s at 94°C for denaturing, 20 s annealing at 55°C, 40 s at 72°C for elongation, and a final elongation for 3 min at 72°C. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5µl aliquots of the PCR products.

**Gel retardation of *amo*A amplificates**

The gel retardation of amplified *amo*A fragments was performed in a Hoefer HE33 submarine gel electrophoresis unit (Pharmacia Biotech, Freiburg, Germany) with cooled base using a modification of the protocol published by WAWER et al., 1995. The 2% agarose gel consisted of 35 ml 0.5 x TBE (0.0445 M Tris/HCl, 0.0445 M boric acid, 0.001 M EDTA, pH 8.3) and 0.7 g Nusieve 3:1 agarose. After cooling down the agarose mixture to 70°C, 35 µl of the DNA ligand bisbenzimide, to which long chains of polyethylene glycol 6000 are covalently coupled (Hanse Analytik Yellow; Hanse Analytik, Bremen, Germany), was added to the gel. The dye bisbenzimide binds preferentially to A + T rich sequence motifs in the DNA and retards them

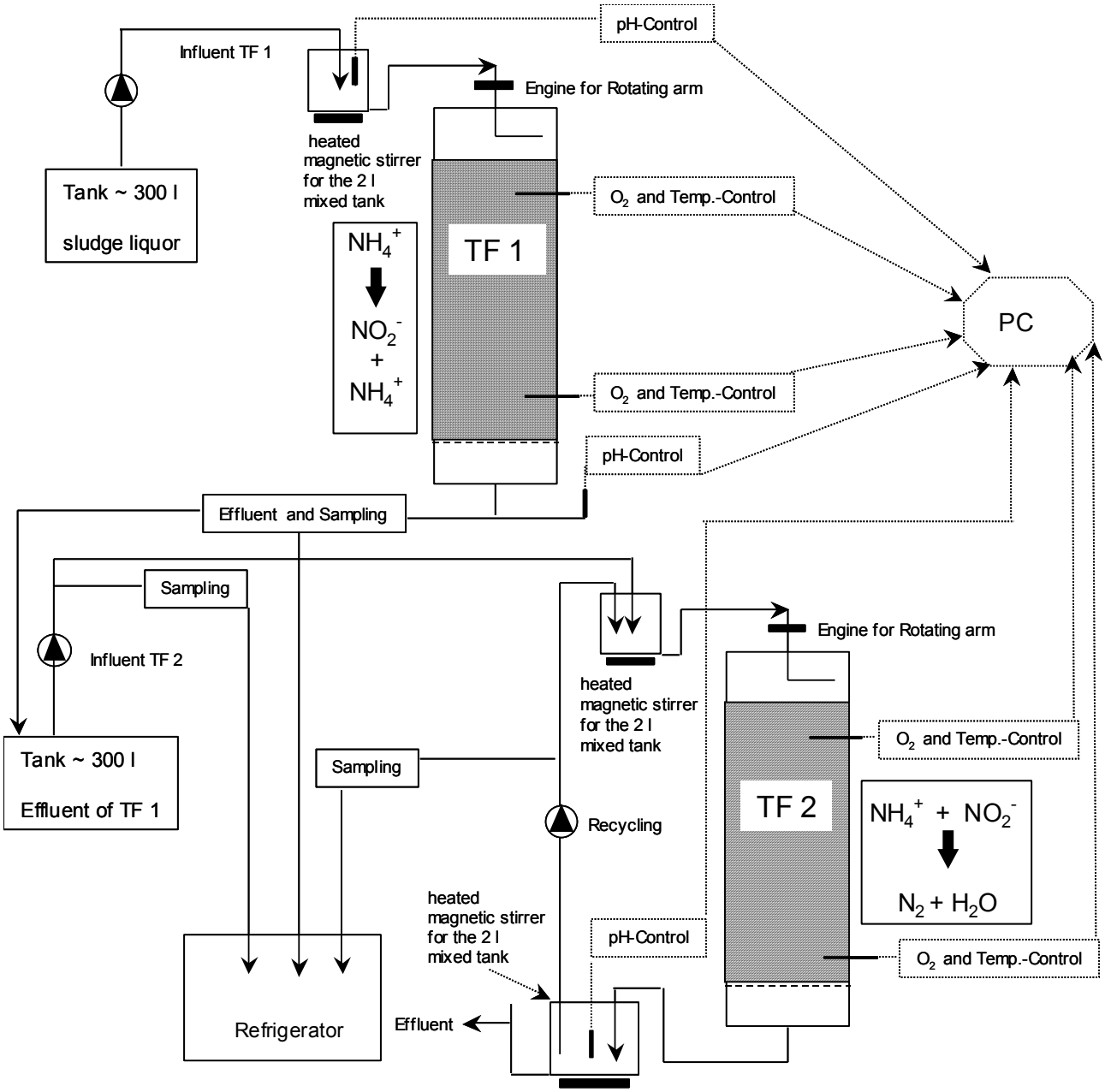


**Fig.1.** Scheme of the semi-technical scale plant at the University of Stuttgart

**Table 2.** Probe sequences, target sites, formamide concentrations in the hybridization buffer required for specific *in situ* hybridization and observed probe hybridization with the trickling filter 2 biofilm.

| Trivial name (reference) | OPD[a] designation | Specificity | Sequence 5'-3' | Target site[b] | % Formamide/ mM [NaCl] | Hybrid-ization with biofilm |
|---|---|---|---|---|---|---|
| Pla 46 (NEEF et al., 1998) | S-P-Planc-0046-a-A-18 | *Planctomycetales* | GACTTGCATGCCTAATCC | 46–63 | 25/159 | + |
| – (STROUS et al., submitted) | S-*-Amx-0156-a-A-18 | Anaer. ammonium oxidizer | CGGTAGCCCCCAATTGCTT | 156–173 | 40/56 | – |
| – (STROUS et al., submitted) | S-*-Amx-0223-a-A-18 | Anaer. ammonium oxidizer | GACATTGACCCCTCTCTG | 223–240 | 40/56 | – |
| – (STROUS et al., submitted) | S-*-Amx-0432-a-A-18 | Anaer. ammonium oxidizer | CTTAACTCCCGACAGTGG | 432–449 | 40/56 | – |
| – (STROUS et al., submitted) | S-*-Amx-0613-a-A-22 | Anaer. ammonium oxidizer | CCGCCATTCTTCCGTTAAGCGG | 613–634 | 40/56 | – |
| – (STROUS *et al.*, submitted) | S-*-Amx-0820-a-A-22 | Anaer. ammonium oxidizer | AAAACCCCTCTACTTAGTGCCC | 820–841 | 40/56 | + |
| – (STROUS et al., submitted) | S-*-Amx-0997-a-A-21 | Anaer. ammonium oxidizer | TTTCAGGTTTCTACTTCTACC | 997–1017 | 20/225 | – |
| – (STROUS et al., submitted) | S-*-Amx-1015-a-A-18 | Anaer. ammonium oxidizer | GATACCGTTCGTCGCCCT | 1015–1032 | 60/14 | – |
| – (STROUS et al., submitted) | S-*-Amx-1154-a-A-18 | Anaer. ammonium oxidizer | TCTTGACGACAGCAGTCT | 1154–1171 | 20/225 | – |
| – (STROUS et al., submitted) | S-*-Amx-1240-a-A-23 | Anaer. ammonium oxidizer | TTTAGCATCCCTTTGTACCAACC | 1240–1262 | 60/14 | – |
| – (this study) | S-*-Kst-1275-a-A-20 | "*Candidatus* Kuenenia stuttgartiensis" | TCGGCTTTATAGGTTTCGCA | 1275–1294 | 25/159 | + |
| Nso 190 (MOBARRY et al., 1996) | S-P-Betao-1225-a-A-19 | Ammonia oxidizers of the beta- Proteobacteria | CGATCCCCTGCTTTTCTCC | 190–208 | 55/20 | + |
| Nso 1225 (MOBARRY et al., 1996) | S-P-Betao-1225-a-A-20 | Ammonia oxidizers of the beta-Proteobacteria | CGCCATTGTATTACGTGTGA | 1225–1244 | 35/80 | + |
| Neu (WAGNER et al., 1995) | S-*-Neu-0653-a-A-18 | Halophilic and halotolerant *Nitrosomonas* sp. | CCCCTCTGCTGCACTCTA | 653–670 | 40/56 | + |
| NmV (JURETSCHKO et al., 1998) | S-S-Ncmob-0174-a-A-18 | *Nitrosococcus mobilis* | TCCTCAGAGACTACGCGG | 174–191 | 35/80 | – |
| Nsv 443 (MOBARRY et al., 1996) | S-F-Nsp-0444-a-A-19 | *Nitrosospira*-cluster | CCGTGACCGTTTCGTTCCG | 444–462 | 30/112 | – |
| NIT 3 (WAGNER et al., 1996) | S-G-Nb-1035-a-A-18 | genus *Nitrobacter* | CCTGTGCTCCATGCTCCG | 1035–1048 | 35/80 | – |
| – (DAIMS et al., in press) | S-G-Ntspa-0662-a-A-18 | genus *Nitrospira* | GGAATTCCGCGCTCCTCT | 662–679 | 35/80 | – |
| Eub 338 (AMANN et al., 1990) | S-D-Bact-0338-a-A-18 | *Bacteria* | GCTGCCTCCCGTAGGAGT | 338–355 | 0/900 | + |
| Eub 338 II (DAIMS et al., 1999) | S-D-Bact-0338-b-A-18 | Refer to DAIMS et al., 1999 | GCAGCCACCCGTAGGTGT | 338–355 | 0/900 | + |
| Eub 338 III (DAIMS et al., 1999) | S-D-Bact-0338-c-A-18 | Refer to DAIMS et al., 1999 | GCTGCCACCCGTAGGTGT | 338–355 | 0/900 | + |

[a]Oligonucleotide probe database (ALM et al., 1996), [b]16S rRNA position, *E. coli* numbering (BROSIUS et al., 1978), [c]Formamid in the hybridization buffer and [NaCl] in the washing buffer, respectively.

compared to DNA sequences with low A+T content. After poring, the gel was solidified in the dark. The electrophoresis was performed applying a voltage of 100V for 80 min with 0.5 x TBE as running buffer. After the run, the gel was stained with SYBR Green I (FMC BioProducts, Rockwell, US) nucleic acid stain (5 µl in 50 ml 0.5 x TBE) for 1 h in the dark. The bands on the gel were visualized using UV illumination (364nm) and cut out with a capillary. The gel fragments were resuspended in 300 µl sterile $H_2O$ for 10 min at 80°C and reamplified using the PCR protocol described above.

#### Cloning and Sequencing

The biofilm-derived 16S rDNA and *amo*A PCR products were cloned directly by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the Quiaprep spin miniprep kit (Quiagen, Hilden, Germany). Plasmids with an insert of the expected size were identified by agaraose (1.0%) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3h at 37°C). Sequencing was done nonradioactively by using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit according to the instructions of the manufacturer (Amersham, Freiburg, Germany). The reaction mixtures were analyzed with an infrared automated DNA sequencer (model LiCor Longreadir DNA 4200, MWG - Biotech, Ebersberg, Germany). The complete sequences of the *amoA* fragments and the 16S rDNA fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site.

#### Phylogenetic analysis

The 16S rDNA and *amoA* sequences retrieved in this study were added to the 16S rDNA and *amoA* sequence databases of the Technischen Universität München (currently encompassing more than 16.000 small subunit rRNA sequences and about 200 *amoA* sequences), respectively, by use of the ARB program package (STRUNK and LUDWIG, 1997). 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection considering the secondary structure of the 16S rRNA. Deduced amino acid sequences for *amo*A were aligned manually by pooling the amino acids into six groups with the GDE 2.2 sequence editor implemented in the ARB software package. Nucleic acid sequences of the *amo*A gene fragments were then aligned in accordance with the amino acid alignment. Phylogenetic analysis of 16S rRNA sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analysis (fast DNAml, MAIDAK et al., 1996) to different data sets. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling) (Phylogeny Inference Package Version 3.5c,University of Washington, Seattle). Checks for chimeric sequences were conducted by independently subjecting the first 5' 454 base positions, the middle 455 base positions, or the last 454 3' base positions for phylogenetic analysis. The reconstruction of phylogenetic trees based on comparative analysis of the AmoA amino acid sequences was performed using protein maximum likelihood with the JTT-f amino acid replacement model (PROTML 2.2; ADACHI and HASEGAWA, 1996), Parsimony, and FITCH (using the Dayhoff PAM 001 matrix as the amino replacement model, randomized input order, and global rearrangements) methods (PHYLIP 3.5c; FELSENSTEIN, 1993) implemented in the ARB software package.

#### Probedesign, fluorescence *in situ*hybridization, DAPI-staining, microscopy, and quantification of probe target bacteria

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridization buffers are displayed in Table 2. Probe S-*-Kst-1275-a-A-20 specific for the retrieved biofilm sequences related to the anaerobic ammonium oxidizer was designed using the probe design tool of the ARB package (Table 2). Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Interactiva (Ulm, Germany). Hybridizations were performed as described by AMANN (1995). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (WAGNER et al., 1994). Optimal hybridization conditions for probe S-*-Kst-1275-a-A-20 were determined by using the hybridization and wash buffers described by MANZ et al. (1992). An *in situ* probe dissociation curve was recorded by measuring the relative fluorescence intensity of biofilm bacteria after hybridization with probe S-*-Kst-1275-a-A-20 at different stringencies as described by DAIMS et al. (1999). Dual staining of cells with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent oligonucleotides was performed as previously described (JURETSCHKO et al., 1998). Surprisingly, probe S-*-Kst-1275-a-A-20 positive bacteria did only show a very weak DAPI-conferred fluorescence. Thus, we post-stained biofilm material hybridized with Cy3-labeled probes with SYBR Green I (FMC Bioproducts, Rockland). For preparation of a working solution SYBR Green I was diluted with $ddH_2O$ 10.000-fold. 20 µl of the SYBR Green I working solution were applied to each well of the microscopic slide and incubated in the dark for 10min at room temperature. Slides were washed briefly with $ddH_2O$, air-dried and embedded in Citifluor (Citifluor Ltd., Canterbury, UK). For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). The EUB/SYBR Green I ratio was determined by using an equimolar mixture of the probes EUB338, EUB338-II, and EUB338-III (30 ng of each probe labeled with Cy3; DAIMS et al., 1999) by applying the digital image analysis procedure described below. For quantification of biofilm bacteria related to the anaerobic ammonium-oxidizer or beta-subclass ammonia-oxidizers Cy3 labeled probes S-*-Kst-1275-a-A-20 or Nso1225 were used together with the Cy5 labeled bacterial probe set (EUB338, EUB338-II, EUB338-III) for simultaneous hybridization. The ratio of the area of cells stained with SYBR Green I or the specific probe, respectively, vs. the ratio of the area of those cells labeled with the bacterial probes was determined for random confocal optical biofilm sections (1 µm) by digital image analysis using the Carl Zeiss Vision KS400 software package together with a newly developed macro (R.A.M., Relative Area Measurement – the macro is available on request). For each probe 20 different microscopic fields completely covered with biofilm material (magnification x400) were analyzed.

#### Nucleotide sequence accession numbers

The sequences obtained in this study are available in GenBank under accession no. AF202655 – AF202663 (16S rRNA of "*Candidatus* Kuenenia stuttgartiensis") and AF202649 – AF202654 (anoxic biofilm *amoA* clones).

## Results

#### Anaerobic ammonium oxidation in trickling filter 2

The semi-technical plant displayed in Figure 1 was inoculated with activated sludge of the denitrification and aeration tank (ratio 1:1) of the waste water treatment

**Table 3.** Average nitrogen balances of trickling filter 2 during the biofilm sampling period

| | Influent [mg $l^{-1}$] | Effluent [mg $l^{-1}$] | Degradation [%] | Formation [%] | Load conversion g $(m^3*d)^{-1}$ | Load conversion g $(m^2*d)^{-1}$ |
|---|---|---|---|---|---|---|
| $NH_4^+$-N | 280 | 33 | 88.3 | | 45,6 | 0.42 |
| $NO_2^-$-N | 336 | 13 | 96.2 | | 58,7 | 0.54 |
| $NO_3^-$-N | 7 | 71 | | 11.3[a] | 11,6 | 0.11 |
| $\sum N_{anorg}$ | 623 | 117 | 81.2 | | 92,6 | 0.85 |
| $TN_B$ | 634 | 127 | 80.0 | | 92,6 | 0.85 |

[a] It should be noted that nitrate formation is consistent with anaerobic ammonium-oxidizing activity (STROUS et al., 1998; VAN DE GRAAF et al., 1996, VAN DE GRAAF et al., 1997 )

plant (designed for 10,000 inhabitants and population equivalents) of the Institute for Sanitary Engineering (Stuttgart, Germany). Sludge liquor containing up to 690 mg $l^{-1}$ N ammonium was used as influent for trickling filter 1. During the start-up phase semisynthetic waste water was successfully used as influent for trickling filter 2 in order to establish anaerobic ammonium-oxidizing activity. Thereafter, the effluent of trickling filter 1 was used as influent for trickling filter 2. In the experimental period more than 88% of the ammonium and 96% of the nitrite present in the influent were removed in trickling filter 2. Furthermore, an average production of 64 mg $l^{-1}$ of nitrate (11.3% formation) was observed (Table 3). Thus, the average total nitrogen removal in trickling filter 2 was 81.2%.

**Biofilm bacteria related to the anaerobic ammonium-oxidizer.**

Nine 16S-rRNA targeted oligonucleotide probes designed for specific *in situ* detection of the previously recognized anaerobic ammonium-oxidizer (STROUS et al., submitted) were used for *in situ* hybridization of biofilm samples of trickling filter 2. While high numbers of densely clustered cells were specifically labeled after hybridization with probe S-*-Amx-0820-a-A-22, no signals could be detected after application of the other eight probes (Table 2). Simultaneous application of the *Planctomycetales*-specific probe Pla46 (NEEF et al., 1998) demonstrated that bacteria detectable with probe S-*-Amx-0820-a-A-22 made up more than 99% of the area

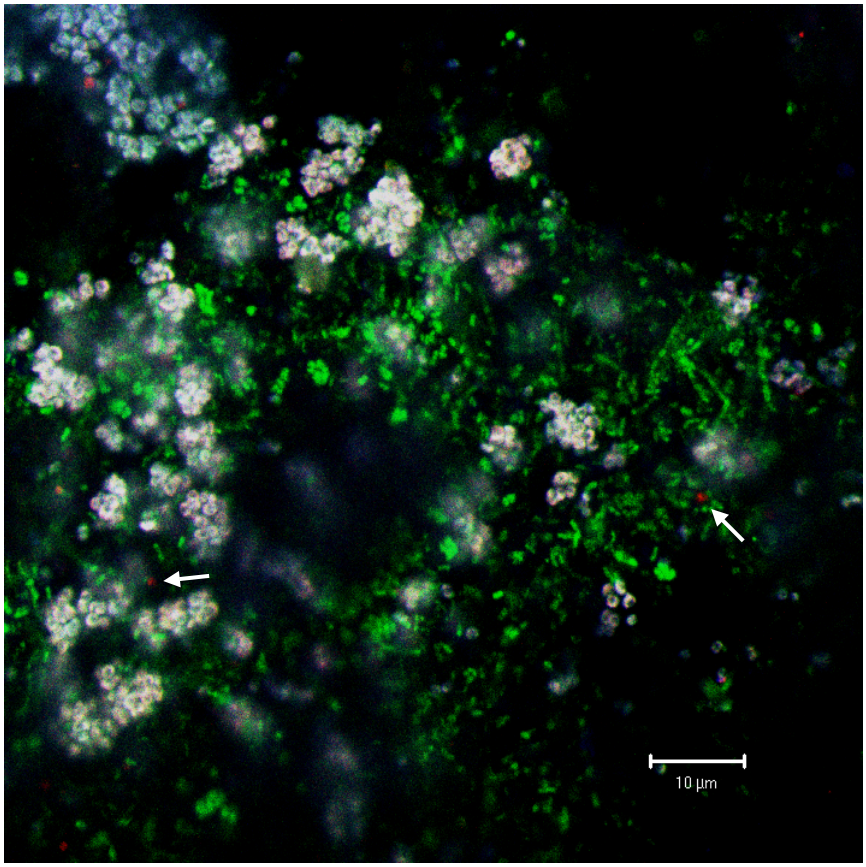


**Fig. 2.** *In situ* identification of "*Candidatus* Kuenenia stuttgartiensis" in biofilm of trickling filter 2 by simultaneous hybridization with Cy3-labeled probe Pla46, Fluos-labeled probe Eub338 and Cy5-labeled probe S-*-Kst-1275-a-A-20. "*Candidatus* Kuenenia stuttgartiensis" appears white because of the overlapping labels. Planctomycetes other than "*Candidatus* Kuenenia stuttgartiensis" are labeled red (locations indicated by arrows). Please note that for illustration purposes probe EUB338 was used without the probe modifications EUB338-II and EUB338-III. As all members of the genus *Isosphaera*, "*Candidatus* Kuenenia stuttgartiensis" possess a single mismatch within the EUB338 probe-target site which does, however, not hamper probe EUB338 binding under the hybridization conditions applied in this study (DAIMS et al., 1999).

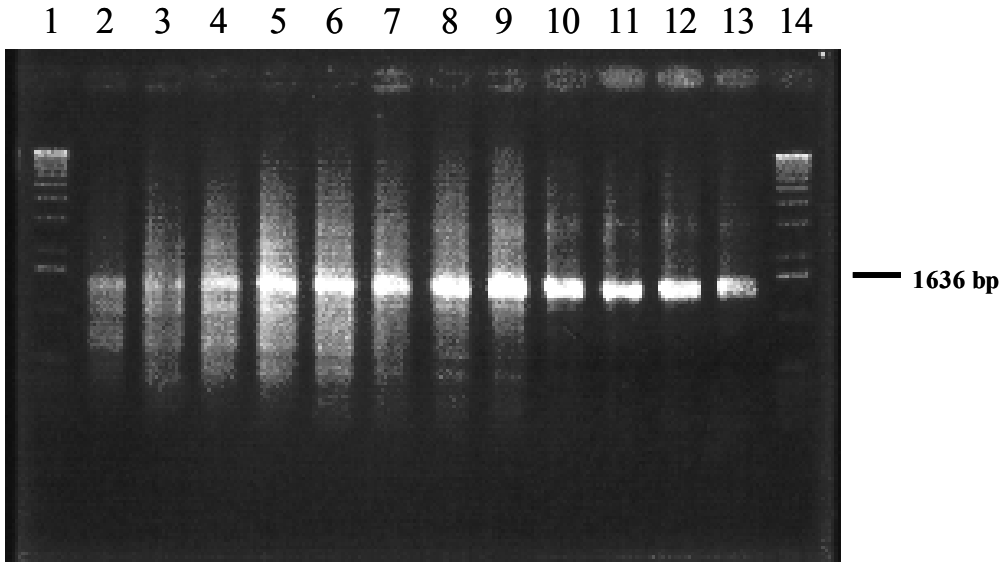


**Fig. 3.** PCR amplification of trickling filter 2 derived 16S rDNA with the primers Pla46 and 1390R using different annealing temperatures. Lanes: 1 and 14, 1-kb DNA ladder, 2:44°C, 3:45.8°C, 4:47.6°C, 5:49.5°C, 6:51.3°C, 7:53.1°C, 8:54.9°C, 9:56.7 °C, 10:58.6°C, 11:60.4°C, 12:62.2°C, 13:64°C.

of those cells stained with probe Pla46 (data not shown). Both, the probe S-*-Amx-0820-a-A-22-positive bacteria and the cells stained exclusively with probe Pla46 had a coccoid morphology (average diameter is 1.5 µm) and showed a ring-shaped hybridization signal indicative for members of the *Planctomycetales* (NEEF et al., 1998; STROUS et al., 1999). Pla46-positive cells which did not hybridize with the Anammox-specific probes occurred as single cells within the biofilm (Figure 2).

To establish a phylogenetic inventory of members of the *Planctomycetales* present within trickling filter 2 probe Pla46 was used as forward primer in combination with the universal reverse primer 1390R for PCR 16S rDNA amplification. The effect of different annealing temperatures on yield and specificity of the PCR reaction was analyzed (Figure 3). At annealing temperatures from 44°C to 56,7°C relatively large amounts of unspecific amplificates were observed. Use of higher annealing temperatures up to 64°C resulted in specific product formation. The PCR product obtained with an annealing temperature of 58,6°C was used for subsequent direct cloning to cover a maximum diversity of 16S rDNA sequences. Surprisingly, only nine of the twenty-five clones analyzed were affiliated with the *Planctomycetales*. These nine sequences (1363-1365 bp in length – primers not counted) were highly similar to each other (more than 98.8% sequence similarity) but only distantly related to all other sequences presently represented in public

**Table 4.** Overall sequence similarities for the retrieved biofilm 16S rRNA sequences and representative species of the *Planctomycetales* and *Chlamydia trachomatis*, respectively.

| rDNA source | % Similarity to rDNA of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. trachomatis* | *P. marina* | *P. limnophilus* | *G. obscuriglobus* | *I. pallida* | Marine agg. 27 | Biofi.18 | An.amm. ox. |
| *Pirellula marina* | 72.4 | | | | | | | |
| *Planctomyces limnophilus* | 71.8 | 82.4 | | | | | | |
| *Gemmata obscuriglobus* | 70.9 | 81.0 | 80.8 | | | | | |
| *Isosphera pallida* | 72.1 | 78.1 | 79.5 | 78.9 | | | | |
| Marine aggregate clone 27 | 72.7 | 77.8 | 76.0 | 75.4 | 75.8 | | | |
| Biofilm clone 18 | 71.4 | 75.0 | 73.0 | 72.2 | 71.9 | 77.7 | | |
| Anaerobic ammonium-oxidizer | 72.7 | 76.1 | 75.9 | 75.7 | 74.7 | 77.6 | 75.0 | |
| **Anoxic biofilm-clone 2-48**[a] | **74.2** | **77.3** | **75.5** | **74.8** | **73.8** | **78.6** | **74.5** | **90.4** |

[a] The molecular isolate 2-48 was selected as representative of the nine almost identical 16S rDNA sequences (>98,9%) retrieved from the anoxic biofilm

Table 5. 16S rRNA signature nucleotides for the subdivisions of the order *Planctomycetales*.

| Position(s) | Reference | *Pirellula* group | *Planctomyces* group | *Gemmata* group | *Isosphaera* | Anaerobic ammonium oxidizer | **Anoxic biofilm clone 2–48**[a] | Biofilm clone 18 | Marine clone 27 Marine cl OM190 |
|---|---|---|---|---|---|---|---|---|---|
| 169 | LIESACK and STACKEBRANDT, 1992 | G | G | G | G | C | C | C | G |
| 291–309 | LIESACK and STACKEBRANDT, 1992 | G-C | G-C | G-C | G-C | C-G | C-G | C-G | G-C |
| 331 | LIESACK and STACKEBRANDT, 1992 | U | U | U | U | U | U | U | U |
| 353 | WOESE, 1987; LIESACK and STACKEBRANDT, 1992 | U | U | U | U | U | U | U | U |
| 538 | LIESACK and STACKEBRANDT, 1992 | $A_{U,G}$[b] | $A_{U}$[b] | A | A | G | G | U | G |
| 570–588 | WOESE, 1987; LIESACK and STACKEBRANDT, 1992 | U-A | U-A | U-A | U-A | G-C | G-C | A-U | A-U |
| 659 | LIESACK and STACKEBRANDT, 1992 | $A_{G,U}$[b] | A | A | A or G | C | C | A | C |
| 686 | LIESACK and STACKEBRANDT, 1992 | G | G | G | G | G | G | U | G |
| 768 | LIESACK and STACKEBRANDT, 1992 | A | A | A | A | A | A | A | A |
| 784–798 | LIESACK and STACKEBRANDT, 1992 | G-C | G-C | G-C | G-C | G-C | G-C | no sequence information | G-C |
| 772–807 | LIESACK and STACKEBRANDT, 1992 | A-U | A-U | A-U | A-U | A-U | A-U | A-U | A-U |
| 811 | LIESACK and STACKEBRANDT, 1992 | C | C | C | C | C | C | C | C |
| 819 | LIESACK and STACKEBRANDT, 1992 | A | A | A | A | A | A | A | A |
| 833–853 | LIESACK and STACKEBRANDT, 1992 | G-C | $G\text{-}C_{A\text{-}U}$[b] | G-C | G-C | G-C | G-C | G-C | A-U, |
| 826–874 | LIESACK and STACKEBRANDT, 1992 | C-G | C-G | C-G | C-G | C-G | C-G | U-A | C-G |
| 933 | WOESE, 1987; LIESACK and STACKEBRANDT, 1992 | A | A | A | A | G | G | A | A |
| 955 | WOESE, 1987; LIESACK and STACKEBRANDT, 1992 | C | C | C | C | C | C | U | U |
| 983:1[c] | WOESE, 1987; LIESACK and STACKEBRANDT, 1992 | U | U | U | U | – | – | C | – |
| 1109 | WOESE, 1987 | A | A | A | A | A | A | C | A |
| 1384 | WOESE, 1987 | U | U | U | U | C | C | U | U |
| 114–313 | FUERST et al., 1997 | A-U | G-C | G-C | U-A | U-A | U-A | U-A | U-A |
| 115–312 | FUERST et al., 1997 | G-C | U-A | C-G | G-C | G-C | G-C | G-C | G-C |
| 668–738 | FUERST et al., 1997 | $U\text{-}A_{A\text{-}U}$[b] | U-A | C-G | G-C | A-U | A-U | U-A | U-A |
| 680–710 | FUERST et al., 1997 | A-U | C-G | $C\text{-}G_{U\text{-}A}$[b] | C-G | C-G | C-G | A-U | C-G |
| 812 | FUERST et al., 1997 | G | G | C | G | G | G | C | G |
| 822-878 | FUERST et al., 1997 | $A\text{-}U_{U\text{-}A}$[b] | A-U | G-C | G-C | G-C | G-C | U-A | G-C |
| 948–1233 | WOESE, 1987; FUERST et al., 1997 | G-C | C-G | C-G | C-G | C-G | C-G | C-G | C-G |
| 976 | FUERST et al., 1997 | G | G | G | A | G | G | G | G |
| 1100 | FUERST et al., 1997 | U | G,A | U | U | U | U | U | U |
| 1361:1[c] | FUERST et al., 1997 | – | C | C | C | – | – | C | C |

[a] The molecular isolate 2–48 was selected as representative of the nine almost identical 16S rDNA sequences (>98.9%) retrieved from the anoxic biofilm
[b] Lowercase letters indicate nucleotides present in less than 85% of members of main line of descent
[c] Colon indicates inserted nucleotide at position 983 and 1361, respectively

16S rRNA databases. Among those the highest similarity values were calculated for the previously recognized anaerobic ammonium-oxidizer (90.2% - 90.5%). Other members of the *Planctomycetales* and all other organisms displayed very low sequence similarities below 79% (Table 4). The nine biofilm 16S rDNA sequences display most but not all the signature nucleotides and nucleotide pairs (Table 5) characteristic of planctomycetes (FUERST et al., 1997; LIESACK and STACKEBRANDT 1992; WOESE, 1987). Consistent results were obtained after application of different treeing methods for phylogenetic analysis of the *Planctomycetales*-related sequences retrieved from the biofilm. Neighbor-joining, maximum-likelihood and maximum parsimony analysis agreed that these sequences form a grouping with the deep-branching anaerobic ammonium-oxidizer within the *Planctomycetales* (Figure 4). Selection of different sets of outgroup organisms and exclusion of highly variable positions prior to treeing analysis (by use of a 50% conservation filter for the *Planctomycetales* and the *Bacteria*, respectively) resulted in identical assignment of the biofilm sequences (data not shown). Bootstrap support for the clustering of the biofilm-retrieved sequences with the anaerobic ammonium oxidizer and for the monophyly of the *Planctomycetales* and the anaerobic ammonium oxidizer related sequences is highly significant (Figure 4). Interestingly, three environmentally derived 16S rRNA sequences from a trickling filter biofilm (VAN DER MEER et al., 1998), marine coastal picoplancton (RAPPE, et al. 1996) and a marine aggregate (DE LONG et al., 1993) group with the anaerobic ammonium-oxidizer and the biofilm sequences if maximum likelihood (only in combination with a filter selecting those sequence positions which share the same nucleotides in at least 50% of the available bacterial sequences) or maximum parsimony methods (only without sequence filter) are applied. However, these methods used with other sequence filters as well as all neighbor joining analyses suggest that these three previously published sequences form a separate lineage which branches not as deep as the anaerobic ammonium oxidizer lineage.

