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Population Structure and Functional Analyses, by *In Situ* Techniques, of Nitrifying Bacteria in Wastewater Treatment Plants

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Abbreviations

2D	two-dimensional
3D	three-dimensional
A	adenine
ARB	*Arbor* (name of a computer program)
FISH	fluorescence *in situ* hybridization
C	cytosine
CFU	colony forming units
CLSM	confocal laser scanning microscope; confocal laser scanning microscopy
COD	chemical demand of oxygen
DAPI	4,6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EBPR	enhanced biological phosphorus removal
EPS	extracellular polymeric substances
et al.	*et alii*
EtOH	ethanol
FA	fluorescent antibodies
Fig.	Figure
FLUOS	5(6)-carboxyfluorescein-N-hydroxysuccinimide ester
G	guanine
GAO	glycogen accumulating organism
LB	Luria-Bertani (medium)
MAR	microautoradiography
MPN	most probable number
NOB	nitrite-oxidizing bacteria
OD	optical density
OTU	operational taxonomic unit
PAO	polyphosphate accumulating organism
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoates
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RU	relative units
SBBR	sequencing batch biofilm reactor
SBR	sequencing batch reactor
SS	suspended solids
SSCP	single strand conformation polymorphism
T	thymine
TIFF	tagged image file format
T-RFLP	terminal restriction fragment length polymorphism
U	uracile
UV	ultraviolet
VBNC	viable-but-nonculturable
WWTP	wastewater treatment plant

General Introduction

1. *In Situ* Structural and Functional Analysis of Microbial Communities

1.1 The limitations of cultivation-dependent techniques

Reliable techniques to detect, identify, and quantify microorganisms are required for analyzing microbial communities in environmental samples. The simplest solution would be the microscopic identification of microbial cells based on morphological criteria. However, in contrast to animals and plants, the morphology of most microorganisms is rather inconspicuous. As a consequence, additional properties like growth with different carbon and energy sources, base composition of the DNA, and cell wall components have been catalogized (in Bergey's Manual of Systematic Bacteriology, Murray *et al*., 1984 and The Prokaryotes, Balows *et al*., 1992) and are used besides cell morphology to identify bacteria. This approach requires that the bacteria in a sample are isolated and grown as pure cultures. Enrichment and isolation are intrinsically selective, because the cultivation media determine which organisms will grow. This selectivity is best demonstrated by attempts to quantify bacteria in environmental samples. For this purpose, usually diluted suspensions of a sample are streaked onto solid media, and colony forming units are counted subsequently. Alternatively, most-probable-number techniques are used to estimate cell concentrations. Direct microscopic counting of the cells in the same samples, however, reveals in most cases that the cell numbers measured by cultivation-dependent methods are far too low. For example, in seawater samples at best 0.1% (Kogure *et al.*, 1979; Kogure *et al.*, 1980; Ferguson *et al.*, 1984), in freshwater only 0.25% (Jones, 1977), in soil samples 0.5% (Torsvik *et al.*, 1990), and in activated sludge not more than 15% (Wagner *et al.*, 1993; Kämpfer *et al.*, 1996) of the indigenous bacteria could be cultivated. All remaining organisms were obviously unable to grow in the media used for enrichment and isolation. The studies performed on activated sludge showed also that nutrient-rich media favored growth of heterotrophic saprophytes and selected against other bacteria, which were far more abundant in the sludge samples. Similar population shifts were noticed after incubation of seawater samples in complex nutrient media (Ferguson *et al.*, 1984). The significant differences between total cell numbers and the fraction of culturable bacteria in environmental samples were early discovered (Jannasch and Jones, 1959), and are today well known as the "great plate count anomaly" (Staley and Konopka, 1985). This phenomenon is most likely caused by our inadequate knowledge of the growth requirements of most microorganisms. In addition,

culturable bacteria may not be detected in a sample, because they have entered a dormant viable-but-nonculturable (VBNC) state due to unfavorable conditions previously (for a review, see Roszak and Colwell, 1987). These findings altogether indicate that only a small fraction of the microorganisms in nature could be isolated and characterized so far. The *Approved List of Bacterial Names* (Skerman *et al.*, 1989) contains at present a few thousand entries, but this number must be an enormous underestimation of the real microbial diversity (Amann *et al.*, 1995). However, ecological studies dealing with structural and functional features of microbial communities depend on possibilities to detect all microorganisms in the habitats examined. Molecular approaches for the cultivation-independent detection of microorganisms have been developed to meet this requirement. Suitable combinations of these techniques allow to analyze the composition of natural microbial populations not only qualitatively, but also quantitatively. Furthermore, they offer even insights into physiological traits, and thereby into the aut- and synecology, of uncultivated organisms. The following sections explain these approaches as far as they were applied in this thesis.

1.2 Comparative sequence analysis of ribosomal RNA

Bacteria can be classified according to a natural system, which reflects their phylogenetic affiliation, by comparative analysis of marker gene sequences (for reviews, see Woese, 1987 and Ludwig *et al.*, 1998). The most frequently utilized phylogenetic markers are the 16S (prokaryotes) and 18S (eukaryotes) small subunit ribosomal RNAs. Accordingly, the affiliation of unknown bacteria can be determined by comparing their 16S rRNA sequences with the 16S rRNA sequences of other, already classified bacteria. This approach has enormous advantages for microbial ecology: Bacterial 16S rRNA genes can be retrieved from practically every sample by DNA extraction, PCR with suitable primers, and cloning of the amplified DNA fragments. Enrichment or isolation steps are not required. In this manner, 16S rRNA gene libraries can be established which represent a molecular inventory of the bacteria in a particular sample. Large 16S rRNA sequence databases exist, which contain already thousands of entries available for comparison with new sequences. Countless different, mostly uncultivated bacteria have been detected in various habitats by using this technique (e.g., Bond *et al.*, 1995; Borneman and Triplett, 1997; Snaidr *et al.*, 1997; Dojka *et al.*, 1998; Hugenholtz *et al.*, 1998b). Moreover, this approach led to the discovery of previously unknown bacterial taxa up to the level of new phyla (e.g., Liesack and Stackebrandt, 1992; Hugenholtz *et al.*, 1998b; for a review, see Hugenholtz *et al.*, 1998a). However, ecological

conclusions cannot be drawn solely based on rRNA sequence data due to biases of the DNA extraction and PCR steps. Not all DNA extraction protocols are equally effective in breaking open bacterial cells (Kuske *et al.*, 1998). Bacteria, which are not targeted by the PCR primers used, will not at all be detected by rRNA gene sequence analysis. DNA from allochthonous organisms, which might be present in a sample or in laboratory reagents (Tanner *et al.*, 1998), could function as PCR template. This will result in a falsified picture of the autochthonous microbial community. Finally, the relative abundance of amplified rRNA genes in the gene library does not necessarily provide any measure of the gene ratios in the original DNA mixture (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Suzuki *et al.*, 1998). Therefore, rRNA sequence analysis must be supplemented by other methods to visualize and quantify bacterial cells *in situ*. A powerful approach to achieve this aim is explained in the following sections.

1.3 Fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes is a cultivation-independent technique that allows to visualize bacteria (or other microorganisms) specifically and directly in their habitats (DeLong *et al.*, 1989; Amann, 1995; Amann *et al.*, 1995). The oligonucleotide probes are specific for single species, whole genera, or even phyla and domains according to the sequence conservation at their target sites on the rRNA (Amann *et al.*, 1995). FISH with rRNA-targeted probes can be combined effectively with comparative rRNA sequence analysis: A first overview of the bacterial community composition in an environmental sample is obtained by hybridization of the sample with existing probes that target different phylogenetic groups of bacteria. In parallel, rRNA gene libraries of the sample are established and screened for sequences of new or otherwise interesting bacteria. Based on these rRNA sequences, new probes are developed which detect the corresponding organisms *in situ*. This "rRNA approach" (Amann *et al.*, 1995) proved to be highly useful for investigating microbial communities in numerous different, natural and artificial habitats. Up to seven different populations can be detected in the same experiment if several oligonucleotide probes, which have been labeled with different fluorochromes, are applied simultaneously (Amann *et al.*, 1996). Probes of nested specificity can be used to distinguish bacterial populations with a successively increasing resolution (Amann *et al.*, 1995; Fig. 1).

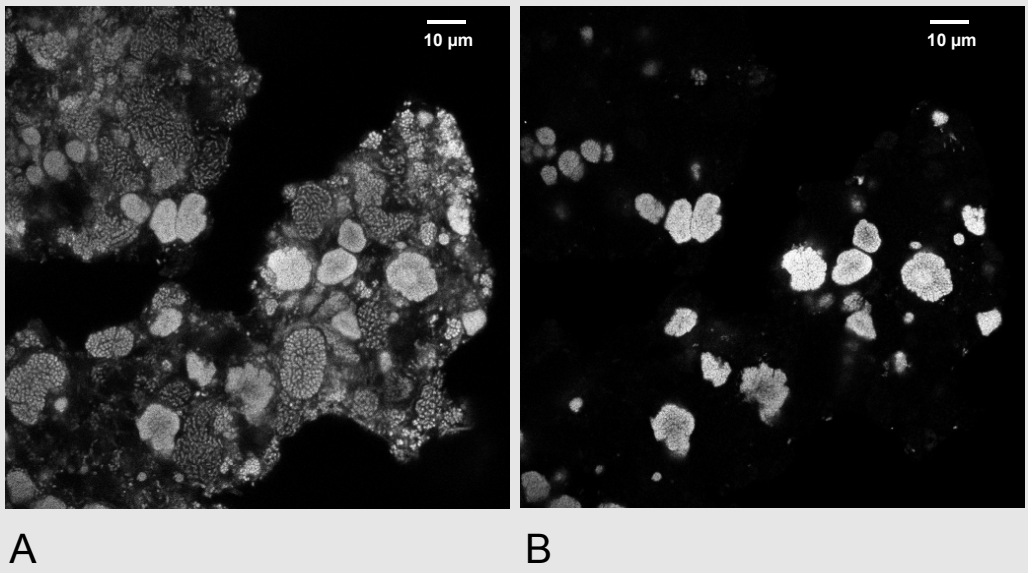

Fig. 1. CLSM micrographs of a nitrifying biofilm after FISH with rRNA-directed oligonucleotide probes of nested specificity. (A) Detection of all bacteria (including *Nitrospira*-like bacteria) by a *Bacteria*-specific probe set. (B) Exclusive detection of *Nitrospira*-like bacteria in the same microscopic field by a probe specific for this particular phylogenetic lineage.

The existing set of rRNA-targeted oligonucleotide probes has been extended continuously. Different probes of broad specificity cover for example the different subclasses of the *Proteobacteria* (Manz *et al.*, 1992), the *Cytophaga-Flavobacterium-Bacteroides* phylum (Manz *et al.*, 1996), gram-positive bacteria with high and low DNA G+C content (Roller *et al.*, 1994; Meier *et al.*, 1999), the planctomycetes (Neef *et al.*, 1998), most *Bacteria* (Amann *et al.*, 1990), and the *Archaea* (Burggraf *et al.*, 1994). In addition, many probes have been designed that detect smaller groups, for example the ammonia-oxidizing bacteria in the beta subclass of *Proteobacteria* (Wagner *et al.*, 1995; Mobarry *et al.*, 1996; Pommering-Röser *et al.*, 1996; Juretschko *et al.*, 1998), diverse filamentous bacteria (Wagner *et al.*, 1994a), or different *Yersinia* species (Trebesius *et al.*, 1998).

The practical value of FISH is perhaps best demonstrated by the numerous studies on microbial consortia in wastewater treatment plants. FISH was applied to examine the high bacterial diversity in activated sludge without the constraints of cultivation-dependent methods (e.g., Manz *et al.*, 1994; Kämpfer *et al.*, 1996; Wagner *et al.*, 1993; Snaidr *et al.*,

1997; Amann *et al*., 1996). Specific probes were used to monitor defined groups of bacteria living in wastewater treatment plants, like nitrifiers (Wagner *et al.*, 1995; Mobarry *et al.*, 1996; Juretschko *et al.*, 1998) or floc-forming bacteria (Wagner *et al.*, 1994a; Rosselló-Mora *et al.*, 1995; Erhart *et al.*, 1997).

The application spectrum of FISH is expanded by combinations with other techniques like confocal laser scanning microscopy, digital image analysis, and microautoradiography. These extensions will be introduced in the following sections.

1.4 Confocal laser scanning microscopy

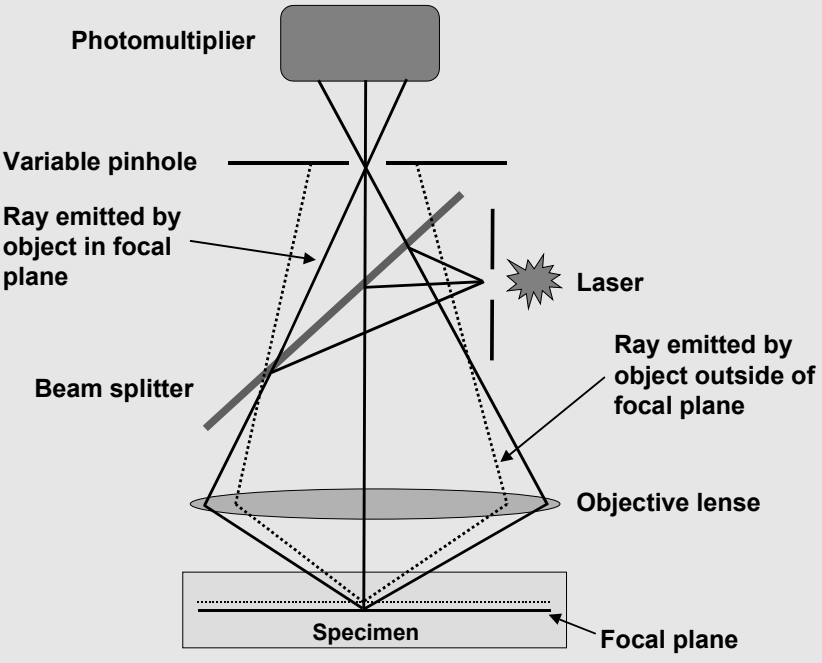

Fig. 2. The confocal principle.

FISH with rRNA-targeted oligonucleotide probes offers the chance to study the spatial organization of microbial consortia with a microscope providing a sufficient optical resolution. Most images acquired with conventional epifluorescence microscopes are blurred due to fluorescence emitted by objects outside of the focal plane. In consequence of this, details like single cells in cell aggregates can often not be resolved. Sectioning the samples with a microtome may sometimes overcome this problem, but reconstructing spatial structures of sectioned objects can be difficult. In contrast, the confocal laser scanning microscope (CLSM) offers possibilities to investigate the three-dimensional architecture of biological

objects in a non-invasive manner (White *et al.*, 1987; Lawrence *et al.*, 1991; Caldwell *et al.*, 1992). Here the specimen is scanned by a point-like light source (the laser) of a specified excitation wavelength. The fluorescence emitted by excited dye molecules is collected by the objective lense and directed to a photomultiplier (the detector). Before the light rays reach the detector they must pass an aperture (the pinhole). This pinhole blocks all light emitted outside of the focal plane (Fig. 2). The diameter of the pinhole is adjustable and regulates, how much of the light emitted above and below the focal plane is recorded by the detector. This confocal principle improves the resolution especially along the z-axis and allows to acquire "optical sections" through an object. The complete three-dimensional structure of a specimen can be reconstructed if stacks of serial optical sections are recorded. Modern confocal microscopes allow distances as short as 0.2 µm between the single optical sections of such stacks. These advantages of the CLSM have already been exploited to examine microbial populations with biochemical and immunological staining methods (Caldwell *et al.*, 1992; Schloter *et al.*, 1993). In combination with FISH, confocal microscopy has been used to study the localization of probe-stained bacteria for example in activated sludge flocs (Wagner *et al.*, 1994a; Wagner *et al.*, 1994b; Juretschko *et al.*, 1998) and biofilms (Møller *et al.*, 1996; Schramm *et al.*, 1996; Okabe *et al.*, 1999). This combination was also applied to visualize prokaryotic endosymbionts directly in their protozoan hosts with a high optical quality (e.g., Embley and Finlay, 1994). The necessary dehydration during the standard FISH protocol (Amann, 1995), however, becomes a substantial problem if the three-dimensional structure of a specimen must be preserved. Biofilms and flocs, which contain large amounts of hydrated extracellular polymeric substances (e.g., Sutherland, 1977; Lawrence *et al.*, 1991), shrink during this step and their native structure is destroyed. This effect can be minimized by embedding the samples (e.g., in acrylamide, Møller *et al.*, 1998) prior to the dehydration.

1.5 Cultivation-independent quantification of microbial populations

As explained in section 1.1, microbial ecology needs cultivation-independent tools to quantify bacteria directly in environmental samples. Manual microscopic counting of probe-stained cells after FISH with rRNA-targeted probes was peformed in numerous studies (e.g., DeLong *et al.*, 1999; Glöckner *et al.*, 1996; Ravenschlag *et al.*, 2001; Wagner *et al.*, 1993; Wagner *et al.*, 1994c; Kämpfer *et al.*, 1996; Manz *et al.*, 1994). In this manner, valuable insight into microbial population structures was obtained, but this straightforward quantification method has important limitations. Manual counting of cells in dense aggregates as found in activated

sludge or biofilm (Fig. 3) is extremely tedious. The cell numbers in such clusters are easily underestimated (Manz *et al.*, 1994), and attempts to break up cell aggregates are not always successful (Manz *et al.*, 1994). Due to the tediousness of manual cell counting, flow cytometry has been applied to quantify probe-stained cells automatically (Amann *et al.*, 1990; Wallner *et al.*, 1995; Wallner *et al.*, 1997). This technique allows to count suspended single cells with high efficiency and accuracy (Wallner *et al.*, 1997). Since cell clusters are counted as single large objects, flow cytometry cannot be used to quantify bacteria in flocs and biofilms (Wallner *et al.*, 1995; Wallner *et al.*, 1997).

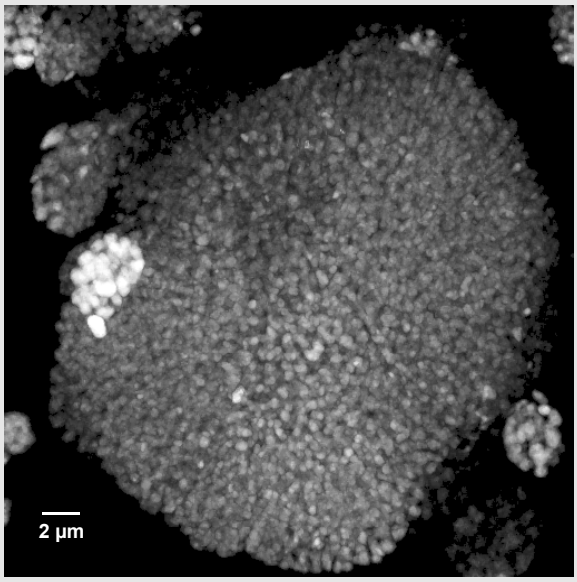

Fig. 3. Cell aggregate of ammonia-oxidizing bacteria as observed frequently in nitrifying biofilms. Individual cells are clearly visible in this CLSM micrograph. The aggregate is formed by several thousand cells.

The semi-automatic quantification of FISH-stained cells by digital image analysis is another alternative to manual counting. For this purpose, high-quality images such as those acquired by a CLSM (section 1.4) are needed. Single, non-clustered cells can be resolved and counted by image analysis software (Bloem *et al.*, 1995; Møller *et al.*, 1995). Automatic counting of the cells in large aggregates is not yet possible due to the limited resolution of light microscopes including the CLSM. Therefore, image analysis programs have been developed to quantify the biovolume of cell aggregates (Kuehn *et al.*, 1998; Schramm *et al.*, 1999; Bouchez *et al.*, 2000; Heydorn *et al.*, 2000; Schmid *et al.*, 2000). Digital image analysis is at present the most flexible approach to quantify bacteria *in situ*. One disadvantage is the required, laborious adaptation and evaluation of the image analysis software to be used in a

particular quantification setup. The design of appropriate image sampling strategies is also critical to ensure that the quantification results are statistically representative.

1.6 Combined FISH and microautoradiography

The population structures of microbial consortia can be characterized by using the techniques described in sections 1.2-1.5. The ecological functions of microbial communities, however, cannot be studied with these tools only. Functional analyses include physiological experiments, which are usually performed with pure cultures. Since most bacteria are uncultured (section 1.1), methods are needed that allow to track physiological processes *in situ*.