The oligonucleotide probe S-*-Kst-1275-a-A-20 was designed complementary to a specific target region shared between the nine (anaerobic ammonium-oxidizer related) 16S rDNA biofilm sequences. Probe S-*-Kst-1275-a-A-20 had at least three mismatches with respect to all other available 16S rRNA sequences (Figure 5). Since no pure culture is available to determine the optimal hybridization stringency for probe S-*-Kst-1275-a-A-20, an *in situ* probe dissociation curve was recorded with fixed biofilm samples using increasingly stringent

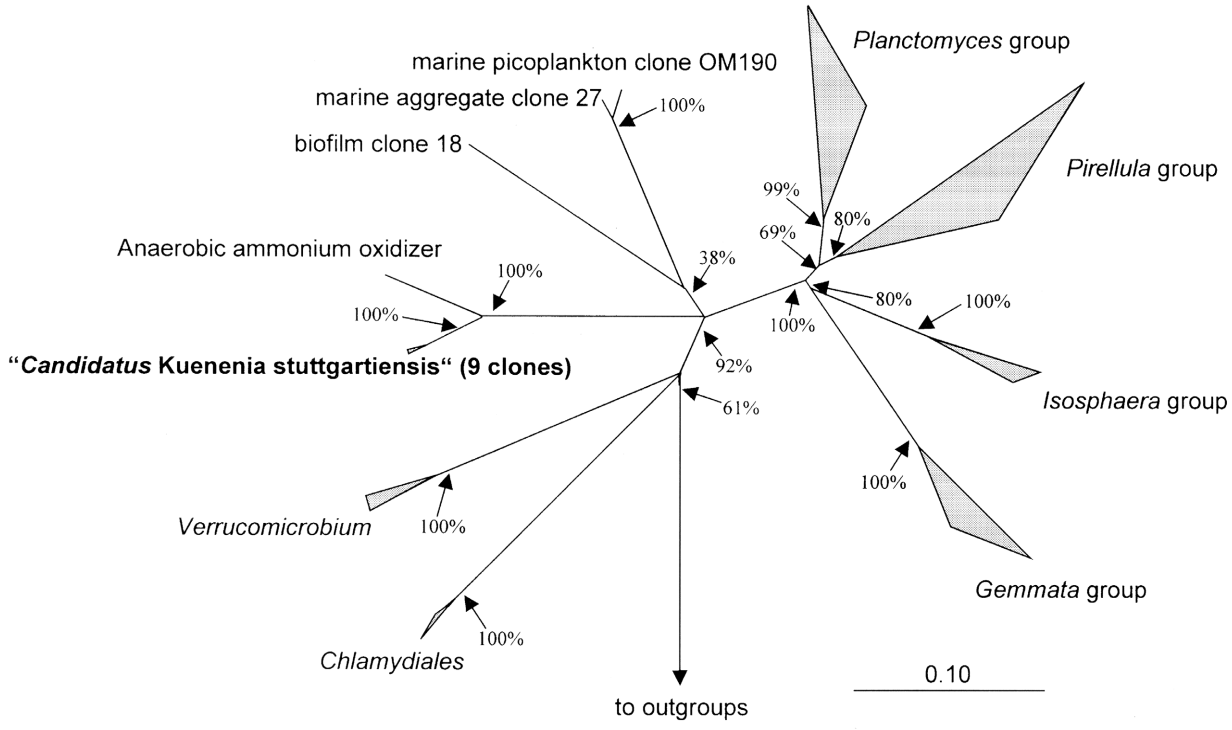


**Fig. 4.** Phylogenetic tree reflecting the relationships of the Stuttgart trickling filter 2 16S rDNA clones, the previously recognized anaerobic ammonium oxidizer (STROUS et al., 1999; AJ131819), the other *Planctomycetales,* and other reference organisms. The triangles indicate phylogenetic groups. The tree is based on the results of maximum likelihood analysis on different data sets. Multifurcations connect branches for which a relative order could not unambiguously be determined applying different treeing methods. Parsimony bootstrap values 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 represents 10% estimated sequence divergence.

| | |
|---|---|
| Probe sequence | 3'-ACGCTTTGGATATTTCGGCT-5' |
| Target sequence | 5'-UGCGAAACCUAUAAAGCCGA-3' |
| "*Candidatus* Kuenenia stuttgartiensis" | ..... |
| Anaerobic ammonium oxidizer | A.....C.....A..A. |
| *Paenibacillus curdlanolyticus* | A.....U.....G |
| *Desulfotomaculum thermosapovorans* | A.....C..G.....G |
| *Thermoanaerobacter lacticus* | A.....C.G.....G |
| *Actinopolyspora halophila* | A.....CGG.....G |
| *Halomonas halodurans* | A.....U..C.....G |
| *Brachybacterium alimentarium* | A.....U....A.....G |
| *Clavibacter michiganense* | A.....U..C.N.....G |
| *Mycobacterium ulcerans* | A.....U...U.....G |
| *Legionella adelaidensis* | A.....U.U..G.....G |
| *Sporotomaculum hydroxybenzoicum* | A.....C..G.G.....G |
| *Thermoanaerobacter brockii* | A.....C.A.....G |
| *Corynebacterium flavescens* | A.....U.G.G.....G |

**Fig. 5.** Difference alignment for probe S-*-Kst-1275-a-A-20. 16S rRNA sequences at the target site of the probe are displayed for representative reference organisms.

conditions (Figure 6). Probe S-*-Kst-1275-a-A-20 yielded strong signals up to 25% (v/v) formamide in the hybridization buffer followed by a decline at 30% (v/v) formamide. Signal intensities dropped to the level of autofluorescence after increasing the formamide concentration in the hybridization buffers to more than 50% (v/v). Cells with highly similar morphology were detected by probe S-*-Kst-1275-a-A-20 under conditions of different stringency. Considering strength and position of the mismatches in other known non-target organisms a formamide concentration of 25% (v/v) was chosen as optimal stringency for S-*-Kst-1275-a-A-20. After simultaneous hybridization of biofilm material with probe S-*-Kst-1275-a-A-20, and probe S-*-Amx-0820-a-A-22 (labeled with different dyes) exclusively double-labeled cells occurring in dense aggregates were observed.

Quantitative *in situ* analysis of the bacteria related to the anaerobic ammonium-oxidizer within the biofilm was performed by applying probe S-*-Kst-1275-a-A-20 with 25% formamide in the hybridization buffer and 159 mM NaCl in the washing buffer (see Materials and Methods). Digital image analysis of confocal biofilm sections simultaneously hybridized with the Cy3-labeled EUB338 probe mixture (DAIMS et al., 1999) and the Cy5-labeled probe S-*-Kst-1275-a-A-20 demonstrated that bacteria related to the anaerobic ammonium oxidizer occupied 49% (+/-12; 95% confidence limit; the relatively high standard deviation is caused by the unequal, cluster-like distribution of the probe target bacteria within the biofilm) of the area of those bacterial cells detectable by *in situ* hybridization (Figure 2). In a control experiment, the relative area of the novel biofilm *Planctomycetales* after hybridization with probe S-*-Amx-0820-a-A-22 was determined to be 50% (+/-8; 95% confidence limit). Since 85% (+/-8; 95% confidence limit) of the total bacterial cells within the biofilm stained with SYBR Green I were simultaneously detectable with the bacterial probe mixture, the bacteria related to anaerobic ammonium-oxidizer constitute almost half of the bacterial biomass in trickling filter 2.

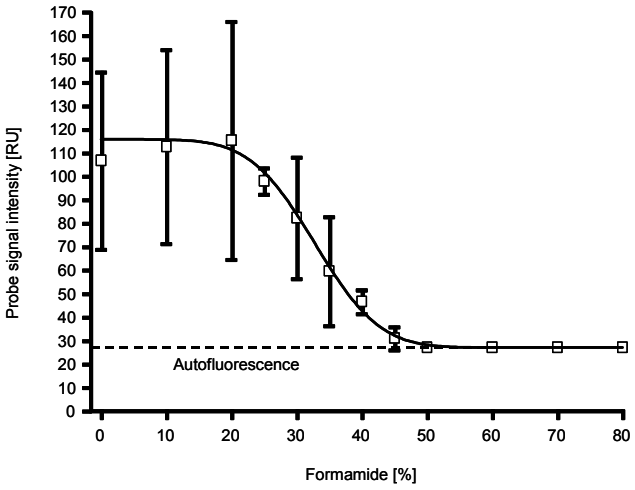


**Fig. 6.** Probe binding profile of probe S-*-Kst-1275-a-A-20. The relative strength of hybridization was determined at increasing concentrations of formamide in the hybridization buffer and decreasing concentrations of NaCl in the washing buffer by quantification of intensities of the fluorescence signals.

### "Aerobic" nitrifiers within the anoxic biofilm

The abundance and diversity of "aerobic" ammonium-oxidizing bacteria of the beta-subclass of Proteobacteria within the anoxic biofilm of trickling filter 2 were investigated by fluorescence *in situ* hybridization with a set of previously developed 16S rRNA-targeted oligonucleotide probes listed in Table 2 (JURETSCHKO et al.,1998; MOBARRY et al., 1996; WAGNER et al., 1995; WAGNER et al., 1996) and by comparative sequence analysis of retrieved fragments of the *amoA* gene (ROTTHAUWE et al., 1997). In confocal biofilm sections 27% (+/-8; 95% confidence limit; the relatively high standard deviation is caused by the unequal, cluster-like distribution of the probe target bacteria within the biofilm) of the area of those cells detectable with the bacterial probe set was occupied by ammonia oxidizers identified as halophilic or halotolerant members of the genus *Nitrosomonas* by simultaneous hybridization with probes Nso 1225, Nso190 and NEU. No signals were observed after hybridization with the *Nitrosococcus mobilis* specific probe NmV or the *Nitrosospira* cluster-specific probe Nsv443. In addition, no

nitrite-oxidizing bacteria of the genera *Nitrobacter* and *Nitrospira* could be visualized after hybridization with the probes NIT3 (WAGNER et al., 1996) and S-G-Ntspa-0662-a-A-18 (DAIMS et al., submitted), respectively.

For 16S rRNA-independent high resolution diversity analysis of beta-subclass ammonia-oxidizers of trickling filter 2 the *amoA* approach was performed (ROTTHAUWE et al., 1997). *AmoA* gene fragments were successfully amplified from trickling filter 2 biofilm derived DNA. After gel electrophoretic separation of the equal-sized *amoA* fragments according to their GC content by using gel retardation, three distinct bands were observed, cloned and sequenced (Figure 7). Phylogenetic analysis of the deduced amino acid sequences of the molecular isolates representing the respective bands demonstrated their grouping with *Nitrosococcus mobilis* (band 1; clones S1, S2, and S4, GC content 43-44%, identity values between 95.7 and 97.2% on the amino acid level to the respective *amoA* fragment of *N. mobilis), Nitrosomonas eutropha* (band 2, clone S5, GC content 47%, identity value of 95.0 % on the amino acid level to the respective *amoA* fragments of *N. eutropha*), and *Nitrosomonas europaea* (band 3, clones S3 and S6, GC content 49%, identity values of 98.6 - 99.3% on the amino acid level to the respective *amoA* fragment of *N. europaea*) (Figure 8).

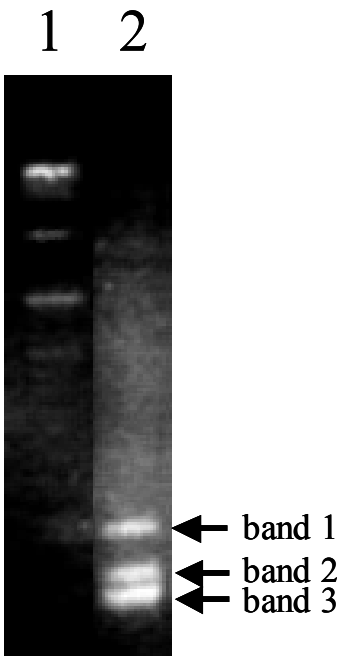


**Fig. 7.** Separation of trickling filter 2 retrieved amoA PCR amplificates according to their GC content by gel retardation. Lane 1: 1-kb DNA ladder (please note that the migration pattern is strongly altered due to the gel retardation); Lane 2: 5µl PCR product. Bands 1, 2 and 3 were excised, cloned and sequenced (See Figure 8).

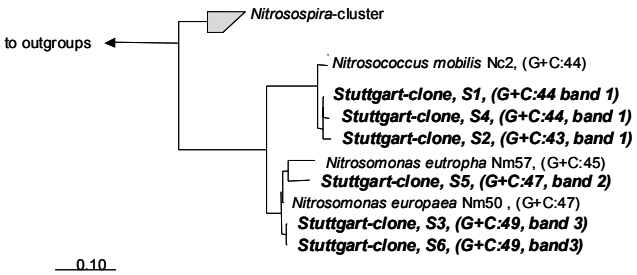


**Fig. 8.** Phylogenetic Fitch-Margoliash tree reflecting the relationships of the trickling filter 2 ammonia oxidizers based on deduced AmoA amino acid sequences. GC contents and the respective gel retardation band are given for each biofilm clone in brackets. The bar indicates 10% estimated sequence divergence.

## Discussion

### Genus-level diversity of anaerobic ammonium oxidizers

On thermodynamical grounds Broda predicted the existence of lithotrophic prokaryotes mediating the anaerobic oxidation of ammonia to nitrogen in nature (BRODA, 1977). Recently, this hypothesis was verified by the identification of a novel, deep-branching planctomycete capable of catalyzing the above mentioned process (STROUS et al., 1999). Our data provide evidence that an additional genus within the *Planctomycetales* capable to anaerobically oxidize ammonium does exist. The suggested novel genus was identified based on nine highly similar 16S rRNA sequences retrieved from a trickling filter biofilm with high anaerobic ammonium-oxidizing activity. Different phylogenetic analyses consistently demonstrated that these sequences group as a clearly separated cluster with the previously described anaerobic ammonium oxidizer. The moderate sequence similarities between the molecular biofilm isolates and the anaerobic ammonium-oxidizer (below 91%), indicate the presence of two different genera (LUDWIG et al., 1998). Consistent with these findings, the biofilm retrieved sequences do not possess fully complementary target sites for eight out of nine 16S rRNA-targeted oligonucleotide probes previously designed for specific *in situ* detection of the anaerobic ammonium-oxidizer (STROUS et al., submitted).

Our results do not provide direct evidence that the identified novel planctomycetes occurring in the trickling filter biofilm do actually perform anaerobic ammonium-oxidation. However, there are two independent experimental results which strongly support the conclusion that these organisms are indeed anaerobic ammonium oxidizers. Firstly, it should be noted that the influent of trickling filter 2 contained significant amounts of ammonium (280 mg $l^{-1}$) and nitrite (336 mg $l^{-1}$) but was very low in total organic carbon (5-10 mg $l^{-1}$). Analysis of the chemical composition of the influent and effluent of the analyzed trickling filter showed that its biofilm possessed a high anaerobic ammonium-oxidizing activity at the

time of sampling. Taken together it appears very likely that the numerically dominant biofilm microorganism, the novel planctomycete-like bacterium which accounts for almost half of the prokaryotic biofilm biomass, are chemolithoautotrophs gaining energy from the anaerobic ammonium oxidation. Secondly, the previously described anaerobic ammonium-oxidizer is the closest known relative of the novel biofilm planctomycete representing together the deepest recognized line of descent within the *Planctomycetales*. Since no other recognized members of the *Planctomycetales* which are capable to perform the anaerobic ammonium oxidation have been described, it is tempting to speculate that the common ancestor of the anaerobic ammonium oxidizer and the novel *Candidatus* genus either developed or acquired (by lateral gene transfer from a yet unknown bacterium) this physiological ability. In this context three environmentally derived 16S rRNA sequences, one from a marine aggregate (DE LONG et al., 1993), marine picoplancton (RAPPE et al., 1996) and one from a rotating contactor disk biofilm (VAN DER MEER et al., 1998), should also be considered. Some but not all treeing methods suggest that these two sequences do also group with the anaerobic ammonium oxidizer and the novel biofilm planctomycete. Future studies are required to show whether these sequences also represent bacteria performing anaerobic ammonium-oxidation. If such is the case a widespread environmental distribution of bacteria with this physiological trait would be expected.

Based on the results of our study we propose, according to MURRAY and SCHLEIFER(1994) provisional classification of the novel biofilm planctomycete as "*Candidatus* Kuenenia stuttgartiensis". The short description of "*Candidatus* Kuenenia stuttgartiensis" is as follows: deep-branching within the *Planctomycetales*; not cultivated; Gram-reaction not applicable; coccus, approximately 1.5 µm in diameter; basis of assignment, 16S rDNA sequences (accession numbers AF202655 – AF202663) and oligonucleotide probe complementary to unique region of 16S rRNA S-*-Kst-1275-a-A-20 (5'-TCGGCTTTATAGGTTTCGCA-3'); free-living (anoxic biofilm); anaerobic ammonium oxidizer, mesophilic; SCHMID et al., this study.

#### Significance of classical "aerobic" ammonia-oxidizers in anoxic habitats

Several studies have demonstrated that "aerobic" ammonia oxidizers of the beta-subclass of Proteobacteria can survive extended periods of anaerobiosis (ABELIOVICH, 1987; BLACKBURN, 1983; JETTEN et al., 1999). In addition, a surprising metabolic versatility of different *Nitrosomonas* strains under oxygen limitation and/or anoxic conditions including anaerobic oxidation of ammonium with nitrite as electron acceptor has been reported (BOCK et al., 1995; DE BRUIJN et al., 1995; POTH, 1986; POTH and FOCHT, 1985, SCHMIDT and BOCK, 1997). Quantitative FISH showed that halophilic or halotolerant ammonia-oxidizers of the genus *Nitrosomonas* constitute about one fourth of the microbial biomass of the anoxic trickling filter biofilm. Comparative sequence analysis of biofilm derived *amoA* fragments indicated the presence of DNA originating from ammonia oxidizers closely related to *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis* within the biofilm. It should be noted that, due to the reamplification of *amoA* fragments after gel-retardation, two rounds of 30 PCR cycles were performed prior to cloning of *amoA* fragments. Thus Taq polymerase induced sequence errors might account for some of the differences in the deduced amino acid sequences between the cloned *amoA* fragments and those of the cultured ammonia oxidizers. While the detection of *amoA* fragments from *Nitrosomonas europaea* and *Nitrosomonas eutropha*-like bacteria is congruent with the FISH results, *Nitrosococcus mobilis* cells were not detectable *in situ*. Possible explanations of this discrepancy are that *Nitrosococcus mobilis*-like cells are not detectable by FISH since they (I) occur in low numbers below the FISH detection limit, (II) possess a low cellular ribosome content, (III) were subjected to a mutation in the NmV probe-target site, or (IV) were lysed within reactor 2 and their extracellular DNA served as template for *amoA* amplification. At present it is difficult to judge the significance of the relatively high *in situ* abundance of classical ammonia oxidizers in the anoxic biofilm for anaerobic ammonium oxidation. It appears to be most likely that aerobic ammonia oxidizers have been transferred via the sewage from the aerobic trickling filter 1 with high ammonia-oxidizing activity to the anoxic trickling filter 2. The high cellular ribosome content of these ammonia-oxidizers in trickling filter 2 observed by FISH is not necessarily an indicator for substantial physiological activity since beta-subclass ammonia-oxidizers maintain high rRNA concentrations per cell even under unfavorable conditions like starvation (MORGENROTH, in press; MORITA, 1993) or chemical inhibition (WAGNER et al., 1995). Consequently, the high ribosome content of trickling filter 2 beta-subclass ammonia-oxidizers could just reflect their previous aerobic activity in trickling filter 1. However, the relatively high numbers of the "aerobic" ammonia-oxidizers observed *in situ* in trickling filter 2 suggest that they might be able to grow under anoxic conditions or under transient periods of microaerophilic conditions (up to 0.08 mg $O_2$ $l^{-1}$ was detectable in trickling filter 2; Table 1). Nevertheless, classical ammonia-oxidizers constituted only approx. one half of the biomass of "*Candidatus* Kuenenia stuttgartiensis" within the trickling filter 2. Keeping in mind that beta-subclass ammonia-oxidizers analyzed to date possess (at least in pure culture) specific rates for anaerobic ammonium oxidation which are more than 20fold lower that the rate measured for the anaerobic ammonium-oxidizer affiliated with the *Planctomycetales* (JETTEN et al., 1999) we conclude that "*Candidatus* Kuenenia stuttgartiensis" is most likely responsi-ble for a large part of the anaerobic ammonium oxidation in trickling filter 2. Future studies will show whether members of the *Planctomycetales* are also responsible for the nitrogen loss without corresponding decrease of BOD which has been recently reported for two rotating biological contactor systems treating landfill leachate

(HELMER and KUNST, 1998; HELMER et al., 1999; SIEGRIST et al., 1998). In a more general perspective these results demonstrate that there is a principal possibility to actually integrate the process of anaerobic ammoniumoxidation in modern waste water treatment for inexpensive N-removal from waste water with high ammonium concentrations and low BOD. We just begin to elucidate the actual natural genetic diversity of bacteria capable to perform anaerobic ammonium-oxidation. How this diversity also reflects differences in physiology remains unresolved but will ultimately be important in both, our fundamental understanding of nitrogen cycling and the design of highly efficient waste water treatment plants.

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## References

ABELING, U., and SEYFRIED, C. F.: Anaerobic-aerobic treatment of high-strength ammonium wastewater - nitrogen removal via nitrite. Wat. Sci. Tech. 26(5-6), 1007-1015 (1992).

ABELIOVICH, A.: Nitrifying bacteria in waste water reservoirs. Appl. Environ. Microbiol. 53, 754-760 (1987).

ADACHI, J., and HASEGAWA, M.: Computer science monographs, no. 28.MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Institute of Statistics and Mathematics, Tokyo, Japan (1996).

ALM, E., OERTHER, D. B., LARSEN, N., STAHL, D. A., and RASKIN, L.: The oligonucleotide database. Appl. Environ. Microbiol. 62, 3557-3559 (1996).

AMANN, R.: *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p.1-15. *In* A.D.C. Akkermans, J.D. van Elsas, and F.J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands (1995).

AMANN, R. I., BINDER, B. J., OLSEN, R. J., CHISHOLM, S. W., DEVEREUX, R., and STAHL, D. A.: Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919-1925 (1990).

BLACKBURN, T. H.: The microbial nitrogen cycle. *In* W. E. KRUMBEIN (ed.), Microbial geochemistry. Blackwell Scientific Publications, Ltd., Oxford (1983).

BOCK, E., SCHMIDT, I., STÜVEN, R., and ZART, D.: Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. 163, 16-20 (1995).

BRODA, E.: Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. 17, 491-493 (1977).

BROSIUS, J., PALMER, M. L., KENNEDY, P. J., and NOLLER, H. F.: Complete sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 75, 4801-4805 (1978).

DAIMS, H., BRÜHL, A., AMANN, R., SCHLEIFER, K.-H., and WAGNER, M.: The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434-444 (1999).

DAIMS, H., NIELSEN, P. H., NIELSEN, J.L., JURETSCHKO, S., and WAGNER, M. Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: Diversity and *in situ* physiology. Wat. Sci. Technol., in press.

DE BRUIJN, P., VAN DE GRAAF, A. A., JETTEN, M. S. M., ROBERTSON, L. A., and KUENEN, J. G.: Growth of *Nitrosomonas europaea* on hydroxylamine. FEMS Microbiol. Lett. 125, 179-184 (1995).

DE LONG, E. F., FRANKS, D. G., and ALLDREDGE, A. L.: Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. 38(5), 924-934(1993).

FELSENSTEIN, J.:PHYLIP(Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle (1993).

FUERST, J. A.: The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology 141, 1493-1506 (1995).

FUERST, J. A., GWILLIAM, H. G., LINDSAY, M., LICHANSKA, A., BELCHER, C., VICKERS, J. E., and HUGENHOLTZ, P.: Isolation and molecular identification of Planctomycete bacteria from postlarvae of the Giant Tiger Prawn, *Penaeus monodon*. Appl. Environ. Microbiol. 63, 254-262 (1997).

HELLINGA C, SCHELLEN, A. A. J. C., MULDER, J. W., VAN LOOSDRECHT, M. C. M., and HEIJNEN, J. J.: The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. Wat. Sci. Tech. 37, 135-142 (1998).

HELMER, C., and KUNST, S.: Simultaneous nitrification/denitrification in an aerobic biofilm system. Water Sci. Technol. 37(4-5), 183-187 (1998).

HELMER, C., KUNST, S., JURETSCHKO, S., SCHMID, M. C., SCHLEIFER, K.-H., and WAGNER, M.: Nitrogen loss in a nitrifying biofilm system. Wat. Sci. Tech. 39(7), 13-21 (1999).

JETTEN, M. S. M., STROUS, M., VAN DE PAS-SCHOONEN, K. T., SCHALK, J., VAN DONGEN, U.G.J. M., VAN DE GRAAF, A., LOGEMANN, S., MUYZER, G., VAN LOOSDRECHT, M. C. M., and KUENEN, J. G.: The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22, 421-437 (1999).

JURETSCHKO, S., TIMMERMANN, G., SCHMID, M., SCHLEIFER, K.-H., POMMERENING-RÖSER, A., KOOPS, H.-P., and WAGNER, M.: Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042-3051 (1998).

KUAI, L., and VERSTRAETE, W.: Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system. Appl. Environ. Microbiol. 64, 4500-4506 (1998).

LIESACK, W., and STACKEBRANDT, E.: Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an australian terrestrial environment. J. Bacteriol. 174, 5072-5078 (1992).

LOGEMANN, S., SCHANTL, J., BIJVANK, S., VAN LOOSDRECHT, M., KUENEN, J. G., and JETTEN, M.: Molecular microbial diversi ty in a nitrifying reactor system without sludge retention. FEMS Microbiol. Ecol. 27, 239-249 (1998).

LUDWIG, W., STRUNK, O., KLUGBAUER, S., KLUGBAUER, N., WEIZEEGGER, M., NEUMAIER, J., BACHLEITNER, M., and SCHLEIFER, K.-H.: Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19, 554-568 (1998).

MAIDAK, B. L., OLSEN, G. J., LARSEN, N., OVERBECK, R., MC CAUGHEY, M. J., and WOESE, C. R.: The ribosomal database project (RDP). Nucleic Acid Res. 24, 82-85 (1996).

MANZ, W., AMANN, R., LUDWIG, W., WAGNER, M., and SCHLEIFER, K.-H.: Phylogenetic oligonucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. 15, 593-600 (1992).

MOBARRY, B. K., WAGNER, M., URBAIN, V., RITTMANN, B. E., and STAHL, D. A.: Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62, 2156-2162 (1996).

MORGENROTH, E., OBERMAYER, A., ARNOLD, E., BRÜHL, A., WAGNER, M., and WILDERER, P. A.: Effect of long term idle periods on the performance of sequencing batch reactors. Wat. Sci. Technol., in press.

MORITA, R. Y. (1993) Bioavailability of energy and starvation state. *In* S. Kjelleberg (ed.) Starvation in bacteria. Plenum Press, New York.

MURRAY, R.G.E., and SCHLEIFER, K.-H.: Taxonomic notes: A proposal for recording the properties of putative taxa of procaryotes. Int. J. System. Bacteriol. 44, 174-176 (1994).

NEEF, A., AMANN, R. I., SCHLESNER, H., and SCHLEIFER, K.-H.: Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144, 3257-3266 (1998).

POTH, M.: Dinitrogen gas production from nitrite by a *Nitrosomonas* isolate. Appl. Environ. Microbiol. 52, 957-959 (1986).

POTH, M., and FOCHT, D. D.: $^{15}N$ kinetic analysis of $N_2O$ production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl. Environ. Microbiol. 49, 1134-1141 (1985).

RAPPE, M.S., KEMP, P.F., AND GIOVANNONI, S.J.: Genbank direct submission. Accession number: U70712. (1996)

ROTTHAUWE, J. H., WITZEL, K.-P., and LIESACK, W.: The ammonia monooxygenase structural gene *amo*A as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704-4712 (1997).

SCHMIDT, I., and BOCK, E.: Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. 167, 106-111 (1997).

SIEGRIST, H., REITHAAR, S., and LAIS, P.: Nitrogen loss in a nitrifying rotating contractor treating ammonium rich leachate without organic carbon. Water Sci. Technol. 37(4-5), 589-591 (1998).

STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R. I., KUENEN, J. G., and JETTEN, M. S. M.: Missing lithotroph identified as new planctomycete. Nature. 400, 446-449 (1999).

STROUS, M., HEIJNEN, J. J., KUENEN, J. G., and JETTEN, M. S. M.: The sequencing batch reactor as a powerful tool to study very slowly growing micro-organisms. Appl. Microbiol. Biotechnol. 50, 589-596 (1998).

STROUS, M., VAN DE PAS-SCHOONEN, K.T., KRAMER, E. H. M., RAGHOEBARSING, A., MUYZER, G., WAGNER, M., KUENEN, J. G., and JETTEN, M. S. M.: New planctomycete responsible for the anaerobic ammonium oxidation (Anammox) process. Submitted to Environmental Microbiology

STRUNK, O., and LUDWIG W.: ARB software program package; http://www.biol.chemie.tu-muenchen.de/pup/ARB/. (1997).

VAN DE GRAAF, A. A., DE BRUIJN, P., ROBERTSON, L. A., JETTEN, M. S. M., and KUENEN, J. G.: Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor. Microbiology. 142, 2187-2196 (1996).

VAN DE GRAAF, A. A., DE BRUIJN, P., ROBERTSON, L. A., JETTEN, M. S. M., and KUENEN, J. G.: Metabolic pathway of anaerobic ammonium oxidation on the basis of N-15 studies in a fluidized bed reactor. Microbiology. 143, 2415-2421 (1997).

VAN DER MEER, J. R., VAN DER MEER, J., WERLEN, C., and SIEGRIST, H.: Genbank direct submission. Accession number: AJ224943. (1998).

WAGNER, M., AMANN, R., KÄMPFER, P., AßMUS, B., HARTMANN, A., HUTZLER, P., SPRINGER, N., and SCHLEIFER, K.-H.: (1994) Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17, 405-417.

WAGNER, M., RATH, G., AMANN, R., KOOPS, H.-P., and SCHLEIFER, K.-H.: *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol. 18, 251-264 (1995).

WAGNER, M., RATH, G., FLOOD, J., KOOPS, H.-P., and AMANN, R. *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water. Sci. Technol. 34(1-2), 237-244 (1996).

WAWER, C., RÜGGEBERG, H., MEYER, G., and MUYZER, G.: A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified DNA fragments. Nucleic Acid Res. 23, 4928-4929 (1995).

WOESE, C. R.: Bacterial evolution. Microbiol. Rev. 51, 221-271 (1987).

ZHENG, D., ALM, E. W., STAHL, D. A., and RASKIN, L.: Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62, 4504-4513 (1996).

ZHOU, J., BRUNS, M. A., and TIEDJE, J. M.: DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62, 316-322 (1996).

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# Appendix C

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.

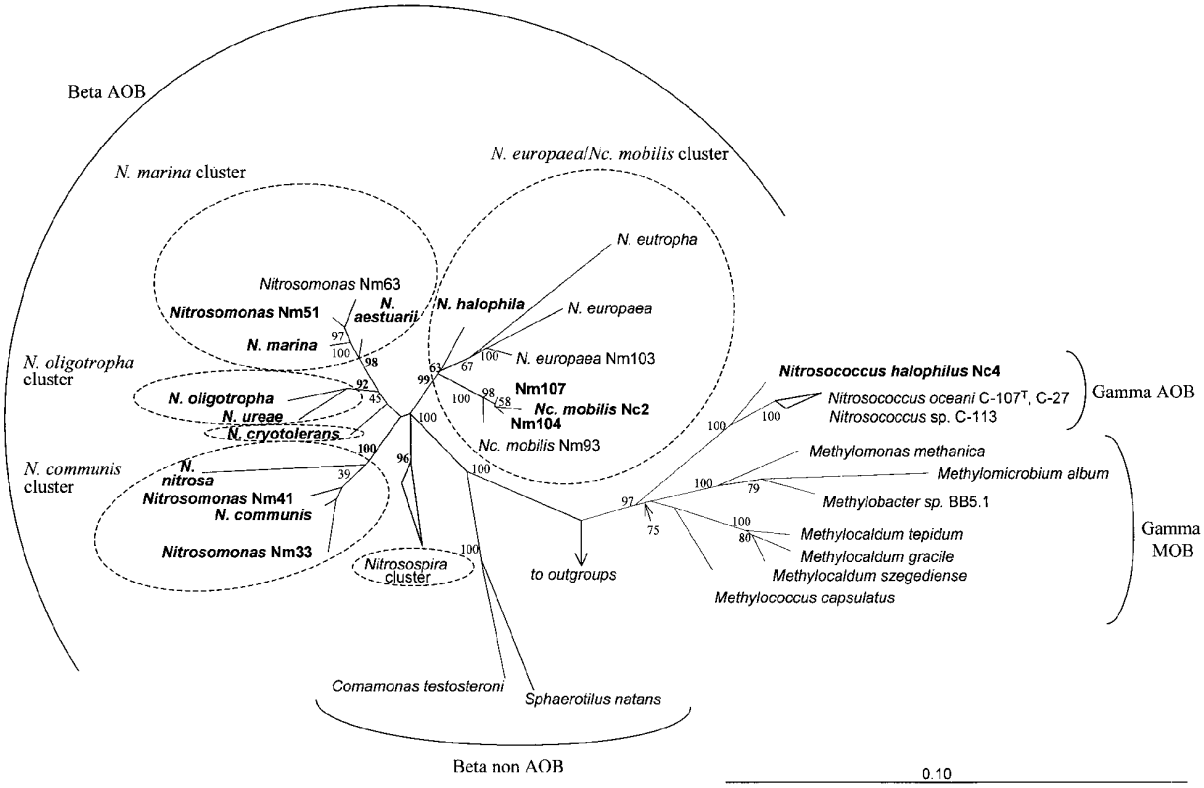


FIG. 1. Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms. The tree is based on results of neighbor-joining analysis using a 50% conservation filter for the *Bacteria*. An encompassing collection of organisms representing all major lineages of the *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*

| *Nitrosomonas cryotolerans* cluster Nm55 | % *amoA* (AmoA) sequence similarity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *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**) |

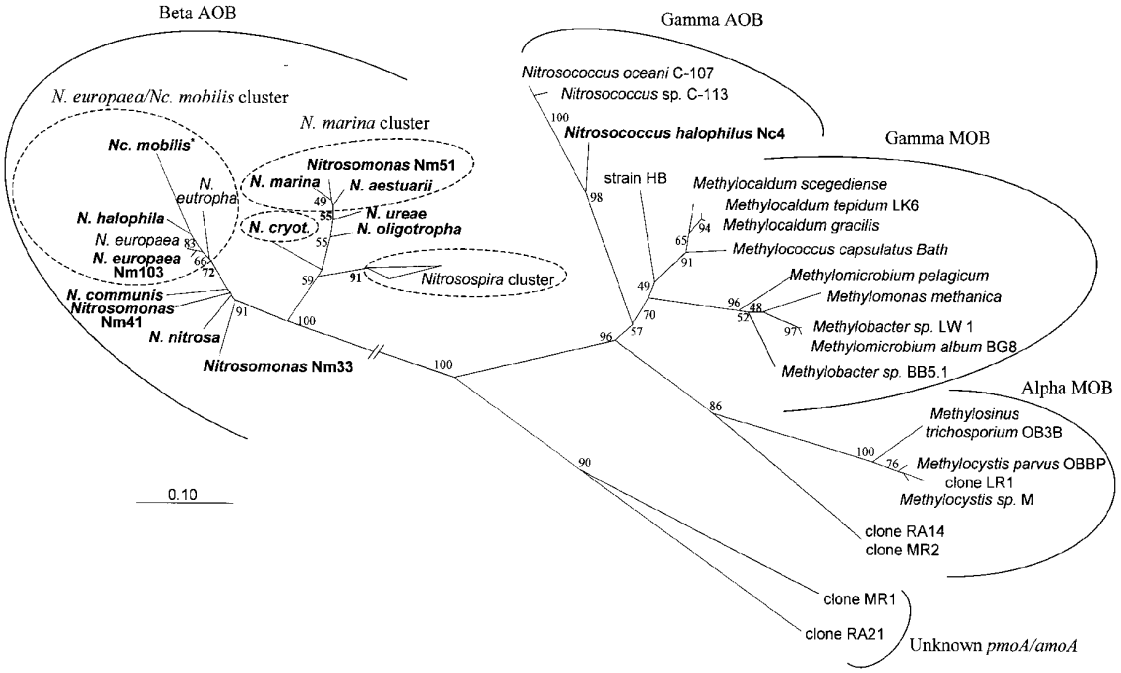


FIG. 2. Phylogenetic Fitch-Margoliash tree (using global rearrangement and randomized input order [7 jumbles]) reflecting the relationships of AOB and methane-oxidizing bacteria (MOB) based on deduced AmoA and PmoA sequences. Parsimony bootstrap values (100 replicates) for branches are reported. Missing 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

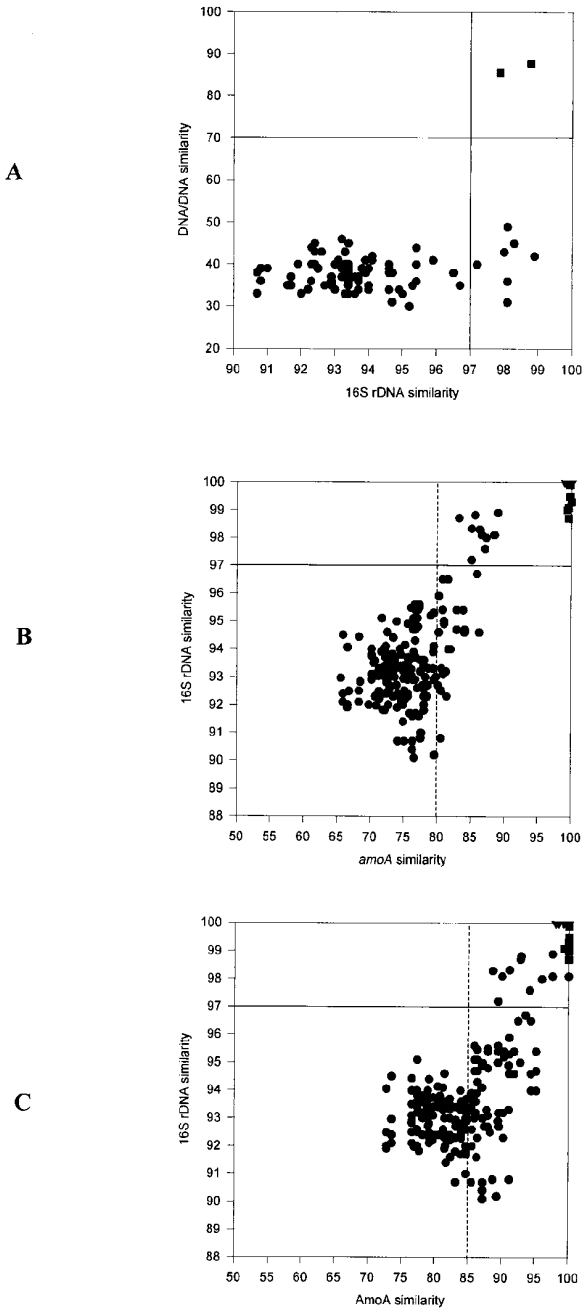


FIG. 3. Correlation plots of DNA-DNA reassociation, 16S rRNA similarity, and *amoA* and AmoA similarity values of AOB. (A) Comparison of 16S rRNA similarity and DNA-DNA similarity values. DNA-DNA hybridization data were obtained from studies by Juretschko et al. (28), Koops et al. (34), Koops et al. (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.

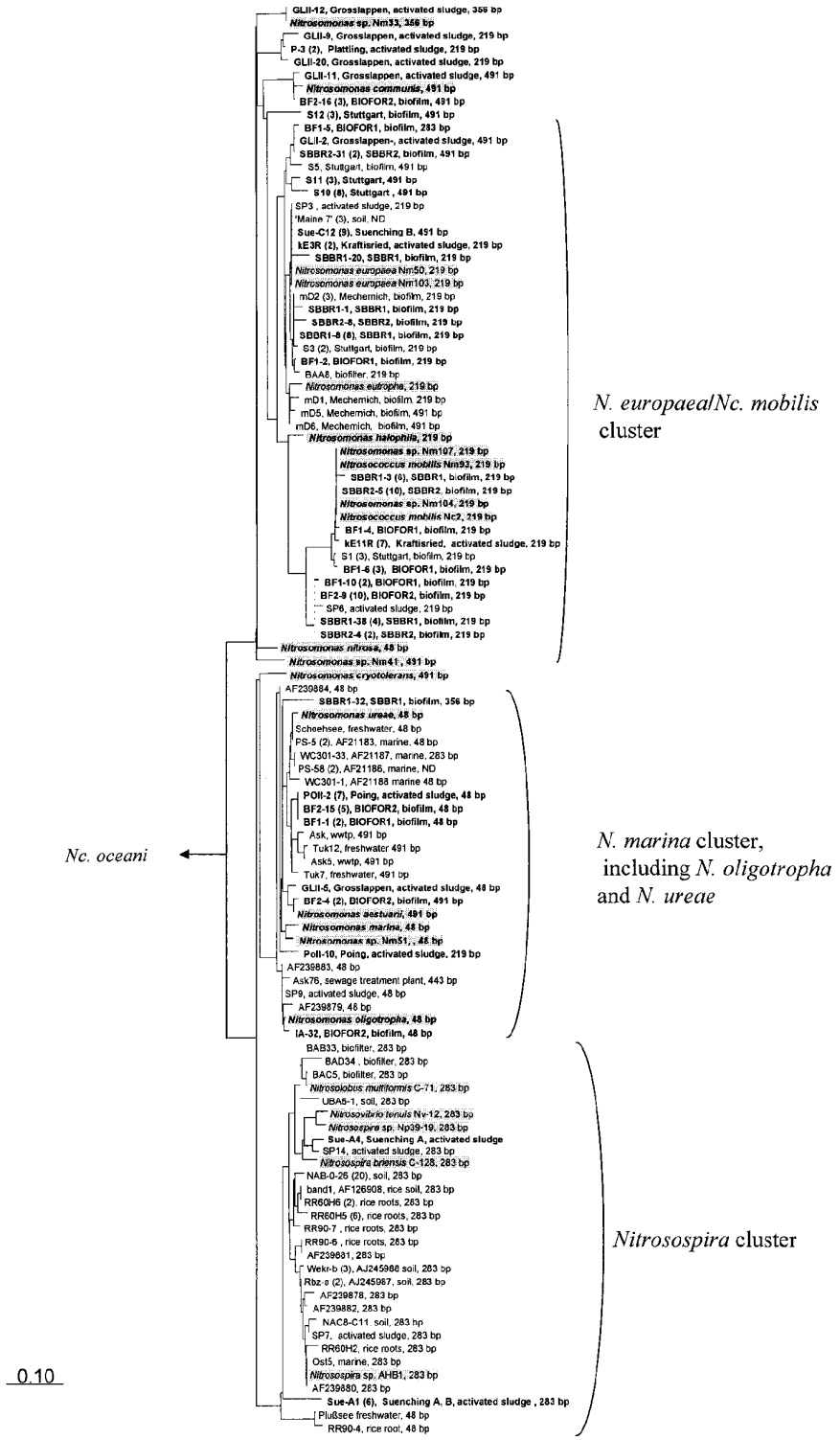


FIG. 4. Phylogenetic Fitch-Margoliash AmoA dendrogram (using global rearrangement and randomized input order [3 jumbles]) showing the positions of cultured ammonia oxidizers (shaded in gray) in relation to environmental sequences recovered from 11 wastewater treatment plants (bold [this study]) and other 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

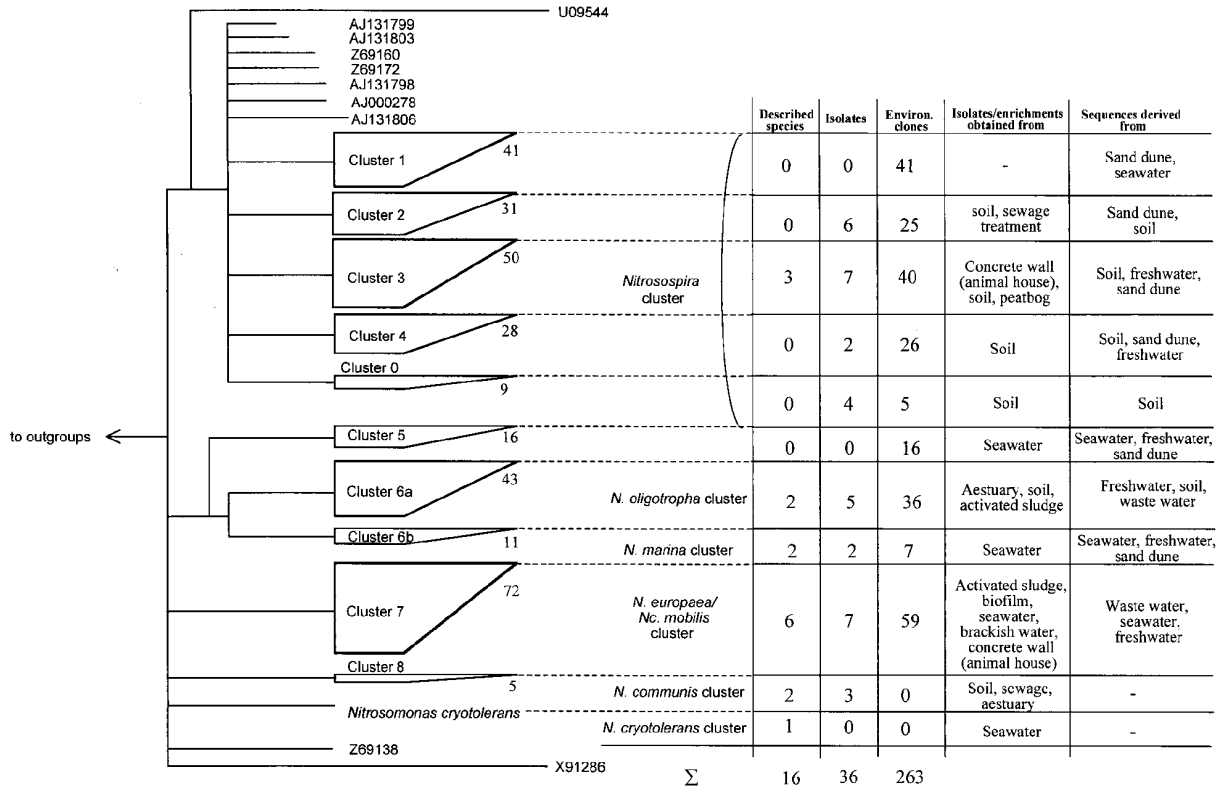


FIG. 5. Schematic 16S rRNA-based phylogenetic classification of the beta-subclass AOB. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The height of each tetragon represents the number of sequences in the cluster. 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 | | | *Nitrospira* 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] | Nsp1 | Np22 | AHB1 | L13 | Nv1 | Nv12 | C128 | C71 | Nc2 | Nm93 | Nm104 | Nm107 | Nm1 | Nm50 | Nm103 | Nm57[b] | Nm55 | 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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## REFERENCES

1. **Aakra, Å., J. B. Utaker, and I. F. Nes.** 1999. RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int. J. Syst. Bacteriol. **49:**123–130.
2. **Aakra, Å., J. B. Utaker, I. F. Nes, and L. R. Bakken.** 1999. An evaluated improvement of the extinction dilution method for isolation of ammonia-oxidizing bacteria. J. Microbiol. Methods **39:**23–31.
3. **Alm, E., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The Oligonucleotide Probe Database. Appl. Environ. Microbiol. **62:**3557–3559.
4. **Alzerreca, J. J., J. M. Norton, and M. G. Klotz.** 1999. The *amo* operon in marine, ammonia-oxidizing gamma-proteobacteria. FEMS Microbiol. Lett. **180:**21–29.
5. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.

6. **Arthur, J. W., C. W. West, K. N. Allen, and S. F. Hedke.** 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bull. Environ. Contam. Toxicol. **38:**324–331.
7. **Bano, N., and J. T. Hollibaugh.** 2000. Diversity and distribution of DNA sequences with affinity to ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in the Arctic Ocean. Appl. Environ. Microbiol. **66:**1960–1969.
8. **Bock, E., R. Atueven, R. Mansch, and M. Vollmer.** 1994. Formation and consumption of nitric oxide by nitrifying bacteria, p. 241–244. *In* L. Alberghina, L. Frontali, and P. Sensi (ed.), ECB6: Proceedings of the 6th European Congress on Biotechnology. Elsevier Science B. V., Amsterdam, The Netherlands.
9. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
10. **Chandler, D. P., J. K. Fredrickson, and F. J. Brockman.** 1997. Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. Mol. Ecol. **6:**475–482.
11. **Chandler, D. P., R. W. Schreckhise, J. L. Smith, and H. Bolton Jr.** 1997. Electroelution to remove humic compounds from soil DNA and RNA extracts. J. Microbiol. Methods **28:**11–19.
12. **Farrelly, V., F. A. Rainey, and E. Stackebrandt.** 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. **61:**2798–2801.
13. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr.** 1992. How close is close: 16S rDNA identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
14. **Frankland, P. F., and G. C. Frankland.** 1890. The nitrifying process and its specific ferment. Part I. Phil. Trans. R. Soc. B **181:**107–128.
15. **Hall, G. H.** 1986. Nitrification in lakes, p. 127–156. *In* J. I. Prosser (ed.), Nitrification, vol. 20. IRL Press, Oxford, United Kingdom.
16. **Hastings, R. C., M. T. Ceccherini, N. Miclaus, J. R. Saunders, M. Bazzicalupo, and A. J. McCarthy.** 1997. Direct molecular biological analysis of ammonia oxidizing bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol. Ecol. **23:**45–54.
17. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **139:**1147–1153.
18. **Helmer, C., S. Kunst, S. Juretschko, M. C. Schmid, K.-H. Schleifer, and M. Wagner.** 1999. Nitrogen loss in a nitrifying biofilm system. Water Sci. Technol. **39:**13–21.
19. **Henckel, T., M. Friedrich, and R. Conrad.** 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol. **65:**1980–1990.
20. **Henckel, T., U. Jackel, S. Schnell, and R. Conrad.** 2000. Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane. Appl. Environ. Microbiol. **66:**1801–1808.
21. **Hiorns, W. D., R. C. Hastings, I. M. Head, A. J. McCarthy, J. R. Saunders, R. W. Pickup, and G. H. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidising bacteria. Microbiology **141:**2793–2800.
22. **Holmes, A. J., A. Costello, M. E. Lidstrom, and J. C. Murrell.** 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. **132:**203–208.
23. **Holmes, A. J., P. Roslev, I. R. McDonald, N. Iversen, K. Henriksen, and J. C. Murrell.** 1999. Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. Appl. Environ. Microbiol. **65:**3312–3318.
24. **Horz, H. P., J. H. Rotthauwe, T. Lukow, and W. Liesack.** 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. J. Microbiol. Methods **39:**197–204.
25. **Hovanec, T. A., and E. F. DeLong.** 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl. Environ. Microbiol. **62:**2888–2896.
26. **Imhoff, K., and K. R. Imhoff.** 1985. Taschenbuch der Stadtentwässerung, 26th ed. Oldenbourg Verlag, Munich, Germany.
27. **Jones, R. D., R. Y. Morita, H. P. Koops, and S. W. Watson.** 1988. A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. Can. J. Microbiol. **34:**1122–1128.
28. **Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Röser, H. P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64:**3042–3051.
29. **Klotz, M. G., and J. M. Norton.** 1995. Sequence of an ammonia monooxygenase subunit A encoding gene from *Nitrosospira* sp. NpAV. Gene **163:**159–160.
30. **Koops, H.-P., B. Böttcher, U. C. Möller, A. Pommerening-Röser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. and *Nitrosomonas halophila* sp. nov. J. Gen. Microbiol. **137:**1689–1699.
31. **Koops, H.-P., and H. Harms.** 1985. Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch. Microbiol. **141:**214–218.
32. **Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov. sp. Arch. Microbiol. **107:**277–282.
33. **Koops, H.-P., and U. C. Möller.** 1992. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2637. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The *Prokaryotes*, 2nd ed., vol. 3. Springer-Verlag, New York, N.Y.
34. **Koops, H.-P., B. Böttcher, U. C. Möller, A. Pommerening-Röser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. **154:**244–248.
35. **Kowalchuk, G. A., P. L. E. Bodelier, G. H. J. Heilig, J. R. Stephen, and H. J. Laanbroek.** 1998. Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. **27:**339–350.
36. **Kowalchuk, G. A., Z. S. Naoumenko, P. J. Derikx, A. Felske, J. R. Stephen, and I. A. Arkhipchenko.** 1999. Molecular analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in compost and composted materials. Appl. Environ. Microbiol. **65:**396–403.
37. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **63:**1489–1497.
38. **Kowalchuk, G. A., A. W. Stienstra, G. H. Heilig, J. R. Stephen, and J. W. Woldendorp.** 2000. Molecular analysis of ammonia-oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiol. Ecol. **31:**207–215.
39. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21:**3021–3023.
40. **Logemann, S., J. Schantl, S. Bijvank, M. v. Loosdrecht, J. G. Kuenen, and M. Jetten.** 1998. Molecular microbial diversity in a nitrifying reactor system without sludge retention. FEMS Microbiol. Ecol. **27:**239–249.
41. **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner, and K.-H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554–568.
42. **MacDonald, R. M.** 1986. Nitrification in soil: an introductory history, p. 1–16. *In* J. I. Prosser (ed.), Nitrification, vol. 20. IRL Press, Oxford, United Kingdom.
43. **McCaig, A. E., T. M. Embley, and J. I. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol. Lett. **120:**363–368.
44. **McCaig, A. E., C. J. Phillips, J. R. Stephen, G. A. Kowalchuk, S. M. Harvey, R. A. Herbert, T. M. Embley, and J. I. Prosser.** 1999. Nitrogen cycling and community structure of proteobacterial beta-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. Appl. Environ. Microbiol. **65:**213–220.
45. **McTavish, H., J. A. Fuchs, and A. B. Hooper.** 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436–2444.
46. **Meincke, M., E. Krieg, and E. Bock.** 1989. *Nitrosovibrio* spp., the dominant ammonia-oxidizing bacteria in building sandstone. Appl. Environ. Microbiol. **55:**2108–2110.
47. **Mendum, T. A., R. E. Sockett, and P. R. Hirsch.** 1999. Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the beta subdivision of the class *Proteobacteria* in arable soils to nitrogen fertilizer. Appl. Environ. Microbiol. **65:**4155–4162.
48. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162. (Erratum, **63:**815, 1997.)
49. **Painter, H. A.** 1986. Nitrification in the treatment of sewage and wastewaters, p. 185–211. *In* J. I. Prosser (ed.), Nitrification, vol. 20. IRL Press, Oxford, United Kingdom.
50. **Phillips, C. J., Z. Smith, T. M. Embley, and J. Prosser.** 1999. Phylogenetic differences between particle-associated and planctonic ammonia-oxidizing bacteria of the β-subdivision of the class *Proteobacteria* in the northwestern Mediterranean Sea. Appl. Environ. Microbiol. **65:**779–786.
51. **Polz, M. F., and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:**3724–3730.
52. **Pommerening-Röser, A., G. Rath, and H.-P. Koops.** 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Syst. Appl. Microbiol. **19:**344–351.
53. **Princic, A., I. Mahne, F. Megusar, E. A. Paul, and J. M. Tiedje.** 1998. Effects of pH and oxygen and ammonium concentrations on the community struc-