Microautoradiography (MAR; Brock and Brock, 1968) is an elegant tool to observe the uptake of radioactively labeled subtrates by bacteria without cultivation. The simultaneous identification of these bacteria is possible by combining MAR with FISH (Lee *et al.*, 1999). At the beginning of this procedure, a native environmental sample is incubated with a radioactive substrate. The bacteria in the sample have time to take up the radioactive substrate during this incubation. Afterwards, the sample is fixed and sliced with a microtome. Thin slices are placed onto microscope cover slips and are hybridized with suitable rRNA-targeted oligonucleotide probes. After FISH is completed, the slips are covered with a radiographic film emulsion. Following exposition and development of the film, the sample is observed in an inverse microscope (Fig. 4). The probe-conferred fluorescence of the cells and the silver grain formation in the film are correlated to identify those bacteria which took up the radioactive substrate during the incubation (Fig. 4). This combination of FISH and MAR allows to study physiological properties of selected organisms directly in their natural environment. The results may not only be relevant for ecological considerations, but can also help to identify essential components of nutrient media used to isolate yet uncultured bacteria.

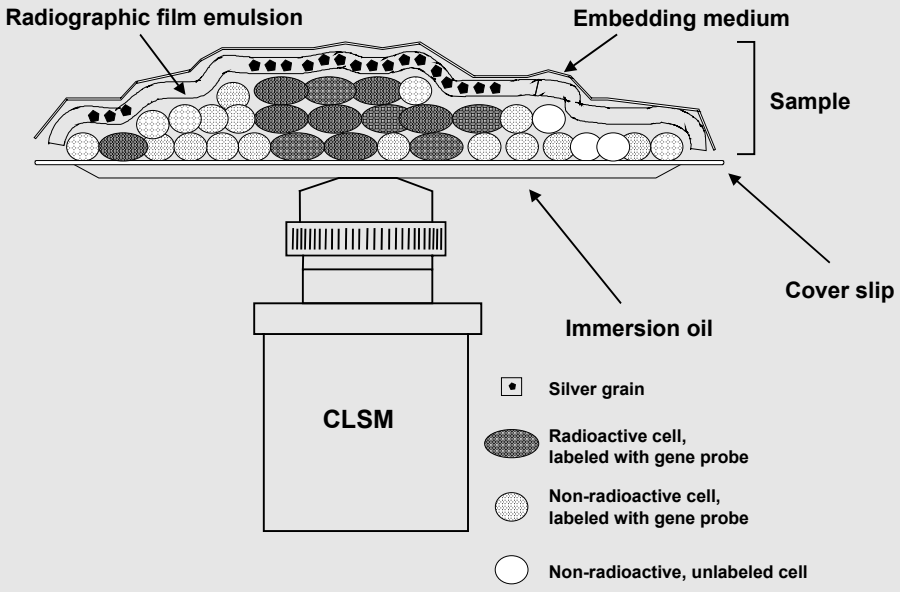

Fig 4. The combination of FISH and MAR.

2. Wastewater Treatment and Nitrification

2.1 Applications of biofilms in wastewater treatment

Biofilms are defined as surface-attached accumulations of microbial cells enceased in extracellular polymeric substances (EPS; Characklis and Wilderer, 1989). In particular, most biofilms are not formed by homogeneous layers of evenly distributed cells. Instead they consist of distinct cell clusters, which are suspended in a complex matrix of varying density (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992). This matrix is frequently interlaced by interstitial voids and channels, which are in contact with the bulk liquid and facilitate the transport of gases and water soluble substances within the biofilm (Robinson *et al.*, 1984; MacLeod *et al.*, 1990; de Beer *et al.*, 1994; Stoodley *et al.*, 1994; Massol-Deyá *et al.*, 1995). Not only the physical structure of biofilms, but also their species composition and the spatial arrangement of the different populations are of special interest. For example, the syntrophy of ammonia- and nitrite-oxidizing bacteria is reflected by their co-localization in nitrifying

biofilms (Schramm *et al.*, 1996; Juretschko *et al.*, 1998; Schramm *et al.*, 1998; Okabe *et al.*, 1999; Fig. 5). Nutrients and gases are not equally distributed in biofilms due to steep chemical gradients (e.g., Kühl and Jørgensen, 1992; Dalsgaard and Revsbech, 1992). Since the microorganisms are localized along these gradients according to their nutritional demands, many biofilms are highly stratified (e.g., Ramsing *et al.*, 1993).

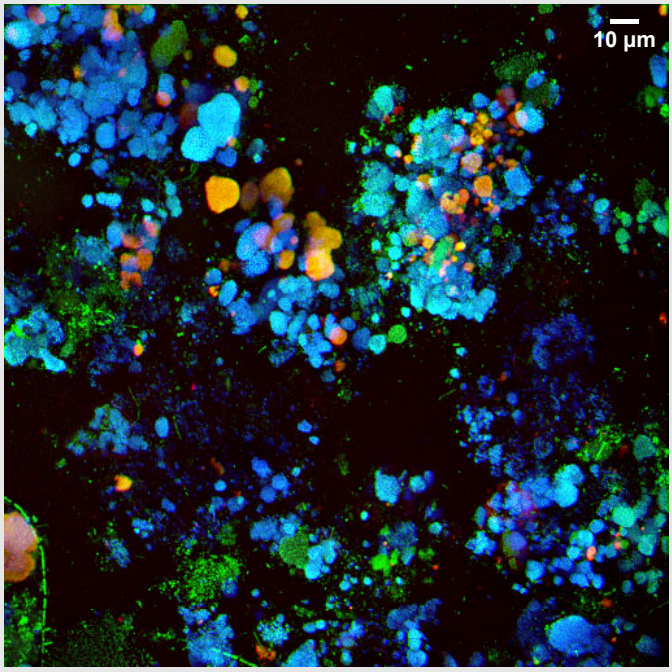

Fig. 5. Architecture of a nitrifying biofilm from a sequencing batch biofilm reactor. FISH was performed with group-specific oligonucleotide probes targeting ammonia-oxidizers (light blue or cyan) and *Nitrospira*-like bacteria (yellow). The other bacteria were stained by the EUB338 probe mix (green). Co-localization of the nitrifiers and large cavities in the biofilm are clearly visible. The biofilm was embedded in agarose to preserve its spatial structure during FISH.

Several wastewater treatment techniques take advantage of the high bacterial density in biofilms. Trickling filters, for example, are widely-used biofilm reactors. They contain a stationary medium as substrate for the biofilm, above which the wastewater is distributed. While the water is trickling down, it has contact with the microorganisms in the biofilm. The purified water is then collected under the substrate.

The activated sludge process is the most important technique in biological wastewater treatment. Activated sludge is a suspended mixed culture of microorganisms, which catalyze the substrate conversions required for wastewater purification. The microorganisms aggregate and form flocs. These flocs consist of filamentous bacteria and cell clusters as "backbone", of single cells, and of EPS as extracellular matrix. Cavities and irregular surfaces are additional

characteristics of activated sludge flocs. Because of the high structural similarities to biofilms, the flocs are often viewed as "suspended" or "mobilized" biofilms in the context of an extended biofilm definition. Oxygen is brought into activated sludge basins by aeration, which also produces turbulence and ensures permanent agitation of the sludge flocs. Conventional activated sludge plants are operated continuously. The aerated process stage is followed by a settling tank, where the purified water is separated from the biomass by gravitational sedimentation of the flocs. The settled flocs are then partly recirculated to the aerated basin. This procedure allows the accumulation of fast-growing as well as slow-growing microorganisms like nitrifying bacteria (Henze *et al.*, 1997). Excessive sludge is removed and transferred to sludge dewatering.

The sequencing batch process introduced by Irvine (e.g., Irvine *et al.*, 1979) combines the aerated and sedimentation stages of the conventional activated sludge process in one reactor (the sequencing batch reactor, SBR). The procedure is a cyclic sequence of (i) filling the SBR with wastewater, (ii) aeration and stirring, (iii) settling of the sludge flocs, and (iv) draining of the purified water. Important parameters like cycle duration or aeration intensity can be adjusted to meet particular requirements. The sequencing batch principle has also been adapted to biofilms that grow on solid substrates. Such sequencing batch biofilm reactors (SBBRs) are operated similarly to SBRs and may be even more effective, because the time-consuming sludge settling phase can be omitted.

2.2 The importance of nitrogen elimination for wastewater treatment

The transformations of nitrogen compounds carried out by microorganisms are key steps of the biogeochemical nitrogen cycle. Reduced nitrogen is released as ammonia primarily during the decomposition of organic substance (ammonification). A part of this released ammonia is directly assimilated and incorporated into biomass, while the remaining ammonia is oxidized to nitrate by aerobic, ammonia-oxidizing and nitrite-oxidizing bacteria (Fig. 6). Thereupon, the nitrate is either assimilated or it is used by facultatively anaerobic bacteria as alternative electron acceptor in the absence of oxygen (denitrification). The end products of denitrification are gaseous dinitrogen and smaller amounts of nitric (NO) and nitrous (N_2O) oxide. Nitrogen-fixing bacteria close the cycle by reducing dinitrogen to ammonia (Fig. 6).

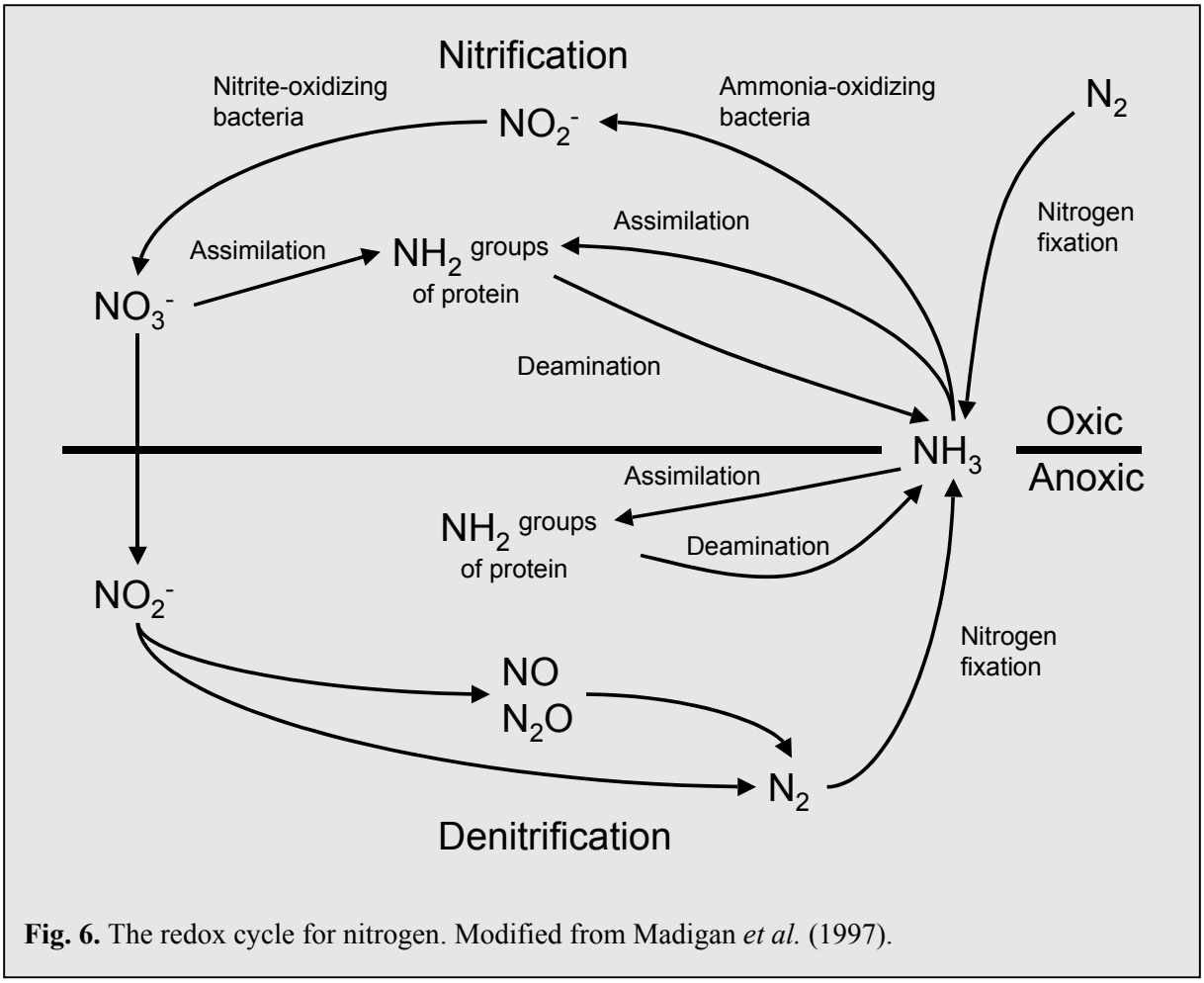

Fig. 6. The redox cycle for nitrogen. Modified from Madigan *et al.* (1997).

These natural processes are influenced strongly by human activities. Nitrogen compounds like ammonia and nitrate are main components of fertilizers and wastewater. Their release in the environment has to be minimized, because ammonia and nitrite are highly toxic to aquatic life (ammonia already at a concentration of 0.01 mg/l, Arthur *et al.*, 1987). Nitrite and nitrate can also be harmful to humans (Schneider and Selenka, 1974). Nitrogen compounds in sewage water contribute to the eutrophication of natural waters, a process which causes incalculable ecological damage. The efficient elimination of nitrogen is therefore one of the most important processes in modern wastewater treatment. It takes place during a two-phase process in biological wastewater treatment plants (e.g., Bever *et al.*, 1995; Henze *et al.*, 1997). In the first stage (nitrification), ammonia is transformed to nitrate by ammonia- and nitrite-oxidizing bacteria under aerobic conditions. The nitrate is reduced to gaseous N_2, nitric and nitrous oxide in the following, anaerobic denitrification stage. The next sections deal with the first of these two phases, nitrification, and with the microorganisms involved in this process.

2.3 The chemolithotrophic nitrifying bacteria

The two oxidation steps of nitrification are catalyzed by different, physiologically as well as phylogenetically well-defined groups of bacteria (the nitrifiers). These organisms grow chemolithoautotrophically with ammonia or nitrite as electron donor and oxygen as electron acceptor. Although once classified as one family, *Nitrobacteraceae* (Buchanan, 1917), the ammonia- and nitrite-oxidizing bacteria are not related. The phylogenetic tree in Fig. 7 illustrates the affiliation of the nitrifiers with major bacterial lines of descent.

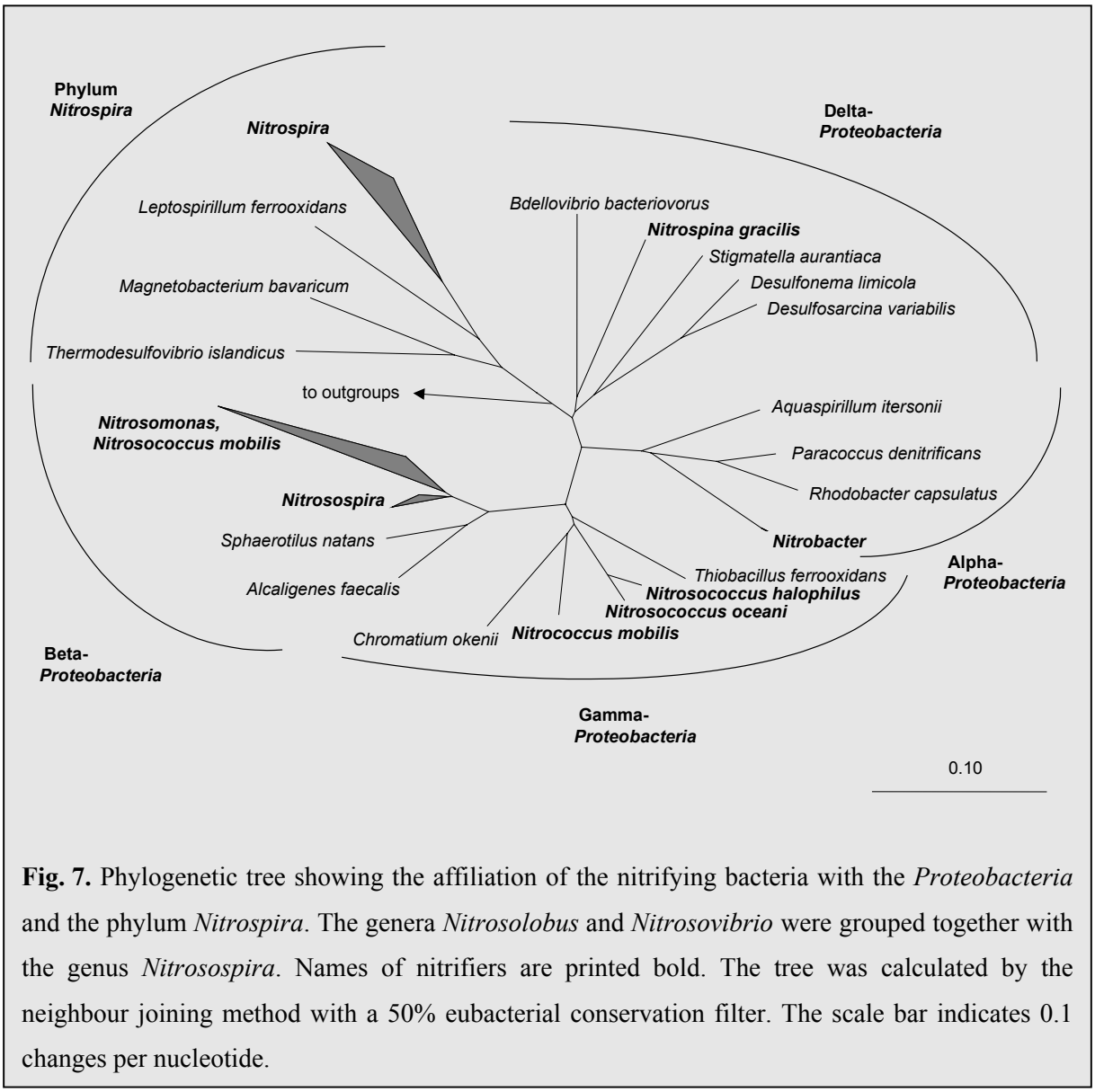

Fig. 7. Phylogenetic tree showing the affiliation of the nitrifying bacteria with the *Proteobacteria* and the phylum *Nitrospira*. The genera *Nitrosolobus* and *Nitrosovibrio* were grouped together with the genus *Nitrosospira*. Names of nitrifiers are printed bold. The tree was calculated by the neighbour joining method with a 50% eubacterial conservation filter. The scale bar indicates 0.1 changes per nucleotide.

Ammonia-oxidizing bacteria perform the first step of nitrification, the oxidation of ammonia to nitrite. Most known ammonia-oxidizers group together in one monophyletic lineage within the beta-subclass of *Proteobacteria*. Four genera belonging to this lineage have been described so far: *Nitrosomonas* (with *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* (Woese *et al.*, 1984; Head *et al.*, 1993; Teske *et al.*, 1994; Utåker *et al.*, 1995; Pommering-Röser *et al.*, 1996). Reclassification of the latter three genera in the single genus *Nitrosospira* has been suggested (Head *et al.*, 1993), but has been discussed controversially due to ultrastructural features (Teske *et al.*, 1994). The only known aerobic ammonia-oxidizing bacteria, which are not members of the beta-subclass of *Proteobacteria*, are *Nitrosococcus oceani* (Watson, 1965; Trüper and de Clari, 1997) and *N. halophilus* (Koops *et al.*, 1990). These two species group with the gamma-subclass of *Proteobacteria* (Woese *et al.*, 1985; Head *et al.*, 1993; Teske *et al.*, 1994).

The chemolithotrophic oxidation of ammonia to nitrite is catalyzed by two enzymes: the membrane-bound ammonia monooxygenase (McTavish *et al.*, 1993; Hooper *et al.*, 1997), which oxidizes ammonia to hydroxylamine (equation 1), and the periplasmatic hydroxylamine oxidoreductase (Bergmann *et al.*, 1994; Sayavedra-Soto *et al.*, 1994), which oxidizes hydroxylamine to nitrite (equation 2). Only the oxidation of hydroxylamine is exergonic and is therefore regarded as the actual energy source in lithotrophic ammonia oxidation (Bock *et al.*, 1992).

Ammonia monooxygenase: $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$ (eq. 1)

Hydroxylamine oxidoreductase: $NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-$ (eq. 2)

The conversion of ammonia to nitrite yields little energy due to the high standard redox potentials of the two redox couples NH_2OH/NH_3 (+899 mV) and NO_2^-/NH_2OH (+66 mV). Consequently, ammonia-oxidizers are slow-growing bacteria. They depend also on reverse electron flow to regenerate reduction equivalents (reduced pyridine nucleotides; Aleem, 1966; Bock *et al.*, 1992). It has to be mentioned, however, that the metabolism of ammonia-oxidizing bacteria is surprisingly versatile. Anoxic reduction of nitrite (denitrification) by *Nitrosomonas europaea* with pyruvate as electron donor has been observed (Abeliovich and Vonhak, 1992), and *N. eutropha* can reduce nitrite with hydrogen as electron donor at low oxygen pressure (Bock *et al.*, 1995). While ammonia-oxidizers are widely distributed in soils,

freshwater, brackish and marine environments, the requirements of individual species for ammonia concentration, oxygen pressure, pH and temperature differ (Koops and Möller, 1992).