ture of nitrifying bacteria from wastewater. Appl. Environ. Microbiol. **64:** 3584–3590.
54. **Reysenbach, A. L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:**3417–3418.
55. **Rotthauwe, J. H., W. de Boer, and W. Liesack.** 1995. Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira* sp. AHB1 and *Nitrosolobus multiformis* C-71. FEMS Microbiol. Lett. **133:**131–135.
56. **Rotthauwe, J. H., K. P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704–4712.
57. **Sakano, Y., and L. Kerkhof.** 1998. Assessment of changes in microbial community structure during operation of an ammonia biofilter with molecular tools. Appl. Environ. Microbiol. **64:**4877–4882.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **7:**5463–5467.
60. **Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K.-H. Schleifer, and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:**93–106.
61. **Schramm, A., D. De Beer, M. Wagner, and R. Amann.** 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. **64:** 3480–3485.
62. **Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K. H. Schleifer.** 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **62:**4641–4647.
63. **Schramm, A., C. M. Santegoeds, H. K. Nielsen, H. Ploug, M. Wagner, M. Pribyl, J. Wanner, R. Amann, and D. de Beer.** 1999. On the occurrence of anoxic microniches, denitrification, and sulfate reduction in aerated activated sludge. Appl. Environ. Microbiol. **65:**4189–4196.
64. **Sinigalliano, C. D., D. Kuhn, and R. D. Jones.** 1995. Amplification of the *amoA* gene from diverse species of ammonium-oxidizing bacteria from an indigenous bacterial population from seawater. Appl. Environ. Microbiol. **61:**2702–2706.
65. **Speksnijder, A. G., G. A. Kowalchuk, K. Roest, and H. J. Laanbroek.** 1998. Recovery of a Nitrosomonas-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. **21:**321–330.
66. **Stackebrandt, E., and M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Bacteriol. **44:**846–849.
67. **Stehr, G. B., B. Böttcher, P. Dittberner, G. Rath, and H. P. Koops.** 1995. The ammonia oxidizing nitrifiying population of the River Elbe estuary. FEMS Microbiol. Ecol. **17:**177–187.
68. **Stephen, J. R., Y. J. Chang, S. J. Macnaughton, G. A. Kowalchuk, K. T. Leung, C. A. Flemming, and D. C. White.** 1999. Effect of toxic metals on indigenous soil beta-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. **65:**95–101.
69. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:** 4147–4154.
70. **Suwa, Y., T. Sumino, and K. Noto.** 1997. Phylogentic relationships of activated sludge isolates of ammonia-oxidizers with different sensitivities to ammonium sulfate. J. Gen. Appl. Microbiol. **43:**373–379.
71. **Suzuki, M., M. S. Rappe, and S. J. Giovannoni.** 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl. Environ. Microbiol. **64:**4522–4529.
72. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
73. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittman, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
74. **Tortoso, A. C., and G. L. Hutchinson.** 1990. Contributions of autotrophic and heterotrophic nitrifiers to soil NO and $N_2O$ emission. Appl. Environ. Microbiol. **56:**1799–1805.
75. **Trüper, H. G., and L. de Clari.** 1997. Taxonomic note: necessary correction of specific epithets formed as substantives (nouns) "in apposition." Int. J. Syst. Bacteriol. **47:**908–909.
76. **Utåker, J. B., L. Bakken, Q. Jiang, and J. Nes.** 1995. Phylogenetic analysis of seven new isolates of ammonia oxidizing bacteria based on 16S rRNA gene sequences. Syst. Appl. Microbiol. **18:**549–559.
77. **Utåker, J. B., and I. F. Nes.** 1998. A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **21:**72–88.
78. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450. (Erratum, **61:**2811.)
79. **Wagner, M., R. Amann, H. Lemmer, and K. H. Schleifer.** 1993. Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
80. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K. H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **18:** 251–264.
81. **Wagner, M., G. Rath, H.-P. Koops, J. Flood, and R. Amann.** 1996. *In situ* analysis of nitrifiying bacteria in sewage treatment plants. Water Sci. Technol. **34:**237–244.
82. **Ward, B. B.** 1996. Nitrification and denitrification: probing the nitrogen cycle in aquatic environments. Microb. Ecol. **32:**247–261.
83. **Ward, B. B., M. A. Voytek, and K. F. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification. Microb. Ecol. **33:**87–96.
84. **Watson, S. W.** 1965. Characteristics of a marine nitrifying bacterium, *Nitrosocystis oceanus* sp. n. Limnol. Oceanogr. **10**(Suppl.)**:**R274–R289.
85. **Watson, S. W., and M. Mandel.** 1971. Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. J. Bacteriol. **107:**563–569.
86. **Watson, S. W., F. W. Valois, and J. B. Waterbury.** 1981. The family *Nitrobacteraceae*, p. 1005–1022. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The *Prokaryotes*, vol. 1. Springer Verlag, Berlin, Germany.
87. **Whitby, C. B., J. R. Saunders, J. Rodriguez, R. W. Pickup, and A. McCarthy.** 1999. Phylogenetic differentiation of two closely related *Nitrosomonas* spp. that inhabit different sediment environments in an oligotrophic freshwater lake. Appl. Environ. Microbiol. **65:**4855–4862.
88. **Winogradsky, S.** 1890. 2nd memoire. Recherches sur les organismes de la nitrification. Ann. Inst. Pasteur. **4:**257–275.
89. **Winogradsky, S.** 1892. Contribution a la morphologie des organismes de la nitrification. Arch. Sci. Biol. **1:**88–137.
90. **Wintzigerode, F., and U. B. Goebel.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
91. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of purple bacteria: the gamma subdivision. Syst. Appl. Microbiol. **6:**25–33.
92. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of purple bacteria: the beta subdivision. Syst. Appl. Microbiol. **5:**327–336.

# Appendix D

Isolation and properties of obligatly chemolithoautotrophic and extremely alkali-tolerant ammonia-oxidizing bacteria from Mongolian soda lakes

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# Isolation and properties of obligately chemolithoautotrophic and extremely alkali-tolerant ammonia-oxidizing bacteria from Mongolian soda lakes

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**Abstract** Five mixed samples prepared from the surface sediments of 20 north-east Mongolian soda lakes with total salt contents from 5 to 360 g/l and pH values from 9.7 to 10.5 were used to enrich for alkaliphilic ammonia-oxidizing bacteria. Successful enrichments at pH 10 were achieved on carbonate mineral medium containing 0.6 M total $Na^+$ and ≤4 mM $NH_4Cl$. Five isolates (ANs1–ANs5) of ammonia-oxidizing bacteria capable of growth at pH 10 were obtained from the colonies developed on bilayered gradient plates. The cells were motile and coccoid, with well-developed intracytoplasmic membranes (ICPM) and carboxysomes. At pH 10.0, ammonia was toxic for growth at concentrations higher than 5 mM $NH_4Cl$. The bacteria were able to grow within the salinity range of 0.1–1.0 M of total $Na^+$ (optimum 0.3 M). In media containing 0.3–0.6 M total $Na^+$, optimal growth in batch cultures occurred in the presence of a bicarbonate/carbonate buffer system within the pH range 8.5–9.5, with the highest pH limit at pH 10.5. At pH lower than 8.0, growth was slower, most probably due to decreasing free ammonia. The pH profile of the respiratory activity was broader, with limits at 6.5–7.0 and 11.0 and an optimum at 9.5–10.0. In pH-controlled, $NH_3$-limited continuous culture, isolate ANs5 grew up to pH 11.3, which is the highest pH limit known for ammonia-oxidizing bacteria so far. This showed the existence of extremely alkali-tolerant ammonia-oxidizing bacteria in the soda lakes. Comparative 16S rDNA sequence analysis of the five isolates demonstrated that they possess identical 16S rDNA genes and that they are closely related to *Nitrosomonas halophila* (sequence similarity 99.3%), a member of the β-subclass of the Proteobacteria. This affiliation was confirmed by comparative sequence analysis of the *amoA* gene, encoding the active-site subunit of the ammonia-monoxygenase, of one of the isolates. DNA-DNA hybridization data further supported that the soda lake isolates are very similar to each other and represent an alkali-tolerant subpopulation of *N. halophila* whose species description is herewith amended.

**Keywords** Alkalitolerant · Ammonia-oxidizing bacteria · Nitrification · Soda lakes · *Nitrosomonas*

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## Introduction

Autotrophic nitrifying bacteria that are able to obtain energy from the oxidation of ammonia and nitrite have been known for more than a century. They were one of the first discovered chemolithoautotrophs (Winogradsky 1890) and have been subject of detailed studies (Laanbroek and Woldendorp 1995). Because of the relatively high specific substrate-conversion rates, they play a key role in nitrogen cycling in many different environments, mostly in aquatic environments, (especially in sediments) and soils (Abeliovich 1987; Laanbroek and Woldendorp 1995). They are also an extremely important component of wastewater treatment systems (Jetten et al. 1997; Juretschko et al. 1998; Purkhold et al. 2000; Wagner et al. 1995, 1996).

Most of the ammonia oxidizers isolated in pure culture grow well at moderate temperature, within a relatively

narrow pH range from neutral to slightly alkaline, and at low to moderate salt concentrations (Koops and Möller 1992). One of the reasons for such restrictions may be the relatively low energy efficiency of nitrification reactions which might not allow these bacteria to survive energetically expensive extreme conditions (Oren 1999). Therefore the ability of nitrifying bacteria to grow under extreme conditions should be limited. In particular, almost nothing is known about ammonia-oxidizing bacteria able to grow under extremely alkaline conditions.

Soda lakes represent a rare example of a natural environment where two extreme factors are combined (high salinity and high pH) due to the presence of alkaline salts at high concentrations, in particular sodium carbonates. Surprisingly, such lakes may be very productive because of the bloom of alkaliphilic nitrogen-fixing cyanobacteria (Melack 1981; Cloern et al. 1983) that facilitate an active nitrogen cycle. Substantial evidence for the presence of an autotrophic ammonia-oxidation process in one specific layer, containing ammonia and small amounts of oxygen, of the stratified alkaline and saline Mono Lake in California has recently been obtained by using specific inhibitors (Joye et al. 1999) and by molecular probing (Ward et al. 2000). The latter investigations indicated members of the *Nitrosomonas europaea* lineage to be present in this environment; however, these results might have been biased due to the limited coverage of the specific primers used for PCR amplification (Purkhold et al. 2000). Furthermore, these studies were not substantiated by the isolation of pure cultures of the responsible bacteria.

In our preliminary investigation on the presence of nitrifying bacteria in soda lakes and soda soils in the southeast Siberian dry steppe and in the Kenyan Rift Valley, some evidence was obtained that both ammonia- and nitrite-oxidizing bacteria are active in these environments (Sorokin 1998). However, only nitrite-oxidizing enrichments from several samples at pH 10 were successful, resulting in the isolation and description of a new alkaliphilic *Nitrobacter* species (Sorokin et al. 1998). The possibility of ammonia oxidation to nitrite at pH ≥10 has been demonstrated so far only in pure culture of *Methylomicrobium* sp., an alkaliphilic methanotrophic bacterium isolated from Kenyan soda lake sediments (Sorokin et al. 2000a).

Here we describe the isolation and properties of extremely alkali-tolerant, lithoautotrophic, ammonia-oxidizing bacteria from Mongolian soda lake sediments that are capable of growth at extremely high pH values.

## Materials and methods

### Samples

Surface sediments from 20 soda lakes in the north-east dry steppe of Mongolia (Choibolsan Province), obtained in September 1999, were used to enrich for alkaliphilic ammonia-oxidizing bacteria. Samples were combined into five groups according to the salt content (Table 1) of the lakes from which the samples were obtained.

**Table 1** Characteristics of the composite samples (sediments/water 1:1) from Mongolian soda lakes (September, 1999) used for the enrichment of alkaliphilic ammonia-oxidizing bacteria

| Isolated bacterium | Number of subsamples | Composite soda lake sediment samples | |
|---|---|---|---|
| | | pH | Total g salts $l^{-1}$ |
| ANs5 | 5 | 9.5–10.5 | 5– 10 |
| ANs1, ANs2 | 7 | 9.4–10.0 | 15– 30 |
| ANs4 | 6 | 9.5–10.2 | 40– 65 |
| None | 4 | 10 –10.2 | 200–220 |
| ANs3 | 2 | 9.8–10.0 | 360–390 |

### Bacterial cultures

Pure cultures of ammonia-oxidizing bacteria of the genus *Nitrosomonas* were used as a reference to carry out comparative studies with the new isolates from the soda lakes. These strains were obtained from the culture collection of the University of Hamburg.

### Culture conditions and media composition

Ammonia-oxidizing bacteria from soda lakes were enriched and routinely cultivated in basal alkaline salt medium containing 0.6 M $Na^+$ [$NaHCO_3$-$Na_2CO_3$ buffer (0.5 M $Na^+$), NaCl (0.1 M) and $K_2HPO_4$ (5 mM)]. After sterilization the pH of this mineral medium was 10.05–10.1 and sufficiently stable during aerobic cultivation: in sterile controls the pH did not change, and in cultures the maximal pH drop did not exceed 0.2 pH units. After sterilization 1 ml $l^{-1}$ of trace element solution (Pfennig and Lippert 1966) and 1 mM $MgSO_4 \cdot 7H_2O$ were added. $NH_4Cl$ was supplied in a fed-batch manner from the 4 M stock solution so that its concentration did not exceed 4 mM. Batch cultivation was performed in serum flasks of 30–1000 ml or in 20-l bottles closed with rubber septa (to prevent ammonia loss) with 1:10 liquid to air ratio. Bottles were incubated at 30 °C with gentle agitation (50–100 rpm). To investigate the influence of pH on growth and respiration activity, 0.1 M HEPES-NaCl buffer for a pH range of 7.0–8.0 and $NaHCO_3$-$Na_2CO_3$ buffer for a higher pH range were used with the same total $Na^+$ content. These allowed prolonged aerobic cultivation at stable pH conditions up to pH 10.3. Short-term activity tests with washed cells were possible up to the highest pH created with sodium carbonate system (11.5).

To study the possibility of growth at pH>10.3, a pH-controlled continuous cultivation was carried out using a fermentor (Applikon, Schiedam, The Netherlands) with a 2 l working volume. The set-point pH was maintained within ±0.05 units by automatic titration with 2 M NaOH. The 2-component medium (acidic and basic components separately) contained 0.25 M total $Na_2CO_3$ and 0.05 M NaCl. Influent $NH_4Cl$ concentration was 22 mM. Dissolved oxygen concentration and temperature were controlled at 50% air saturation and at 30 °C.

Colonies of ammonia-oxidizing bacteria from soda lakes were obtained on solid medium with an ammonia gradient. The vertical gradient was created as follows: the bottom layer contained 4% agar and 10 mM $NH_4Cl$. This layer was covered with a mineral alkaline agar (2% final agar concentration) of pH 10, prepared by 1:1 mixing of the carbonate mineral base medium and 4% agar at 50 °C. The plates were allowed to equilibrate for 24 h before use.

The potential for heterotrophic growth of the isolates was tested in 0.3 M $Na^+$ mineral base medium at pH 10 using nitrate as nitrogen source. Carbon was supplied as 0.5 g yeast extract $l^{-1}$ or 5 mM pyruvate or 5 mM acetate.

### Activity measurements

The oxidation kinetics of $NH_3$ and $NH_2OH$ was assayed either directly with a chemostat culture or with washed cells using the

buffer systems mentioned above. Oxygen consumption rates were monitored with a Clark dissolved-oxygen electrode (Yellow Spring Instruments, Ohio, USA) in a thermostated chamber with a magnetic stirrer (total volume of 5 ml) at 30 °C. Cells were collected by centrifugation, washed with sodium carbonate buffer and resuspended in appropriate buffers at a protein concentration of 10 mg $ml^{-1}$. For measurements the cells were diluted 100-fold in buffer.

### Electron microscopy

Cells were collected from batch or chemostat cultures by centrifugation, washed and resuspended in 0.5 M NaCl, and fixed with glutaraldehyde (3% final). After removal of glutaraldehyde, preparations were post-fixed with $OsO_4$ (1% final) containing 0.5 M NaCl, dehydrated and embedded into resin. Thin sections were double stained with lead citrate and uranyl acetate. Cells for total preparations were stained with 1% uranyl acetate after fixation with glutaraldehyde.

### Biochemical analysis

Total cell proteins of soda lake isolates and neutrophilic *Nitrosomonas* species were compared by SDS-PAGE under denaturing conditions. The cells were sonicated, and the resulting total protein extracts were denatured in sample buffer by boiling for 5 min. Acrylamide (10%) gels were used to separate the proteins. Nitrite, ammonium, and nitrate in the supernatants were measured colorimetrically (Sorokin et al. 1998). The biomass protein was determined by the method of Lowry (1951).

### DNA extraction

Cell pellets of the isolates ANs1–ANs5 were suspended in 280 µl TE-buffer [10 mM Tris-HCl (pH 7.4), 10 mM sodium EDTA (pH 7.4)], 40 µl SDS and 80 µl 5 M NaCl. For cell lysis, the mixture was incubated for 1 h at 65 °C. After addition of 400 µl phenol-chloroform-isoamyl alcohol (25:24:1) and 10 s vortexing the mixture was centrifuged for 10 min at 10,000×*g*. The aqueous phase was carefully transferred to a fresh tube, mixed with one volume of chloroform-isoamyl alcohol by vortexing and again centrifuged for 10 min at 10,000×*g*. Subsequently, the aqueous phase was transferred to a fresh tube, and the DNA was allowed to precipitate by incubation with 0.6 volumes of isopropanol for 1 h at room temperature. After centrifugation for 20 min at 10,000×*g* the pellet was washed with 1 ml of 70% ethanol, dried and finally resuspended in 50 µl double-distilled $H_2O$.

### PCR amplification, sequencing and phylogeny of 16S rDNA and *amo*A

To obtain almost full-length 16S rDNA PCR products, the DNA of the isolates ANs1–ANs5 was amplified with the forward primer 616V (*Escherichia coli* positions 8–27; 5′-AGAGTTTGATYMTGGCTCAG-3′) and the reverse primer 630R (*E. coli* positions 1529–1545: 5′-CAKAAAGGAGGTGATCC-3′). PCR was performed in a 96-microwell plate with a gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 3 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 50 s, and elongation at 72 °C for 3 min. Cycling was completed by a final elongation step at 72 °C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of 5-µl aliquots of the PCR products. The pure culture-derived 16S rDNA PCR products were sequenced directly using a previously published procedure (Purkhold et al. 2000).

The *amoA* gene fragment of isolate ANs1 was amplified, cloned and sequenced as described in Purkhold et al. (2000).

Phylogenetic analyses of the retrieved 16S rDNA and *amoA* sequences were performed by use of the ARB program package (Felsenstein 1993; Maidak et al. 1996; Strunk and Ludwig 1997) as described in Purkhold et al. (2000).

The isolation of complete genomic DNA and subsequent DNA-DNA hybridization were carried out according to Marmur (1961) and De Ley et al. (1970), respectively.

### Nucleotide sequence accession numbers

The 16S rDNA sequences of isolates ANs1–5 obtained in this study are available in GenBank under accession no. AY026313-AY026317. The *amoA* sequence of isolate ANs1 received the GenBank accession no. AY026907

## Results

### Enrichment and isolation of pure cultures

Alkaliphilic $NH_3$-oxidizing bacteria were enriched with soda-based mineral medium at pH 10.05 containing 0.6–2 M total $Na^+$ using five combined sediment samples from Mongolian soda lakes (see Table 1). Positive results (nitrite production concomitant with ammonia consumption) were obtained in 2–4 weeks with all five samples on medium containing 0.6 M total $Na^+$ and with a $NH_4Cl$ concentration less than 4 mM. The most rapid nitrite formation was observed with sample group 2 from the lakes containing 15–30 g salts $l^{-1}$. Stable development of the nitrifying population was achieved using a fed-batch mode of $NH_4Cl$ supply (2 mM doses). But even after three to four repeated 100-fold dilutions of the enrichments, heterotrophic bacteria outnumbered the nitrifiers in subsequent dilution series. Pure cultures were successfully purified and isolated by using double-layered solid medium with an ammonia gradient. The plates were incubated in closed jars under an atmosphere containing 99% argon and 1% $O_2$. A total of five isolates (ANs1–5) of obligately autotrophic ammonia-oxidizing bacteria were obtained (see Table 1). The isolates did not show any potential for heterotrophic growth. Addition of a small amount of organic compounds even inhibited ammonia oxidation.

### Morphology

Cells of the isolates grown at pH 10 were mostly coccoid and 1–2.5 µm in diameter. Some of the cells were motile by a tuft of flagella (Fig. 1a). In old cultures most of the cells became refractile under the light microscope. The cell ultrastructure of isolates ANs1 and ANs5 was typical for ammonia-oxidizing bacteria of the genus *Nitrosomonas.* The cells contained extensive multiple layers of intracytoplasmic membranes and carboxysome-like bodies (Fig. 1b). Electron-transparent granules of storage material could be seen in some of the cells. It was not clear whether or not the cells of the new isolates contained an

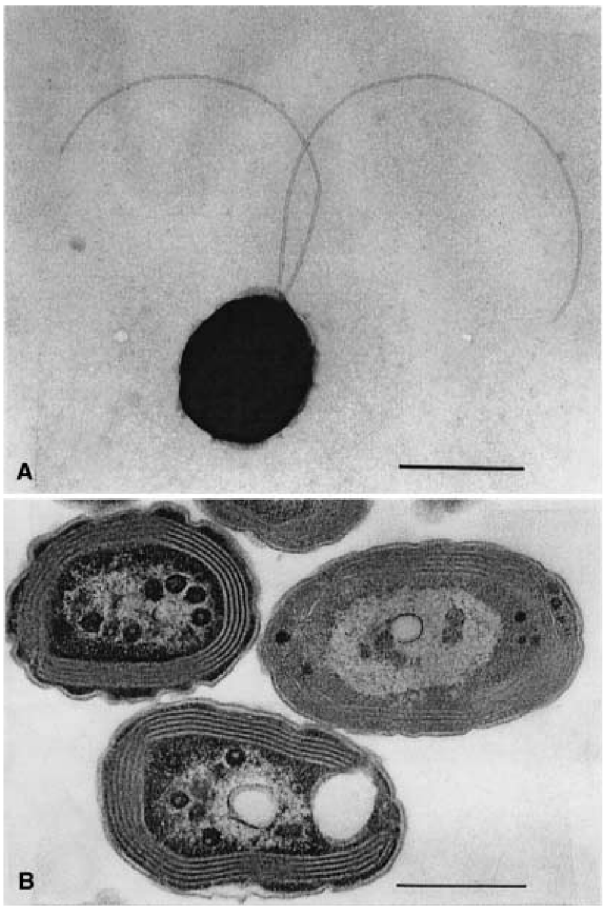


**Fig. 1a, b** Morphology of the soda lake ammonia-oxidizing bacterium isolate ANs1 grown at pH 10 and salt content 0.6 M $Na^+$. **a** Negatively stained whole cell preparation; *bar* 1 µm. **b** Thin-section; *bar* 1 µm

S-layer. Fragments of lysed cells that looked similar to subunit layers were observed, but it was not possible to see them in cell sections. Isolates ANs1, ANs2 and ANs4 formed tiny (1 mm) reddish colonies covered with a skin-like surface layer. In liquid cultures they formed aggregates. Isolates ANs3 and ANs5 formed much larger (up to 5 mm) reddish colonies with a refractile center. Compared to isolates ANs1, ANS2, and ANs4, they grew homogeneously and notably faster in liquid cultures.

## Ammonia toxicity for growth at pH 10

At pH 10 more than 90% of the added $NH_4Cl$ was present as $NH_3$, which is known to be a very toxic compound for ammonia-oxidizers. The importance of this fact for growth of soda lake ammonia-oxidizing bacteria was already observed during isolation of pure cultures. Isolate ANs1 was tested for the ability to start growth at pH 10 with 2–10 mM $NH_4Cl$. Growth started without a lag phase only at ≤4 mM $NH_4Cl$. At 5–8 mM, a 2- to 5-day lag phase preceded growth. At concentrations above 9 mM $NH_4Cl$ the isolate failed to grow. Therefore all subsequent batch-cultivation experiments were performed using a fed-batch ammonia supply of 4 mM $NH_4Cl$ doses after at least 80% oxidation of the previous dose. By using this strategy, high-density cultures were obtained that consumed up to 80 mM $NH_3$.

## Influence of salts on growth in batch culture at pH 10

Isolate ANs1 was capable of growth at pH 10 in sodium-carbonate-based medium within the range of 0.1–0.8 M total $Na^+$. If sodium-carbonate base was completely replaced by NaCl, adjusted up to pH 10 by NaOH, no growth was observed. The minimal carbonate concentration supporting growth on 0.3 M NaCl-based medium had to be above 0.05 M $Na^+$ (Fig. 2a). Maximum salt tolerance was observed on medium containing 0.2 M $Na^+$ carbonates and NaCl (Fig. 2b) with a growth optimum around 0.3–0.5 M $Na^+$. Overall, the results demonstrated relatively low salt tolerance of the ANs isolates (even those

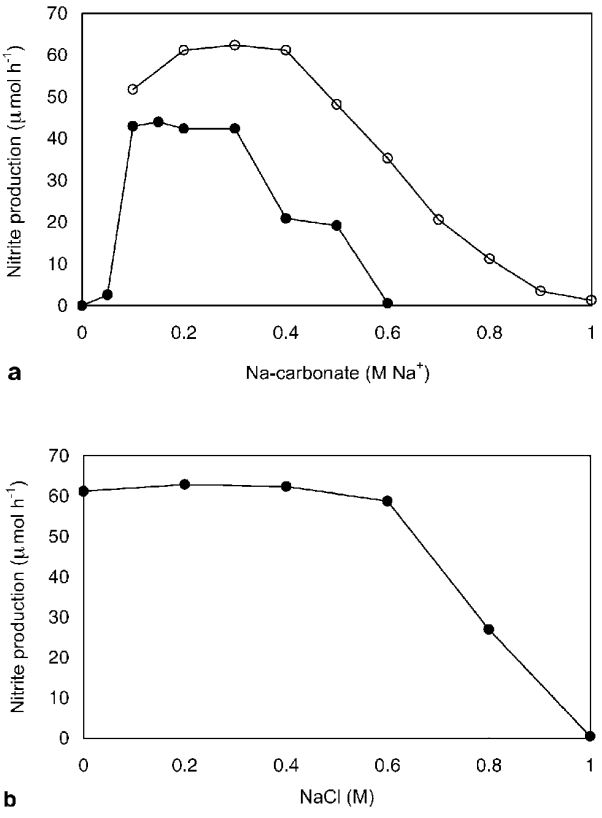


**Fig. 2** The influence of sodium carbonate (**a**) and sodium chloride (**b**) on growth of ammonia-oxidizing isolate ANs1 in batch culture at pH 10. The basic medium contained no NaCl (○) or 0.3 M NaCl (●) in variant (**a**) and 0.2 M $Na^+$ sodium carbonates in variant (**b**)

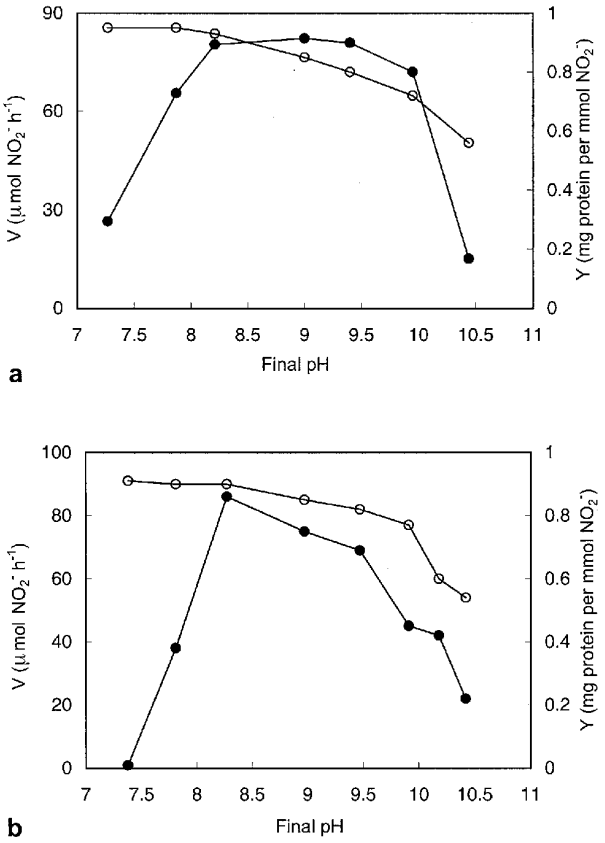


**Fig. 3a** The pH profiles for growth of ammonia-oxidizing isolates ANs1 (**a**) and ANs5 (**b**) in batch culture at 0.6 M $Na^+$. ○ Molar growth yield, ● average rate of nitrite production (*V*). Buffering system for pH range 7–8 was 0.1 M HEPES+0.6 M NaCl and for higher pH values sodium bicarbonate/carbonate buffer, 0.6 M total $Na^+$

isolated from lakes containing more than 50 g salt $l^{-1}$) with an optimum corresponding to salt concentrations of diluted soda lakes.

## Influence of pH on growth in batch culture at 0.6 M $Na^+$

All five ANs isolates grew best on sodium bicarbonate-/carbonate-buffered medium within the pH range 8.5–9.5 (Fig. 3). Isolates ANs1 and ANs2 had a lower pH limit at pH 7.2, while the other three isolates started to grow at pH≥7.5. The maximum pH limit achieved in batch cultures was 10.5, but the buffering capacity of carbonate base was not sufficient to keep the pH constant: it already started to drop after cultures had oxidized only 3–4 mM $NH_3$. The growth yield was within the range of 0.6–1 mg protein per mmol $NO_2^-$ produced with a clear tendency to decrease at pH>10.

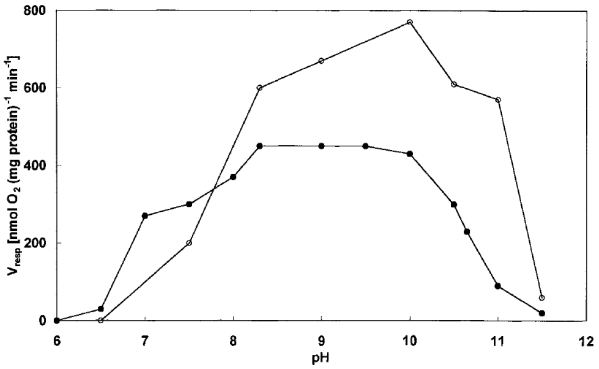


**Fig. 4** The pH profiles for respiratory activity ($V_{resp}$) of ammonia-oxidizing isolate ANs5 grown in batch culture at pH 10 and 0.6 M $Na^+$ (●) and in $NH_3$-limited and pH-controlled continuous culture at pH 11.0 and 0.3 M $Na^+$ (○). The buffering system was as described in Fig. 3, except that for cells from the chemostat buffers contained 0.3 M $Na^+$

## Influence of pH on respiratory activity of washed cells

In general, the pH profiles for $NH_3$-dependent oxygen consumption by washed cells of ANs isolates grown at pH 10 were similar to their growth pH profiles with somewhat higher tolerance at pH>10.5 (Fig. 4). Some isolates were even active up to pH 11.0. In sodium bicarbonate/carbonate buffers, the optimum pH was between 8.2 and 10. If the biomass was obtained from cultures in which most of the cells entered refractile phase, a very long lag phase (up to 20 min) was observed before oxygen consumption started. Ammonia toxicity for respiration increased with increasing pH. $K_i$ 50 values of 8, 4.5 and 0.5 mM were measured at pH 9.0, 10.0, and 11.0, respectively. $K_m$ values for ammonia estimated from the V (rate of substrate oxidation) vs S (substrate concentration) plot at pH 10 were in the range of 40–50 µM for all ANs isolates. With hydroxylamine, the pH dependence of the oxidation was difficult to measure because of its high spontaneous auto-oxidation at high pH. Measured for cells of isolate ANs4, $NH_2OH$ rates of oxidation were much lower than rates of ammonia oxidation and had an pH optimum of 9.0.

## Growth of isolate ANs5 in ammonia-limited continuous culture at pH>10

As was mentioned above, it was not possible to keep the pH constant during batch cultivation of ANs isolates at a pH higher than 10.3–10.4. Moreover ammonia toxicity increased dramatically at these pH values. To avoid these problems and to reveal the true pH limits for growth of the soda lake isolates, strain ANs5 was grown in ammonia-limited and pH-controlled continuous culture in medium containing 0.3 M total $Na^+$. Under these conditions the bacteria exhibited a remarkable potential to grow at extremely high pH values (Table 2). The cultures grew very

**Table 2** Parameters of growth of alkaliphilic ammonia-oxidizing bacterium isolate ANs5 in ammonia-limited continuous culture at pH>10 containing ammonia, 22.5 mM; temperature 30 °C; salts: $Na_2CO_3$, 0.25 M $Na^+$+0.05 M NaCl). *Q* Ammonia-dependent respiration activity measured directly in the culture taken from the fermentor (culture) or after centrifugation and resuspension of the cells in buffers containing 0.3 M total $Na^+$, $\mu$ specific growth rate, *Y* molar growth yield

| Culture pH | μ ($h^{-1}$) | Y (mg protein $mmol^{-1}$) | Q [nmol $O_2$ (mg protein)$^{-1}$ $min^{-1}$] | | |
|---|---|---|---|---|---|
| | | | Culture | Washed cells | |
| | | | | Culture pH | pH 10 |
| 10.05 | 0.110 | 1.23 | 1970 | 300 | 300 |
| 10.52 | 0.092 | 1.12 | 1670 | 620 | 610 |
| 10.70 | 0.075 | 0.95 | 1290 | 480 | 620 |
| 10.90 | 0.052 | 0.68 | 1180 | 410 | 640 |
| 11.02 | 0.035 | 0.64 | 1080 | 480 | 770 |
| 11.18 | 0.028 | 0.57 | 1050 | 150 | 220 |
| 11.28 | 0.012 | 0.35 | 820 | Nd | Nd |

fast at pH 10, with a $\mu_{max}$ 0.11 $h^{-1}$, which is within the highest rate known for autotrophic ammonia-oxidizing bacteria, and were still able to grow actively up to pH 11.2. The other interesting fact revealed in chemostat culture was the much higher activity of ammonia-dependent respiration in the cell suspensions taken directly from the chemostat than from washed cells (Table 2). It is clear that the washing step partially inactivated the respiratory activity of ANs isolates. The cells grown at pH 11.0 in a chemostat showed a definite alkaline shift in their respiratory pH profile as compared to cells grown at pH 10, which was especially pronounced at pH 11.0 (Fig. 4).

## Phylogenetic analyses

All five ANs isolates possessed a 16S rDNA gene with identical sequence. Comparative 16S rDNA sequence analysis demonstrated that the isolates are affiliated with the *Nitrosomonas europaea* lineage, which comprises besides *Nitrosomonas europaea* the species *Nitrosomonas eutropha* and the halophilic species *Nitrosomonas halophila* (Fig. 5). The low sequence divergence of the 16S rDNA (below 1%) with the latter species indicated that the new alkali-tolerant isolates might belong to the species *N. halophila.* Consistent with these results the *amoA* sequence of the isolate ANs1 showed the highest sequence similarity (95.6% on the nucleic acid level and

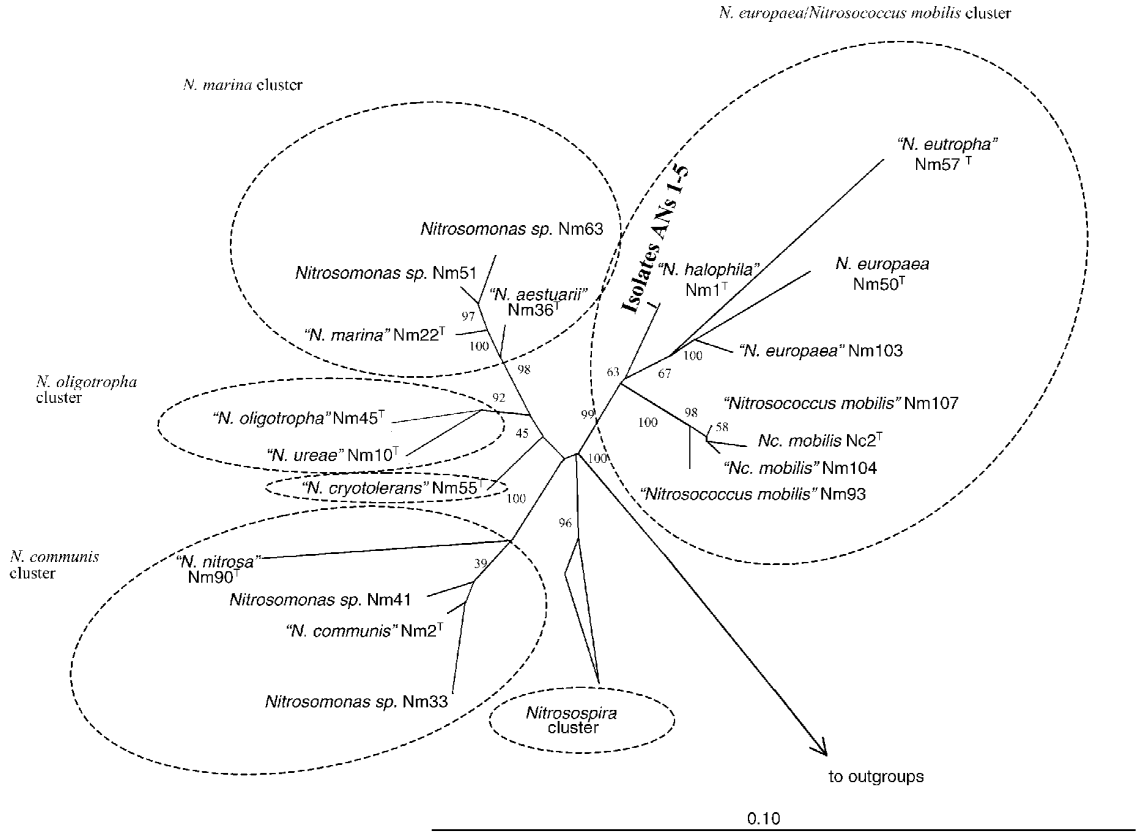


**Fig. 5** Phylogenetic neighbor-joining 16S rRNA tree showing the affiliation of the ammonia-oxidizing isolates ANs1–5 from the Mongolian soda lakes to recognized ammonia-oxidizers of the β-subclass of *Proteobacteria*. *Bar* 10% sequence divergence

98.6% on the amino acid level) with the respective gene of *N. halophila.* DNA-DNA hybridization experiments finally proved that the isolates indeed belong to the species *N. halophila*, since the observed DNA-DNA similarity values of the isolates with the type strain of *N. halophila* were higher than 70% (73–87%).

## Discussion

In a recent investigation of the total bacterial DNA collected from the water of alkaline and saline Mono Lake, a number of 16S rDNA clones were identified that belonged to the *Nitrosomonas europaea* cluster (Ward et al. 2000). Some of the clones were almost identical to the species *N. europea* and *N. eutropha*, respectively. These two species have no obligate salt requirement, but both reveal relatively strong salt tolerance (Koops and Harms 1985; Koops et al. 1991). Clearly, the molecular approach by itself was not sufficient for the characterization and understanding of specific adaptation of the ammonia-oxidizing population to specific conditions of the highly alkaline and saline environment.

Our previous physiological studies with methanotrophic (Sorokin et al. 2000a) and nitrite-oxidizing (Sorokin et al. 1998) bacteria isolated from soda lake sediments revealed that both stages of nitrification can be carried out at much higher pH values than was previously recognized. The isolation of specific autotrophic ammonia-oxidizing bacteria able to grow well at pH values above 10 confirmed that nitrifying bacteria can play an active role in the soda lake microbial system. Taking into account that at a high pH ammonia has an increasing potential to escape into the atmosphere and the toxicity of ammonia at high pH, the role of the alkali-tolerant ammonia-oxidizing bacterial population in nitrogen cycling of the soda lakes may be very important. The physiological properties of the ammonia-oxidizing strains isolated from the Mongolian soda lakes demonstrated that they indeed can be active under conditions common for diluted soda lakes.

Based on their tolerance to extremely high pH values, the new ammonia-oxidizing isolates may be considered as absolute leaders among alkaliphilic chemolithoautotrophic bacteria isolated so far from soda lakes (Sorokin 1998; Sorokin et al. 1998, 2000a, b, c). There is only one example described in the literature demonstrating the ability of a heterotrophic *Bacillus* species to grow up to pH 11.4 in chemostat under pH-controlled conditions (Sturr et al. 1994). This value seems to be a trustful alkaline pH limit for growth of bacteria. Above this limit, the maintenance of pH homeostasis became impossible even for highly effective heterotrophic energy metabolism. Therefore, the ammonia-oxidizing isolates from the soda lakes belong to the most alkali-tolerant groups of bacteria known yet. One of the reasons for the limitation of growth of alkaliphilic autotrophs at pH>10.5 might be carbon limitation due to the dominance of carbonate ion over bicarbonate. Assuming the much bigger cell volume of ammonia-oxidizing isolates from soda lakes than of other types of chemolithoautotrophic alkaliphiles, it might be speculated that the former can overcome the carbon limitation at pH>10.5 by increasing the specific number of carboxysomes. Comparison of the thin sections prepared from the cells of isolate ANs5 grown at pH 10 and 11 revealed a sharp increase in the number of carboxisomes per cell (from 2–4 to 10–15) with a concomitant decrease of their volume.

The salt concentration seems to be a much more crucial factor for ANs isolates than the high pH values. In this respect, the new ammonia-oxidizing alkali-tolerant isolates resemble the low-salt-tolerant nitrite-oxidizing *Nitrobacter alkalicus,* also obtained from the soda lake environments (Sorokin et al. 1998). In contrast, some of the sulfur-oxidizing alkaliphiles from soda lakes are able to grow up to saturating salt concentrations (Sorokin et al. 2001). One of the reasons for the low salt tolerance of the autotrophic nitrifying bacteria may be their relatively low efficiency of energy production, which might limit their ability to synthesize energetically expensive organic compatible solutes (Oren 1999).

Phylogenetic analysis clearly demonstrated that the soda lake isolates do not represent a new bacterial species but rather represent a specific alkali-tolerant subpopulation of *Nitrosomonas halophila*, which was originally isolated from the North Sea. It is very interesting to note that in environmental surveys, including marine ecosystems, *N. halophila* 16S rDNA sequences were never detected among the many sequences retrieved for other ammonia-oxidizing bacteria (Purkhold et al. 2000). This might indicate that the soda lakes are the actual habitat of this bacterium. The type strain *N. halophila* Nm1 was described as a neutrophilic bacterium but revealed a relatively strong salt tolerance (Koops et al. 1991). The genetic relation on the species level among isolates obtained from substantially different environments is surprising. Our careful reexamination of the influence of pH on growth and activity of *N. halophila* Nm 1, however, demonstrated a remarkable tolerance of this marine isolate to high pH. This tolerance was missing among another species of the *N. europea* lineage, *N. eutropha*. *N. halophila* Nm1 grew up to pH 9.5 with an optimum at pH 8.5–9 in bicarbonate-based medium with a total $Na^+$ concentration of 0.3 M. But, in contrast to the soda lake ANs isolates, *N. halophila* Nm1 was not able to grow at pH 10 and higher in carbonate-dominating medium. Accordingly, here we suggest a modification of the species description of *Nitrosomonas halophila* (based on the original description of Koops et al. 1991), including the properties of the new alkali-tolerant representatives from the soda lakes:

*Nitrosomonas halophila* (hal.o'phi.la. Gr. n. *hal,* salt; Gr. adj. *philos*, loving; L. fem. adj. *halophila*, salt-loving).

Cells are 1.1–1.8×1.5–2.5 µm in size. In some isolates cells are motile by a tuft of flagella. Carboxysomes and intracytoplasmic membranes are present. Have an obligate salt requirement with a growth optimum around 0.3 M $Na^+$ and upper limit of 0.8 M $Na^+$. The marine-type isolate can grow up to pH 9.5, and soda lake isolates are extremely alkali-tolerant, with a maximum pH limit for

growth up to 11.2. Utilization of urea not observed. The G+C content in DNA is 53.8 mol % ($T_m$). Habitat: the type strain Nm 1 was isolated from the North Sea; isolates ANs1–5 were obtained from the Mongolian soda lakes. Type strain: Nm 1 is deposited and maintained in the culture collection of the Institut fur Allgemeine Botanik der Universitaet Hamburg. Mikrobiologische Abteilung, Germany. ANs1–5 isolates are maintained in the same collection and in the culture collection of the Department of Biotechnology, Kluyver Laboratory, TU Delft, Delft, The Netherlands.

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## References

Abeliovich A (1987) Nitrifying bacteria in wastewater reservoirs. Appl Environ Microbiol 53:754–760

Brosius J, Palmer ML, Kennedy PJ, Noller HF (1978) Complete sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci USA 75:4801–4805

Cloern JE, Cole BE, Oremland RS (1983) Autotrophic processes in meromictic Big Soda Lake, Nevada. Limnol Oceanogr 28: 1049–1061

De Ley JH, Cattoir H, Reynaerts A (1970) The quantitative measurements of DNA hybridization from renaturation rates. Eur J Biochem 12:133–140

Felsenstein J (1993) PHYLIP (phylogeny inference package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Jetten MSM, Logemann S, Muyzer G, Robertson LA, DeVries S, VanLoosdrecht MCM, Kuenen JG (1997) Novel principles in the microbial conversion of nitrogen compounds. Antonie van Leeuwenhoek 71:75–93

Joye SB, Conell TL, Miller LG, Oremland RS, Jellison RS (1999) Oxidation of ammonia and methane in an alkaline, saline lake. Limnol Oceanogr 44:178–188

Juretschko S, Timmermann G, Schmid M, Schleifer K-H, Pommerening-Röser A, Koops H-P, Wagner M (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol 64:3042–3051

Koops H-P, Harms H (1985) Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch Microbiol 141:214–218

Koops H-P, Möller UC (1992) The lithotrophic ammonia-oxidizing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes. Springer, Berlin, Heidelberg, New York, pp 2625–2637

Koops H-P, Böttcher B, Möller UC, Pommering-Röser A, Stehr G (1991) Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov., *Nitrosomonas halophila* sp. nov. J Gen Microbiol 137:1689–1699

Laanbroek HJ, Woldendorp JW (1995) Activity of chemolithotrophic nitrifying bacteria under stress in natural soils. Adv Microb Ecol 14:275–304

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275

Maidak BL, Olsen GJ, Larsen N, Overbeck R, Mc Caughey MJ, Woese CR (1996) The ribosomal database project (RDP). Nucleic Acid Res 24:82–85

Marmur J (1961) A procedure for isolation of DNA from microorganisms. J Mol Biol 3:208–214

Melack JM (1981) Photosynthetic activity of phytoplankton in tropical African soda lakes. Hydrobiologia 81:71–85

Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348

Pfennig N, Lippert KD (1966) Über das Vitamin $B_{12}$ – Bedürfnis phototropher Schwefelbakterien. Arch Mikrobiol 55:245–256

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid MC, Koops HP, Wagner M (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, 66:5368–5382

Sorokin DY (1998) Occurrence of nitrification in extremely alkaline environments. Microbiology (Moscow, English translation) 67:335–339

Sorokin DY, Muyzer G, Brinkhoff T, Kuenen JG, Jetten MSM (1998) Isolation and characterization of a novel facultatively alkaliphilic *Nitrobacter* species, *N. alkalicus* sp. nov. Arch. Microbiol. 170:345–352

Sorokin DY, Jones B, Kuenen JG (2000a) A novel methane-oxidizing bacterium from highly alkaline environment. Extremophiles 4:145–155

Sorokin DY, Robertson LA, Kuenen JG (2000b) Isolation and characterization of obligately chemolithoautotrophic alkaliphilic sulfur-oxidizing bacteria. Antonie van Leeuwenhoek 77:251–262

Sorokin DY, Tourova TP, Kuenen JG (2000c) A novel facultatively autotrophic hydrogen-oxidizing bacterium from alkaline environment. Extremophiles 4:237–245

Sorokin DY, Lysenko AM, Mityushina LL, Tourova TP, Jones BE, Rainey FA, Robertson LA, Kuenen JG (2001) *Thioalkalimicrobium sibiricum*, *Thioalkalimicrobium aerophilum*., and *Thioalkalivibrio versutus*, *Thioalkalivibrio nitratis* and *Thioalkalivibrio denitrificans* gen. nov., sp. nov – new alkaliphilic obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes. Int J Syst Evol Microbiol 51:565–580

Strunk O, Ludwig W (1997) ARB software program package; http://www.biol.chemie.tu-muenchen.de/pup/ARB/

Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF 4. J Bacteriol 176: 3111–3116

Wagner M, Rath G, Amann R, Koops H-P, Schleifer K-H (1995) In situ identification of ammonia oxidizing bacteria. Syst Appl Microbiol 18:251–264

Wagner M, Rath G, Flood J, Koops H-P, Amann R (1996) In situ analysis of nitrifying bacteria in sewage treatment plants. Water Sci Technol 34:237–244

Ward BB, Martino DP, Diaz MC, Joye SB (2000) Analysis of ammonia-oxidizing bacteria from a hypersaline Mono Lake, California, on the basis of 16S rRNA sequences. Appl Environ Microbiol 66:2873–2881

Winogradsky S (1890) Recherches sur les organismes de la nitrification. Ann Inst Pasteur 4:213–231

# Appendix E

16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium oxidizing bacteria: implications for phylogeny and in situ detection

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# 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and *in situ* detection

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**Summary**

**Recently, anaerobic ammonium-oxidizing bacteria (AAOB) were identified by comparative 16S rDNA sequence analysis as a novel, deep-branching lineage within the *Planctomycetales*. This lineage consists currently of only two, not yet culturable bacteria which have been provisionally described as *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis'. In this study, a large fragment of the rDNA operon, including the 16S rDNA, the intergenic spacer region (ISR) and approximately 2000 bases of the 23S rDNA, was polymerase chain reaction (PCR) amplified, cloned and sequenced from both AAOB. The retrieved 16S rDNA sequences of both species contain an insertion at helix 9 with a previously overlooked pronounced secondary structure (new subhelices 9a and 9b). This insertion, which is absent in all other known prokaryotes, is detectable by fluorescence *in situ* hybridization (FISH) and thus present in the mature 16S rRNA. In contrast to the genera *Pirellula*, *Planctomyces* and *Gemmata* which possess unlinked 16S and 23S rRNA-genes, both AAOB have the respective genes linked together by an ISR of approximately 450 bp in length. Phylogenetic analysis of the obtained 23S rRNA genes confirmed the deep branching of the AAOB within the *Planctomycetales* and allowed the design of additional specific FISH probes. Remarkably, the ISR of the AAOB also could be successfully detected by FISH via simultaneous application of four monolabelled oligonucleotide probes. Quantitative FISH experiments with cells of *Candidatus* 'Brocadia anammoxidans' which were inhibited by exposure to oxygen for different time periods demonstrated that the concentration of transcribed ISR reflected the activity of the cells more accurately than 16S or 23S rRNA concentration. Thus the developed ISR probes might become useful tools for *in situ* monitoring of the activity of AAOB in their natural environment.**

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## Introduction

The order *Planctomycetales* first described in 1986 by Schlesner and Stackebrandt (Schlesner and Stackebrandt, 1986) currently includes four genera (*Planctomyces, Pirellula, Gemmata* and *Isosphaera*) and seven described species represented by pure cultures isolated from various aquatic systems (Schlesner, 1994, Fuerst 1995; Ward *et al*., 1995; Griepenburg *et al.,* 1999). Molecular 16S rDNA-based diversity surveys of different environments demonstrated that the naturally occurring diversity within this order is not adequately represented by the cultured species (Liesack and Stackebrandt, 1992, De Long *et al*., 1993; Bond *et al*., 1995; Ward *et al.,* 1995; Rappe *et al*., 1997; Sekiguchi *et al*., 1998; Van der Meer *et al*., 1998; Bowman *et al*., 1999; Griepenburg *et al.,* 1999; Li *et al*., 1999; Ravenschlag *et al*., 1999, Strous *et al*., 1999; LaPara *et al*., 2000; Schmid *et al*., 2000; Derakshani *et al*., 2001) (Fig. 1). Among the not yet cultured *Planctomycetales* are two recently identified new *Candidatus* genera containing the provisionally named species *Candidatus* 'Brocadia anammoxidans' (Strous *et al*.,1999; Strous, 2000) and *Candidatus* 'Kuenenia stuttgartiensis' (Schmid *et al.,* 2000). These bacteria are the only known organisms which derive their energy from anaerobic oxidation of ammonium with nitrite as electron acceptor (Jetten *et al.,* 1999). According to comparative 16S rRNA analysis, they form a deep branching lineage within the *Planctomycetales* to the exclusion of all other members of this order. Although *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' are organisms with an approved physiotype within the order *Planctomycetales* virtually nothing is known about their genetics and ecology. An improved understanding of these unique bacteria is not only of interest from an fundamental point of view but also of practical importance as anaerobic ammonium oxidation can successfully be used for nitrogen removal in waste water treatment plants (Strous, 2000; Schmid *et al*., 2000).

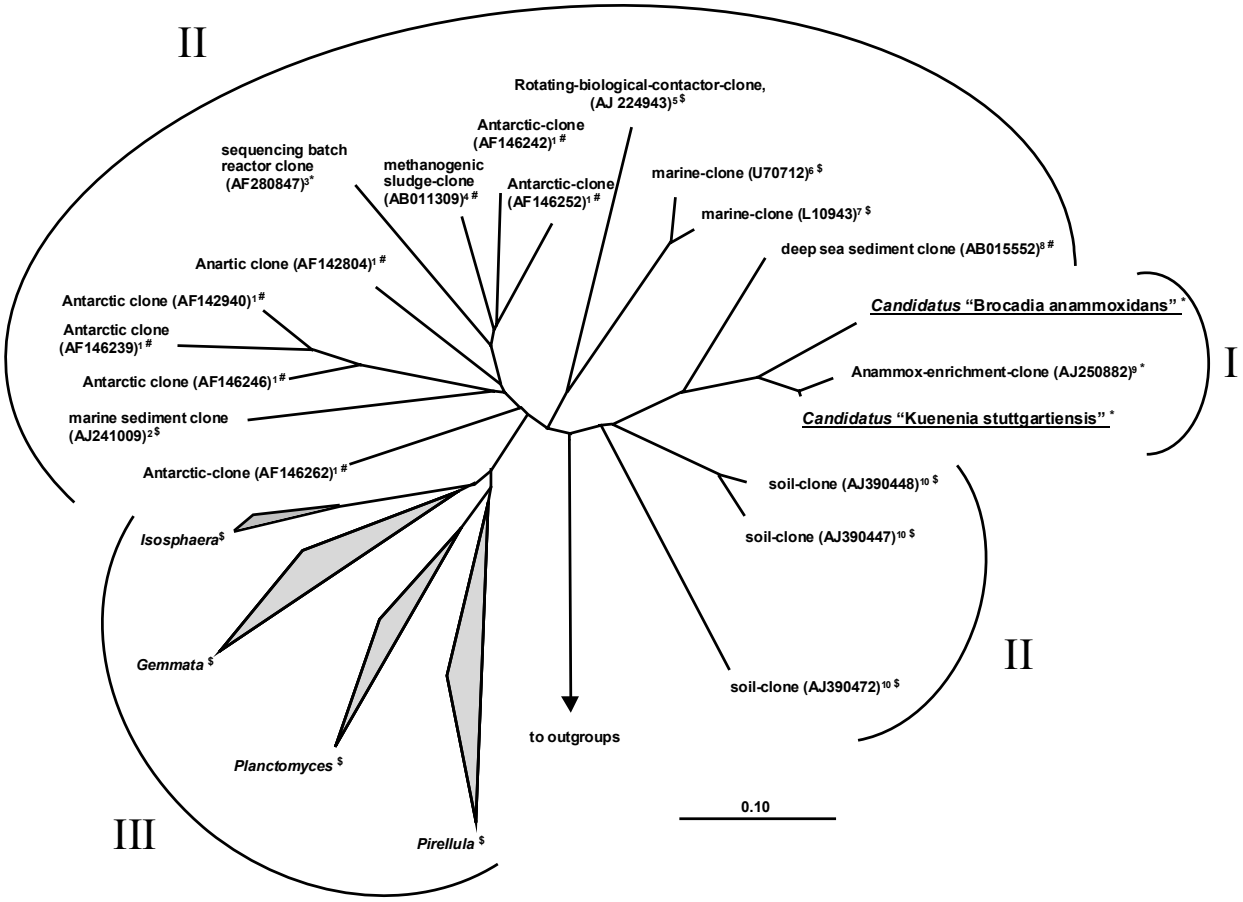


**Fig. 1.** 16S rDNA based phylogenetic tree reflecting the relationships of *Candidatus* 'Kuenenia stuttgartiensis' and *Candidatus* 'Brocadia anammoxidans' (I), environmentally derived 16S rDNA sequences of organisms with unknown physiology affiliated to the order *Planctomycetales* (II), the 'classical" planctomycetes (III), and other reference organisms. The triangles indicate phylogenetic groups. The tree is based on results of maximum likelihood analyses on different data sets. The bar represents 10% estimated sequence divergence. Sequences containing or missing the insertion at helix 9 are labeled by * and $, respectively. Partial sequences not covering helix 9 are marked with #. References: [1] Bowman *et al*., 1999; [2] Ravenschlag *et al*., 1999; [3] LaPara *et al*., 2000; [4] Sekiguchi *et al*., 1998; [5] Van der Meer *et al*., 1998; [6] Rappe *et al*., 1997; [7] DeLong *et al*., 1993; [8] Li *et al*., 1999; [9] Egli *et al*., 2001 ; [10] Derakshani *et al*., 2001.

The current study is based on rRNA operon sequence analysis of *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis'. We detected a previously overlooked secondary structure of an insertion within helix 9 of the 16S rRNA of these organisms and developed a specific oligonucleotide probe for its *in situ* detection. Furthermore, the retrieved 23S rRNA sequences were used for extended phylogenetic analysis of the anaerobic ammonium-oxidizing bacteria (AAOB) and for probe design. Interestingly, the AAOB differ from most other members of the *Planctomycetales* (Liesack and Stackebrandt, 1989; Menke *et al*., 1991; Ward *et al*., 2000) by having the 16S and 23S rRNA genes adjacent to each other within a single operon. Both genes are separated by an intergenic spacer region (ISR) of about 450 bp. Specific by fluorescence in situ hybridization (FISH) probes for the *in situ* detection of the ISR transcript were developed and applied. Compared to FISH with 16S or 23S rRNA-targeted probes, the signal intensity obtained with the ISR probes was demonstrated to be more directly linked with the physiological activity of the cells.