Nitrite-oxidizing bacteria perform the second step of nitrification, the oxidation of nitrite to nitrate. This physiological group is phylogenetically more heterogenous than the ammonia-oxidizers as all four described genera of nitrite-oxidizers belong to different lines of descent. The genera *Nitrobacter*, *Nitrococcus* and *Nitrospina* are *Proteobacteria*, but group with different subclasses of this phylum. The genus *Nitrobacter* (Winogradsky, 1892) with the four described species *N. winogradskyi* (Winslow *et al.*, 1917; Watson, 1971), *N. hamburgensis* (Bock *et al.*, 1983), *N. vulgaris* (Bock *et al.*, 1990), and *N. alkalicus* (Sorokin *et al.*, 1998) belongs to the alpha-subclass of *Proteobacteria* (Stackebrandt *et al.*, 1988). The genera *Nitrococcus* und *Nitrospina* (Watson and Waterbury, 1971) contain to date only one species, respectively: *Nitrococcus mobilis* is a member of the gamma-subclass of *Proteobacteria*, while *Nitrospina gracilis* groups with the delta-subclass of *Proteobacteria* (Teske *et al.*, 1994). The nitrite-oxidizers of the genus *Nitrospira* form a distinct phylum in the domain *Bacteria* together with the genera *Leptospirillum*, *Thermodesulfovibrio* and "*Magnetobacterium bavaricum*" (Ehrich *et al.*, 1995). Two species, which were found in completely different habitats, have been assigned to this genus so far: *N. marina* was isolated from ocean water (Watson *et al.*, 1986), whereas *N. moscoviensis* was obtained from a heating system in Moscow (Ehrich *et al.*, 1995). Except for *Nitrospira*, only *Nitrobacter* species occur in various habitats like soils, building stones, freshwater, brackish water, and even in acid sulfidic ores (Bock and Koops, 1992). In contrast, *Nitrospina* and *Nitrococcus* appear to be obligately halophilic and hence exclusively marine (Watson and Waterbury, 1971).

The integral membrane enzyme nitrite oxidoreductase catalyzes the chemolithotrophic oxidation of nitrite to nitrate in *Nitrobacter* cells (Tanaka *et al.*, 1983; Sundermeyer-Klinger *et al.*, 1984). This reaction is reversible and the oxygen, which is incorporated into nitrate, stems from water (equation 3):

Nitrite oxidoreductase: $NO_2^- + H_2O \leftrightarrow NO_3^- + 2H^+ + 2e^-$ (eq. 3)

The nitrite oxidoreductase of *Nitrobacter* has been studied extensively. The holoenzyme consists of three subunits in *N. hamburgensis* (Sundermeyer-Klinger *et al.*, 1984), but only of

two subunits in *N. winogradskyi* and *N. vulgaris* (Bock *et al.*, 1990). Nitrite oxidoreductase contains molybdenum, iron-sulfur clusters, and manganese (Ingledew and Halling, 1976; Sundermeyer-Klinger *et al.*, 1984; Fukuoka *et al.*, 1987; Krüger *et al.*, 1987; Bock *et al.*, 1992). Much less is known about the composition of the nitrite-oxidizing systems of the other nitrite-oxidizers. Biochemical data indicate substantial differences between the nitrite oxidoreductase of *Nitrobacter* and the nitrite-oxidizing systems of *Nitrospira marina* and *N. moscoviensis* (Watson *et al.*, 1986; Ehrich *et al.*, 1995). While *Nitrococcus* and *Nitrospina* seem to be obligate chemolithotrophs (Watson and Waterbury, 1971), *Nitrobacter* and *Nitrospira* possess alternative metabolic pathways. Organotrophic growth in absence of nitrite, for example with acetate or pyruvate, was reported for *Nitrobacter* (Smith and Hoare, 1968; Bock, 1976). *Nitrospira marina* cultures reached higher cell densities in media containing nitrite and pyruvate than in pure nitrite medium, indicating that this species can grow mixotrophically (Watson *et al.*, 1986). *N. moscoviensis* is able to reduce nitrate with hydrogen as electron donor under anoxic conditions (Ehrich *et al.*, 1995). *Nitrobacter* can also grow by denitrification in anoxic environments (Freitag *et al.*, 1987; Bock *et al.*, 1988) and possesses a dissimilatoric nitrite reductase, which reduces nitrite to nitric oxide (NO; Ahlers *et al.*, 1990). This reaction might be a link between dissimilatoric and assimilatoric pathways, because nitric oxide can serve as electron donor for the reduction of NAD^+ (Freitag and Bock, 1990). With respect to energy metabolism, the nitrite-oxidizers are confronted with similar problems as the ammonia-oxidizers. The standard redox potential of the NO_3^-/NO_2^- couple is extremely high (+420 mV). Consequently, the growth rates of nitrite-oxidizing bacteria are very low.

Chemolithotrophic, anaerobic oxidation of ammonia to N_2 is carried out by physiologically specialized planctomycetes (ANAMMOX organisms; Strous *et al.*, 1999; Schmid *et al.*, 2000). Although this process may in future be exploited in wastewater treatment, at present no large-scale reactors exist that were designed specifically for anaerobic ammonia oxidation.

2.4 The key nitrite-oxidizers in wastewater treatment plants are uncultured bacteria

According to a traditional concept, *Nitrosomonas* and *Nitrobacter* are responsible for nitrification in wastewater treatment plants (e.g., Bever *et al.*, 1995; Henze *et al.*, 1997). This opinion is based on the experience that *Nitrosomonas* and *Nitrobacter* species can be isolated from practically every activated sludge. In contrast, *Nitrobacter* was not detected in aquarium

biofilters by quantitative dot blot (Hovanec and DeLong, 1996) or in activated sludge by FISH (Wagner *et al.*, 1996) with rRNA-targeted probes. These findings indicated that other nitrite-oxidizers could be more important for the nitrification process in wastewater treatment. This hypothesis was corroborated when *Nitrospira*-related bacteria were detected in a nitrite-oxidizing, laboratory-scale reactor by rRNA sequence analysis (Burrell *et al.*, 1998). Ribosomal RNA sequences affiliated to *Nitrospira* were also retrieved from freshwater aquaria (Hovanec *et al.*, 1998). Quantitative dot blot hybridization of total rRNA with *Nitrospira*-specific probes was performed in the same study. These experiments confirmed the high abundance of *Nitrospira*-like bacteria in the aquarium samples. Finally, FISH of activated sludge with *Nitrospira*-specific probes demonstrated for the first time that *Nitrospira*-like bacteria were a dominant population in a full-scale wastewater treatment plant (Juretschko *et al.*, 1998). Although *Nitrobacter* was not detectable in this sludge by FISH, a *Nitrobacter* strain could be isolated from the same sample. Attempts to isolate the *Nitrospira*-like bacteria were not successful (Juretschko *et al.*, 1998). These results unmasked the dogma claiming that *Nitrobacter* species were the important nitrite-oxidizers in wastewater treatment as a mere artifact of cultivation. Later studies confirmed this conclusion repeatedly by using FISH and microsensors to correlate the spatial localization of *Nitrospira*-like bacteria with zones of active nitrite oxidation in biofilms (Schramm *et al.*, 1998; Okabe *et al.*, 1999; Schramm *et al.*, 1999; Schramm *et al.*, 2000).

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Aims of the Thesis

Recent studies demontrated that wastewater treatment plants harbour a high diversity of nitrifying bacteria, and that *Nitrobacter* does not contribute significantly to nitrification in these systems (Wagner *et al.*, 1995; Wagner *et al.*, 1996; Burrell *et al.*, 1998; Hovanec *et al.*, 1998; Juretschko *et al.*, 1998; Schramm *et al.*, 1998; Okabe *et al.*, 1999; Purkhold *et al.*, 2000). In contrast, the current models used to operate nitrifying bioreactors are based on countless physiological experiments with pure cultures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*. These models must be updated to match the real composition of nitrifying bacterial communties. In many wastewater treatment plants, nitrification is unstable and suffers from unpredictable performance breakdowns. Measures to prevent such failure are overdue, but can be planned only based on in-depth structural and functional analyses of the nitrifiers. As detailed in the general introduction, cultivation-dependent methods are of limited use in studies dealing with complex microbial communities. This restriction applies in particular on nitrifying populations: The key nitrite-oxidizers in bioreactors, *Nitrospira*-like bacteria, have resisted all cultivation attempts (Juretschko *et al.*, 1998; Bartosch *et al.*, 1999). The enrichment and isolation of culturable nitrifiers are extremely time-consuming due to the slow growth of these bacteria. Finally, it is highly questionable whether all results obtained in pure culture experiments can be transferred to natural or engineered habitats.

The aim of this thesis was to gain more insight into the microbiology of nitrifying bacteria in activated sludge and biofilm by using improved *in situ* techniques. Since no comprehensive set of rRNA-targeted probes existed for the *in situ* detection of the genus and phylum *Nitrospira*, one task was to design new probes of nested specificity that covered these phylogenetic lineages. These probes should be applied together with other, already existing probes to detect and quantify ammonia- and nitrite-oxidizers. The accuracy of the existing quantification methods had to be improved, and a technique to measure absolute cell concentrations of aggregated bacteria had to be developed. The spatial arrangement of nitrifying bacteria in flocs and biofilms reflects their physiological properties and ecological interactions. This can be investigated only by using a protocol that preserves the native three-dimensional structure of flocs, biofilms, and individual cell aggregates during FISH. Such a protocol had to be developed and combined with confocal laser scanning microscopy and digital image analysis. *Nitrospira*-like bacteria are the dominant nitrite-oxidizers in wastewater treatment, but very little is known about their physiology. Specific measures to

stabilize their populations in bioreactors depend on such knowledge. The combination of FISH and MAR should be applied to monitor the uptake of different carbon sources by *Nitrospira*-like bacteria in wastewater treatment plants. These experiments should be performed to estimate the metabolic versatility of *Nitrospira*-like bacteria under the growth conditions in bioreactors.

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Publication Summaries

The Domain-specific Probe EUB338 is Insufficient for the Detection of all *Bacteria*: Development and Evaluation of a more Comprehensive Probe Set

HOLGER DAIMS, ANDREAS BRÜHL, RUDOLF AMANN, KARL-HEINZ SCHLEIFER, AND MICHAEL WAGNER

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In situ hybridization with rRNA-targeted oligonucleotide probes has become a widely applied tool for direct analysis of microbial population structures of complex natural and engineered systems. In such studies probe EUB338 (Amann *et al.* (1990), Appl. Environ. Microbiol. 56: 1919-1925) is routinely used to quantify members of the domain *Bacteria* with a sufficiently high cellular ribosome content. Recent reevaluations of probe EUB338 coverage based on all publicly available 16S rRNA sequences, however, indicated that important bacterial phyla, most notably the *Planctomycetales* and *Verrucomicrobia*, are missed by this probe. The 16S rRNA sequences of these organisms contain between one and three mismatches to the sequence of probe EUB338 in the target region of this probe. We therefore designed and evaluated two supplementary versions (EUB338-II and EUB338-III) of probe EUB338 for *in situ* detection of most of those phyla not detected with probe EUB338. *Planctomyces limnophilus* and *Verrucomicrobium spinosum*, which are target bacteria of probes EUB338-II and III, respectively, were cultivated. *In situ* dissociation curves of the new probes and of EUB338 with these organisms were recorded under increasing stringency to optimize hybridization conditions. For that purpose a digital image software routine, which allows to quantify the fluorescence intensity of single microbial cells, was developed. Additional dissociation curves were recorded with *Bacillus stearothermophilus*, which is a target organism of the original probe EUB338. Based on all obtained probe dissociation curves, hybridization conditions were defined that allow to differentiate the target organisms of probes EUB338, EUB338-II and EUB338-III in environmental samples. *In situ* hybridization of a complex biofilm community with the three EUB338 probes demonstrated the presence of significant numbers of probe EUB338-II and EUB338-III target organisms. The application of EUB338, EUB338-II and EUB338-III should allow a more accurate quantification of members of the domain *Bacteria* in future molecular ecological studies.

The full text of this publication is reprinted in appendix 1.

Cultivation-independent, Semiautomatic Determination of Absolute Bacterial Cell Numbers in Environmental Samples by Fluorescence *In Situ* Hybridization

HOLGER DAIMS, NIELS B. RAMSING, KARL-HEINZ SCHLEIFER, AND MICHAEL WAGNER

Published in Applied and Environmental Microbiology **67** (12) : 5810-5818 (2001)

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes has found widespread application for analyzing the composition of microbial communities in complex environmental samples. Although bacteria can quickly be detected by FISH, a reliable method to determine absolute numbers of FISH-stained cells in aggregates or biofilms has, to our knowledge, never been published. In this study we developed a semi-automated protocol to measure the concentration of bacteria (in cells per volume) in environmental samples by a combination of FISH, confocal laser scanning microscopy, and digital image analysis. The quantification is based on an internal standard, which is introduced by spiking the samples with known amounts of *Escherichia coli* cells. This method was initially tested with artificial mixtures of bacterial cultures and subsequently used to determine the concentration of ammonia-oxidizing bacteria in a municipal nitrifying activated sludge. The total number of ammonia-oxidizers was found to be $9.8 \times 10^7 \pm 1.9 \times 10^7$ cells ml^{-1}. Based on this value, the average *in situ* activity was calculated to be 2.3 fmol of ammonia converted to nitrite per ammonia-oxidizer cell per hour. This activity is within the previously determined range of activities measured with ammonia-oxidizer pure cultures, demonstrating the utility of the developed quantification method to enumerate bacteria in samples where cells are not homogeneously distributed.

The full text of this publication is reprinted in appendix 2.

In Situ Characterization of *Nitrospira*-like Nitrite-oxidizing Bacteria Active in Wastewater Treatment Plants

HOLGER DAIMS, JEPPE L. NIELSEN, PER H. NIELSEN, KARL-HEINZ SCHLEIFER, AND MICHAEL WAGNER

Published in Applied and Environmental Microbiology **67** (11) : 5273-5284 (2001)

Uncultivated *Nitrospira*-like bacteria in different biofilm and activated sludge samples were investigated by cultivation-independent, molecular approaches. Initially, the phylogenetic affiliation of *Nitrospira*-like bacteria in a nitrifying biofilm was determined by 16S rDNA sequence analysis. For this purpose, a 16S rDNA library was established from biofilm DNA, and 129 of the cloned, almost full-length 16S rDNA fragments were sequenced and subjected to phylogenetic analyses. Subsequently, a phylogenetic consensus tree of the *Nitrospira* phylum including all publicly available sequences was constructed. This analysis revealed that the genus *Nitrospira* consists of at least four distinct sublineages. Based on these data, two 16S rRNA-directed oligonucleotide probes specific for the phylum and genus *Nitrospira*, respectively, were developed and evaluated for their application for fluorescence *in situ* hybridization (FISH). Optimal hybridization conditions for these probes were determined by recording probe dissociation profiles with cells of *Nitrospira moscoviensis*, which is a target organism of both probes. The hybridization conditions were further optimized by verifying the specificity of the new probes and by recording additional probe dissociation profiles with other members of the phylum *Nitrospira* (*Leptospirillum ferrooxidans* and *Thermodesulfovibrio yellowstonii*), which were cultivated for this purpose, and with the non-target organisms *Bacillus stearothermophilus* and *Desulfovibrio desulfuricans*. The newly developed probes were used to investigate the *in situ* architecture of cell aggregates of the *Nitrospira*-like nitrite-oxidizers in wastewater treatment plants by FISH, confocal laser scanning microscopy and computer-aided 3D visualization. The 3D visualization was realized by using a self-written image analysis and visualization program. Cavities and a network of cell-free channels inside the *Nitrospira* microcolonies were detected, which were water permeable as demonstrated by fluorescein-staining. The uptake of different carbon sources by *Nitrospira*-like bacteria within their natural habitat under different incubation conditions was studied by combined FISH and microautoradiography. Under aerobic conditions, the *Nitrospira*-like bacteria in bioreactor samples took up CO_2 and pyruvate but not acetate, butyrate, and propionate suggesting the capability of these bacteria to grow mixotrophically in the presence of pyruvate. In contrast, no uptake of any of the tested carbon sources could be observed for the *Nitrospira*-like bacteria under anoxic or anaerobic conditions.

The full text of this publication is reprinted in appendix 3.

Nitrification in Sequencing Biofilm Batch Reactors: Lessons from Molecular Approaches

HOLGER DAIMS, ULRIKE PURKHOLD, LOTTE BJERRUM, EVA ARNOLD, PETER A. WILDERER, AND MICHAEL WAGNER

Published in Water Science and Technology **43**(3) : 9-18 (2001)

The nitrifying microbial diversity and population structure of a sequencing biofilm batch reactor receiving sewage with high ammonia and salt concentrations (SBBR 1) was analyzed by the full-cycle rRNA approach. The diversity of ammonia-oxidizers in this reactor was additionally investigated using comparative sequence analysis of a gene fragment of the ammonia monooxygenase (*amoA*), which represents a key enzyme of all ammonia-oxidizers. Despite of the "extreme" conditions in the reactor, a surprisingly high diversity of ammonia- and nitrite-oxidizers was observed to occur within the biofilm. In addition, molecular evidence for the existence of novel ammonia-oxidizers is presented. Quantification of ammonia- and nitrite-oxidizers in the biofilm by Fluorescence *In situ* Hybridization (FISH) and digital image analysis revealed that ammonia-oxidizers occurred in higher cell numbers and occupied a considerably larger share of the total biovolume than nitrite-oxidizing bacteria. In addition, ammonia oxidation rates per cell were calculated for different WWTPs following the quantification of ammonia-oxidizers by competitive PCR of an *amoA* gene fragment. The morphology of nitrite-oxidizing, unculturable *Nitrospira*-like bacteria was studied using FISH, confocal laser scanning microscopy (CLSM) and three-dimensional visualization. Thereby, a complex network of microchannels and cavities was detected within microcolonies of *Nitrospira*-like bacteria. Microautoradiography combined with FISH was applied to investigate the ability of these organisms to use CO_2 as carbon source and to take up other organic substrates under varying conditions. Implications of the obtained results for fundamental understanding of the microbial ecology of nitrifiers as well as for future improvement of nutrient removal in wastewater treatment plants (WWTPs) are discussed.

The full text of this publication is reprinted in appendix 4.

Activated Sludge – Molecular Techniques for Determining Community Composition

ALEXANDER LOY, HOLGER DAIMS, AND MICHAEL WAGNER

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(John Wiley & Sons, Inc., New York)

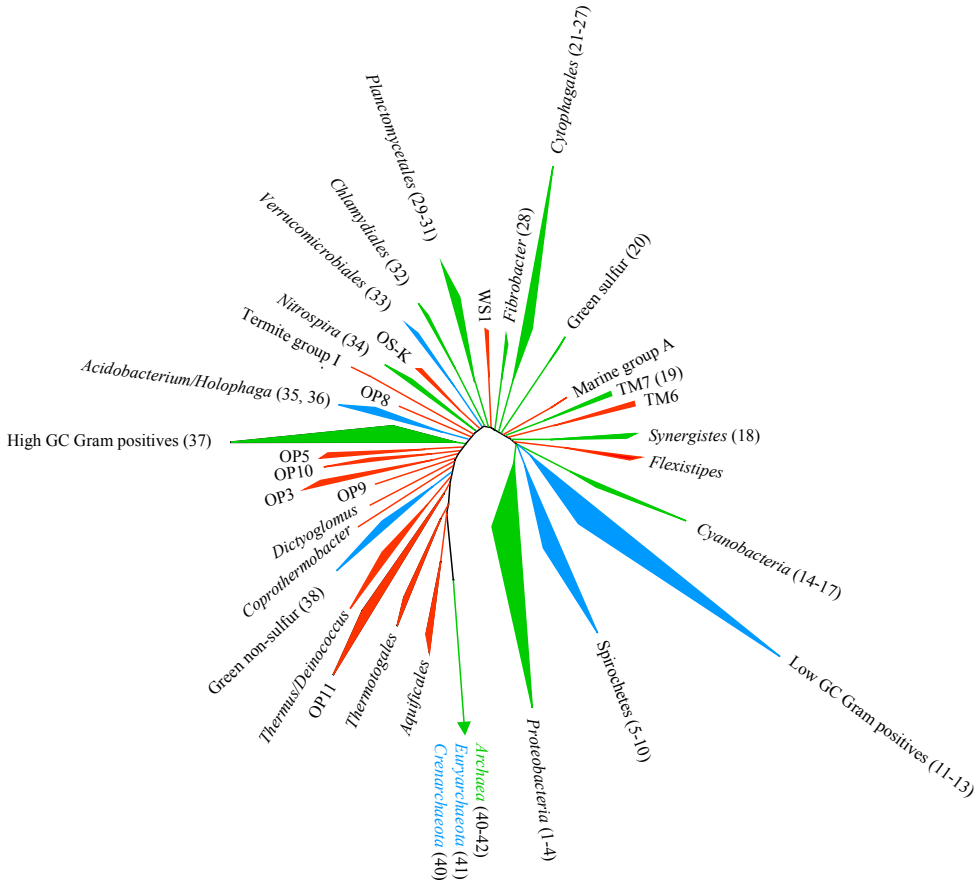
Wastewater treatment is one of the most important biotechnological processes which is used worldwide to treat polluted sewage and to ameliorate anthropogenically induced damage to the environment. Dependent on the treatment goals different types of sewage treatment plants are used. This book chapter focuses on the microbiology of the activated sludge process, which is most commonly used and in which the microbial biomass is aerated and kept in suspension during the treatment process. In addition we also cover activated sludge and biofilm nutrient removal plants in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and/or biologically enhanced phosphorus removal. It is common knowledge that in all types of wastewater treatment plants prokaryotic microorganisms dominate and represent the “causative agent” responsible for the observed conversions. On the other hand, certain filamentous microorganisms cause the most frequently encountered problems in wastewater treatment. Thus, the efficiency and robustness of a wastewater treatment plant mainly depends on the composition and activity of the microbial communities present in its different stages. Although biological wastewater treatment has been intentionally used for more than a century, due to methodological limitations knowledge on the microbiology of this process was scarce till the end of the ‘80s. Consequently, these microbial communities were considered generally as a "black box", and progress in the design and control of wastewater treatment plants was derived mainly from empirical research in civil engineering. Only after the introduction of molecular techniques in microbial ecology during the last decade, it became possible to determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different process types. It is the aim of this book chapter to review these new insights and to provide some guidance on how this knowledge could be extended by implementing newly developed methods and be used for future improvement of wastewater treatment.

The full text of this publication is reprinted in appendix 5.

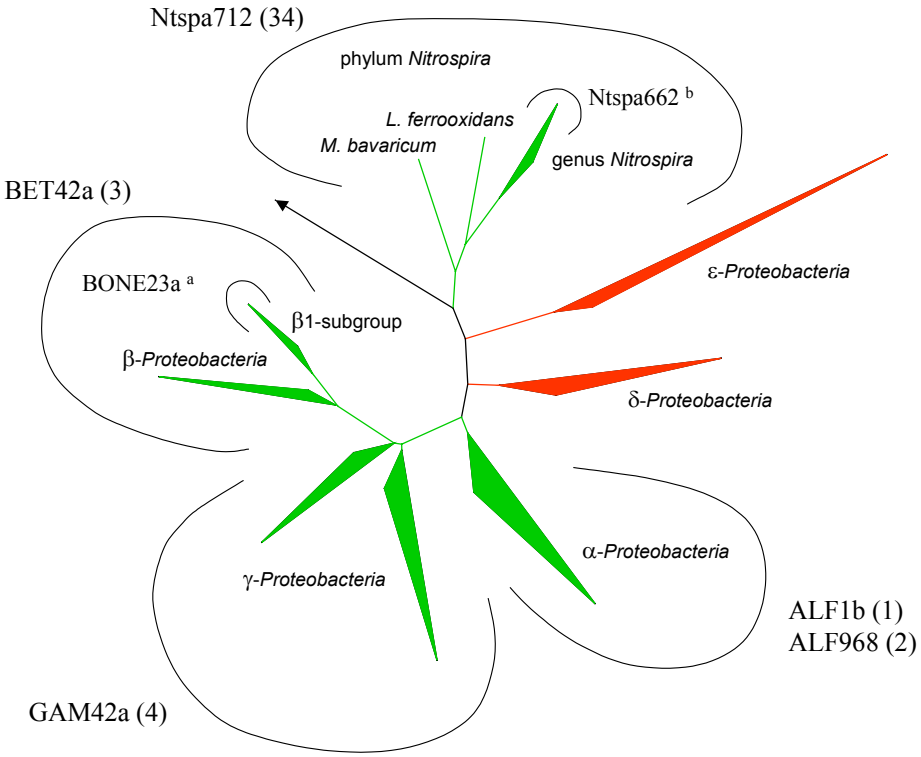
Discussion

New rRNA-targeted oligonucleotide probes specific for the genus and the phylum *Nitrospira* were developed in this thesis (appendix 3). These probes cover all known *Nitrospira*-like, nitrite-oxidizing bacteria as well as most organisms in the other two main lineages of the *Nitrospira* phylum (the genus *Leptospirillum* and the *Thermodesulfovibrio* / "*Magnetobacterium bavaricum*" group). Other *Nitrospira*-specific probes were developed in previous studies (Hovanec *et al.*, 1998; Juretschko *et al.*, 1998; Schramm *et al.*, 1998), but these probes were optimized to detect only parts of the genus *Nitrospira*. The new *Nitrospira*-specific probes and the new EUB338 variants (also developed in this thesis; appendix 1) belong to the set of group- and domain-specific oligonucleotide probes targeting main prokaryotic lines of descent. This probe set must be expanded further in future studies, because most bacterial phyla are still incompletely or not at all covered by group-specific probes suitable for *in situ* hybridization. The current coverage of the *Bacteria* and *Archaea* by group-specific probes is summarized in Table 1 and Fig. 1a. In addition to the probes of broad specificity, which target whole phyla, numerous subphylum-, genus-, and species-specific probes are already available for more detailed analyses of microbial communities. This can be illustrated by the coverage of the nitrifying bacteria by oligonucleotide probes on different taxonomic levels. The nitrite-oxidizers of the genus *Nitrospira* can be distinguished from all other known bacteria by the *Nitrospira*-phylum- and genus-specific probes developed in this thesis (Fig. 1b). All other known, aerobic, lithotrophic nitrifiers belong to different subclasses of the *Proteobacteria*. The α, β and γ-subclasses of this phylum are covered by subphylum-specific probes (Fig. 1b). Additional probes target particular groups within these subclasses, like the genus *Nitrobacter* in the alpha-subclass (Wagner *et al.*, 1996) or the ammonia-oxidizers in the beta-subclass. Most members of the latter group are detected by the probes Nso190 and Nso1225 (Fig. 2). They can be further differentiated by means of subgroup- and species-specific probes. For example *Nitrosococcus mobilis* (which groups phylogenetically with the genus *Nitrosomonas*; Teske *et al.*, 1994; Purkhold *et al.*, 2000) is targeted by the species-specific probe NmV and is detected also by probe NEU (specific for a sublineage of the genus *Nitrosomonas*) and probe Nsm156, which is specific for the genus *Nitrosomonas* (Fig. 2). Such probe binding patterns are used in microbial ecology like fingerprints to detect and quantify single bacterial species or higher taxa by *in situ* or membrane hybridization. This

A


Planctomycetales (29-31)
Cytophagales (21-27)
Chlamydiales (32)
Verrucomicrobiales (33)
Fibrobacter (28)
WS1
Green sulfur (20)
Nitrospira (34)
Termite group 1
OS-K
Marine group A
TM7 (19)
Acidobacterium/Holophaga (35, 36)
OP8
TM6
High GC Gram positives (37)
Synergistes (18)
OP5
OP10
Flexistipes
OP3
OP9
Cyanobacteria (14-17)
Dictyoglomus
Coprothermobacter
Green non-sulfur (38)
Low GC Gram positives (11-13)
Thermus/Deinococcus
OP11
Thermotogales
Aquificales
Spirochetes (5-10)
Archaea (40-42)
Euryarchaeota (41)
Crenarchaeota (40)
Proteobacteria (1-4)

B


Ntspa712 (34)
phylum Nitrospira
Ntspa662 b
L. ferrooxidans
M. bavaricum
genus Nitrospira
BET42a (3)
ε-Proteobacteria
BONE23a a
β1-subgroup
β-Proteobacteria
δ-Proteobacteria
α-Proteobacteria
γ-Proteobacteria
ALF1b (1)
ALF968 (2)
GAM42a (4)

approach was also applied in different parts of this thesis, for example to detect the high diversity of ammonia-oxidizing bacteria in a sequencing batch biofilm reactor (appendix 4).

The abundance of bacteria stained by FISH with specific probes is usually expressed as a fraction of the total bacterial population in an environmental sample ("relative abundance"). For this purpose, the total bacterial population is quantified by counting the cells which have been labeled by a *Bacteria*-specific probe, mostly by probe EUB338 (Amann *et al.*, 1990). Therefore, the quantification accuracy is strongly influenced by the coverage of the *Bacteria* by this probe. Previous studies demonstrated repeatedly that in environmental samples a significant fraction of the cells stained by the DNA-binding dye DAPI was not detected by EUB338 (appendix 1). The new probes EUB338-II and EUB338-III (developed in this thesis; appendix 1) cover two additional bacterial phyla (the planctomycetes and the verrucomicrobia) and a few members of other bacterial lineages, which are not targeted by the original probe EUB338. In total less than 2% of all 16S rRNA sequences in the current Arb database are complementary to EUB338-II and III at the probe target site (Table 1). However, some of the organisms in the target groups of these probes were found to be abundant in various natural and engineered habitats and could thus be of major ecological importance. Planctomycetes were isolated from freshwater and marine samples (Hirsch and Müller, 1985; Schlesner, 1986) and were detected in activated sludge, biofilm and terrestrial habitats by molecular methods (Liesack and Stackebrandt, 1992; Bond *et al.*, 1995; Snaidr *et al.*, 1997; Zarda *et al.*, 1997; Neef *et al.*, 1998; Schmid *et al.*, 2000). Verrucomicrobia were found not only in aquatic environments (Staley *et al.*, 1976; Schlesner, 1987; Hiorns *et al.*, 1997; Zwart *et al.*, 1998) but also in sediments and soils (Liesack and Stackebrandt, 1992; Janssen *et al.*,

Fig. 1. A. Diagrammatic representation of the main prokaryotic lines of descent and their coverage by group-specific, rRNA-targeted oligonucleotide probes. At least 75% of the known organisms in the phyla depicted green are covered by existing group-specific probes. Blue coloring indicates that less than 75% of a phylum is covered by such probes. The lineages depicted red are not targeted by any group-specific probe published so far. The numbers in parentheses refer to the probe numbers in the first column of Table 1. **B.** Diagrammatic representation of the *Proteobacteria* and the phylum *Nitrospira* showing the coverage of these phyla by group-specific, rRNA-targeted oligonucleotide probes. Coloring is like in (A). The numbers in parentheses refer to the probe numbers in Table 1. Additional probes were designed by Amann *et al.* (1996) [a] and in this thesis [b].

1997; Wise *et al.*, 1997). An uncultured bacterium belonging to the verrucomicrobia was identified as a main metabolizer in a grassland soil (Felske and Akkermans, 1998). The importance of EUB338-II and III is also emphasized by the high abundance of different bacterial populations, which were detected by these probes but not by EUB338, in a biofilm sample (appendix 1). Moreover, almost all DAPI-stained cells in this biofilm could be assigned to the *Bacteria* by using a mixture of all three probes EUB338, EUB338-II and EUB338-III. Consequently, the new EUB338 variants should be used together with probe EUB338 in all quantification experiments to obtain more accurate results. They were already applied in this thesis (appendices 2 and 4) and in another study (Schmid *et al.*, 2000) to quantify the total bacterial populations in samples taken from various nitrifying bioreactors.

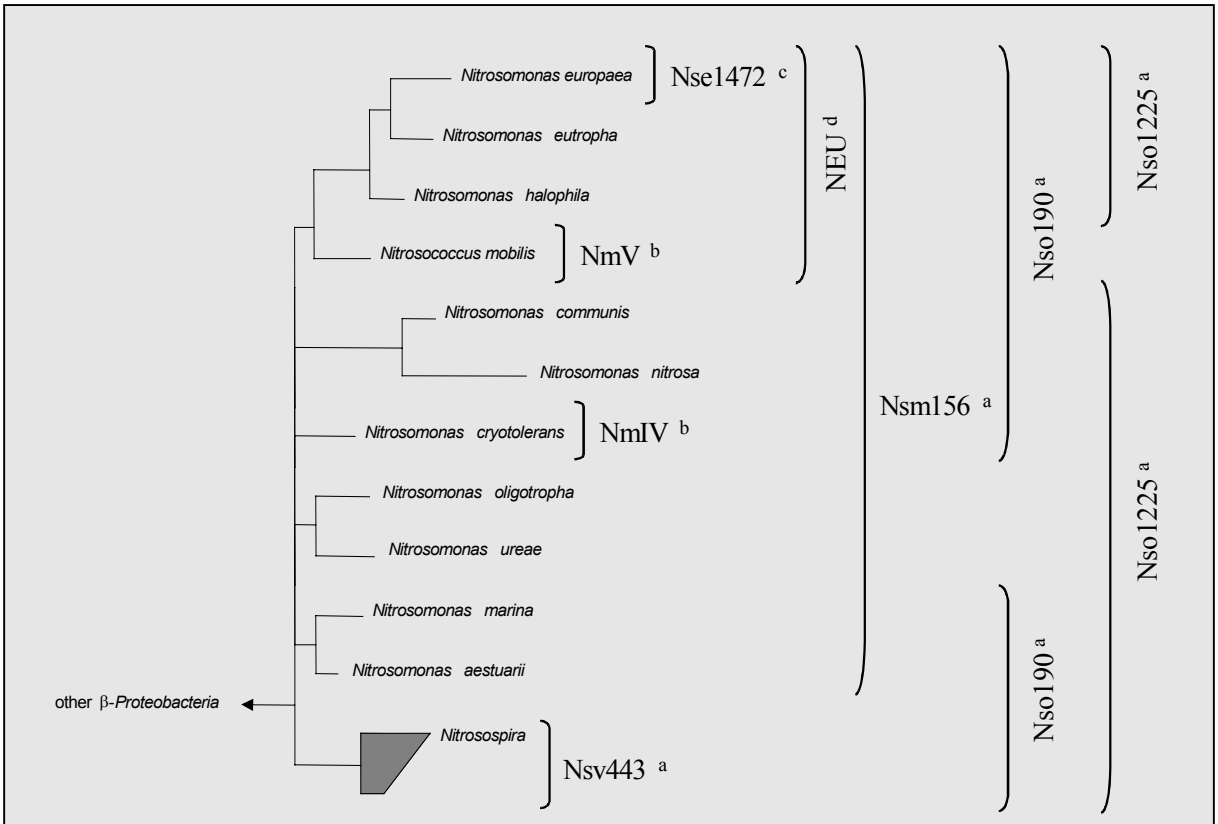


Fig. 2. Coverage of the ammonia-oxidizers in the beta-subclass of *Proteobacteria* by rRNA-targeted oligonucleotide probes. The dendrogram contains only representative members of the phylogenetic clades proposed by Purkhold *et al.* (2000). The brackets indicate the coverage of the probes. Probe names are located on the right of the corresponding brackets. Probes were designed by Mobarry *et al.* (1996) [a], Pommering-Röser *et al.* (1996) [b], Juretschko *et al.* (1998) [c], and Wagner *et al.* (1995) [d].

Table 1. Coverage of the main prokaryotic lines of descent by group-specific, rRNA-targeted oligonucleotide probes. Probes of more narrow specificity (for genera or species) are listed only for those phyla which are not targeted by any published phylum- or subphylum-specific probe. Sensitivity and specificity of the probes were determined by using the Arb "probe match" module on current Arb databases of almost complete 16S and 23S rRNA sequences.

No.	Probe	Sensitivity[a] (%)	Specificity[b]	Reference	Target group and total coverage (%)[c]
1	ALF1b	37.6	1,105	Manz *et al.*, 1992	α-*Proteobacteria*
2	ALF968	76.6	132	Neef, 1997	84.7
3	BET42a[f]	92.6	22 [d]	Manz *et al.*, 1992	β-*Proteobacteria* 92.6
4	GAM42a[f]	90.8	45 [d]	Manz *et al.*, 1992	γ-*Proteobacteria* 90.8
5	TRE I	4.9	0	Choi *et al.*, 1997	Spirochetes 12.9
6	TRE II	2.1	0	Moter *et al.*, 1998	
7	TRE IV	2.1	0	Moter *et al.*, 1998	
8	DDK4	0.4	0	Choi *et al.*, 1997	
9	Pilosi209	2.1	0	Boye *et al.*, 1998	
10	SER1410	1.7	0	Boye *et al.*, 1998	
11	LGC A	41.1	9	Meier *et al.*, 1999	Low G+C gram-positive bacteria 52.1
12	LGC B	29.1	7	Meier *et al.*, 1999	
13	LGC C	10.9	0	Meier *et al.*, 1999	
14	CYA762	53.7	38	Schönhuber *et al.*, 1999	*Cyanobacteria* 92.2
15	CYA664	35.3	0	Schönhuber *et al.*, 1999	
16	CYA361	86.9	8	Schönhuber *et al.*, 1999	
17	CIV/V1342	6.2	5	Schönhuber *et al.*, 1999	
18	DHP1006	100	0	McSweeney *et al.*, 1993	*Synergistes* 100
19	TM7905	100	2	Hugenholtz *et al.*, 2001	TM7 100
20	GSB532	75	0	Tuschak *et al.*, 1999	Green sulfur bacteria 75

No.	Probe	Sensitivity[a] (%)	Specificity[b]	Reference	Target group and total coverage (%)[c]
21	CFB286	49.3	0	Weller *et al.*, 2000	*Cytophagales* 90.5
22	CFB563	27.2	0	Weller *et al.*, 2000	
23	CFB719	31.1	4	Weller *et al.*, 2000	
24	CFB972	27.2	96	Weller *et al.*, 2000	
25	CFB1082	39.2	2	Weller *et al.*, 2000	
26	CF319	41.9	28	Manz *et al.*, 1996	
27	BAC303	33.1	0	Manz *et al.*, 1996	
28	Fibro	100	0	Lin and Stahl, 1995	*Fibrobacter* 100
29	Pla46	92.6	6	Neef *et al.*, 1998	*Planctomycetales* 95.1
30	Pla886	82.7	2,561 [d]	Neef *et al.*, 1998	
31	EUB338-II	75.3	1 [d,e]	this thesis	
32	Chls523	97.8	3 [d]	M. Horn, personal communication	*Chlamydiales* 97.8
33	EUB338-III	70	31 [d,e]	this thesis	*Verrucomicrobiales* 70
34	Ntspa712	85	0 [d]	this thesis	*Nitrospira* 85
35	IRog1	38.4	1	Ludwig *et al.*, 1997	*Acidobacterium*/*Holophaga* 44.2
36	IRog2	44.2	0	Ludwig *et al.*, 1997	
37	HGC[f]	82.5	10	Roller *et al.*, 1994	High G+C gram-positive bacteria 82.5
38	SAR202B	1.9	0	Giovannoni *et al.*, 1996	Green non-sulfur 1.9
39	EUB338	90.4	0	Amann *et al.*, 1990	domain *Bacteria* 91.8
31	EUB338-II	0.8	0	this thesis	
33	EUB338-III	0.6	0	this thesis	
40	CREN499	30.6	0	Burggraf *et al.*, 1994	*Crenarchaeota* 30.6
41	EURY498	50	2	Burggraf *et al.*, 1994	*Euryarchaeota* 50
42	Arch915	88.3	0	Stahl and Amann, 1991	*Archaea* 88.3
					domain *Archaea* 89.8

[a] The fraction of the sequences within the target group which have not more than 0.4 weighted mismatches to the probe sequence.

[b] The number of non-target sequences which have up to 0.4 weighted mismatches to the probe sequence.

[c] Total coverage of the target group by a combination of all listed probes which are specific for this group.

[d] The probe specificity is improved by a competitor oligonucleotide as detailed in the cited publication.

[e] The probe detects parts of the specified group and additional bacteria, because it belongs to the *Bacteria*-specific probe set.

[f] The probe target site is located on the 23S rRNA.