## Results

### *AAOB contain an insertion at helix 9 of their 16S rRNA*

16S rDNA sequences of the AAOB *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' have been presented in previous studies (Strous *et al.,* 1999; Schmid *et al.,* 2000). In this study we obtained for both organisms clones of a large fragment of the rDNA operon (approximatly 4000 bp), including the 16S rDNA, the intergenic spacer region (ISR) and about 2000 bases of the 23S rDNA. Inspection of the 16S rDNA sequences revealed a previously overlooked insertion with 20 nucleotides in length located within helix 9 (beginning at *E. coli* position 157). A pronounced secondary structure

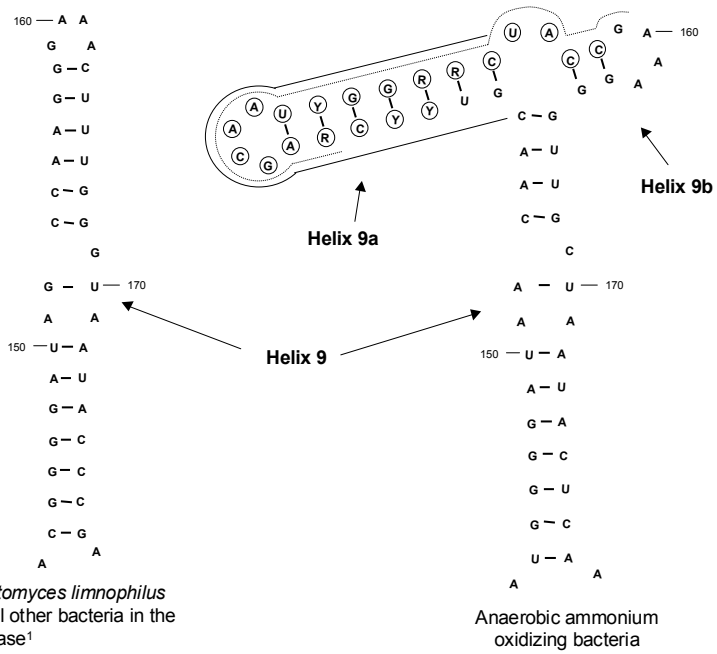


**Fig. 2.** Comparison of the secondary structure of helix 9 of the 16S rDNA of the AAOB *Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans" (right) and *Planctomyces limnophilus* (as an example for all other bacteria) (left). Inserted nucleotides are encircled. Differences in sequence between *Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans" are displayed using the IUP ambiguity codes (R= A/G, Y= U/C). Positions of probes S-S-Ban-0162(B.anam.)-a-A-18 and S-S-Kst-0157-a-A-18 are indicated by dotted and straight lines, respectively. Numbering refers to the respective *E. coli* positions.

can be predicted for this insertion leading to the proposal of the two new subhelices 9a and 9b (Fig. 2). With the exception of the *Planctomycetales*-affiliated environmental 16S rDNA clone AF280847 (LaPara *et al*., 2000; Fig. 1) which contains 14 inserted nucleotides at this site, this insertion is absent from all other 16S rDNA sequences in the database of the ARB program package for sequence analysis (Strunk and Ludwig, 1997; about 18.000 entries; status March, 2001). The presence of the insertion in the mature 16S rRNA of the AAOB was demonstrated using the insertion-targeted oligonucleotide probes S-S-Ban-0162(B.anam.)-a-A-18 (specific for *Candidatus* 'Brocadia anammoxidans') and S-S-Kst-0157-a-A-18 (specific for *Candidatus* 'Kuenenia stuttgartiensis') for FISH. Both probes hybridized specifically to their respective target organisms with an intensity comparable to the 16S rRNA-targeted probe S-*-Amx-0820-a-A-22 (data not shown). The newly designed probe S-S-Kst-0157-a-A-18 has at least four mismatches with all other available 16S rRNA sequences including *Candidatus* 'Brocadia anammoxidans'. As no pure culture is available to determine the optimal hybridization stringency for probe S-S-Kst-0157-a-A-18, an *in situ* probe dissociation curve was recorded with biofilm samples containing *Candidatus* 'Kuenenia stuttgartiensis' using increasingly stringent conditions. Probe S-S-Kst-0157-a-A-18 yielded strong signals with up to 25% (v/v) formamide in the hybridization buffer followed by a decline at 30% (v/v) formamide. Signal intensities dropped to the level of autofluorescence after increasing the formamide concentration in the hybridization buffers to more than 40% (v/v). At 25% (v/v) formamide no cross reaction of probe S-S-Kst-0157-a-A-18 with cells from the enrichment of *Candidatus* 'Brocadia anammoxidans' could be observed (data not shown). Thus the use of a formamide concentration of 25% (v/v) is suggested for *in situ* hybridizations with probe S-S-Kst-0157-a-A-18.

*AAOB contain 23S rDNA and 16S rDNA in one operon.*

In contrast to most other recognized members of the *Planctomycetales* (Liesack and Stackebrandt, 1989; Menke *et al*., 1991; Ward *et al*., 2000) the 23S rDNA sequence of both investigated AAOB is directly linked by an ISR (approx. 450 bp) to the 16S rDNA. Furthermore, helix 58 is not deleted in the 23S rDNA of the AAOB as described for some other species of the *Planctomycetales* and *Verrucomicrobiales* (Ward *et al.,* 2000). The obtained 23S rDNA sequences were used for design of the oligonucleotide probe L-*-Amx-1900-a-A-21 specific for both anaerobic ammonia-oxidizers (Table 1). Probe L-*-Amx-1900-a-A-21 has at least three mismatches with respect to all other available 23S rRNA sequences. The optimal hybridization stringency for probe L-*-Amx-1900-a-A-21 was determined by recording an *in situ* probe dissociation curve with an enrichment culture of *Candidatus* 'Brocadia anammoxidans' and biofilm material containing *Candidatus* 'Kuenenia stuttgartiensis' using increasingly stringent conditions. L-*-Amx-1900-a-A-21 yielded strong signals up to 30% (v/v) formamide in the hybridization buffer followed by a decline at 35% (v/v) formamide. Signal intensities dropped to the level of autofluorescence after increasing the formamide concentration in the hybridization buffers to more than 45% (v/v) (data not shown). Thus a formamide concentration of 30%

**Table 1.** Probes used in this study

| Trivial name (reference) | OPD[a] designation | Specificity | Sequence 5′-3′ | Target site | %Formamide /mM NaCl[e] |
|---|---|---|---|---|---|
| This study | I-*-Ban-0071(*B. anam.*)-a-A-18 | *Candidatus* 'Brocadia anammoxidans' | CCCTACCACAAACCTCGT | 71-88[b] | 10/450 |
| This study | I-*-Ban-0108(*B. anam.*)-a-A-18 | *Candidatus* 'Brocadia anammoxidans' | TTTGGGCCCGCAATCTCA | 108-125[b] | 10/450 |
| This study | I-*-Ban-0222(*B. anam.*)-a-A-19 | *Candidatus* 'Brocadia anammoxidans' | GCTTAGAATCTTCTGAGGG | 222-240[b] | 10/450 |
| This study | I-*-Ban-0389(*B. anam.*)-a-A-18 | *Candidatus* 'Brocadia anammoxidans' | GGATCAAATTGCTACCCG | 389-406[b] | 10/450 |
| This study | I-*-Kst-0031(*K. stutt.*)-a-A-18 | *Candidatus* 'Kuenenia stuttgartiensis' | ATAGAAGCCTTTTGCGCG | 31-48[b] | 10/450 |
| This study | I-*-Kst-0077(*K. stutt.*)-a-A-18 | *Candidatus* 'Kuenenia stuttgartiensis' | TTTGGGCCACACTCTGTT | 77-94[b] | 10/450 |
| This study | I-*-Kst-0193(*K. stutt.*)-a-A-19 | *Candidatus* 'Kuenenia stuttgartiensis' | CAGACCGGACGTATAAAAG | 193-211[b] | 10/450 |
| This study | I-*-Kst-0288(*K. stutt.*)-a-A-20 | *Candidatus* 'Kuenenia stuttgartiensis' | GCGCAAAGAAATCAAACTGG | 288-297[b] | 10/450 |
| This study | L-*-Amx-1900-a-A-21 | Both AAOB | CATCTCCGGCTTGAACAA | 1900-1483[c] | 30/112 |
| This study | S-S-Kst-0157-a-A-18 | *Candidatus* 'Kuenenia stuttgartiensis' | GTTCCGATTGCTCGAAAC | 16S rRNA insertion | 25/159 |
| Schmid *et al.* 2000 | S-S-Ban-0162(*B. anam.*)-a-A-18[f] | *Candidatus* 'Brocadia anammoxidans' | CGGTAGCCCCAATTGCTT | 16S rRNA insertion | 40/56 |
| Schmid *et al.* 2000 | S-*-Amx-0820-a-A-22 | Both AAOB | AAAACCCCTCTACTTAGTGCCC | 820-841[d] | 40/56 |
| Eub 338 (Amann *et al.*, 1990) | S-D-Bact-0338-a-A-18 | *Many but not all* Bacteria | GCTGCCTCCCGTAGGAGT | 338-355[d] | 0/900 |
| Eub 338 II (Daims *et al.*, 1999) | S-D-Bact-0338-b-A-18 | To be used in combination with probe EUB338 | GCAGCCACCCGTAGGTGT | 338-355[d] | 0/900 |
| Eub 338 III (Daims *et al.*, 1999) | S-D-Bact-0338-c-A-18 | To be used in combination with probe EUB338 | GCTGCCACCCGTAGGTGT | 338-355[d] | 0/900 |

**a.** Oligonucleotide probe database (Alm *et al.*, 1996).
**b.** Position of intergenic spacer region (ISR) with regard to the respective target organism.
**c.** 23S rRNA position, *E. coli* numbering (Brosius *et al.*, 1980).
**d.** 16S rRNA position, *E. coli* numbering (Brosius *et al.*, 1978)
**e.** Percentage of formamid in the hybridization buffer and mM NaCl in the washing buffer, respectively, required for specific *in situ* hybridization.
**f.** Erroneously called S-*-Amx-0156-a-A-18 in Schmid *et al.* 2000.

(v/v) was chosen as optimal stringency for probe L-*-Amx-1900-a-A-21.

*Phylogenetic analysis of the rDNA operon of the AAOB.*

*Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' possess 90.5% and 87.8% sequence similarity on the 16S and 23S rRNA level, respectively. 16S and 23S rRNA sequence similarities between AAOB and all other planctomycetes are below 78% and 72%, respectively (Table 2). Phylogenetic analyses were performed independently for 16S rRNA and 23S rRNA as well as for a concatenated 16S and 23S rRNA data set. Independent from the data set analysed and the treeing method used, the AAOB form a stable, deep branching line of descent within the order *Planctomycetales* (Fig. 3). Exclusion of highly variable positions prior to treeing (only those positions were considered which are conserved in at least 50% of the *Planctomycetales* and the *Bacteria*, respectively) did not influence the phylogenetic affiliation of the AAOB (data not shown).

*In situ detection of AAOB using intergenic spacer region (ISR) targeted oligonucleotide probes.*

The approximately 450 bp ISRs between the 16S and 23S rDNA of *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis contain genes for the tRNAs tAlanine and tIsoleucine. ISR regions located outside of the tRNA genes were used as target for oligonucleotide probes. Four probes were designed for each of the AAOB and applied in FISH (Table 1). Each probe of both sets had at least four mismatches with all 16S and 23S rRNA sequences and all other sequences at GenBank (www.ncbi.nlm.nih.gov). For initial FISH experiments, physiologically active AAOB cells of both genera were used. *In situ* hybridization of these cells with single ISR-targeted probes did result in detectable but very weak fluorescence (data not shown). For increasing signal intensities the four ISR probes for each species (labelled with the same dye) were applied together. This resulted in strong, specific hybridization signals for *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis'. No addition of formamide to the hybridization buffer was required to obtain specific

**Table 2.** 16S and 23S rDNA sequence similarities of the AAOB *Candidatus* 'K. stuttgartiensis' and *Candidatus* 'B. anammoxidans' with *Verrucomicrobium spinosum*, *Chlamydia trachomatis*, and representative species of the order *Planctomycetales*. Similarity values were determined after excluding insertions and deletions from the data set.

| | % Similarity to rDNA of | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *P. marina* | | | *P. limnophilus* | | | G. obscuriglobus | | | *I. pallida* | | | *C.* 'K. stuttgartiensis' | | | *C.* 'B. anammoxidans' | | | *V. spinosum* | | |
| rDNA source | 16S | 23S | C[a] | 16S | 23S | C[a] | 16S | 23S | C[a] | 16S | 23S | C[a] | 16S | 23S | C[a] | 16S | 23S | C[a] | 16S | 23S | C[a] |
| *Planctomyces limnophilus* | 82.4 | 80.6 | 81.2 | | | | | | | | | | | | | | | | | | |
| *Gemmata obscuriglobus* | 80.5 | 76.4 | 77.8 | 80.4 | 76.8 | 78.0 | | | | | | | | | | | | | | | |
| *Isosphera pallida* | 78.3 | 74.4 | 75.7 | 79.6 | 74.5 | 76.3 | 79.2 | 74.3 | 76.0 | | | | | | | | | | | | |
| *C.* 'Kuenenia stuttgartiensis' | 77.6 | 70.8 | 73.7 | 75.6 | 71.8 | 73.5 | 74.9 | 71.5 | 73.1 | 73.6 | 67.2 | 70.1 | | | | | | | | | |
| *C.* 'Brocadia anammoxidans' | 76.3 | 66.3 | 70.4 | 76.7 | 68.0 | 71.6 | 75.6 | 67.1 | 70.7 | 74.6 | 64.0 | 68.5 | 90.5 | 87.8 | 89.0 | | | | | | |
| *Verrucomicrobium spinosum* | 72.3 | 67.9 | 69.4 | 72.3 | 68.5 | 70.1 | 71.7 | 68.2 | 69.4 | 71.4 | 67.6 | 68.9 | 73.5 | 65.7 | 69.2 | 73.3 | 61.9 | 66.7 | | | |
| *Chlamydia trachomatis* | 72.4 | 69.8 | 70.7 | 71.8 | 70.0 | 70.7 | 70.3 | 69.9 | 70.1 | 71.6 | 67.5 | 69.0 | 74.2 | 67.1 | 70.2 | 72.4 | 64.2 | 67.7 | 73.4 | 69.0 | 70.6 |

**a.** C: combined 16S and 23S rDNA sequence data.

signals. The specificity of the ISR probes was confirmed by simultaneous application of 16S and 23S rRNA-targeted probes (S-*-AMX-820-a-A-20 and L-*-Amx-1900-a-A-21, labeled with different dyes) specific for the AAOB. The suitability of the ISR probes to measure activity changes of AAOB was evaluated with cells of *Candidatus* 'Brocadia anammoxidans' which were inhibited by exposure to oxygen for different time periods (Fig. 4). Active cells (maintained at anoxic conditions) are detectable by both the rRNA and the ISR-targeted probes (see above). However, after 1 h of oxygen exposure only a minor percentage (8 ± 1 %) of the AAOB cells showed ISR-probe conferred fluorescence while cells could still be detected with specific 16S and 23S rRNA-targeted probes. After 4 h of oxygen incubation no ISR probe signals were detectable while the mean 16S and 23S rRNA-probe conferred fluorescence was only reduced to 42 ± 1 % (16S rRNA-targeted probe S-S-Ban-0162-a-A-18) and 72 ± 1 % (23S rRNA-targeted probe L-*-Amx-1900-a-A-21) respectively. Even after one day of complete physiological inhibition by oxygen exposure, the AAOB cells were still detectable with 16 and 23S rRNA probes while again no ISR probe signal was recorded (Fig. 4).

## Discussion

### *Phylogeny and unusual rDNA operon structure of AAOB*

Phylogenetically the AAOB *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' represent the deepest branch within the *Planctomycetales*. This holds true for 16S rDNA (Fig. 1), 23S rDNA, or concatenated 16S/23S rDNA data sets (Fig. 3) and is independent from the treeing method applied. The 16S rDNA sequences of both recognized AAOB contain a 20 bp insertion at helix 9 which is, according to FISH analysis, present in the mature 16S rRNA and presumably forms the two novel subhelices 9a and 9b (Fig. 2). This insertion is also found in a recently published 16S rDNA sequence almost identical to *Candidatus* "Kuenenia stuttgartiensis' (sequence similarity >99%; Fig. 1; Egli *et al*., 2001) and in truncated form (14 bp) in a 16S rDNA sequence amplified from an industrial waste water treatment plant (AF280847; LaPara *et al*., 2000) which is affiliated to another deep branching lineage within the *Planctomycetales* (Fig. 1; 16S rDNA sequence similarity to AAOB <75%). All other prokaryotic 16S rDNA sequences in publicly accessible data bases do not contain subhelices 9a and 9b. According to the 16S rDNA phylogeny of the *Planctomycetales*, the most parsimonious interpretation of these data is that this insertion emerged in two independent events in the clone

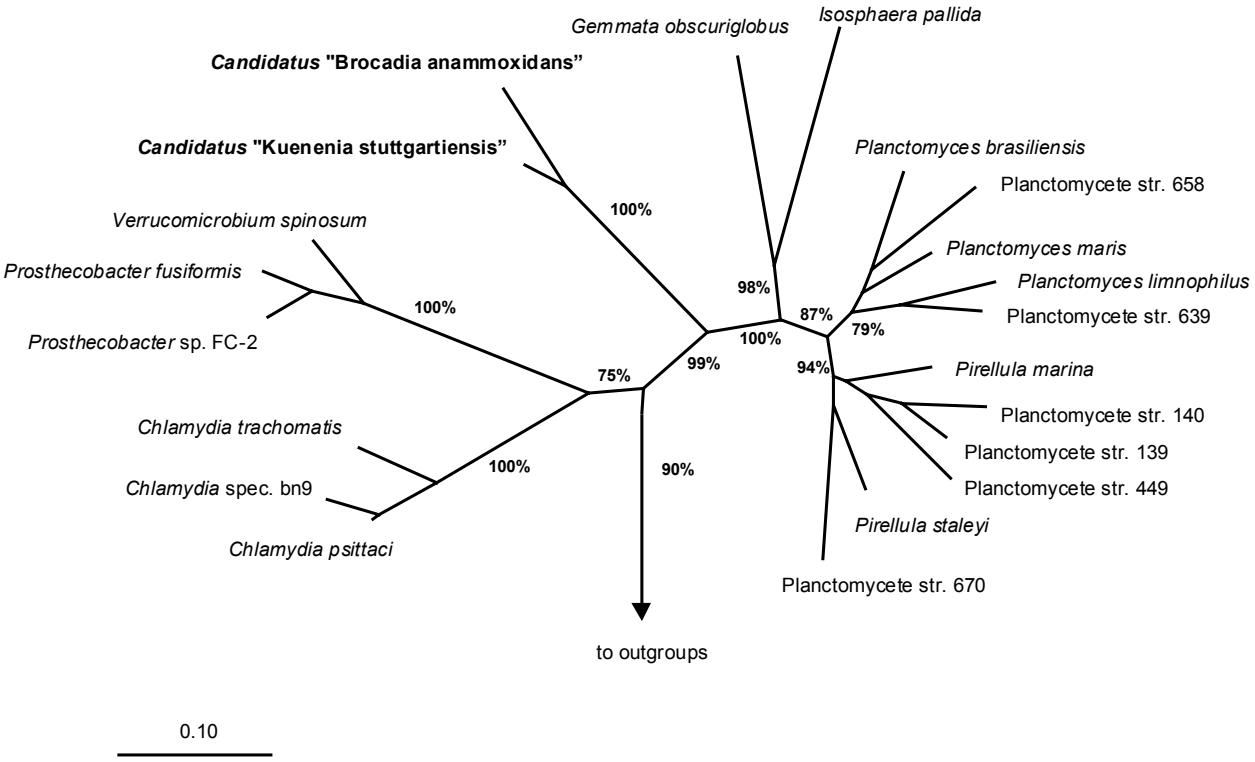


**Fig. 3.** Phylogenetic tree based on combined sequence data of 16S and 23S rDNA reflecting the relationships of the AAOB *Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans", the cultivated members of the order *Planctomycetales,* and other reference organisms. The tree is based on results of maximum likelihood analysis. Parsimony bootstrap values for the major branches are reported. The bar represents 10% estimated sequence divergence.

AF280847 lineage and in the common ancestor of the candidatus genera Brocadia and Kuenenia.

Another feature that differs between the AAOB *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' and most recognized members of the *Planctomycetales* is the genomic arrangement of the 16S and 23S rDNA genes. The AAOB and members of the genus *Isosphaera* possess linked 16S and 23S rDNA genes while members of the genera *Planctomyces, Pirellula* and *Gemmata* (Liesack and Stackebrandt, 1989; Menke *et al*., 1991; Ward *et al*., 2000) contain separated 16S and 23S rDNA operons.

*In situ detection of the intergenic spacer region in AAOB as measure of activity*

The FISH signal intensity of a microbial cell labeled with a rRNA-targeted oligonucleotide probe is proportional to the concentration of ribosomes and precursor-rRNA molecules per cell. Since the cellular rRNA content is known to depend on the rate of growth, FISH signal intensities have been used to infer growth rates of defined microorganisms within their natural environment (e.g. Poulsen *et al*., 1993). However, the links of cellular rRNA content with physiological activity differ significantly between organisms (Flärdh *et al*., 1992) and also vary dependent upon conditions of growth and starvation (Oda *et al*., 2000). Consequently, a high cellular rRNA content is not always indicative for physiological activity. For example, ammonia-oxidizing bacteria of the beta-subclass of *Proteobacteria* maintain high cellular rRNA contents even after prolonged periods of starvation (Morita, 1993; Morgenroth *et al*., 2000) or inhibition (Wagner *et al*., 1995). As precursor rRNA concentrations are more reflective of the physiological activity of a microbial cell than the mature rRNA contents (Cangelosi and Brabant, 1997), targeting the intergenic spacer region (ISR) between the 16S rRNA and 23S rRNA with fluorescently labeled oligonucleotide probes allows a more direct probing of cellular activity (Oerther *et al*., 2000). However, this approach has up to now only been applied for the fast growing heterotrophic bacteria *E. coli* and *Acinetobacter calcoalceticus.* In the present study we sequenced the ISR of the AAOB *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' and designed probes for ISR *in situ* detection. The suitability of the ISR probes to *in situ* monitor changes in activity of AAOB was evaluated using oxygen-inhibited cells. AAOB immediately arrest their metabolism after exposure to oxygen (Jetten *et al*., 1999) but maintain

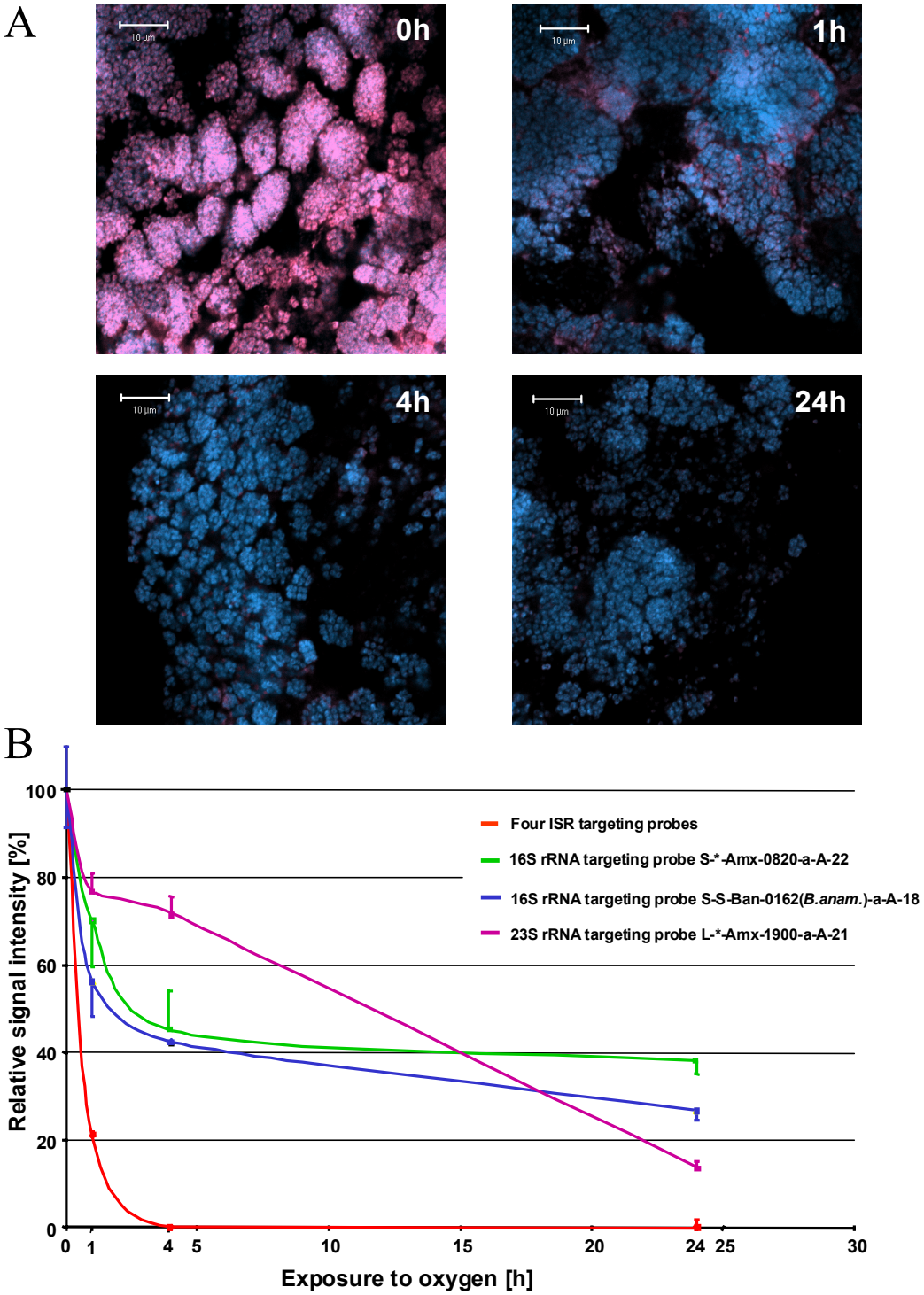


**Fig. 4.** Panel A. *In situ* hybridization of *Candidatus* "Brocadia anammoxidans" with the 16S-rRNA targeted probe S-*-Amx-0820-a-A-22 (labeled in blue) as well as the ISR-targeted probes I-*-Ban-0071(*B.anam.*)-a-A-18, I-*-Ban-0108(*B.anam.*)-a-A-18, I-*-Ban-0222(*B.anam.*)-a-A-19, and I-*-Ban-0389(*B.anam.*)-a-A-18 (all labeled in red) after different exposure times to oxygen (0 h, 1 h, 4 h, 24 h). Cells stained by rRNA and ISR-targeted probes appear magenta due to the overlap of colors. Panel B. Signal intensities of *Candidatus* "Brocadia anammoxidans" exposed for different time periods to oxygen after *in situ* hybridization with 16S-, 23S- and ISR-targeted probes. The mean relative signal intensities were calculated by determining the mean pixel intensity per microscopic image in the respective channel. Bars indicate standard errors calculated from 5 independent measurements.

detectable levels of 16S and 23S rRNA even 24 h after a switch to oxic conditions. In contrast, the ISR-probe-conferred signals dramatically drop within the first hour of oxygen inhibition and are below the detection limit after 4h of aerobic incubation (Fig. 4) suggesting that the developed ISR-targeted probes might become useful tools for directly investigating physiological responses of the still unculturable AAOB to changes in environmental conditions.

## Experimental Procedures

### *DNA extraction from biofilm and enrichment cultures*

High molecular weight DNA of biofilm material from the trickling filter 2 of the semitechnical plant in Stuttgart containing *Candidatus* 'Kuenenia stuttgartiensis' was isolated as described by Schmid *et al.* (2000). The sample of an enrichment culture of Candidatus 'Brocadia anammoxidans' was harvested from a sequencing batch reactor (Strous *et al.,* 1998). Total genomic DNA was isolated with the Fastprep Bead-beater (BIO 101, Vista) and FastDNA kit (BIO 101, Vista) according to the standard protocol provided with the instrument.

### *PCR amplification of a stretch of the rDNA operon*

For preferential PCR amplification of rDNA of members of the *Planctomycetales* the recently published phylum-specific probe Pla46 (16S rDNA *E. coli* positions 46-63; Neef *et al.,* 1998; Table 3) was used unlabeled as a forward primer in combination with the universal reverse primer 1037R (23S rDNA *E. coli* positions 1929-1953; Table 3). PCR was performed in a 96 micro well plate (Biorad, München, Germany) with the gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 4 min at 94°C, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 50 s, and elongation at 72 °C for 3 min. Cycling was completed by a final elongation step at 72°C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of amplification products were determined by agarose (0.8%) gel electrophoresis of 5 µl aliquots of the PCR products.

### *Cloning and Sequencing*

The biofilm-derived rDNA PCR products were cloned by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the Quiaprep spin miniprep kit (Quiagen, Hilden, Germany). Plasmids with an insert of the expected size were identified by agaraose (0.1%) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3h at 37°C). Sequencing was done nonradioactively by using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit according to the instructions of the manufacturer (Amersham, Freiburg, Germany). The reaction mixtures were analyzed with an infrared automated DNA sequencer (model LiCor Longreadir DNA 4200, MWG - Biotech, Ebersberg, Germany). The complete sequences of the rDNA operon fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site and internal primers located on the 16S and 23S rDNA, respectively (Table 3).

### *Phylogenetic analysis*

Sequences obtained in this study were split in 16S rDNA, 23S rDNA and ISR and added separately to the respective sequence databases of the Technischen Universität München (currently encompassing more than 18.000 small subunit rRNA sequences and more than 1200 large subunit rRNA sequences) by use of the ARB program package (Strunk and Ludwig, 1997). While ISR sequences were not aligned, 16S and 23S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection. Additionally, a combined 16S and 23S rRNA sequence database was generated including all members of the *Planctomycetales* for which 16S and 23S rRNA sequence information is available (Ward *et al.,* 2000). Phylogenetic analyses of rRNA sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analyses (fast DNAml, Maidak *et al.,* 1996) to different data sets. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling) (Phylogeny Inference Package Version 3.5c, University of Washington, Seattle).