Since quantifying probe-stained cells by counting them manually in the microscope is tedious, time-consuming and often inaccurate, quantification procedures were developed that allow to measure the biovolumes of fluorescently labeled cells by digital image analysis (Kuehn *et al.*, 1998; Bouchez *et al.*, 2000; Heydorn *et al.*, 2000; Schmid *et al.*, 2000). Like manual cell counting, most of these approaches determine the relative abundance of a bacterial population (which then refers to the total bacterial biovolume instead of total bacterial cell numbers). However, relative abundance data obtained for different samples by any method cannot be compared directly, because the bacterial biomass content of the samples may differ. In contrast, absolute cell numbers per sample volume or weight are comparable. Absolute cell concentrations are also needed to determine key physiological parameters like cell division rates and average per-cell substrate turnover activities. In this thesis, a quantification method was developed that allows to measure absolute cell concentrations of uncultivated bacteria semi-automatically and with a satisfying accuracy even if the quantified cells are tightly clustered (appendix 2). This feature was exploited to quantify ammonia-oxidizers forming large cell aggregates in activated sludge in order to estimate their average per-cell ammonia oxidation rate (appendix 2). The result (approx. 2.3 fmol NH_4^+ $cell^{-1}$ h^{-1}) was about one order of magnitude higher than per-cell ammonia oxidation activities estimated previously for the ammonia-oxidizers in other biofilm and activated sludge samples (Wagner *et al.*, 1995; Schramm *et al.*, 1999). This discrepancy might result from the use of different cell quantification methods in these two studies and this thesis. While Wagner *et al.* (1995) counted the ammonia-oxidizer cells in microscope viewing fields manually, Schramm *et al.* (1999) combined manual cell counting with non-automated cell area measurement by digital image analysis. Only a limited number of microscope viewing fields or images could be

evaluated by these two approaches due to the manual counting steps. In contrast, the method developed and applied in appendix 2 avoids manual counting of large cell numbers. Therefore it allowed to quantify the ammonia-oxidizers in a significantly larger number of images. The cell concentrations of ammonia-oxidizers determined by this method, and consequently the activity estimation, may thus be more accurate. The different per-cell activities estimated in the three studies (including this thesis) could also reflect different environmental conditions or nitrifying community compositions in the investigated habitats. In a recent publication, the per-cell activities of ammonia-oxidizers in a nitrifying biofilm reactor were estimated to be 0.65-1.5 fmol NH_4^+ $cell^{-1}$ h^{-1} based on manual cell counts (Gieseke *et al.*, 2001). Although slightly lower, these estimates are more similar to the value obtained here. Furthermore, Gieseke and coworkers stated that they most probably underestimated the per-cell activity of the ammonia-oxidizers in their biofilm. Interestingly, the values obtained by Gieseke *et al.* (2001) and in this thesis are within the range of average per-cell activities measured with pure cultures of *Nitrosomonas europaea* (1.24-23 fmol NH_4^+ $cell^{-1}$ h^{-1}; Laanbroek and Gerards, 1993).

Starving or chemical inhibition do not cause a measurable decrease of the cellular ribosome content in ammonia-oxidizing bacteria (Wagner *et al.*, 1995; Morgenroth *et al.*, 2000). Therefore, active and inactive ammonia-oxidizer cells cannot be distinguished by FISH with rRNA-targeted probes, and the assumption that all quantified, probe-stained cells were metabolically active leads to unprecise activity estimations. This problem can be circumvented only by methods to detect active cells specifically. Cangelosi and Brabant (1997) reported that fluctuations in the precursor rRNA level reflected growth phase changes of *E. coli* cells much better than the mature ribosome content. Oerther *et al.* (2000) used DNA probes targeting the intergenic spacers on the rRNA precursors to monitor the activities of *Acinetobacter* cells *in situ*. They showed that rRNA production in *Acinetobacter* corresponds to cell activity, but is regulated differently than in *E. coli*. Future experiments must verify whether metabolic activity and precursor rRNA synthesis correlate also in nitrifying bacteria. Moreover, it will be important to test whether the precursor rRNA levels in nitrifier cells are sufficiently high to obtain clearly visible fluorescence after FISH with pre-rRNA targeted probes. If these tests were positive then the quantification protocols presented in this thesis and in previous studies could be used without major modifications to determine average per-cell activities of nitrifiers more precisely. A significant disadvantage of this approach arises from the low sequence conservation of the intergenic spacer regions, which may require that

new strain-specific probes be designed for each investigated environmental sample. The combined application of FISH and MAR is an alternative way to determine the active fraction of nitrifier populations by using existing, rRNA-targeted probes. This technique was applied in this thesis (appendix 3) to count the actively CO_2-fixing microcolonies of *Nitrospira*-like bacteria in activated sludge. Although this experiment demonstrated clearly and for the first time that most *Nitrospira*-like bacteria in the sludge were active, it suffered from certain limitations of the FISH / MAR combination. If MAR signals existed above the smallest *Nitrospira* colonies formed by only a few cells, then they were extremely weak and could not be distinguished from background noise. Thus it was impossible to decide whether the cells in these colonies had fixed CO_2. On the other hand, prolonged exposition of the radiographic film resulted in increased background signals which hampered the microscopic evaluation of the experiment. Therefore, more sensitive techniques than MAR may be needed to detect the radiation emitted by single cells or small cell clusters of slow-growing bacteria after incubation with radioactive substrates. Highly sensitive CCD cameras are available which detect light signals emitted by a radioactive sample that has been covered with a scintillation membrane. The resolution of these systems (approx. 50 µm) is too low for use with most environmental samples, but future detectors may have a higher resolution. Since these cameras do not only detect, but also count photons, they could be applied to quantify the amounts of radioactive substrate taken up by different cells (or cell clusters) during the incubation period. However, a prerequisite for their use will be a method to detect the radioactivity together with the fluorescence emitted by the FISH-stained cells. Otherwise the bacteria which took up the radioactive substrate cannot be identified. The quantification methods developed in this thesis and in previous studies provide a basis for future improvements, which should focus on reliable techniques to quantify active microbial cells *in situ*. The obtained data would be extremely useful to optimize nitrifying bioreactors and to improve the mathematical models of nitrification as well as to study other functional groups of bacteria important for wastewater treatment.

The discovery that various activated sludge plants contained, if any, only very low cell numbers of *Nitrobacter* (Wagner *et al.*, 1996; Juretschko *et al.*, 1998; Bartosch *et al.*, 1999; and appendix 3) came as a surprise, because *Nitrobacter* spp. had been regarded as the key nitrite-oxidizers in wastewater treatment (e.g., Bever *et al.*, 1995; Henze *et al.*, 1997). The factors that cause the dominance of nitrite-oxidizing *Nitrospira*-like bacteria over *Nitrobacter* in most bioreactors have not yet been elucidated. Schramm and coworkers (1999) postulated

that *Nitrobacter* spp. could be r-strategists with a low substrate affinity for nitrite and relatively high growth rates at increased nitrite concentrations. On the contrary, *Nitrospira* spp. could then be K-strategists with a high substrate affinity for nitrite and lower growth rates. *In situ* analyses of nitrite-oxidizing bacteria and exact measurements of nitrite concentrations have so far been combined in only a few studies on nitrifying biofilms (Table 2). Three of the reactors investigated in such detail contained significant amounts of *Nitrobacter* above the detection limit of FISH (10^3-10^4 cells per ml). In two of these three biofilms, also *Nitrospira*-like bacteria were found. One reactor (Schramm *et al.*, 1996) was examined with *Nitrobacter*-specific oligonucleotide probes only, and the parallel occurrence of *Nitrospira*-like bacteria in this reactor cannot be ruled out. The maximum nitrite concentrations in the three reactors containing *Nitrobacter* (Table 2) were close to or far higher than the $K_S(NO_2^-)$ values of pure *Nitrobacter* cultures (60-600 µM; Prosser, 1989; Hunik *et al.*, 1993). Only *Nitrospira*-like bacteria, but not *Nitrobacter*, were detected in biofilm reactors where the maximum nitrite concentrations were lower than the $K_S(NO_2^-)$ values of *Nitrobacter* spp. (Table 2). Thus it seems that *Nitrobacter* was outcompeted by *Nitrospira* in the biofilm systems with low nitrite concentrations exactly as the "K/r hypothesis" mentioned above would suggest. On the other hand, only *Nitrospira* was detected in one alternately aerobic and anaerobic phosphate-removing reactor (Gieseke *et al.*, 2001) and in reactor Biofor 2 (appendix 3) although the nitrite concentrations in these reactors were just within the range of the $K_S(NO_2^-)$ values of *Nitrobacter* spp. (Table 2). This indicates that additional factors could be decisive in reactors with maximum nitrite concentrations around the lowest $K_S(NO_2^-)$ values of *Nitrobacter* (see below).

Table 2. Maximum nitrite concentrations and nitrite-oxidizing bacteria (NOB) detected in different biofilm reactors.

Reactor	NO_2^- (µM)	detected NOB	Reference
membrane reactor	1,200	*Nitrobacter* *Nitrospira*	Schramm *et al.*, 2000
trickling filter	approx. 50	*Nitrobacter*	Schramm *et al.*, 1996
SBBR 1	5,100	*Nitrobacter* *Nitrospira*	this thesis (appendices 3 and 4)
fluidized bed reactor	20	*Nitrospira*	Schramm *et al.*, 1998
fluidized bed reactor	10	*Nitrospira*	Schramm *et al.*, 1999
rotating disc reactor	30	*Nitrospira*	Okabe *et al.*, 1999
phosphate-removing SBBR	90	*Nitrospira*	Gieseke *et al.*, 2001
Biofor 2	70	*Nitrospira*	this thesis (appendix 3)

But which factors can cause the accumulation of nitrite in bioreactors? Nitrite is an intermediary product of nitrification. Therefore, the nitrite concentration will increase only if the ammonia-oxidizers produce more nitrite than the nitrite-oxidizers can consume. The pilot-scale reactor SBBR 1 received reject water from sludge dewatering with high loads of ammonia (300-500 mg l^{-1} NH_4^+ - N). These high ammonia concentrations supported a particularly large population of ammonia-oxidizing bacteria. The quantification performed in this thesis (appendix 4) revealed that the biovolume of the ammonia-oxidizers in this reactor amounted to more than 50% of the total bacterial biovolume, being almost seven times larger than the biovolume of the nitrite-oxidizers. Consequently, the rate of ammonia oxidation exceeded the rate of nitrite oxidation, and nitrite accumulated during each operational cycle of the reactor (appendix 3). Although *Nitrospira*-like bacteria were the dominant nitrite-oxidizing population in SBBR 1, *Nitrobacter* occurred also in significant amounts (appendix 4). The *Nitrospira*-like bacteria were obviously not inhibited by the elevated nitrite concentrations, and *Nitrobacter* took advantage of the surplus nitrite. On the other hand, one might expect that *Nitrobacter* spp. should have overgrown *Nitrospira* in SBBR 1 due to their presumably higher growth rate at increased nitrite concentrations. From this point of view it appears clear that additional factors must be responsible for the dominance of *Nitrospira* in reactor SBBR 1. The abrupt nitrite concentration changes caused by the sequencing batch operation of the reactor could be such a factor: nitrite accumulated towards the end of the cycles, but its concentration dropped significantly when the reactor was flushed and filled with new wastewater (appendix 3). Thus, the growth conditions might have been advantageous for *Nitrobacter* only during the later parts of each cycle while *Nitrospira* could grow faster when the nitrite concentration was lower at the beginning of the cycles. This hypothesis is contradicted by the fact that the lowest nitrite concentration measured during one cycle in reactor SBBR 1 (950 µM) was still far higher than all $K_S(NO_2^-)$ values of *Nitrobacter* species. Thus the dominance of *Nitrospira* in SBBR 1 may rather be completely independent from the nitrite concentration. The ability of *Nitrospira*-like bacteria to take up pyruvate was demonstrated in this thesis (appendix 3). It is well possible that these organisms can also grow mixotrophically with other organic carbon sources, which are present in wastewater but cannot be used by *Nitrobacter*. Furthermore, due to the lack of pure cultures virtually nothing is known about the potential of *Nitrospira*-like bacteria to use other energy sources than nitrite and about their biosynthetic capabilities. The phylum *Nitrospira* is physiologically diverse as it contains not only nitrite-oxidizers, but also iron-oxidizers and sulfate-reducers. Although nitrite oxidation is the most obvious phenotype displayed by the

Nitrospira-like bacteria in bioreactors, it is tempting to speculate that parts of their genome might code for alternative pathways of energy metabolism. Such properties, if really present, could confer selection advantages and might render *Nitrospira*-like bacteria more competitive than *Nitrobacter* under the growth conditions in bioreactors. Cultivation-independent cloning and sequencing of *Nitrospira* DNA by an environmental genomics approach (e.g., Schleper *et al.*, 1998; Rondon *et al.*, 2000) could significantly expand our knowledge on the physiological potentials of these organisms. A lower $K_S(O_2)$ value may also promote the dominance of *Nitrospira*-like bacteria. Nitrite-oxidizers in bioreactors have to compete for oxygen not only with ammonia-oxidizers, but also with fast-growing heterotrophs. Therefore, the ability to tolerate low oxygen partial pressures can be a pivotal advantage. Significant amounts of *Nitrospira*-like bacteria were indeed found in zones of low oxygen availability in stratified biofilms (Okabe *et al.*, 1999; Schramm *et al.*, 2000). *Nitrospira*-like bacteria were also detected by rRNA sequence analysis and by FISH in SBRs with aerobic and anaerobic cycle phases (Bond *et al.*, 1995; Gieseke *et al.*, 2001). *Nitrospira moscoviensis*, which is relatively closely related to the *Nitrospira*-like bacteria that occur in bioreactors (appendix 3), is able to oxidize H_2 with nitrate as electron acceptor in absence of O_2 (Ehrich *et al.*, 1995). If *Nitrospira*-like bacteria could survive at lower oxygen partial pressures than *Nitrobacter*, they might be able to outcompete *Nitrobacter* despite of high nitrite concentrations. This could be decisive particularly in reactor SBBR 1, where the large population of ammonia-oxidizers most probably consumes the bulk of the available oxygen. Dependence of *Nitrobacter* on higher oxygen concentrations was already assumed by Schramm *et al.* (2000), because they found *Nitrobacter* mainly in the oxygen-rich biofilm zones of a membrane reactor. As a conclusion, *Nitrobacter* spp. may be r-strategists which can persist in biofilm reactors where nitrite is not growth-limiting, but there they may be confined to special microenvironments where the selection advantages of *Nitrospira*-like bacteria are less effective. Accordingly, *Nitrobacter* would be absent from biofilms that lack suitable niches. The aforementioned phosphate-removing, aerobic / anaerobic SBR (Gieseke *et al.*, 2001) might be an example of such a reactor: *Nitrobacter* species were not found in this system although the nitrite concentration was within the range of their $K_S(NO_2^-)$ values.

Based on these considerations, the low cell numbers of *Nitrobacter* in activated sludge plants become feasible, too. Firstly, the growth conditions in activated sludge basins are more uniform than in biofilm reactors. Microhabitats are numerous in most sessile biofilms, but are most likely rare in activated sludge flocs. The flocs are permanently moved within the basin

and are frequently disintegrated due to shear stresses. Therefore, selection advantages of *Nitrospira*-like bacteria are probably even more effectful in activated sludge than in biofilms. Secondly, the steady state concentration of nitrite is lower than 70 µM in most conventionally operated activated sludge plants with intact nitrification (like the AAV plant, appendix 3). This concentration is already at the lower end of the $K_S(NO_2^-)$ values of *Nitrobacter*. Therefore, nitrite is most likely the growth-limiting factor for *Nitrobacter* if aeration is sufficient. If the "K/r hypothesis" applied and *Nitrospira*-like bacteria competed more efficiently than *Nitrobacter* for these small amounts of nitrite, their nitrite-oxidizing activity would render the growth conditions for *Nitrobacter* even more unfavorable. As a result, the growth rates of *Nitrobacter* would become so low that *Nitrobacter* would be simply washed out of the continuously operated activated sludge systems. The easiness to isolate *Nitrobacter* from activated sludge may be attributed to allochthonous *Nitrobacter* cells, which are brought into the reactors with the influent wastewater stream or which stem from the soils surrounding the activated sludge plant.

In addition to the possible causes discussed above, influences of other organisms or viruses could also support the dominance of *Nitrospira*-like bacteria in wastewater treatment plants. For example, *Nitrospira* could be more resistant to grazing by protozoa or to bacteriophages, which may reduce the cell numbers of *Nitrobacter*. Finally, *Nitrospira*-like bacteria could be able to cope better with the sudden changes of the growth conditions, which occur frequently in many nitrifying plants. This would require a physiological flexibility, which can hardly be assessed before pure cultures of *Nitrospira*-like bacteria have been isolated from wastewater treatment plants. Although physiological data are scarce, the complex morphology of the *Nitrospira* microcolonies (appendix 3) indicates a certain adaptability. The cavities and channels most likely facilitate the exchange of gases, nutrients, and metabolic end products with the surrounding medium. Nothing is known about the mechanisms of channel formation that take effect in these cell clusters. Simple starvation and lysis of cells in the colony center do not explain the continuous network of channels protruding to the colony surface. One may speculate that the morphogenesis of the colonies could be regulated by some kind of cell to cell communication. The importance of autoinducers, which modulate gene expression depending on cell density ("quorum sensing"), for biofilm formation by other bacteria has already been demonstrated (for a review see Eberl, 1999).

The composition and dynamics of nitrifying microbial communities are not only important for microbiologists, but also for engineers. Many nitrifying wastewater treatment plants suffer from breakdowns of nitrification performance, which are caused by often unpredictable changes of the growth conditions in the plant. Examples for such disturbances are changes of wastewater composition or pH and temperature shifts. Failure of nitrification is extremely problematic due to the toxicity of ammonia and nitrite, and can be costly since penalties must be paid if untreated wastewater has to be dumped into natural waters. One attempt to restore the nitrifying activity once it has broken down is bioaugmentation (Rittmann and Whiteman, 1994), i.e. the addition of activated sludge taken from another nitrifying plant. This measure is often unsuccessful, because the added bacteria cannot get established in the plant, and nitrification activity does not increase until the indigenous nitrifiers have recovered after a while. The failure of such a bioaugmentation step was followed by using FISH and *in situ* biovolume quantification recently (Bouchez *et al.*, 2000). Molecular studies revealed also that the ammonia-oxidizer populations in different reactors often consist of different strains or species (Purkhold *et al.*, 2000), and that a bioreactor with extreme growth conditions is dominated by those ammonia-oxidizers which are best adapted to the these conditions (Juretschko *et al.*, 1998). Hence it is not surprising that bioaugmentation of any plant with the "wrong" nitrifiers will not have the desired effect. These bacteria are not adapted to the conditions in the augmented plant and cannot replace the indigenous nitrifiers. Therefore, indexing the nitrifying populations in wastewater treatment plants by means of FISH, rRNA and *amoA* sequence analyses (Purkhold *et al.*, 2000) would be a meaningful measure. If such catalogues were updated frequently enough they could be invaluably useful: bioreactors suffering from low nitrification performance could be augmented with sludge taken from other plants that contain similar ammonia- and nitrite-oxidizers. A different, but possibly more effectful strategy aims at stabilizing a high diversity of indigenous nitrifier populations in bioreactors instead of replacing the nitrifiers after a breakdown. In the sequencing batch biofilm reactor SBBR 1 a relatively high diversity of ammonia-oxidizing bacteria was detected by FISH (appendix 4) and by *amoA* sequence analysis (Purkhold *et al.*, 2000). As mentioned above, different nitrite-oxidizers (*Nitrobacter* and *Nitrospira*-like bacteria) were also found in this reactor. Although SBBR 1 is a pilot-scale research reactor and the operational mode was slightly altered on different occasions, the nitrification performance of this system was surprisingly stable for almost three years (Eva Arnold, Technische Universität München, personal communication). This stability could be caused by the high diversity of the nitrifiers. For example, SBBR 1 contained ammonia-oxidizers related to the

moderately halotolerant species *Nitrosomonas europaea* as well as strains related to *Nitrosococcus mobilis* (appendix 4). *N. mobilis* is a marine ammonia-oxidizer, which was first detected in an industrial plant receiving wastewater with very high salt and ammonia concentrations (Juretschko *et al.*, 1998) and which seems to cope particularly well with peak concentrations of ammonia. While some nitrifiers living in a reactor that harbours a high nitrifier diversity may be negatively affected by changes of the environmental conditions, other nitrifiers present in the same reactor may grow even better under the altered conditions. As a consequence, nitrification does not cease. This could have happened in SBBR 1 for example when the ammonia concentration in the influent changed, because the ammonia-oxidizer population contained moderately and highly ammonia-tolerant strains. Such population shifts should be reversible without significant losses of nitrification performance as long as all species can maintain a minimum population density. Under these circumstances they can reach higher cell numbers within a short time after the environmental conditions have become more favourable for their growth. Different scenarios are imaginable where a high nitrifier diversity would be advantageous, reaching from ammonia or nitrite concentration shifts to bacteriophage attacks, which may have impact on some but not all populations of a diverse community. But is there any practicable strategy to establish a high nitrifier diversity in bioreactors? One possibility may be to introduce small, non-destructive disturbances intentionally during the start-up phase of a nitrifying treatment plant. This could ensure that different nitrifier populations instead of a monoculture get established in the reactor. However, the maintenance of this diversity during the later, normal reactor operation might be more difficult. In this context it seems that biofilm reactors like SBBR 1 could be more useful to realize this strategy than activated sludge plants. As mentioned above, most sessile biofilms contain more different microhabitats than activated sludge. This variety of ecological niches is likely to support a higher diversity of nitrifiers that can occupy these niches. In addition, populations that grow slowly under the current conditions are not washed out of a biofilm system as quickly as they would be removed from an activated sludge reactor. These organisms, although confined to particular biofilm zones and less abundant than the currently dominant populations, can take the position of the key nitrifiers when the environmental conditions and therewith the kind and distribution of the microhabitats in the biofilm change. The effects of well-directed disturbances on the stability and performance of nitrifying bioreactors should certainly be evaluated by well-designed experiments with laboratory-scale reactors. The applicability of this approach on a technical scale will depend strongly on the possibilities to monitor population development and dynamics in large

reactors. The molecular methods applied in this thesis are the tools of choice to accomplish this task of reactor control as well as they are essential for future basic studies on the microbiology of nitrifying bacteria. The many remaining questions can be answered only by combined molecular and physiological approaches. Such efforts are urgently required, because the capacity of the existing wastewater treatment plants is certainly not inexhaustible. Although we are just beginning to understand the ecology of complex microbial communities as found in activated sludge and biofilm, it is already foreseeable that microbiological studies will substantially contribute to the design of more efficient, stable, and economical bioreactors.