**Table 3.** Sequences and target sites of primers used for PCR amplification and sequencing.

| Trivial name | Reference | Sequence 5′-3′ | Target site |
|---|---|---|---|
| Pla46 | Neef *et al.* (1998) | GACTTGCATGCCTAATCC | 46-63[a] |
| 630R[b] | Juretschko *et al.* (1998) | CAKAAAGGAGGTGATCC | 1529-1545[a] |
| 1035R[b] | Ludwig *et al.* (1992) | TTCGCTCGCCRCTAC | 242-256[c] |
| 1274V[b] | Ludwig *et al.* (1992) | GCGTRCCTTTTGTAKAATG | 559-577[c] |
| 1020R[b] | Ludwig *et al.* (1992) | TCTGGGYTGTTYCCCT | 975-990[c] |
| 1037R | Ludwig *et al.* (1992) | CGACAAGGAATTTCGCTAC | 1930-48[c] |

**a.** 16S rRNA position, *E. coli* numbering (Brosius *et al.*, 1978).
**b.** Primer exclusively used for sequencing.
**c.** 23S rRNA position, *E. coli* numbering (Brosius *et al.*, 1980).

### Probe design, FISH and microscopy

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridization buffers are displayed in Table 1. Probe design was performed with the respective tool of the ARB package. Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Interactiva (Ulm, Germany). Hybridizations were performed as described by Amann (1995). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (Wagner *et al.,* 1994). Optimal hybridization conditions for probes S-S-Kst-0157-a-A-18 and L-*-Amx-1900-a-A-21 were determined by using the hybridization and wash buffers described by Manz *et al.* (1992). The *in situ* probe dissociation temperatures were evaluated by measuring the relative fluorescence intensity of biofilm bacteria after hybridization with probes S-S-Kst-0157-a-A-18 and L-*-Amx-1900-a-A-21 at different stringencies as described by Daims *et al.* (1999). Slides were washed briefly with $ddH_2O$, air-dried and embedded in Citifluor (Citifluor Ltd., Canterbury, UK). For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). FISH signal intensities were quantified using the procedure described by Daims *et al.* (1999).

### Nucleotide sequence accession numbers

The sequences obtained in this study are available in GenBank under accession nos. AF375994 (16S rRNA, ISR, and 23S rRNA of '*Candidatus* Brocadia anamoxidans') and AF375995 (16S rRNA, ISR, and 23S rRNA of '*Candidatus* Kuenenia stuttgartiensis').

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**References**

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., and Raskin, L. (1996) The oligonucleotide probe database. *Appl Environ Microbiol* **62:** 3557-3559.

Amann, R. (1995) *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular microbial ecology manual*. Akkermans, A.D.C., van Elsas, J.D. and de Bruijn, F.J. (eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp.1-15.

Amann, R.I., Binder, B.J., Olsen, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919-1925.

Brosius, J., Palmer, M.L., Kennedy, P.J., and Noller, H.F. (1978) Complete sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75:** 4801-4805.

Brosius, J., Dull, T.J., Noller, H.F. (1980) Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **77:** 201-204.

Bond, P., Hugenholtz, P., Keller, J., and Blackall, L.L. (1995) Bacterial community structure of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. *Appl. Environ Microbiol* **61:** 1910-1916.

Bowman, J.P., Rea, S.M., McCammon, S.A. and McMeekin, T.A. (1999) Genbank direct submission. Accession numbers: AF142804, AF 142939, AF142940, AF146246, AF146252, AF146262.

Cangelosi, G.A, Brabant, W.H. (1997) Depletion of pre-16S rRNA in starved *Escherichia coli* cells. *J Bacteriol* **179:** 4457-4463.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22:** 434-444.

De Long, E.F., Franks, D.G., and Alldredge, A.L. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol Oceanogr* **38:** 924-934.

Derakshani, M., Lukow, T., and Liesack, W. (2001) Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl Environ Microbiol* **67:** 623-631.

Egli, K., Fanger, U., Alvarez, P.J.J., Siegrist, H., Van der Meer, J.R., and Zehnder, A.J.B. (2001) Enrichment and characterization of a new Anammox bacterium from a rotating biological contactor treating an ammonium-rich leachate. *Arch Microbiol* **175:** 198-207.

Flärdh, K., Cohen, P.S., and Kjelleberg, S. (1992) Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. *J Bacteriol* **174:** 6780-6788.

Fuerst, J.A. (1995) The planctomycetes: emerging models for microbial ecology, evolution and cell biology. *Microbiology* **141:** 1493-1506.

Griepenburg, U., Ward-Rainey, N., Mohamed, S., Schlesner, H., Marxen, H., Rainey, F.A., Stackebrandt, E., and Auling, G. (1999) Phylogenetic diversity, polyamine pattern and DNA base composition of members of the order *Planctomycetales*. *Int J Syst Bacteriol* **49:** 689-696.

Jetten, M.S.M., Strous, M., van de Pas-Schoonen, K.T., Schalk, J., van Dongen, U.G.J.M., van de Graaf, A., Logemann, S., Muyzer, G., van Loosdrecht, M.C.M., and Kuenen, J.G. (1999) The anaerobic oxidation of ammonium. *FEMS Microbiol Rev* **22:** 421-437.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* **64:** 3042-3051.

LaPara T.M., Nakatsu C.H., Pantea L., and Alleman J.E. (2000) Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Appl Environ Microbiol* **66:** 3951-3959.

Li, L., Kato, C., and Horikoshi, K. (1999) Bacterial diversity in deep-sea sediments from different depths. *Biodivers Conserv* **8:** 659-677.

Liesack, W., and Stackebrandt, E. (1989) Evidence for unlinked rrn operons in the planctomycete *Pirellula marina*. *J Bacteriol* **171:** 5025-5030.

Liesack, W., and Stackebrandt, E. (1992) Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J Bacteriol* **174:** 5072-5078.

Ludwig, W., Kirchhof, G., Klugbauer, N., Weizenegger, M., Betzl, D., Ehrmann, M., Hertel, C., Jilg, S., Tatzel, R., Zitzelsberger, H., Liebl, S., Hochberger, M., Shah, J., Lane, D., Wallnöfer, P. R., and Schleifer, K.-H. (1992) Complete 23S ribosomal RNA sequences of Gram-positive bacteria with a low DNA G+C content. *Syst Appl Microbiol* **15:** 487-501.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeck, R., Mc Caughey, M. J., and Woese, C. R. (1996) The ribosomal database project (RDP). *Nucleic Acid Res* **24:** 82-85.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligonucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst. Appl. Microbiol.* **15:** 593-600.

Menke, M.A.O.H., Liesack, W., and Stackebrandt, E. (1991) Ribotyping of 16S and 23S rRNA genes and organization of rrn operons in members of the bacterial genera *Gemmata, Planctomyces, Thermotoga, Thermus and Verrucomicrobium. Arch Microbiol* **155:** 263-271.

Morgenroth, E., Obermayer, A., Arnold, E., Brühl, A., Wagner, M., and Wilderer, P.A. (2000) Effect of long term idle periods on the performance of sequencing batch reactors. *Wat Sci Technol* **41:** 105-113.

Morita, R. Y. (1993) Bioavailability of energy and starvation state. In *Starvation in bacteria*. Kjelleberg, S. (ed.). New York: Plenum Press.

Neef, A., Amann, R. I., Schlesner, H., and Schleifer, K.-H. (1998) Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* **144:** 3257-3266.

Oda, Y., Slagman, S.-J., Meijer, W. G.,Forney, L. J., and Gottschal, J. C. (2000) Influence of growth rate and starvation on fluorescent *in situ* hybridization of *Rhodopseudomonas palustris. FEMS Microbiol Ecol* **32:** 205-213

Oerther, D. B., Pernthaler, J., Schramm, A., Amann, R., and Raskin, L. (2000) Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. *Appl Environ Microbiol* **66:** 2154-2165.

Poulsen, L. K., Ballard, G., and Stahl, D. A. (1993) Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl Environ Microbiol* **59:** 1354-1360.

Rappé, M.S., Kemp, P.F., and Giovannoni S.J. (1997) Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnol Oceanogr* **42(5):** 811-826.

Ravenschlag, K., Sahm, K., Pernthaler, J., and Amann, R. (1999) Genbank direct submission. Accession number: AJ241009.

Schlesner, H. (1994) The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. *Syst Appl Microbiol* **17:** 135-145.

Schlesner, H., and Stackebrandt, E. (1986) Assignment of the genera *Planctomyces* and *Pirella* to a new family *Planctomycetaceae* fam. nov. and description of the order *Planctomycetales* ord. nov. *Syst Appl Microbiol* **8:** 174-176.

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.-H., and Wagner, M. (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Syst Appl Microbiol* **23:** 93-106.

Sekiguchi Y., Kamagata Y., Syutsubo K., Ohashi A., Harada H., Nakamura K. (1998) Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiology* **144:** 2655-2665.

Strous, M. (2000) Microbiology of anaerobic ammonium oxidation. PhD thesis, Delft, The Netherlands: Delft University Press, ISBN 90-9013621-5.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R.I., Kuenen, J.G., and Jetten, M.S.M. (1999) Missing lithotroph identified as new planctomycete. *Nature* **400:** 446-449.

Strous, M., Heijnen, J. J., Kuenen, J. G., and Jetten, M. S. M. (1998) The sequencing batch reactor as a powerful tool to study very slowly growing micro-organisms. *Appl Microbiol Biotechnol* **50:** 589-596.

Strunk, O., and Ludwig W. (1997) ARB software program package; http://www.biol.chemie.tu-muenchen.de/pup/ARB/.

Van der Meer, J. R., Werlen, C., and Siegrist, H. (1999) Genbank direct submission. Accession number: AJ224943

Wagner, M., Amann, R., Kämpfer, P., Aßmus, B., Hartmann, A., Hutzler, P., Springer, N., and Schleifer, K.-H. (1994) Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. *Syst Appl Microbiol* **17:** 405-417.

Wagner, M., Rath, G., Amann, R., Koops, H.-P., and Schleifer, K.-H. (1995) *In situ* identification of ammonia oxidizing bacteria. *Syst Appl Microbiol* **18:** 251-264.

Ward, N., Rainey, F.A., Stackebrandt, E. and Schlesner, H. (1995) Unraveling the extent of diversity within the order *Planctomycetales*. *Appl Environ Microbiol* **61:** 2270-2275.

Ward, N.L., Rainey, F.A., Hedlund, B.P., Staley, J.T., Ludwig, W., and Stackebrandt, E. (2000) Comparative phylogenetic analyses of members of the order *Planctomycetales* and the division *Verrucomicrobia*: 23S rRNA gene sequence analysis supports the 16S rRNA gene sequence-derived phylogeny. *Int J Syst Evol Microbiol* **50:** 1965-1972.

# Appendix F

Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis

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# Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis

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**Abstract**

In this study we present a new approach to determine the volume, the heterogeneity factors, and the composition of the bacterial population of activated sludge flocs by 3D confocal imaging. After staining the fresh flocs with FITC (fluorescein-isothiocyanate), 75 stacks of images (containing approx. 3000 flocs) were acquired with a Zeiss LSM 510 confocal laser scanning microscope. The self-developed macro 3DVASD (3 dimensional volume and surface determination) for the Zeiss Vision KS 400 software combined the images of one stack to a 3D image and calculated the real floc volume and surface. We determined heterogeneity factors like the ratio of real floc surface to the surface of a sphere with the respective volume and the fractal dimension ($D_f$). According to their significant influence on floc integrity and quality, we also investigated the chemical composition of the flocs and quantified their bacterial population structure by using group-specific rRNA-targeted probes for fluorescence in situ hybridization. By a simple settling experiment we enriched flocs with poor settling properties and determined the above mentioned parameters for different stages of the enrichment. This approach revealed shifts in floc volume, heterogeneity, and bacterial and chemical composition according to the settling quality of the flocs.

*Keywords:* sludge settling, confocal laser scanning microscopy (CLSM), floc volume, floc heterogeneity, fluorescence in situ hybridization (FISH)

## 1. Introduction

A prominent problem when wastewater is treated using activated sludge treatment plants is poor settling properties of activated sludge flocs in the secondary clarifier. This often leads to a decreased performance of the treatment plant and in the worst case to environmental pollution.
The settling properties of activated sludge can be affected by a number of factors such as the median floc size and floc heterogeneity [1, 2], by growth of filamentous or zoogloeal bacteria [e.g. 3, 4, 5, 6], and by the amount and composition of extracellular polymeric substances (EPS) in the sludge [7]. The relative importance of these factors is, however, not always well understood [8], so reliable methods to study settling characteristics and floc properties are important. The settling properties of the sludge are often characterized by measurement of the sludge volume index (SVI). However, this method does not provide any detailed knowledge about the floc properties, only about macroscopic settling properties [9]. Therefore, various studies have focused on other methods for a more comprehensive characterization of activated sludge flocs.
Floc size distributions were previously determined by light microscopy [10, 11, 12]. This approach revealed that most particles have a diameter lower than 5 µm while the major part of the volume is

reflected by flocs with a diameter in the range of 68-183 µm. Since light microscopic studies are tedious, sizes of bacterial aggregates and their heterogeneity in terms of fractal dimensions were recently characterized by a light scattering approach [13]. This technique, though fast, provides only an indirect insight into physical floc properties with no information about the microbial community structure or EPS composition.

In this study, we have combined confocal laser scanning microscopy (CLSM) with image analysis to provide a direct and fast determination of floc volume and architecture. We applied this new approach to characterize activated sludge flocs with good and poor settling properties. Furthermore, CLSM based applications for quantitative fluorescence in situ hybridization (FISH) were used to detect the population structure of the activated sludge flocs. An additional determination of the chemical composition of the flocs allowed us to detect differences in the physical structure, chemical composition, and in the microbial composition in poor settling flocs compared to the average flocs from industrial wastewater treatment plants.

## 2. Materials and Methods

### *2.1. Sampling*

Activated sludge samples were taken from the aeration basins from the plants listed and described in Table 1. The samples were immediately stored on ice.

Table 1
Characteristics of analyzed municipal activated sludge samples [Sludge volume index (SVI) and suspended solids (SS) data were determined in the laboratories of the respective wastewater treatment plant (WWTP)]. PE = population equivalent. ND = not determined.

| Sludge samples analyzed | PE | SVI | Suspended Solids [gSS/l] |
|---|---|---|---|
| Semitechnical plant Großlappen | ND | 125 | ND |
| WWTP Dietersheim; high load stage | 2100000 | 72 | 3.7 |
| WWTP Großlappen; high load stage | 2000000 | 103 | 3.3 |
| WWTP Poing | 110000 | 137 | 2.9 |

### *2.2. Fractionation of activated sludge flocs*

For the separation of good and poor settling activated sludge flocs, respectively, we used the strategy displayed in Figure 1. Each of the undiluted sludge samples was applied to three cylinders with a height of 22 cm and a diameter of 8 cm. The flocs were allowed to settle, so the poor settling flocs were enriched in the upper part of the supernatant. Samples were taken using a glass pipette (without tip to avoid shearing effects) at a height of about 5 cm within the supernatant at the beginning of the experiment. Since previous settling experiment showed that an almost complete solid-liquid separation took place after about 20 min, samples were taken after 20 minutes of settling. Aliquots of these samples were immediately processed for analysis of the physical, chemical and biological parameters and compared to unsettled samples.

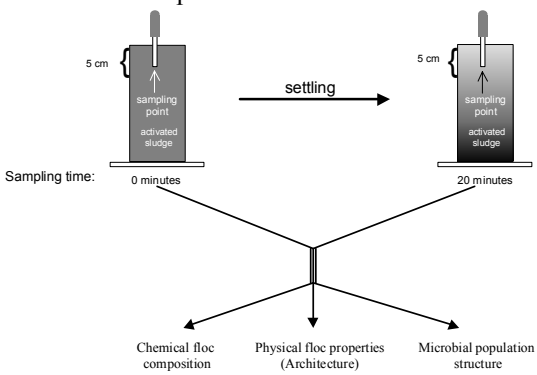


Fig.1. Experimental setup used for enrichment of flocs with poor settling properties.

### *2.3. Staining with FITC*

Samples were vortexed for 5 sec prior to and after staining with FITC (Fluorescein-isothiocyanate, Merk Darmstadt, Germany) to destroy low - energy aggregation between flocs. Staining was performed for 3 hour by addition of 25µl of FITC - stock solution (1% FITC in dimethyl - formamide, Merck Darmstadt, Germany) to 1 ml of the native sample. All floc structures were best stained by FITC, while the Fluorescent Brightener 28 (Sigma-Aldrich, Steinheim, Germany), and the negative staining with fluoresceine (Sigma-Aldrich, Steinheim, Germany) yielded poorer results (data not shown). Excess dye was removed by carefully replacing the supernatant of the settled stained samples with fresh, 0.2 µm - filtered sample liquid. Samples were diluted in 0.2 µm – filtered sample liquid to gain single flocs and immediately viewed with a 40x objective (C-Apochromat, Zeiss, Jena, Germany) using a confocal laser scaning microscope (CLSM).

*2.4. Fluorescence in situ hybridization*

The oligonucleotide probes used are listed in Table 2. They were purchased as Cy3, and Cy5 labeled derivatives from Thermo Hybaid Interactiva division (Ulm, Germany). Flocs were fixed and analyzed by FISH as described by Amann [14].

Table 2
Oligonucleotide probes used in this study. The bacterial target group of each probe is listed together with the appropriate reference. The bacterial nomenclature proposed in the taxonomic outline (released 1. April 2001) of the second edition of Bergey's manual of systematic bacteriology (http://www.cme.msu.edu/bergeys/) was used.

| Oligonucleotide probe | Specificity | **Ref.** |
|---|---|---|
| Alf968 | many *Alphaproteobacteria* | [30] |
| Bet42a | *Betaproteobacteria* | [31] |
| Gam42a | *Gammaproteobacteria* | [31] |
| Pla46 | *Planctomycetes* | [32] |
| CF319a | *many* Bacteroidetes | [33] |
| Hgc69a | *Actinobacteria* | [34] |
| Lgcb+c[1)] | many *Firmicutes* | [35] |
| Eub338 | *many but not all* Bacteria | [36] |
| Eub338II | Bacterial lineages not covered by probe EUB338 | [37] |
| Eub338III | Bacterial lineages not covered by probe EUB338 and EUB338II | [37] |

[1)] An equimolar mixture of Lgcb and Lgcc was used for FISH

*2.5. Microscopy, image analysis, and quantification of probe target bacteria*

For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364nm), an Ar ion laser (458 and 488nm), and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). For floc volume and surface determinations 3D image stacks were acquired with the CLSM and processed by the macro 3DVASD (3 Dimensional Volume and Surface Determination) for the Carl Zeiss Vision KS400 software package, developed in this study. The floc volume was computed from the acquired 3D image stacks by enlargement of the pixels of each single slice by half of the slice distance in both directions of the z-axis. Resulting voxels which form a structure without gaps were counted and multiplied by the volume of one voxel given by the dimension of the pixels of the slices in x- and y-direction and the slice distance. Classes were defined in order to group the flocs according to their volume (Table 3). The overall surface of a floc was determined by adding voxel surfaces of one floc, which did not touch other voxels. Non-stained enclosures within the floc structure were filled out by the software. The surface information measured by the macro 3DVASD from CLSM acquired images was also used to determine the ratio of the real surface of a floc to the surface of a sphere with the respective volume ($S_{floc}$/ $S_{sphere}$). Since the Carl Zeiss Vision KS400 software package is not able to compute the original diameters of the flocs, diameters to the corresponding volume classes were determined with the following equation:

$$d_{floc} = \sqrt{S_{floc} / S_{sphere}} * d_{ulvc}$$

$d_{floc}$: deduced diameter of an original floc with the volume of the upper limit of the respective volume class

$S_{floc}/S_{sphere}$: ratio of the original surface computed by the Kontron KS 400 software and the surface of a sphere with the respective volume of the floc (see below).

$d_{ulvc}$: diameter of the sphere with the volume of the upper volume class limit.

The $d_{floc}$ values were taken as the upper diameter class limit corresponding to the respective volume class and plant. Diameters and heterogeneity factors ($S_{floc}/S_{sphere}$) to the corresponding volume classes and plants are given in Table 3.

The fractal dimension ($D_f$) was deduced out of single 2D image sections from the CLSM acquired 3D image stacks by using the two-point correlation function mentioned in Thill *et al.* [15]. For each analysis, the $D_f$ value of 20 randomly selected sections was determined.
The filament indices were determined according to [4]. Quantification of probe-labeled bacterial populations was performed as described by Schmid *et al*. [16] and Daims *et al.* [17].

*2.6. Determination of the chemical composition*

The chemical composition of the activated sludges in term of total protein, carbohydrate, DNA, and humic substances was measured as described by Frølund *et al.,* [18].

Table 3.
Transfer of volume classes into the respective diameters

| | Dietersheim | | | | Großlappen | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 min | | 20 min | | 0 min | | 20 min | |
| Volume classes [µm³] | $S_{floc}/S_{sphere}$ | Diameter classes [µm] | $S_{floc}/S_{sphere}$ | Diameter classes [µm] | $S_{floc}/S_{sphere}$ | Diameter classes [µm] | $S_{floc}/S_{sphere}$ | Diameter classes [µm] |
| 17-32 | 2.00 | 4.51-5.57 | 2.12 | 4.65-5.74 | 2.16 | 4.69-5.79 | 2.22 | 4.75-5.86 |
| 33-64 | 2.01 | 5.58-7.03 | 2.13 | 5.75-7.25 | 2.19 | 5.80-7.34 | 2.29 | 5.87-7.51 |
| 65-128 | 1.98 | 7.04-8.79 | 2.13 | 7.26-9.13 | 2.27 | 7.35-9.43 | 2.41 | 7.52-9.71 |
| 129-256 | 2.02 | 8.80-11.2 | 2.11 | 9.14-11.5 | 2.32 | 9.44-12.0 | 2.56 | 9.72-12.61 |
| 257-512 | 2.09 | 11.2-14.3 | 2.16 | 11.5-14.6 | 2.38 | 12.0-15.3 | 2.72 | 12.6-16.4 |
| 513-1024 | 2.07 | 14.4-18.0 | 2.32 | 14.6-19.1 | 2.44 | 15.3-19.6 | 2.91 | 16.4-21.3 |
| 1025-2048 | 2.11 | 18.0-22.9 | 2.31 | 19.0-24.0 | 2.61 | 19.6-25.5 | 3.10 | 21.4-27.8 |
| 2049-4096 | 2.39 | 22.9-30.7 | 2.36 | 24.0-30.5 | 2.86 | 25.5-33.6 | 3.10 | 27.8-35.0 |
| 4097-8192 | 2.47 | 30.7-39.3 | 2.72 | 30.5-41.2 | 3.26 | 33.6-45.2 | 3.02 | 35.0-43.5 |
| 8193-16390 | 2.26 | 39.3-47.4 | 2.69 | 41.24-51.7 | 3.52 | 45.2-59.1 | 3.83 | 43.5-61.7 |
| 16390-32770 | 2.56 | 47.4-63.6 | 2.82 | 51.7-66.7 | 4.38 | 59.1-83.1 | 4.36 | 61.7-82.9 |
| 32770-65540 | 2.81 | 63.6-83.9 | 3.56 | 66.7-94.3 | 5.00 | 83.1-112 | 4.61 | 82.9-107 |
| 65540-131100 | 3.24 | 83.9-113 | 4.31 | 94.3-131 | 4.91 | 112-140 | 5.39 | 107 -146 |
| 131100-262100 | 3.63 | 113-151 | 5.31 | 131-183 | 5.64 | 140-189 | 7.30 | 146-215 |
| 262100-524300 | 4.88 | 151-221 | 5.44 | 183-233 | 6.78 | 189-261 | 9.13 | 215-302 |
| 524300-1049000 | 5.43 | 221-294 | 6.50 | 233-321 | 8.28 | 261-363 | 11.38 | 302-425 |
| 1049000-2097000 | 6.96 | 294-419 | 7.58 | 321-437 | 8.64 | 363-467 | 12.34 | 425-558 |

## 3. Results

### *3.1. Evaluation of floc volume determination by CLSM and image analysis*

To determine the number of flocs which need to be analyzed to get a reliable statistical floc volume distribution, the volumes of 600, 3000, 6000, and 15000 randomly selected flocs originating from the same activated sludge sample from Dietersheim wastewater treatment plant (wwtp) were measured. Pronounced differences were observed in the floc volume distributions (plotted versus total volume of all flocs or total floc number) inferred from 600, 3000 and 6000 analyzed flocs, while almost identical volume distributions were obtained from 6000 and 15000 measured flocs (Figure 2 A/B). The 6000 flocs corresponded to approx. 75 confocal images. Consequently, the time required for sample treatment (staining, wash steps etc.) and image acquisition (without data analysis) was approx. 7 hours for a single sample. In this period the floc volume distribution within a sample remained almost constant (see below).

The largest distance between confocal optical sections which can be applied without significant decrease of the accuracy of the analysis, was defined by taking 20 CLSM stack images of an activated sludge sample using 1 µm distance intervals between the optical sections (distances <1 µm were not analyzed since they are not practicable due to bleaching effects caused by the extended excitation time). After data acquisition, individual sections were removed from each stack image to obtain modified data sets with distances between the sections of 2, 4, and 8 µm. The use of section distances of 1, 2, and 4 µm resulted in only marginal variations in the determined floc volumes and distributions (Figure 3 A/B). The total volumes of all measured flocs using 1, 2, and 4 µm section distance, respectively, differed only by 3.5%. However, increase of section distance to 8 µm led to significant alterations of floc volumes and distributions [e.g. the smallest volume class 17-32 $\mu m^3$ (diameter 4.51-5.57 µm; Table 3) is not represented any more, but a new largest volume class 262145 - 524288 $\mu m^3$ (diameter 151-221 µm; Table 3) is generated - see Figure 3 A/B]. This is also reflected by a 25% overestimation

of the total floc volume using 8 µm as section distance.

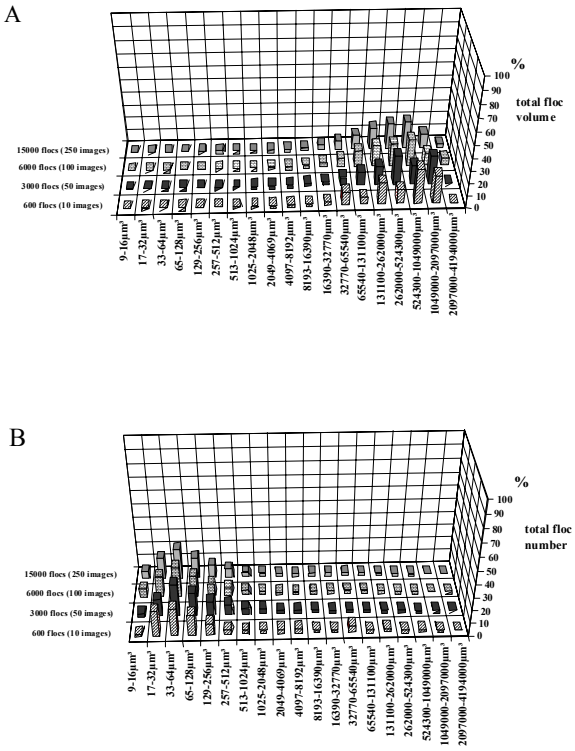


Fig. 2. Influence of the number of analyzed flocs on floc volume distributions. For each volume class the percentage of the total floc volume (A) and the total number of analyzed flocs (B) is given.

Transport and storage of activated sludge samples might influence the number, shape, and volume of the activated sludge flocs. Thus, the influence of sample storage was investigated with sludge from Dietersheim wwtp, which is located closely to the CLSM laboratory. An aliquot was stained and observed immediately after sampling. Other aliquots were examined after storage times of 6 hours, 1, 2, 3 and 5 days at 4°C, respectively. For each analysis, 6000 flocs were recorded at room temperature. Figure 4 shows the floc volume distributions plotted against total floc volume (A) and total floc number (B) after the different storage times. The storage did not cause any pronounced shifts in the volume distributions. However, slight variations were observed especially for larger flocs [65537 - 2097152 µm$^3$ (diameter 83,8-419 µm; Table 3); Figure 4 A].

*3.2. Physical floc properties*

The physical floc properties were determined for the semitechnical plant in Großlappen and the Dietersheim wwtp. Generally, the small flocs were

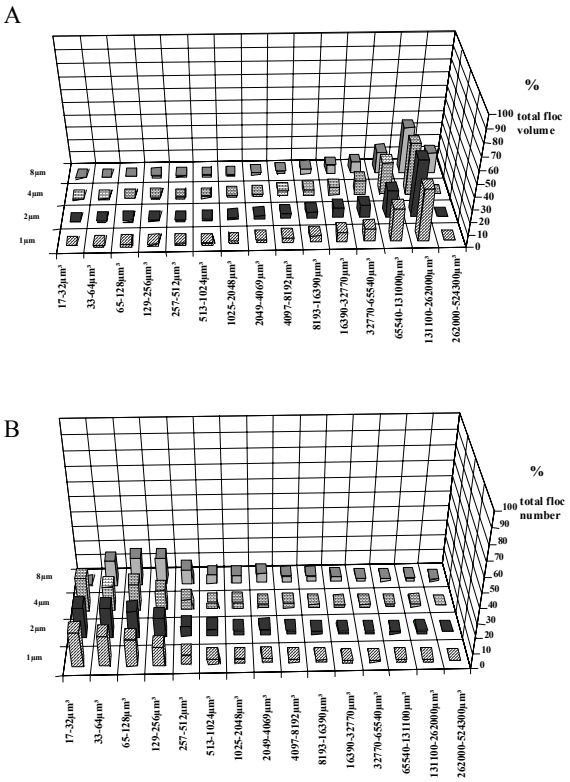


Fig. 3. Influence of section distance on floc volume distribution. For each volume class the percentage of the total floc volume (A) and the total number of analyzed flocs (B) is given

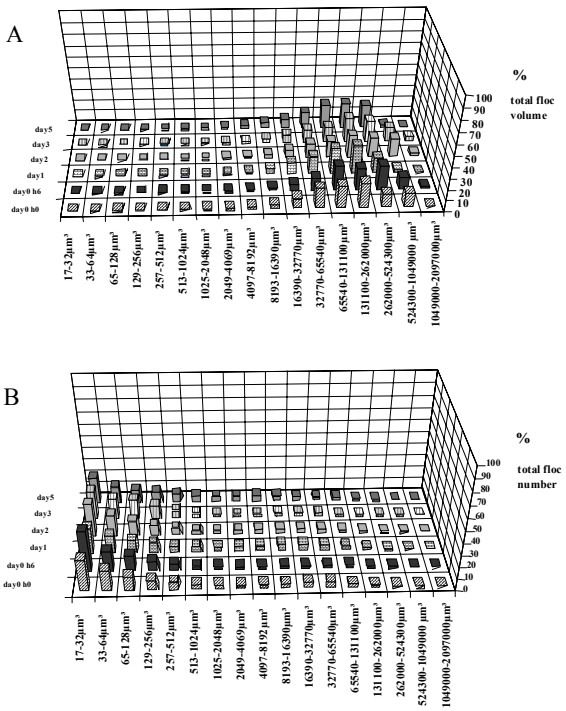


Fig. 4. Influence of storage time on floc volume distribution. For each volume class the percentage of the total floc volume (A) and the total number of analyzed flocs (B) is given.

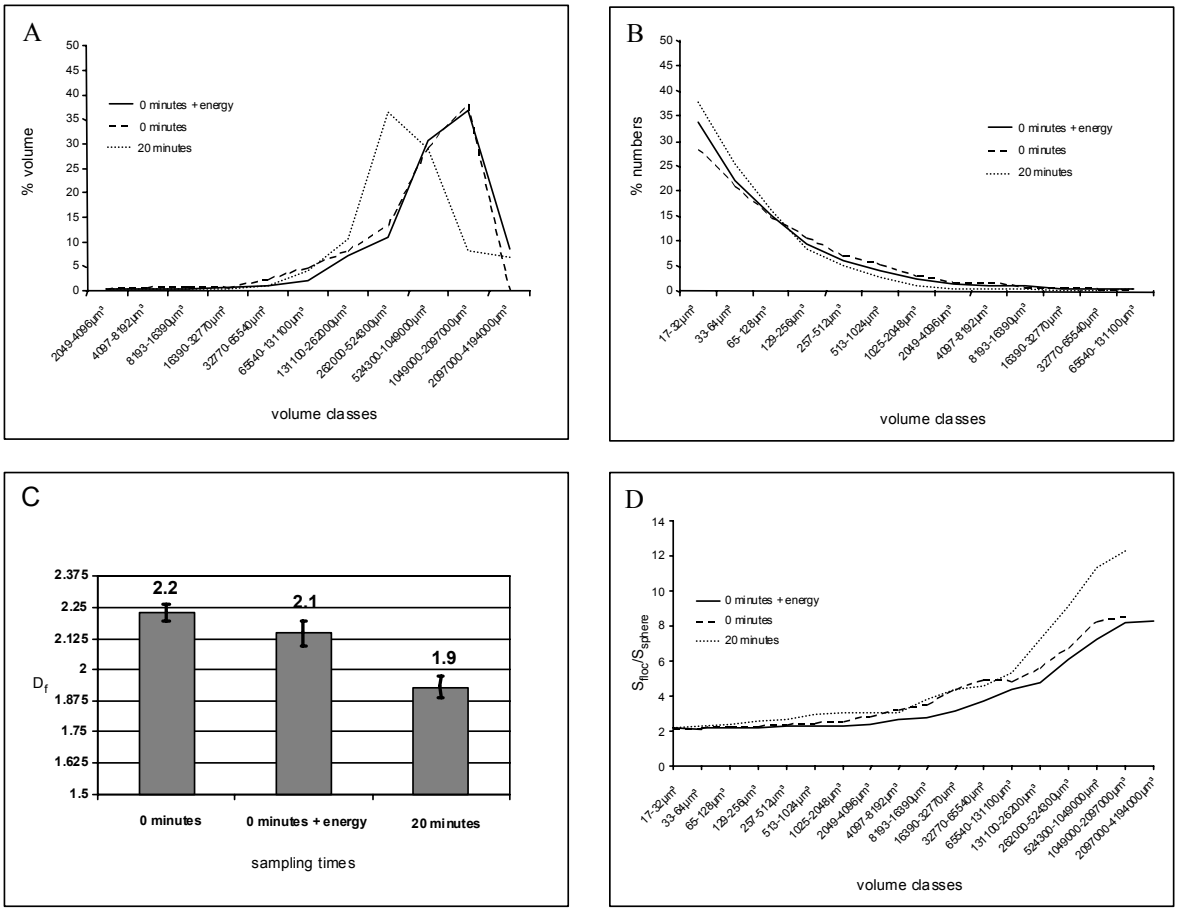


Figure 5. A/B: Volume distribution of activated sludge flocs from Großlappen after enrichment for flocs with poor settleability and application of energy (by vortexing to break up weak adheasions of flocs), respectively. For each volume class the relative contribution to the total volume of all flocs (A) and the relative contribution to the total number of all flocs (B), which were analyzed, is given. C: $D_f$ values of activated sludge flocs after enrichment for flocs with low settleability and application of energy, respectively. For each analysis, the $D_f$ of twenty activated sludge flocs was determined. Error bars indicate the standard error. D: $S_{floc}/S_{sphere}$ of activated sludge flocs after enrichment for flocs with poor settleability and application of energy, respectively

most abundant [below 256 µm$^3$ (diameter of about 9 µm; Table 3) Figure 5 and Figure 6 A and B], while the large ones comprised almost the complete volume [above 131073 µm$^3$ (diameter above about 140 µm; Table 3)]. The poor settling flocs remaining in the supernatant after 20 minutes of settling in Dietersheim were slightly more voluminous (Figure 6A) and the abundance of small flocs decreased (Figure 6B). In contrast, the poor settling flocs in Großlappen showed a shift to smaller volumes in the larger volume classes [above 131073µm$^3$ (diameter above about 140 µm; Table 3); Figure 6A]. A significant increase in the number of small flocs was not visible (Figure 6B).

In both plants, the fractal dimension ($D_f$) of the poor settling flocs had mean values (1.9 for Großlappen and 1.8 for Dietersheim) lower than the $D_f$ of the original sludge flocs (2.2 for Großlappen and 2.1 for Dietersheim), (Figure 5 C and 6 C), indicating that the poor settling flocs were more heterogeneous. This is also supported by measurement of the ratio of the floc surface to the surface of a sphere with an identical volume ($S_{floc}/S_{sphere}$), which showed a marked increase for poor settling flocs in both plants (Figure 5 D and 6 D).

Table 4
Percental share of DNA, carbohydrates, proteins, and humic substances of the total chemical composition of activated sludge flocs of wwtps Dietersheim, Großlappen, and Poing prior to and after enrichment for flocs with bad settling properties.

| | | DNA | Carbo-hydrates | Proteins | Humic substances |
|---|---|---|---|---|---|
| Dietersheim | 0 min | 0.43 | 10.8 | 26.4 | 62.4 |
| | 20 min | 0.92 | 6.3 | 34.3 | 58.5 |
| Großlappen | 0 min | 0.18 | 15.9 | 24.9 | 59.0 |
| | 20 min | 2.64 | 14.2 | 27.2 | 55.9 |
| Poing | 0 min | 0.20 | 17.8 | 28.6 | 53.4 |
| | 20 min | 1.20 | 12.8 | 27.0 | 59.1 |

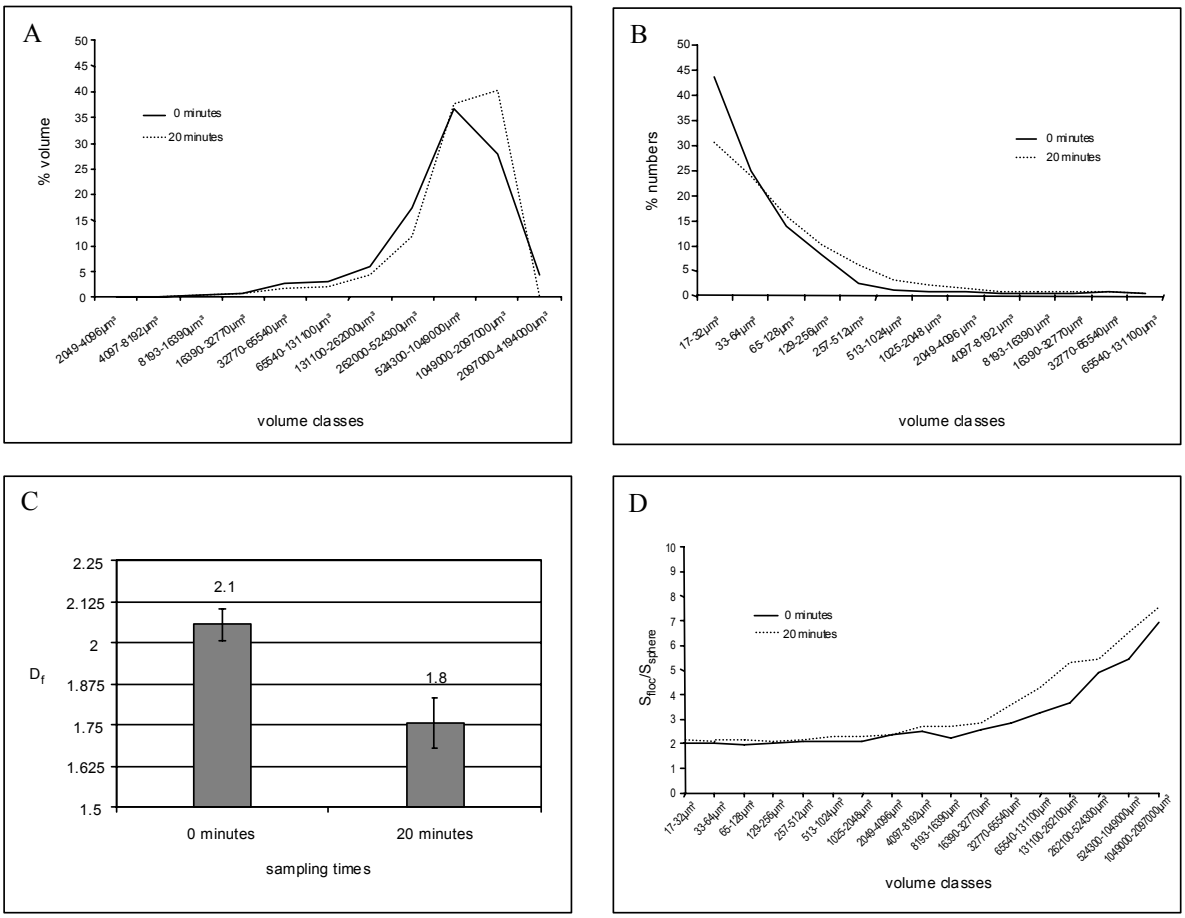


Fig. 6. A/B: Volume distribution of wwtp Dietersheim activated sludge flocs after enrichment for flocs with poor settleability. For each volume class the relative contribution to the total volume of all flocs (A) and the relative contribution to the total number of all flocs (B), which were analyzed, is given. C: $D_f$ values of activated sludge flocs after enrichment for flocs with poor settleability. For each analysis, the $D_f$ of twenty activated sludge flocs was determined. Error bars indicate the standard error. D: $S_{floc}/S_{sphere}$ of activated sludge flocs of wwtp Dietersheim after enrichment for flocs with bad settleability and application of energy, respectively.

### *3.3. Chemical composition and population structure of activated sludge*

The chemical composition of the solids was determined in the activated sludge samples from the three wwtps (Table 4). In all plants the relative carbohydrate content decreased while the DNA content increased when the poor settling flocs were enriched. In contrast, the change in the content of humic substances and proteins did not show a general pattern as it increased, decreased or remained constant in the wwtps investigated.

In this study, the group-specific probes listed in Table 2 were applied to investigate the microbial population structure of the activated sludge of the different wwtps (Figure 7). The bacterial composition differed significantly. Activated sludge from all three plants was dominated by *Betaproteobacteria* and *Actinobacteria* (Figure 7). In Poing also *Firmicutes* were numerous. In medium amounts, members of the *Alpha-* and *Gammaproteobacteria* could be detected in Dietersheim and Poing. *Bacteroidetes* and *Planctomycetes* played only a minor role in these two sludges (Figure 7 A/C). The Großlappen sludge contained the most heterogeneous bacterial population (Figure 7 B) and a relatively high proportion of the bacteria could not be identified by any of the probes applied (18%).

After enrichment for poor settling sludge two major shifts in the population could be observed in the Dietersheim sludge (Figure 7 A). The *Betaproteobacteria* decreased from about 62% to 40%, and the percentage of *Alphaproteobacteria* was only about half of the original (about 15% to 7%).

In poor settling sludge from Großlappen all bacteria could be detected with the applied group-specific oligonucleotide probes and the relative abundance of the *Alpha*, *Beta-Gammaproteobacteria* and *Firmicutes* raised accordingly. This increase was most significantly for *Gammaproteobacteria* (from about 6% to 12%).

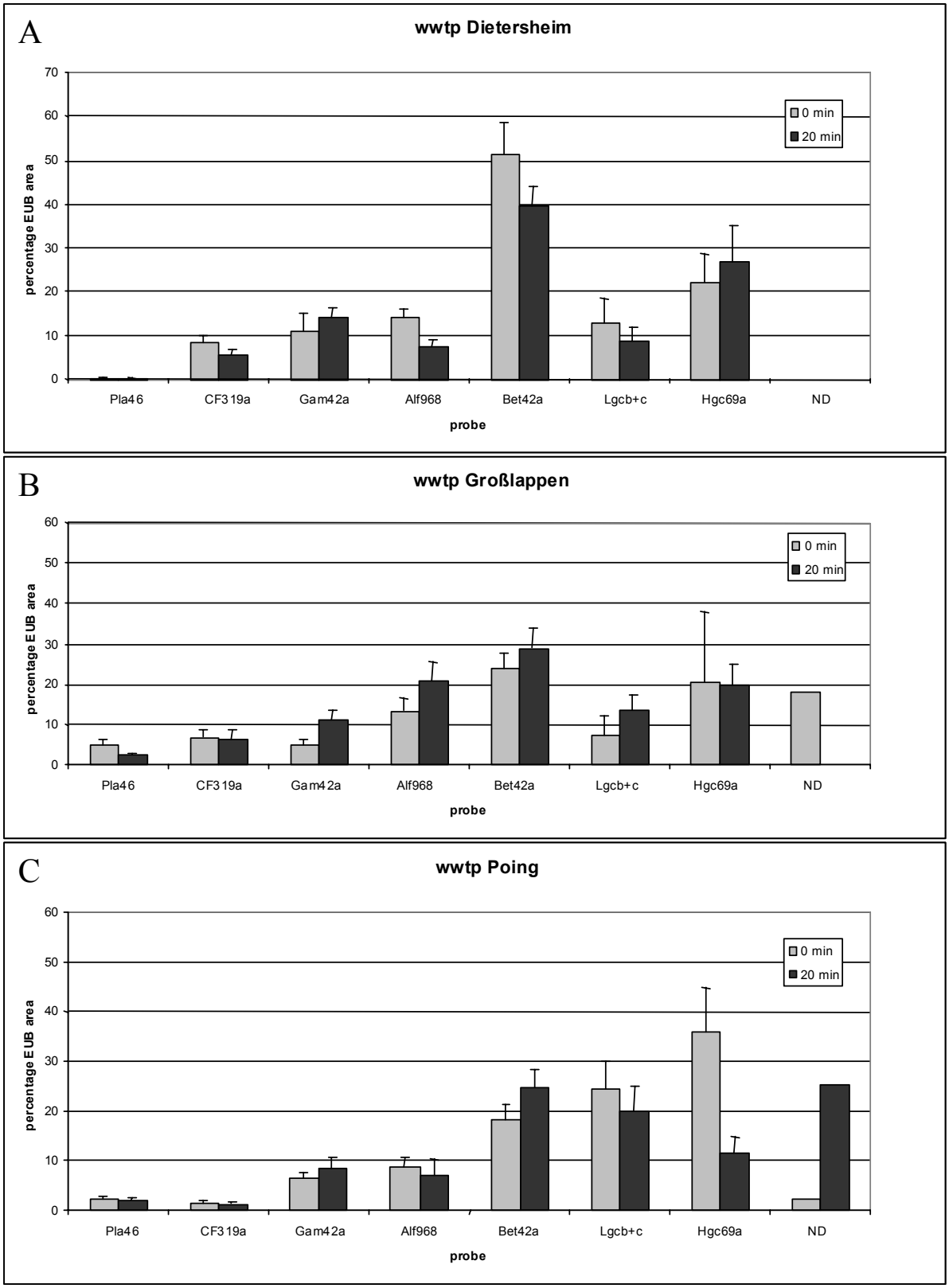


Fig. 7. Microbial population structure of the activated sludge samples prior to and after enrichment for flocs with poor settling properties of wwtps **A:** Dietersheim, **B:** Großlappen and **C:** Poing. (for probe details please refer to Table 2). ND := not determined = bacterial population detected by the EUB probe mix but not affiliated to any of the bacterial groups for which specific probes were applied. Error bars indicate the standard error.

In the poor settling flocs from Poing the abundance of *Actinobacteria* dropped significantly (from 35% to 10% of all bacteria with the EUB probe mixture) after enrichment for poor-settling flocs. A large fraction (about 25%) of the bacterial population in the poor settling flocs could be detected only with the EUB probe mixture (Figure 7 C) and must thus be affiliated with bacterial lingeages for which no specific probes were applied.
Additionally, a general shift in the filament index to higher values (from 2 to 4 for Dietersheim and Großlappen, and from 3 to 5 for Poing, respectively) could be observed for all wwtps after enrichment for poor settling flocs.

## 4. Discussion

### *4.1. Floc volume determined by CLSM and image analysis*

Compared to previous approaches to determine floc structure [13, 19] the use of the CLSM is relatively fast and flexible. To obtain statistically reliable results 75 stacks with ca. 6000 flocs had to be acquired. It took a few hours which potentially could have influenced the floc structure, but as long as the sludge was cooled down or not supplied with substrate, no changes could be detected during data acquisition or during a couple of days storage.
The accuracy of the floc volume determinations depends on the distances between the optical sections. The lower limit for the CLSM is 0.1 µm, but we found a section distance of 4 µm as an optimal compromise as smaller section distances resulted in (i) time intensive measurements, (ii) extended excitation times causing strong fluorochrome bleaching, and (iii) accumulation of large amounts of digital data.
Previous studies used diameter classes [11, 12] rather than volume classes for the description of floc sizes. This method though not combined with a high technological effort does not take into account that it deals with three dimensional structures. A volume measurement is in this respect certainly more accurate. However, the software applied in this study was not able directly to compute the equivalent diameters and therefore they had to be deduced from volume and heterogeneity data in order to compare with previously published data (see materials and methods section). It should be noted that these diameter values only allow a relatively rough comparison and should not be taken as absolute values.

### *4.2. Physical and chemical properties of activated sludge flocs*

The typical floc size distribution is described in various publications [e.g. 11, 12, 20] as a curve with a peak at small particles with a diameter of about 0.5 to 5 µm and one for large particles with a diameter of 30 to 1000 µm. Our results generally obey these findings, but pronounced differences in the floc size distributions and heterogeneity could be found after enrichment for poor settling sludge.
The poor settling flocs in Großlappen were characterized to be smaller flocs compared to the original sludge. Furthermore, there was a significant increase in the number of filaments as indicated by a change in filament index from 2 (few filaments) to 4 (many filaments). In Dietersheim only a slight shift in the floc volume could be detected while the number of filaments also increased significantly. Thus, there seemed to be an enrichment of filaments in the poor settling flocs in both plants.
In contrast to the volume determinations (see above), consistent trends were observed for the fractal dimension of the flocs during enrichment for flocs with poor settleability. $D_f$ of the flocs with a bad settleability were in both plants characterized by a more heterogeneous structure ($D_f$ of 1.9 and 1.8, respectively; Figure 5 C and 6 C) than the flocs from the original activated sludges ($D_f$ of 2.2 and 2.1, respectively; Figure 5 C and 6 C). The ratio of the floc surface to the surface of a sphere of an identical volume ($S_{floc}/S_{sphere}$) also describes the structural heterogeneity of activated sludge flocs. Interestingly, the overall value for $S_{floc}/S_{sphere}$ of large flocs was significantly higher for Großlappen than for Dietersheim indicating a higher heterogeneity in the overall floc structure in Großlappen. This finding is not supported by the $D_f$ values. In this respect it seems that the $D_f$ reflects a tendency within one sludge plant, but cannot be used to compare different plants. It is tempting to speculate that a higher $S_{floc}/S_{sphere}$ of large flocs is indicative for a higher SVI since the sludge from the Großlappen plant has a higher SVI (125) compared to the sludge from Dietersheim (72). The results might indicate that the difference in the overall settling properties as indicated by SVI (Großlappen 125 and Dietersheim 72) was due mainly to larger and more heterogeneous flocs.
The chemical analysis of the activated sludge showed a composition similar to other sludge types with protein as the major compound [8]. For all treatment plants, the relative carbohydrate content

decreased and the relative DNA content increased if flocs with poor settleability were enriched. Changes in the content of humic substances and proteins in all wwtps investigated seemed not to obey general rules. Carbohydrate is likely connected to EPS in tight microcolonies or intracellular storage material (as glycogen), so this could indicate that this biomass fraction is enriched in good settling flocs. However, this assumption is inconsistent with the observed increase in DNA content in poor settling flocs. Thus, there may not exist any substantial chemical difference between the different floc types, or the categories total humic substances, carbohydrates, proteins and DNA are too broad a measure to provide sufficient resolution for linking chemical composition with floc sizes or floc structure.

*4.3. Population structure of activated sludge flocs*

Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is the current gold standard for cultivation-independent in situ identification of microorganisms. In combination with confocal laser scanning microscopy and digital image analysis quantitative data of the composition of the microbial populations in activated sludge flocs and biofilms can be obtained [17, 21, 22, 23]. In accordance with previous investigations [24, 25, 26, 27, 28] two of the activated sludge samples analyzed were dominated by *Betaproteobacteria*. Members of this subclass are for example most lithoautotrophic ammonia-oxidizers, *Zoogloea spp.*, *Sphaerotilus natans*, and *Azoarcus* spp., the latter genus was recently identified to encompass important denitrifiers in wwtps [23]. *Actinobacteria* (e.g. *Nocardia* spp.; *Rhodococcus* sp.) also played a numerically important role in all samples analyzed and dominated in the Poing plant.

Pronounced shifts in the microbial population structure of the activated sludge flocs from three different wastewater treatment plants were observed after enrichment for poor settling flocs (Figure 7). These shifts demonstrate links between the settling property of a floc and its microbial community composition. However, using the group specific probes the community shifts induced by enrichment for poor settling flocs did not follow a general tendency. This finding most likely reflects that different bacterial populations influence the settling properties in the different wwtps investigated. Furthermore, the application of group specific probes does not allow to observe population shifts within the respective bacterial groups. Future research should attempt to apply the full-cycle rRNA approach [23, 29] for a high resolution, comparative analysis of the microbial community composition of activated sludge prior to and after enrichment for poor settling flocs. This approach will almost certainly allow to identify bacterial key populations enriched in flocs with good or bad settleability.

**5. Conclusions**

1. Confocal laser scanning microscopy in combination with image analysis is a powerful method for direct determination of the floc volume, heterogeneity factors and the population structure of activated sludge flocs.
2. The physical, chemical and microbial floc properties are more or less specific for each treatment plant. Therefore, a detailed understanding of variations in sludge settling properties in different treatment plants, e.g. a certain malfunction, requires information about all factors.
3. Certain bacterial groups are enriched in flocs with good or poor settling properties in a specific treatment plant.

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**References**

[1] Sadalgekar VV, Mahajan BA, Shaligram AM. Evaluation of sludge settleability by floc characteristics. J Wat Poll Cont Fed 1988; 60:1862-63.

[2] Andreadakis AD. Physical and chemical properties of activated sludge floc. Water Res 1993;27:1707-14.

[3] Wanner J, Grau P. Filamentous bulking in nutrient removal activated sludge systems. Wat Sci Tech 1988;20:1-8.

[4] Jenkins D, Richard MG, Daigger GJ. Manual on the causes and control of activated sludge bulking and foaming. 2. Edition. Lewis Publishers Michigan 48118 U.S.A. 1993.

[5] Novak L, Larrea L, Wanner J, Garcia-Heras JL. Non-filamentous activated sludge bulking caused by *Zoogloea*. Wat Sci Tech 1994;29:301-04.

[6] Wanner J. Activated sludge bulking and foaming control. Technomic Publishing Company, Lancaster U.S.A. 1994.

[7] Urbain V, Block JC, Manem J. Bioflocculation in activated sludge: an analytical approach. Water Res 1993;27:829-38.

[8] Nielsen PH. The activated sludge floc. Encyclopedia in Environmental Microbiology. Ed. G. Bitton. 2002.

[9] Dick RI, Vesilind PA. The sludge volume index – what is it? J Wat Poll Cont Fed 1969;41:1285-91.

[10] Parker DS, Kaufmann WJ, Jenkins D. Physical conditioning of activated sludge flocs. J Wat Poll Cont Fed 1971;43:1817-33.

[11] Li D-H, Ganczarczyk, JJ. Size distribution of activated sludge flocs. Research J Wat Poll Cont Fed 1991;63:806-14.

[12] Jorand F, Zartarian F, Thomas F, Block JC, Bottero JY, Villemin G, Urbain V, Manem J. Chemical and structural (2D) linkage between bacteria within activated sludge flocs. Water Res 1995; 29:1639-47.

[13] Guan, J, Waite, T. D., and Amal, R. Rapid structure characterization of bacterial aggregates. Environ Sci Technol 1998;32:3735-42.

[14] Amann RI. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p. 1-15. In A.D.C. Akkerman, J.D. van Elsas and F.J. de Bruijn (ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dortrecht, Netherlands. 1995.

[15]Thill A, Veerapaneni S, Simon B, Wiesner M, Bottero JY, Snidaro D. Determination of Structure of Aggregates by Confocal Scanning Laser Microscopy. J Colloid Interface Sci 1998;204:357-62.

[16]Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer K-H, Wagner M. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 2000;23:93-106.

[17]Daims H, Ramsing NB, Schleifer K-H, Wagner M. Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence *in situ* hybridization. Appl Environ Microbiol 2001;67:5810-18.

[18]Frølund B, Palmgren R, Keiding K, Nielsen PH. Extraction of exopolymers from activated sludge using a cation exchange resin. Water Res 1996;30:1749-58.

[19]Zartarian F, Mustin C, Villemin G, Ait-Ettager T, Thill A, Bottero JY, Mallet JL, Snidaro D. Three-dimensional modeling of an activated sludge floc. Langmuir 1997;13:35-40.

[20]Barbusinski K, Koscielniak H. Influence on substrate loading intensity on floc size in activated sludge process. Water Res 1995;29:1703-10.

[21] Wagner M, Hutzler P, Amann R. 3-D analysis of complex microbial communities by combining confocal laser scanning microscopy and fluorescence in situ hybridization (FISH).1. In: Digital Image Analysis of Microbes (Wilkinson, M.H.F.; Schut, F., eds.). John Wiley & Sons, Chichester, England. 1998. p. 467-86.

[22] Bouchez T, Patureau D, Dabert P, Juretschko S, Doré J, Delgenès P, Moletta R, Wagner M. Ecological study of a bioaugmentation failure. Environmental Microbiology 2000;2: 179-90.

[23] Juretschko S, Loy A, Lehner A, Wagner M. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst Appl Microbiol 2002;25:84-99.

[24]Wagner M, Amann R, Lemmer H, Schleifer KH. Probing activated sludge with oligonucleotides specific for *Proteobacteria*: inadequacy of culture-dependent methods for describing microbial community structure. Appl Environ Microbiol 1993;59:1520-25.

[25]Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D, Schleifer KH. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl Environ Microbiol 1994;60:792-800.

[26] Kämpfer P, Erhart R, Beimfohr C, Wagner M, Amann R. Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. Microb Ecol 1996;32:101-21.

[27] Wagner M, Amann R. Molecular techniques for determining microbial community structures in activated sludge. In: Microbial community analysis: The key to the design of biological wastewater treatment systems. (T.E. Cloete and N.Y.O. Muyima, eds.) pp.61-72. IAWQ Scientific Technical Report No. 5, University Press, Cambridge. 1997.

[28] Snaidr J, Amann R, Huber I, Ludwig W, Schleifer K-H. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl Environ Microbiol 1997;63:2884-96.

[29] Amann RI, Ludwig W, Schleifer K-H. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 1995;59:143-69.

[30] Neef A. Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Doctoral thesis (Technische Universität München). 1997.

[31] Manz W, Amann R, Ludwig W, Wagner M and Schleifer K-H. Phylogenetic oligonucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst Appl Microbiol 1992;15:593-600.

[32] Neef A, Amann RI, Schlesner H, Schleifer K-H. Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 1998;144:3257-66.

[33] Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer K-H Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology 1996;142:1097-106

[34] Roller C, Wagner M., Amann R, Ludwig W, Schleifer K-H. In situ probing of Gram positive bacteria with high DNA G+C content using 23S

rRNA-targeted oligonucleotide probes. Microbiology 1994;140:2849-58.

[35] Meier H, Amann R, Ludwig W, and Schleifer KH. Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content. Syst Appl Microbiol 1999;22,:186-96.

[36] Amann RI, Binder BJ, Olsen RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 1990;56:1919-25.

[37] Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 1999;22:434-43.

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