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Appendix 1

The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set

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The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set

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In situ hybridization with rRNA-targeted oligonucleotide probes has become a widely applied tool for direct analysis of microbial population structures of complex natural and engineered systems. In such studies probe EUB338 (Amann *et al.*, 1990) is routinely used to quantify members of the domain *Bacteria* with a sufficiently high cellular ribosome content. Recent reevaluations of probe EUB338 coverage based on all publicly available 16S rRNA sequences, however, indicated that important bacterial phyla, most notably the *Planctomycetales* and *Verrucomicrobia*, are missed by this probe. We therefore designed and evaluated two supplementary versions (EUB338-II and EUB338-III) of probe EUB338 for *in situ* detection of most of those phyla not detected with probe EUB338. *In situ* dissociation curves with target and non-target organisms were recorded under increasing stringency to optimize hybridization conditions. For that purpose a digital image software routine was developed. *In situ* hybridization of a complex biofilm community with the three EUB338 probes demonstrated the presence of significant numbers of probe EUB338-II and EUB338-III target organisms. The application of EUB338, EUB338-II and EUB338-III should allow a more accurate quantification of members of the domain *Bacteria* in future molecular ecological studies.

Keywords: Bacteria-specific probes, EUB338, fluorescent *in situ* hybridization, digital image analysis

Introduction

A central aspect in microbial ecology is the identification and quantification of those microorganisms that occur in the examined habitats. This task is far from trivial because lack of distinctive morphological traits of most prokaryotes hampers their direct microscopic identification (Woese, 1987). Cultivation approaches do, due to limited knowledge of nutritional and other growth requirements for the majority of microorganisms, only offer a limited and biased inventory of the naturally existing microbial diversity (for example Wagner *et al.*, 1993). The advent of rRNA-targeted oligonucleotide probe-based quantitative dot blot and fluorescent *in situ* hybridization (FISH) techniques allows for cultivation-independent insights into community structure and dynamics in microbial ecology (Wagner *et al.*, 1994; Amann *et al.*, 1995). Species-, genus-, group- and domain-specific probes were designed and successfully applied to study microbial consortia present in various natural and engineered systems (e.g. Manz *et al.*, 1992; Raskin *et al.*, 1994; Wagner *et al.*, 1994; Glöckner *et al.*, 1996; Harmsen *et al.*, 1996; Mobarry *et al.*, 1996; Zarda *et al.*, 1997; Felske *et al.*, 1998; Juretschko *et al.*, 1998; Llobet-Brossa *et al.*, 1998; Møller *et al.*, 1998; Moter *et al.*, 1998; Neef *et al.*, 1998; Meier *et al.*, 1999). In such studies probe EUB338 (Amann *et al.*, 1990), which binds to the highly conserved region 338-355 (*E. coli* numbering) of the 16S rRNA molecule, has been generally used to quantify the domain *Bacteria*.

Recent studies indicated that at least one group of bacteria that is increasingly realized to be environmentally important, the *Planctomycetales*, are not detected by EUB338 (Zarda *et al.*, 1997; Neef *et al.*, 1998). Probe EUB338 was designed in 1990 (Amann *et al.*, 1990). Since then extensive comparative 16S rRNA sequence analysis has enormously improved our perspective on prokaryotic diversity. Several new phyla and divisons within the bacterial domain were recognized as being widely distributed in the environment (for a review see Hugenholtz *et al.*, 1998a). Future studies in microbial ecology should not ignore, but rather track those poorly, if at all characterized bacteria. Therefore, we reevaluated the coverage of all currently available 16S rRNA sequences of bacterial origin by probe EUB338 and developed (and evaluated) two modified versions of probe EUB338 which allow, if used in addition to EUB338, for a more complete detection of *Bacteria*.

Materials and Methods

Reference organisms, culture conditions, cell fixation, and biofilm sampling

All reference strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *Bacillus stearothermophilus* (DSM 22) was grown overnight in modified HD Medium (1% (w/v) tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose, 0.7% (w/v) NaCl, pH 7.4) at 55°C under agitation. *Verrucomicrobium spinosum* (DSM 4136) and *Planctomyces limnophilus* (DSM 3776) were cultured as described previously (Hirsch and Müller, 1985; Schlesner, 1987). Cultures were prepared for *in situ* hybridization by washing them once in phosphate buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer pH 7.2) and fixing them for three hours in paraformaldehyde (Sigma, Deisenhofen, Germany) as described by Amann (1995) with the exception that the final paraformaldehyde concentration was 2% (w/v). *Bacillus stearothermophilus* was not fixed in paraformaldehyde but instead the cells were transferred to a 1:1 mixture of PBS and 96% ethanol (v/v) immediately after the washing step (Roller *et al.*, 1994). Following the fixation, paraformaldehyde-fixed cells of the other species were washed once again in PBS and were then resuspended in the PBS:ethanol mixture, too. Paraformaldehyde-fixed cells of the *Parachlamydia*-like endosymbiont of *Acanthamoeba* spec. UWE25 (Fritsche *et al.*, submitted) were kindly provided by Matthias Horn, Technische Universität München. All fixed cultures were stored at -20°C. Expanded clay beads covered by nitrifying biofilm were sampled from a pilot-scale reactor treating municipal sewage (500 population equivalents, Biofor2, Ingolstadt, Germany). For fixation they were incubated for five hours in 3% (w/v) paraformaldehyde (final concentration) immediately after sampling. Subsequently, the biofilm was detached by manually swirling the beads within the fixative. Loosened biofilm was collected by centrifugation, resuspended in a 1:1 mixture of PBS and 96% (v/v) ethanol and stored at -20°C.

Oligonucleotide probes

The software package ARB (Strunk and Ludwig, http://www.biol.chemie.tu-muenchen.de/pub/ARB/) was used to evaluate the coverage of probe EUB338 (Amann *et al.*, 1990) on all 16S rRNA sequences in the TUM 16S rRNA ARB database (December 1998

database release, including approx. 10.500 bacterial and 500 archaeal 16S rRNA sequences) using the probe match tool. The specificity of the newly designed probes EUB338-II and EUB338-III was checked with the same program and database. For *in situ* hybridization, the probes were labeled at the 5'-end with the dye FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimide ester) or with one of the sulfoindocyanine dyes Cy3 and Cy5. When used as competitors in the probe dissociation experiments, the probes were applied as unlabeled oligonucleotides. Labeled and unlabeled probes were obtained from MWG (Ebersberg, Germany) or Interactiva (Ulm, Germany). Probe sequences and conditions required for specific hybridization are specified in the Results section.

In situ hybridization, DAPI-staining, and microscopy

Fixed reference cells were spotted onto microscopic slides (Paul Marienfeld GmbH, Bad Mergentheim, Germany). For immobilization of biofilm samples gelatin-coated slides were used (Amann, 1995). Slides were dried at 46 °C for 10 min and dehydrated in 50, 80 and 96% (v/v) ethanol. Whole cell and *in situ* hybridization experiments were performed as described previously (Manz *et al.*, 1992). Final probe concentrations in the hybridization buffer were 3 $ng \cdot \mu l^{-1}$ for probes labeled with Cy3 or Cy5, and 5 $ng \cdot \mu l^{-1}$ for probes labeled with FLUOS. Competitor oligonucleotide probes were used in equimolar amounts. Optimization of hybridization conditions for the newly developed probes EUB338-II and EUB338-III was performed as described by Manz *et al.* (1992) using different concentrations of formamide and sodium chloride in the hybridization and wash buffers, respectively. Probe dissociation curves were obtained by digital image analysis of signal intensities of reference strains after *in situ* hybridization at different stringencies (see below). Following *in situ* hybridization, biofilm samples were stained at room temperature for five minutes with a 0.1% (w/v) solution of the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) (Sigma, Deisenhofen, Germany) in order to visualize all cells present in the sample regardless of their EUB338 target site sequences. Excess DAPI was removed with distilled water and the slides were air-dried. Prior to microscopic observation, slides were embedded in Citifluor (Citifluor, Canterbury, U.K.). Images were recorded using a LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with one UV laser (351 and 364 nm), one Argon laser (450 to 514 nm) and two Helium-Neon lasers (543 and 633 nm,

respectively). For image reconstruction and printing the software package delivered with the microscope (Zeiss LSM 5 version 2.01) was used together with a Kodak 8650 PS printer.

Quantification of single cell fluorescence

Following *in situ* hybridization of suitable reference cells, for each probe and hybridization condition series of images of fluorescent cells were recorded by confocal laser scanning microscopy applying identical scanning parameters. Parameter settings were such that the brightest cell signals observed did not cause saturation (pixel grey values < 255) while low signal intensities (after applying highly stringent hybridization conditions) were still detectable. Subsequently, the intensity of single cell fluorescence was determined using the Zeiss Kontron KS400 image processing software (version 3.0) together with a newly developed macro (S.I.M., Signal Intensity Measurement). In brief, the first step performed by this macro is the mapping of all color information in an image to grey values of corresponding intensities. The resulting grey scaled image is the so-called "density image". Thereupon another grey scaled version of the original image is calculated, but this time with enhanced pixel intensities. This so-called "mask image" allows the user to spot even the darkest objects on the screen. The user then manually selects the smallest and largest cells in the "mask image" in order to define threshold values for the size range (in this case, the pixel area) of single cells. After the program has automatically selected all objects in the image with pixel areas between these thresholds, remaining artifacts can be manually marked for exclusion from further processing. At this point, special care must be taken to eliminate clustered cells, because they appear brighter than corresponding single cells. Thereafter the program measures the mean brightness of the selected cells in the "density image". This yields one mean intensity value for each cell. These values were imported automatically into suitable spreadsheet software (e.g. Excel, Microsoft, Redmond, Wa) for further processing and graphical visualization. The developed macro is available upon request.

Results

Coverage of currently available bacterial 16S rRNA sequences by probe EUB338

The TUM ARB sequence database, currently containing more than 10.500 bacterial 16S rRNA sequences, was screened to identify those bacteria displaying mismatches within the 18 nucleotide stretch (*E. coli* positions 338-355) representing the probe EUB338 target site. Despite the presence of probe EUB338 target site in 97% of the bacterial 16S rRNA sequences, this search revealed that only 6 of its 18 nucleotides (*E. coli* positions 338, 343, 346, 352, 354, 355) are completely conserved throughout the domain *Bacteria*. None of these represents a signature for the domain *Bacteria*, since identical nucleotides are found at the respective positions in the domains *Archaea* and/or *Eucarya*.

In addition to probe EUB338, other rRNA targeted oligonucleotide probes for detection of the domain *Bacteria* have been published (for a list of domain specific probes, refer to Amann *et al.*, 1995). Screening of the TUM ARB database for those sequences which are fully complementary to each of these probes revealed most encompassing coverage of the domain *Bacteria* by probe EUB338. Therefore we decided to restrict our further analyses to probe EUB338 which offered the best starting-point for the design of a more comprehensive probe set.

Different types of sequence variations within the probe EUB338 target site could be detected for sequences affiliated with the phyla *Aquificales*, Spirochetes, green non-sulfur bacteria, Gram-positive bacteria with low and high G+C content of the DNA, *Planctomycetales*, *Chlamydiales*, *Verrucomicrobia*, *Acidobacterium*/*Holophaga*, *Cytophaga*/*Flavobacterium*/*Bacteroides* (CFB), *Proteobacteria*, and the candidate phylum OP11 (Fig. 1, Table 1). Among these phyla probe EUB388 coverage is still almost complete for the *Proteobacteria* (mismatches are found only for 3 sequences affiliated to the alpha-subclass), the *Cytophaga*/*Flavobacterium*/*Bacteroides* phylum (a single partial sequence of a molecular isolate from activated sludge does not possess the target site), the *Acidobacterium*/*Holophaga* phylum (only two molecular isolates differ within the probe EUB338 target site), and the Spirochetes (*Treponema maltophilum* and *Critispira pectinis* have mismatches to EUB338). We cannot exclude that these gaps in otherwise well covered groups originate from

sequencing errors. Higher numbers of representatives lacking full complementarity to EUB338 were found among the Gram-positive Bacteria with low and high G+C content of the DNA and green non-sulfur bacteria phyla as well as the candidate phylum OP11. Furthermore, none of the sequences affiliated to the phyla *Planctomycetales*, *Aquificales*, *Chlamydiales,* and *Verrucomicrobia* possess a target region fully complementary to probe EUB338.

In the following paragraph we present a detailed description of sequence variations of the probe EUB338 target site for the phyla *Planctomycetales*, *Chlamydiales,* and *Verrucomicrobia*. Within the *Planctomycetales*, two types of sequence variation at the *E. coli* positions 338-355 were observed. Sequence variation type I, represented by the genus *Isosphaera*, is characterized by an A -> U transversion at *E. coli* position 353. This sequence variation is not restricted to the *Planctomycetales* but is also found for all but one member of the *Chlamydiales*, some representatives of the Gram-positive Bacteria with low G+C content of the DNA, three molecular isolates of the *Verrucomicrobia* phylum, and one molecular isolate of candidate phylum OP11 (Fig. 1A). Sequence variation type II, characterized by an A -> U transversion at *E. coli* position 353, an A -> U transversion at *E. coli* position 349, and a U -> A transversion at *E. coli* position 340, is with exception of the genus *Isosphaera*, present in all other *Planctomycetales* and in two environmentally derived 16S rDNA clones affiliated to candidate phylum OP11, but is absent from other bacteria in the database. Concerning the *Chlamydiales*, as mentioned above, all members except for *Chlamydia psittaci*, do possess sequence variation type I. Within the phylum *Verrucomicrobia* a third type of sequence variation of *E. coli* positions 338-355 was identified. Sequence variation type III differs from the probe EUB338 target site due to a U -> A transversion at *E. coli* position 340, and an A -> U transversion at *E. coli* position 349. This sequence variation type is found in all but three 16S rRNA sequences belonging to the *Verrucomicrobia* and is also present in some members of the green non-sulfur Bacteria, and in the only partially sequenced molecular isolates SMKN41 and clone SBR 1082 tentatively clustering together with the Gram-positive bacteria with high G+C content of the DNA and the CFB, respectively. Figure 1A summarizes the distribution of the EUB338 target sequence and of the three above described types of variations among the phyla of the domain *Bacteria*.

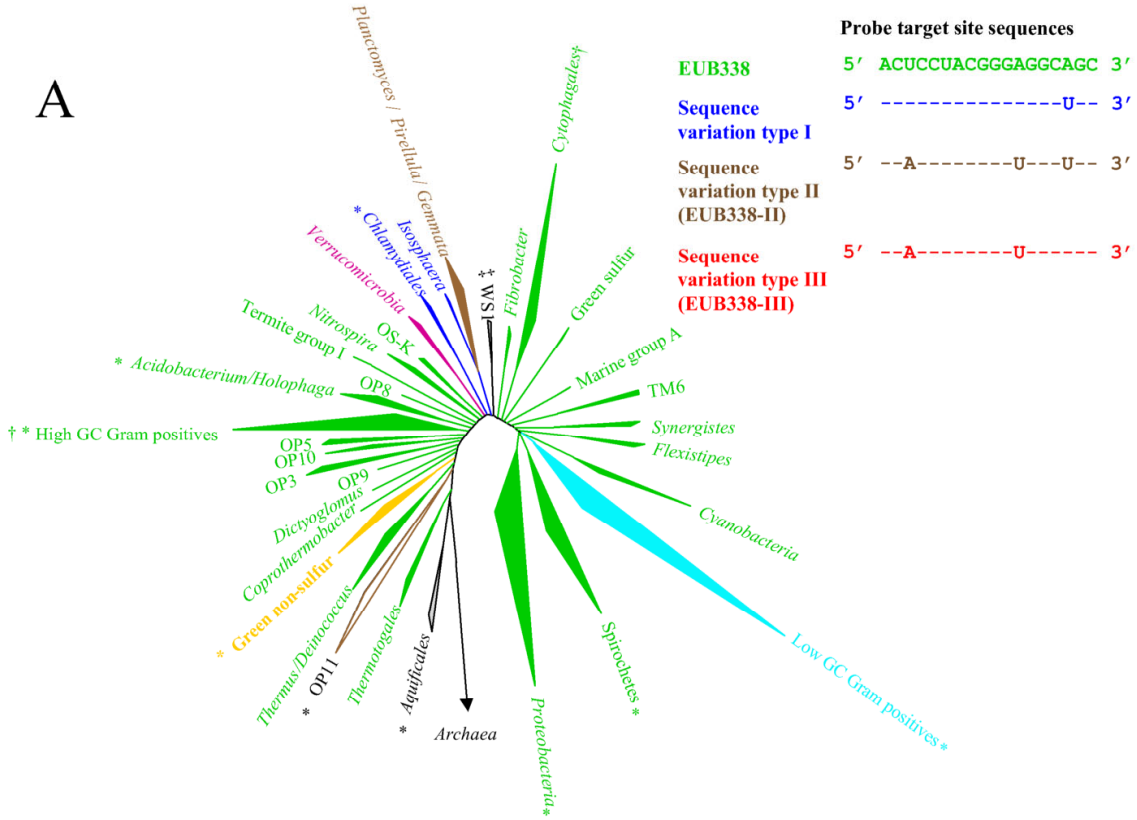
Table 1. 16S rRNA sequences with variations at the target site of probe EUB338 that are different from sequence variation types I-III. Positions of mismatches to original EUB338 are printed in bold. For environmentally-derived cloned sequences accession numbers are given.

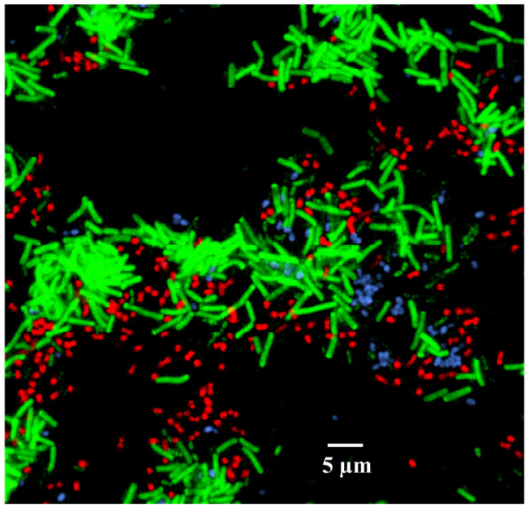
Phylogenetic division / organisms or clones	EUB338 target site sequence variation original EUB338 target site: ACUCCUACGGGAGGCAGC
Aquificales	
1) *Aquifex pyrophilus, Calderobacterium hydrogenophilum*, *Hydrogenobacter thermophilus*, *H. acidophilus*, *Thermocrinis ruber*, clones NAK14 (AB005738), OPB13 (AF027098), OPS1 (AF018186), OPS4 (AF018189), OPS5 (AF018190), OPS6 (AF018191), OPS7 (AF018192), OPS10(AF 018194), OPS14 (AF018196), strEM_17 (U05661)	**--C--------G------**
2) clone GANI4 (AB005736)	**--AA-------G------**
candidate division OP11	
clone LGd14 (AF047573)	**--AU------AU------**
green non-sulfur bacteria	
1) *Herpetosiphon geysericola*, *H. aurantiacus*	**----------G------**
2) clones SJA-35 (AJ009460), WCHB1-50 (AF050571)	**--G--------U------**
High GC Gram positives	
Prauseria hordei, *Nocardiopsis alba*, *Thermomonospora alba*, clones 56AT148 (Z73374), 56AT1203 (Z73373)	**------G----------**
Acidobacterium/Holophaga	
clones "unidentified bacterium 29" (AB004577), "unidentified bacterium 30" (AB004578)	**-------U----------**
Chlamydiales	
Chlamydia psittaci	**---U------A----U--**
Low GC Gram positives	
1) *Mycoplasma iowae*, *M. muris*, *M. penetrans*, *M. volis*, *Eubacterium timidum*, *Spiroplasma* group IX (M24474)	**---U------A-------**
2) *Eperythrozoon suis*, *Haemobartonella felis*, *H. muris*	**-U----------A-----**
3) *Haemaphysalis longcornis*	**----U----A--------**
4) clones RF8 (AF001741), RF9 (AF001742), RF39 (AF001770)	**-------------A----**
Spirochetes	
1) *Critispira pectinis*	**--CA------UG------**
2) *Treponema maltophilum*	**---U--------------**
Proteobacteria	
1) *Orientia tsutsugamushi*	**----------G------**
2) clones Alpha9 (L35460), Alpha12 (L35465)	**---U------A-------**

Experimental evaluation of the detection of *Bacteria* by probe EUB338

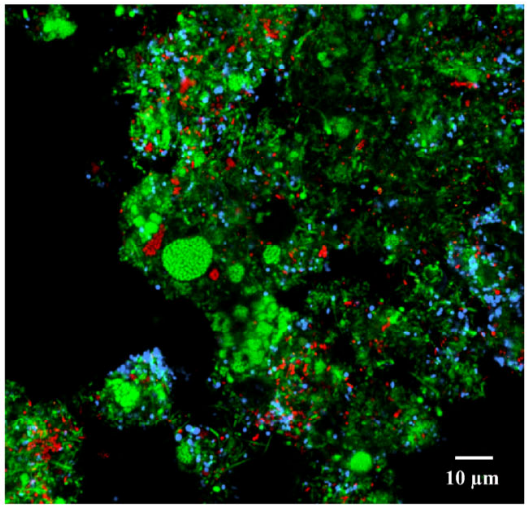
EUB338 coverage was experimentally analyzed by separate whole cell hybridization of *Bacillus stearothermophilus*, *Parachlamydia*-like *Acanthamoeba* symbiont UWE25, *Planctomyces limnophilus*, and *Verrucomicrobium spinosum* representing bacteria which possess the full match EUB338 target site, and the above described type I, II, and III sequence variations of the target site, respectively. Probe dissociation curves were recorded for increasingly stringent hybridization and washing conditions adjusted by increasing formamide concentrations in the hybridization buffer and accordingly decreasing NaCl concentrations in the wash buffer (Figure 2A-D). As expected, hybridization of *B. stearothermophilus* (representative with unaltered EUB338 target site) with probe EUB338 resulted in intense fluorescent labeling of the cells over a broad range of formamide concentrations from 0 to 70% (v/v, Fig. 2A). Further increase of stringency resulted in significant loss of signal intensity of the cells, however even the maximum applied stringency (80% formamide in the hybridization buffer and no NaCl in the wash buffer) was not sufficient to cause dissociation of 50% of the probe (equivalent to the T_d of the probe). Hybridization of the *Parachlamydia*-like *Acanthamoeba* symbiont UWE25, which has the type I sequence variation at the *E. coli* positions 338-355, with probe EUB338 at increasingly stringent conditions showed that the single T-U mismatch caused 50% probe dissociation already at 60.5% formamide but did not hamper probe binding at lower stringency (Fig. 2B). Lower signal intensities of the *Parachlamydia*-like symbiont compared to *B. stearothermophilus* within the stringency range from 0% formamide to 50% formamide were most likely caused by a lower cellular ribosome content of the symbiont cells. In contrast to bacteria with sequence variation of type I, reference cells with sequence variations of type II and III (*P. limnophilus* and *V. spinosum*, respectively) displaying three and two mismatches to probe EUB338, respectively, did not show detectable hybridization of probe EUB338 under the different conditions applied (Fig, 2C,D). These results demonstrate that a quantification of *Bacteria* in environmental samples solely by probe EUB338 would miss most members of the phyla *Planctomycetales* and *Verrucomicrobia* as well as several green non-sulfur bacteria and members of the candidate phylum OP11.

A


Probe target site sequences
EUB338
5′ ACUCCUACGGGAGGCAGC 3′
Sequence variation type I
5′ ----------U-- 3′
Sequence variation type II (EUB338-II)
5′ --A--------U---U-- 3′
Sequence variation type III (EUB338-III)
5′ --A--------U------ 3′
Planctomyces / Pirellula / Gemmata
Cytophagales†
* Chlamydiales
Isosphaera
Verrucomicrobia
WS1 *
Fibrobacter
Green sulfur
Nitrospira
OS-K
Termite group 1
Marine group A
* Acidobacterium/Holophaga
OP8
TM6
† * High GC Gram positives
Synergistes
OP5
OP10
Flexistipes
OP3
OP9
Dictyoglomus
Cyanobacteria
Coprothermobacter
* Green non-sulfur
Thermus /Deinococcus
OP11 *
Thermotogales
Aquificales *
Archaea
Proteobacteria*
Spirochetes *
Low GC Gram positives *


5 µm

B


10 µm

C

Fig. 1. (A). Schematic representation of the phyla within the domain *Bacteria* showing the distribution of the original EUB338 target site sequence (colored green) as well as the sequence variation types I (blue), II (brown), and III (red). Phyla containing more than one sequence variation type are depicted in the resulting additive mixed color (for example the phylum *Verrucomicrobia* is labeled purple due to the presence of sequence variation type I and III, the low G+C Gram positives are colored turquoise due to the presence of the original EUB338 target site and sequence variation type I, and the candidate phylum OP11 is depicted white with brown border due to the presence of the original EUB338 target site and its three variations). The phylum *Aquificales* is given in grey with black border to illustrate that all recognized members of this lineage of descent possess additional sequence variations. Please note that the branching order of the bacterial phyla shown in this dendrogram is phylogenetically not representative.

* Additional sequence variations were observed for certain species and/or 16S rRNA sequences retrieved from environmental samples. Refer to table 1 for details.

† Sequence variation type III was observed for the environmental clones strSMKN41 (X78664, High GC gram positives) and SBR1082 (X84513, *Cytophagales*), but since only short partial sequences are available their phylogenetic affiliation could not unambiguously be resolved.

‡ The publicly available sequences representing candidate division WS1 are incomplete and do not contain *E. coli* positions 338-355.

(**B).** Whole cell hybridization of an artificial mixture of *Bacillus stearothermophilus*, *Planctomyces limnophilus*, and *Verrucomicrobium spinosum* under stringent hybridization conditions with probes EUB338-FLUOS (green), EUB338-II-Cy5 (blue), and EUB338-III-Cy3 (red).

(C). *In situ* hybridization of a nitrifying biofilm from a wastewater treatment plant at Ingolstadt with probes EUB338-FLUOS (green), EUB338-II-Cy5 (blue), and EUB338-III-Cy3 (red). Some cells overlap spatially with neighbouring cells and appear therefore in mixed colors (yellow or white).

Design of probes EUB338-II and EUB338-III

Two variations of probe EUB338 that are fully complementary to the sequence variations of type II and III, EUB338-II and EUB338-III, were designed (Table 2) to allow for more encompassing coverage of the bacterial domain by *in situ* hybridization. Evaluation of probe specificities and optimal hybridization conditions were performed using the reference organisms *B. stearothermophilus*, *P. limnophilus*, and *V. spinosum* as representatives with

16S rRNA full-match target sites for probes EUB338, EUB338-II, and EUB338-III, respectively.

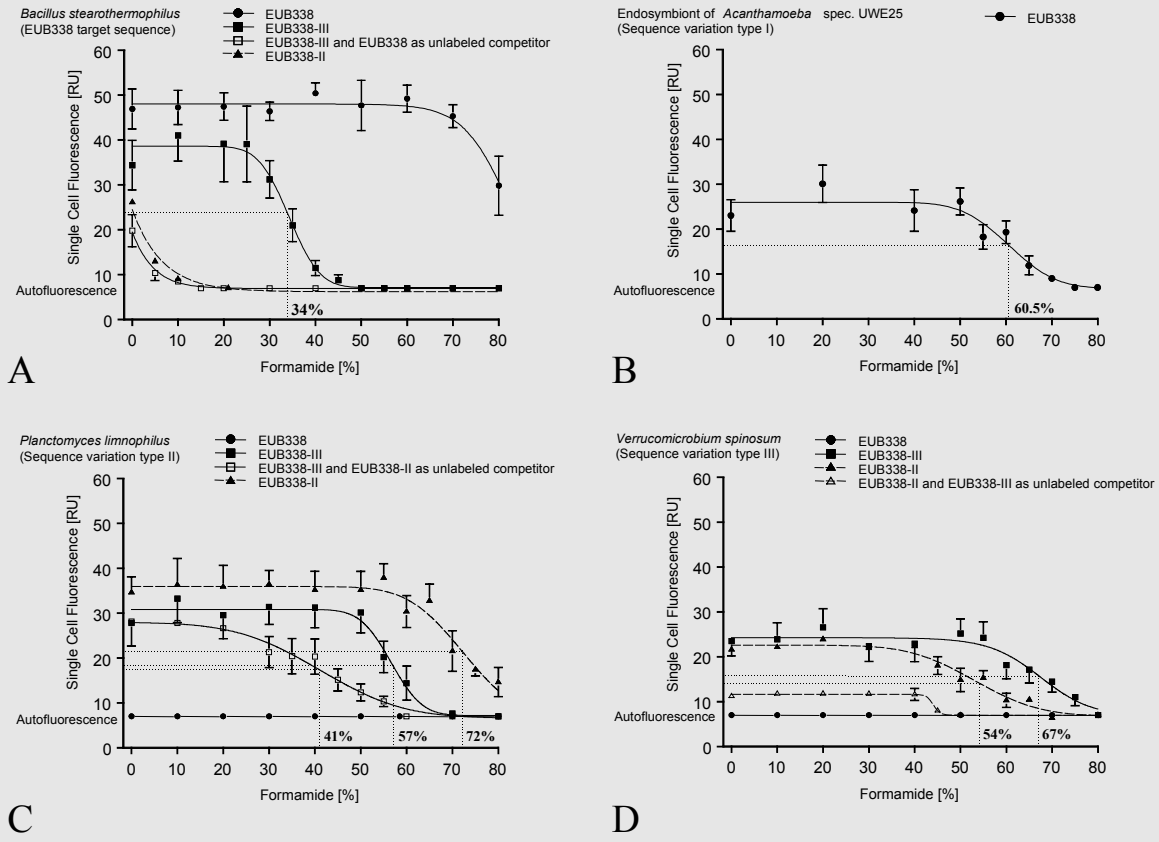


Fig. 2. Probe dissociation curves of probes EUB338, EUB338-II, and EUB338-III determined with suitable reference organisms under increasingly stringent hybridization and washing conditions. For each data point the mean fluorescence intensity of at least 100 cells was determined. Error bars indicate the standard deviation. The formamide percentage in the hybridization buffer required to cause 50% probe dissociation is given where appropriate. Organisms are (A) *Bacillus stearothermophilus* (full match to EUB338), (B) the *Parachlamydia*-like endosymbiont of *Acanthamoeba* spec. strain UWE25 (type I sequence variation), (C) *Planctomyces limnophilus* (type II sequence variation), and (D) *Verrucomicrobium spinosum* (type III sequence variation). Autofluorescence values for all reference cells under all hybridization conditions were determined to be below 10 relative units (RU).

As expected, probe EUB338-II showed strong binding to the target cells of *P. limnophilus* (Fig. 2C). At 72% formamide in the hybridization buffer and without NaCl in the wash buffer probe dissociation was 50%. It should be noted, however, that the mean cellular signal

intensity of the *P. limnophilus* cells after application of probe EUB338-II was, due to the lower cellular ribosome content, lower compared to the respective values of *B. stearothermophilus* with probe EUB338. Probe EUB338-II binding to *B. stearothermophilus*, which has 3 mismatches within the probe target site, was only observed at a low hybridization stringency and could be completely prevented by applying moderately stringent conditions (20% formamide in the hybridization buffer and 0.225 M NaCl in the wash buffer; Fig. 2A). In contrast, *V. spinosum* cells having only a single T-U mismatch within the probe EUB338-II target site showed binding of probe EUB338-II unless highly stringent hybridization conditions (more than 70% formamide in the hybridization buffer and no NaCl in the wash buffer) were used. Addition of equimolar amounts of unlabeled EUB338-III as competitors, however, suppressed binding of probe EUB338-II to *V. spinosum* at formamide concentrations higher than 47% in the hybridization buffer (Fig. 2D). A typical sigmoid dissociation curve was obtained after hybridization of sequence type III representative *V. spinosum* with probe EUB338-III. Half-maximal probe binding was measured at 67% formamide in the hybridization buffer and without NaCl in the wash buffer (Fig. 2D). Observed mean signal intensities for *V. spinosum* with probe EUB338-III were significantly lower compared to the values measured for *P. limnophilus* with probe EUB338-II and *B. stearothermophilus* with probe EUB338. Probe EUB338-III hybridized to *B. stearothermophilus* (two mismatches in the probe target site) under low and medium stringency conditions. After addition of an equimolar amount of probe EUB338, hybrid formation between probe EUB338-III and *B. stearothermophilus* 16S rRNA was only observed under conditions of very low stringency (Fig. 2A). Hybridization results obtained with probe EUB338-III and the *P. limnophilus* reference cells reflected the presence of the single marginal T-U mismatch in the probe target site of this species. At low and medium stringency (from 0 to 50% formamide in the hybridization buffer) probe conferred fluorescence per cell was not significantly reduced compared to hybridization with the full-match probe EUB338-II. At higher stringency the one-mismatch probe EUB338-III showed less binding than probe EUB338-II. The discrimination got more pronounced when equimolar amounts of unlabeled probe EUB338-II were added as competitors to the hybridization buffers (Fig. 2C).

Whole cell hybridization experiments were performed with artificial mixtures of *B. stearothermophilus*, *P. limnophilus* and *V. spinosum* cells and probes EUB338, EUB338-II,

and EUB338-III labeled with different fluorescent dyes in order to confirm the results of the previously described formamide concentration series. As expected, specific hybridization of the reference cells with the respective probes could be achieved by applying stringent hybridization conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer; Fig. 1B).

Table 2. Sequences and target sites of 16S rRNA directed oligonucleotide probes.

Probe	Sequence (5'-3')	Target site [b] (16S rRNA positions)
EUB338[a]	GCTGCCTCCCGTAGGAGT	338-355
EUB338-II	GCAGCCACCCGTAGGTGT	338-355
EUB338-III	GCTGCCACCCGTAGGTGT	338-355

[a] Amann *et al.* (1990)

[b] *E. coli* numbering (Brosius *et al.*, 1981)

Application of the bacterial probe set for fluorescent *in situ* hybridization of a biofilm sample

A biofilm sample originating from a continuos nitrifying reactor of a pilot wastewater treatment plant (Ingolstadt, Germany) was analyzed by simultaneous hybridization with probes EUB338, EUB338-II, and EUB338-III labeled with different fluorescent dyes under stringent conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer). Double or triple hybridization of cells was not observed under these conditions. All organisms stained by probe EUB338-II were spherical or ovoid cocci and occurred more frequently as cell clusters or chains than as single cells. The probe-conferred fluorescence was distributed in a ring-shaped manner as previously observed for cells detected *in situ* with planctomycetes specific probes (Neef *et al.*, 1998). The bacteria stained by probe EUB338-III were more diverse with respect to their morphology. Cocci occurring in groups or as single cells were frequently observed (Fig. 1C). In addition, spindle-shaped cell pairs attached to each other at one pole and, to lower frequency, short rods and thin filaments were detected. Interestingly, almost all DAPI-stained cells in the sample were detectable by fluorescent *in situ* hybridization with the combination of the three probes EUB338, EUB338-II, and EUB338-III.

Discussion

Probe EUB338 is commonly used in rRNA-hybridization based molecular ecological studies in order to quantify the members of the domain *Bacteria* within complex samples. In the *in situ* format, probe EUB338 hybridization is often applied simultaneously with DNA-binding fluorescent dyes (e.g. DAPI) to determine the bacterial fraction of total cell counts which can be visualized by *in situ* hybridization (for references see Table 3). Furthermore, probe EUB338 is frequently applied together with other probes (labeled with different fluorescent dyes) with more narrow specificities to determine the relative abundance of probe-defined populations. The encompassing database check of probe EUB338 specificity performed in this study revealed that several members of the bacterial domain do not possess a fully complementary target site of probe EUB338. Interestingly, all members of the bacterial phyla *Aquificales*, *Verrucomicrobia, Chlamydiales* and *Planctomycetales* show differences to the EUB338 target. Among the observed variations of the probe EUB338 target sites three sequence variation types were abundant. Sequence variation type I (present in 1.5% of the 16S rRNA database entries), which can be found within the phyla *Chlamydiales*, the Gram-positive bacteria with a low DNA G+C content, the candidate phylum OP11, and the genus *Isosphera* is characterized by a single mismatch to probe EUB338. Whole cell hybridization experiments demonstrated that over a wide range of hybridization conditions (0-60% formamide in the hybridization buffer and 0.9-0.008 M NaCl in the wash buffer) hybrid destabilization due to this mismatch is too weak to hamper probe EUB338 binding. Thus, bacteria whose 16S rRNA molecules possess this sequence variation will still be targeted by probe EUB338 under the given hybridization conditions. However, additional hybridization experiments demonstrated that all bacteria characterized by sequence variation types II and III (found in 0.8% and 0.7% of the 16S rRNA database entries, respectively) cannot be detected *in situ* by using probe EUB338. Sequence variation types II and III are present within the candidate phylum OP11, the green non-sulfur bacteria, the *Verrucomicrobia*, and the *Planctomycetales.* Recent molecular analysis demonstrated a previously not recognized importance of these bacterial phyla for many ecosystems. rRNA sequences of the candidate phylum OP11, for which no cultured members are available, were successfully amplified from a Yellowstone hot spring (Hugenholtz *et al.*, 1998b), Carolina Bay sediment (Wise *et al.*, 1997), and Amazonian soil (Borneman and Triplett, 1997). Recent unpublished data discussed

Table 3. Studies where probe EUB338 was used to quantify bacteria in environmental samples and where the amount of cells or rRNA detected by EUB338 was compared to the total cell number or the total amount of extracted rRNA, respectively.

Publication	Samples or habitat examined	Total cell or rRNA detection method	percentage of cells or rRNA detected by EUB338
Alfreider *et al.*, 1996	winter cover and pelagic layers of high mountain lake	DAPI	70 - 85%
Fry *et al.*, 1997	anaerobic, alkaline aquifers	quantitative dot blot with universal probe	64 - 92%
Glöckner *et al.*, 1996	planctonic cells from oligo- and mesotrophic lakes	DAPI	29 - 64%
Hicks *et al.*, 1992	bacterioplancton from artificial ponds	DAPI	35 - 67%
Kalmbach *et al.*, 1997	drinking water biofilms	DAPI	80 - 90%
Kämpfer *et al.*, 1996	activated sludge	DAPI	70 - 82%
Llobet-Brossa *et al.*, 1998	marine sediments	DAPI	30 - 73%
Manz *et al.*, 1994	activated sludge	DAPI	74 - 89%
Manz *et al.*, 1993	drinking water biofilms and free-water-phase	DAPI	40-70%
Neef *et al.*, 1996	biofilms from denitrifying sand filters	DAPI	40-80%
Pernthaler *et al.*, 1998	limnic picoplancton and filaments	DAPI	26 - 100%
Ramsing *et al.*, 1996	water column of Mariager Fjord, Denmark	ethidium bromide staining	50%
Ramsing *et al.*, 1996	water column of Golfo Duce, Costa Rica	ethidium bromide staining	10 - 20%
Snaidr *et al.*, 1997	activated sludge	DAPI	81%
Stoffels *et al.*, 1998	wastewater, biofilm	DAPI	68 - 90%
Wagner *et al.*, 1994	activated sludge	DAPI	78 - 83%
Wagner *et al.*, 1993	activated sludge	DAPI	70 - 90%
Wallner *et al.*, 1995	activated sludge	Hoechst 33342 staining	70-80%
Weiss *et al.*, 1996	limnetic, organic aggregates	DAPI	55 - 100%

by Hugenholtz *et al.* (1998a) indicate OP11 occurrence in hydrocarbon-contaminated soils and deep-subsurface water, too. Application of the full cycle rRNA approach revealed high diversity and significant abundance of *Herpetosiphon*-like bacteria (members of the green non-sulfur bacteria) in an industrial activated sludge (Juretschko *et al.*, in preparation). Of particular note are the *Planctomycetales* and the *Verrucomicrobia* which have been shown to be more widespread in nature than initially expected. All described species of the *Planctomycetales* are freshwater or marine bacteria (e.g. Hirsch and Müller (1985), Schlesner (1986)), but meanwhile members of this phylum have been detected in activated sludge (Bond *et al.*, 1995; Snaidr *et al.*, 1997; Neef *et al.*, 1998, Juretschko et al, in preparation) and even in terrestrial habitats (Liesack and Stackebrandt, 1992; Zarda *et al.*, 1997). The *Verrucomicrobia* seem to be similarly widespread in nature as they occur not only in aquatic environments (Staley *et al.*, 1976; Schlesner, 1987; Hiorns *et al.*, 1997; Zwart *et al.*, 1998) but also in sediments (Wise *et al.*, 1997) and soils (Liesack and Stackebrandt, 1992; Janssen *et al.*, 1997; Felske *et al.*, 1998). Moreover, Felske and Akkermans (1998) reported that a member of this phylum might be among the most active metabolizers in a terrestrial habitat.

Past research performed in various ecosystems has consistently demonstrated that a significant fraction of total cells was missed by *in situ* or dot blot hybridization with probe EUB338 (Table 3). By FISH this fraction could rarely be assigned to the domains *Archaea* or *Eucarya* by probes for those domains and therefore it was speculated that bacterial cells with a low ribosome content or impermeable cell peripheries are responsible for this (Amann *et al.*, 1995). It was indeed shown that specific cell fixation and permeabilization protocols (Manz *et al.*, 1994; Erhart *et al.*, 1997; Schuppler *et al.*, 1998) may significantly increase the fraction of detected cells. Keeping in mind the potential environmental significance of bacteria with sequence variation types II and III it appears to be likely that these bacteria also contribute to the observed differences between EUB338 and total cell counts. Consequently, we designed the supplementary probes EUB338-II and EUB338-III which are fully complementary to the sequence variation types II and III. Optimal hybridization conditions were determined for both probes by whole cell hybridization of suitable reference strains. When used simultaneously under stringent conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer) all three EUB338 probes specifically bound to their respective target sites. In a biofilm sample from a sewage treatment plant, nearly all DAPI-stained cells were detected by *in situ* hybridization when the three EUB338 probes were

applied in combination. In the future, it should be tested whether a 1:1:1 mixture of Cy3-labeled probes EUB338, EUB338-II and EUB338-III also detects significantly higher fractions of DAPI-stained cells in other environments. If the three probes are applied with different fluorescent dyes, additional information about abundance and distribution of the little-known bacterial phyla *Planctomycetales*, *Verrucomicrobia*, green-non sulfur bacteria, and OP11 in nature can be obtained. It should, however, be stressed, that other sequence variations of probe EUB338 target site than those analyzed in this study do exist in a considerable number of bacteria (Table 1). Keeping in mind our limited knowledge of the extend of microbial diversity it is possible or even likely (i) that sequence variation types II and III are also present in novel yet not known bacterial phyla, and (ii) that yet not known bacteria with additional variations do exist in nature. Consequently, the coverage of the bacterial probes, like for all other rRNA-targeted probes, has to be continuously reevaluated and probe modifications or new probes have to be designed if necessary.

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Appendix 2

Cultivation-independent, Semiautomatic Determination of Absolute Bacterial Cell Numbers in Environmental Samples by Fluorescence *In Situ* Hybridization

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Cultivation-independent, Semiautomatic Determination of Absolute Bacterial Cell Numbers in Environmental Samples by Fluorescence *In Situ* Hybridization

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Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes has found widespread application for analyzing the composition of microbial communities in complex environmental samples. Although bacteria can quickly be detected by FISH, a reliable method to determine absolute numbers of FISH-stained cells in aggregates or biofilms has, to our knowledge, never been published. In this study we developed a semi-automated protocol to measure the concentration of bacteria (in cells per volume) in environmental samples by a combination of FISH, confocal laser scanning microscopy, and digital image analysis. The quantification is based on an internal standard, which is introduced by spiking the samples with known amounts of *Escherichia coli* cells. This method was initially tested with artificial mixtures of bacterial cultures and subsequently used to determine the concentration of ammonia-oxidizing bacteria in a municipal nitrifying activated sludge. The total number of ammonia-oxidizers was found to be $9.8 \times 10^7 \pm 1.9 \times 10^7$ cells ml^{-1}. Based on this value, the average *in situ* activity was calculated to be 2.3 fmol of ammonia converted to nitrite per ammonia-oxidizer cell per hour. This activity is within the previously determined range of activities measured with ammonia-oxidizer pure cultures, demonstrating the utility of the developed quantification method to enumerate bacteria in samples where cells are not homogeneously distributed.

Introduction

FISH using rRNA-targeted oligonucleotide probes is frequently applied to quantify the composition of microbial communities in different environments (e.g. 1, 17, 21, 23, 32, 34). In such studies cell numbers are generally obtained by manual counting in an epifluorescence microscope. Usually the relative abundance of a probe target population is determined by comparison of the obtained numbers i) with counts of all bacterial cells detectable by FISH (via simultaneous hybridization with a bacterial probe (e.g. 10, 11, 30, 33) or probe set (9)), or ii) with counts of all organisms containing DNA (by simultaneous application of nucleic acid staining dyes (16, 29, 34, 37)). Although quantitative FISH has provided novel insights into the structure and dynamics of microbial communities, it suffers from its tediousness and its limited accuracy for samples containing densely aggregated cells like activated sludge flocs or biofilms. The latter problem can in part be ameliorated by the use of confocal laser scanning microscopy (CLSM) for the detection of probe-labeled cells (35). However, even if optical CLSM sections are recorded, it is not feasible to count manually a sufficient cell number in each hybridization experiment in a reasonable time period to obtain statistically reliable results. This limitation has two reasons. Firstly, manual counting itself is very time-consuming and thus generally not more than a few thousand cells per hybridization experiment were counted in previous publications. Secondly, manual counting requires high magnification CLSM-sections which allow single cell resolution within clusters. However, such images only contain relatively few cells and therefore many images need to be recorded, rendering the procedure even more time consuming. Therefore, more precise methods are required to quantify the composition of the microflora in samples containing clustered cells. In principal, flow cytometry is a more efficient and accurate alternative for quantification of fluorescently labeled bacterial cells (38). However, for the analysis of microbial flocs and biofilms flow cytometry is of limited use since it necessitates efficient dispersion of clustered bacteria prior to the measurement, a requirement which frequently cannot be fulfilled (37, 38).

To overcome the limitations of manual cell counting procedures, semi-automated digital image analysis tools were recently developed which quantify fluorescently labeled bacteria in environmental samples (6, 29). But such solutions are not able to efficiently count cells in dense clusters or biofilms since single cell recognition within these structures cannot be automized. This problem can be circumvented by measuring the areas of specifically stained

bacteria in randomly acquired optical CLSM sections. This approach only requires the software to differentiate between labeled biomass (including cell clusters) and unlabeled background but does not rely on single cell recognition within clusters. The abundance of a particular population is then expressed as fraction of the area occupied by all bacteria (8, 31). For this purpose, an environmental sample is hybridized simultaneously with different rRNA-targeted oligonucleotide probes: one specific probe that targets the population which is to be quantified, and one domain-specific probe set that detects most bacteria. The population-specific and the domain-specific probes are labeled with different fluorochromes. Following FISH, the fluorescence conferred by the different probes is recorded in separate CLSM images. The areas of the labeled cells in these images are measured by digital image analysis. Since this approach analyses low magnification images and can partly be automized it allows to rapidly quantify high numbers of bacteria thereby significantly improving the statistical accuracy of the measurement (8, 31).

Ecological studies of complex microbial communities may attempt not only to determine relative abundances of probe-defined bacterial populations, but also the respective cell concentrations in a sample. This is particularly important if different samples which differ in their prokaryotic biomass content are to be compared. Furthermore, cell concentrations per volumetric or weight unit of an environmental sample are needed to calculate key functional attributes of bacterial populations like *in situ* growth rates or *in situ* substrate turnover rates per cell. Despite their importance, cell concentrations of FISH-stained bacterial populations have rarely been determined for biofilms or activated sludge flocs by manual counting since these measurements required additional time-consuming and bias-introducing homogenization and membrane filtration steps (e.g. 17, 24, 26, 36). In addition, it is impossible to directly apply the above mentioned area-based quantification methods (8, 31) to semi-automatically determine absolute cell numbers in a sample after membrane filtration since these methods cannot accurately measure the entire biovolume of all cells of a probe-labeled population in the filtered biomass on top of defined filter areas. Recently CLSM-based methods to semi-automatically measure the biovolume of fluorescently labeled bacteria (15, 19) were published. These methods could theoretically be applied to determine absolute cell numbers of probe-defined bacterial populations on membrane filters. However, biovolume-based quantification is only accurate if serial optical sections are recorded using small vertical step intervals and subsequently are combined to image stacks. This procedure is extremely time consuming and leads to significant bleaching of FISH labeled bacterial cells.

In this study we thus developed a semi-automated procedure for determining cell concentrations of bacterial populations in complex samples by FISH and CLSM using the area-based quantification method (8, 31). Spiking of the samples with known amounts of *E. coli* cells, which were used as internal standards for the subsequent FISH analysis, allowed to infer the absolute cell numbers of probe-target bacteria from their measured areas by digital analysis of CLSM images.

Materials and Methods

Test strains, culture conditions, cell fixation, and activated sludge sampling

Type strains of *Comamonas testosteroni* (DSM 1622) and *Gluconobacter asaii* (DSM 7148) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells of *C. testosteroni* and *G. asaii* were grown overnight under agitation at 30°C in medium DSM M1 (0.5% (w/v) peptone, 0.3% (w/v) meat extract, pH 7.0) and DSM M626 (5% (w/v) D-sorbitol, 1% (w/v) yeast extract, 1% (w/v) peptone, pH 6.0), respectively. Pure cultures of *Nitrosomonas europaea* were maintained as described by Koops *et al.* (18). *Escherichia coli* TOP10F' cells (Invitrogen, San Diego, Calif., U.S.A.) were grown overnight in Luria Bertani (LB) medium (1% (w/v) casein, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% agar (optional), pH 7.0) at 37°C under agitation. For fixation, cells of all species were washed in phosphate buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and then incubated for three hours in 3% paraformaldehyde (Sigma, Deisenhofen, Germany) as described by Amann (3). Fixed cells were washed again in PBS and stored at 4°C in PBS until they were used (normally within three days after fixation). For long-term storage, the cells were resuspended in a 1:1 mixture of PBS and 96% (vol/vol) ethanol and were kept at -20 °C.

Activated sludge was obtained from the secondary aerated nitrification basin (27,144 m^3) of the Munich II wastewater treatment plant (one million population equivalents). 12.5 ml of activated sludge was fixed immediately after sampling by adding 37.5 ml 4% paraformaldehyde. After 5 h of incubation at 4°C the activated sludge was centrifuged (5 min; 4,550 g) and the supernatant containing the fixative was discarded. Subsequently, the sludge

pellet was washed with PBS and finally resuspended in 12.5 ml (the original sample volume) of a 1:1 mixture of PBS and 96% (vol/vol) ethanol. Samples were stored at -20°C.

Cell concentration of pure cultures

The numbers of cells per ml in the fixed pure cultures of *C. testosteroni*, *G. asaii*, and *N. europaea* were determined with a Neubauer cell counting chamber (Paul Marienfeld GmbH, Bad Mergentheim, Germany) following the instructions of the manufacturer.

The cell concentrations of *E. coli* cultures were inferred from the optical density at 600 nm (OD_{600}) using a DU 650 spectrophotometer (Beckman, Fullerton, U.S.A.). For calibration, an *E. coli* TOP10F' overnight culture was diluted by factors of 10^{-5}-10^{-7}, the OD_{600} of these dilutions was measured, and aliquots were streaked onto petri dishes with solid LB medium. The numbers of CFU were correlated with the OD_{600} for each dilution, and the resulting conversion factor ($5.5 \cdot 10^8$ CFU·ml^{-1} per OD_{600} unit) was used to calculate the concentration of *E. coli* cultures based on their OD_{600} in all following experiments. It is important to note that *E. coli* LB overnight cultures contain insignificant numbers of cells which do not form colonies (e.g. (39)). In addition, microscopic observation of the *E. coli* culture used showed that the vast majority of cells occurred as single cells.

Spiking of pure culture mixtures and activated sludge with *E. coli* cells

100 ml overnight cultures of *C. testosteroni* and *G. asaii* were harvested by centrifugation (10 min; 4,550 g) and were fixed with paraformaldehyde as described above. The cell densities in these concentrated stock solutions were determined using the Neubauer chamber. Cell densities of *E. coli* overnight cultures were determined photometrically and *E. coli* cells were concentrated and fixed with paraformaldehyde as described above. Subsequently, different 1 ml cell mixtures containing $6.3 \cdot 10^7$ *C. testosteroni* and $3.7 \cdot 10^7$ *G. asaii* cells as well as 10^6, 10^7, 10^8, or 10^9 *E. coli* cells were prepared in 50% PBS/EtOH (vol/vol). In addition a 1 ml cell mixture containing $6.3 \cdot 10^7$ *C. testosteroni* and $3.7 \cdot 10^7$ *G. asaii* cells but without *E. coli* cells was prepared in 50% PBS/EtOH (vol/vol).

Nitrifying activated sludge was spiked with different amounts of *E. coli* using the following protocol. One ml of paraformaldehyde-fixed activated sludge samples was centrifuged (5 min;

10,000 g). The supernatant was removed, and the activated sludge was resuspended in 1 ml PBS containing either 10^6, 10^7, 10^8, or 10^9 paraformaldehyde fixed *E. coli* cells and mixed by vortexing (10 sec). In an additional experiment, $1.7 \cdot 10^8$ paraformaldehyde fixed *N. europaea* cells were added to the paraformaldehyde fixed sludge prior to the centrifugation step. Finally, all spiked activated sludge samples were centrifuged (5 min; 10,000 g). The supernatant was carefully removed and the sludge was resuspended in 1 ml of 50% PBS/EtOH (vol/vol).

Fluorescence *in situ* hybridization

The defined mixtures of pure cultures were spotted onto microscope slides (Paul Marienfeld GmbH, Bad Mergentheim, Germany) and allowed to dry at 46 °C. Afterwards, the slides were immersed for 2-3 sec in molten 0.5% agarose (GibcoBRL ultraPure agarose, Life Technologies, Paisley, Scotland) at 37°C. The slides were then placed on ice until the agarose had solidified. Excess agarose on the backside of the slides was removed, and the samples were dehydrated in 50, 80 and 96% (v/v) ethanol for 5 min each. The agarose coating was applied to minimize cell loss during the following hybridization and washing steps. After an additional drying step at room temperature, whole cell hybridization was performed as described by Manz *et al.* (22). A different protocol was developed for the treatment of activated sludge samples prior to FISH. The sludge was prepared on the same type of microscope slides, but the spotting and drying procedures were repeated for three times to obtain a thick layer of sludge flocs on the slide surface. Afterwards, the slides were immersed in agarose and hybridized as described above. The thick layer of sludge flocs was required (i) to record as many target cells as possible in order to improve the accuracy of the measurements and (ii) to avoid bias in the quantification of planctonic cells which otherwise would accumulate on the slide surface.

The rRNA-directed oligonucleotide probes used for FISH were 5'-labeled with the dye FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimide ester) or with one of the sulfoindocyanine dyes Cy3 and Cy5. Labeled probes and unlabeled competitor oligonucleotides were obtained from MWG (Ebersberg, Germany) or Thermo Hybaid (Interactiva Division, Ulm, Germany). In all experiments, group-specific probes labeled with Cy3 or FLUOS were used together with the Cy5-labeled EUB338 probe mix (consisting of

probes EUB338, EUB338-II, and EUB338-III) covering the domain *Bacteria* (9). The used probes, their sequences and their specificities are listed in Table 1.

Table 1. Oligonucleotide probe sequences and target organisms.

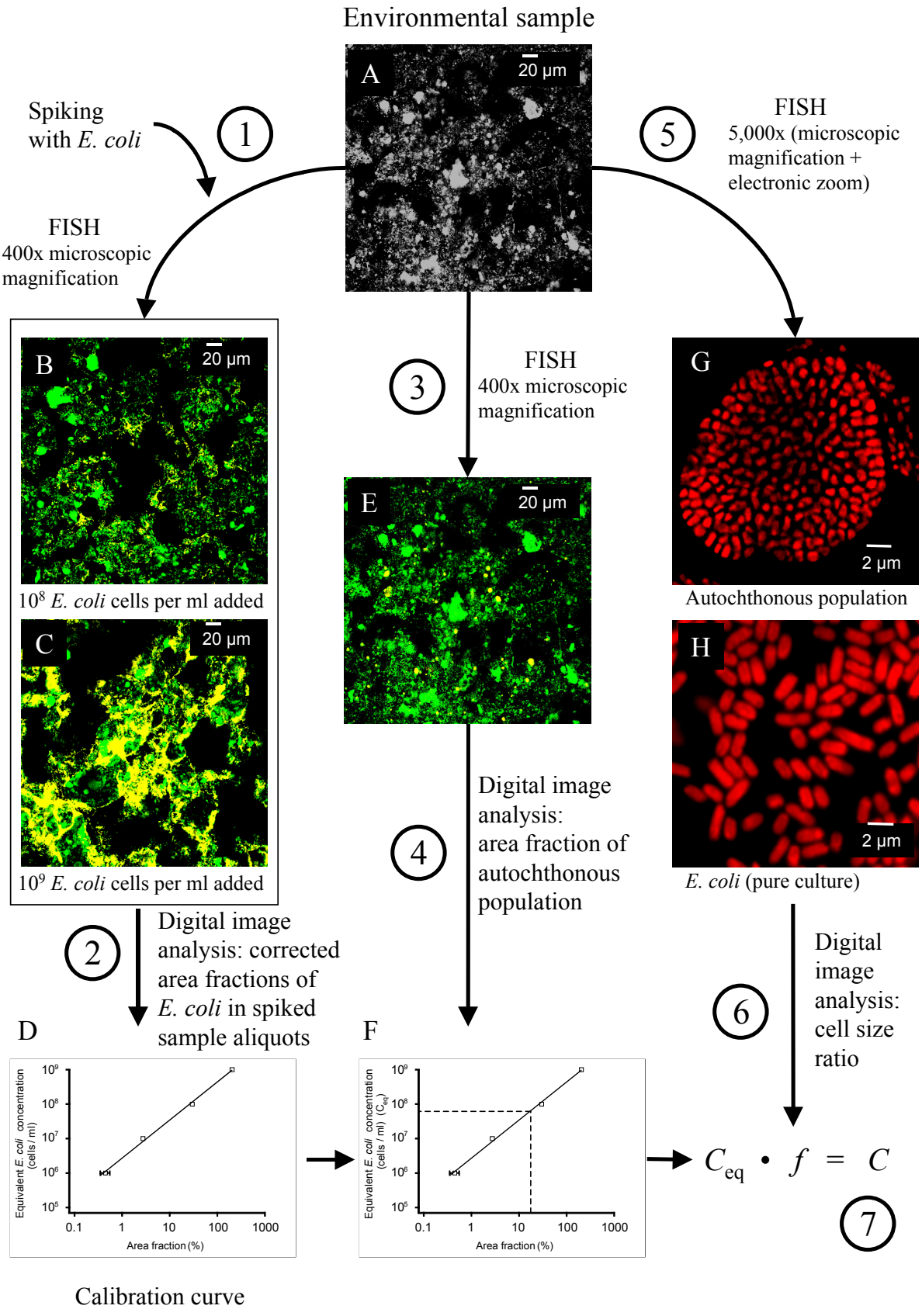
Probe	Sequence (5'-3')	Target organisms	Reference
EUB338	GCTGCCTCCCGTAGGAGT	most *Bacteria*	(4)
EUB338-II	GCAGCCACCCGTAGGTGT	*Planctomycetales* and other *Bacteria* not detected by EUB338	(9)
EUB338-III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales* and other *Bacteria* not detected by EUB338	(9)
NEU	CCCCTCTGCTGCACTCTA	most halophilic and halotolerant ammonia-oxidizers in the beta-subclass of *Proteobacteria*	(36)
CTE	TTCCATCCCCCTCTGCCG	used as unlabeled competitor with probe NEU	(36)
Nso1225	CGCCATTGTATTACGTGTGA	all known ammonia-oxidizers in the beta-subclass of *Proteobacteria* with the exception of *N. mobilis*	(25)
ALF1b	CGTTCG(C/T)TCTGAGCCAG	alpha-subclass of *Proteobacteria*	(22)
BET42a	GCCTTCCCACTTCGTTT	beta-subclass of *Proteobacteria*	(22)
GAM42a	GCCTTCCCACATCGTTT	gamma-subclass of *Proteobacteria*	(22)

Microscopy and digital image analysis

After *in situ* hybridization, the microscope slides were embedded in Citifluor (Citifluor, Canterbury, U.K.). Pictures of fluorescent cells were recorded using a CLSM (LSM 510, Zeiss, Oberkochen, Germany). For detection of Cy3- and Cy5-labeled cells, two Helium-Neon lasers (543 nm and 633 nm, respectively) and for FLUOS-labeled cells an Argon laser (450-514 nm) was used. For each microscope field fluorescence conferred by the different probes was recorded in separate images. For each hybridization experiment 30 microscope

fields at random positions and in random focal planes were recorded using a Zeiss Plan-Neofluar 40x/1.3 Oil objective. This procedure (30 images at low magnification) allows to record a high number of probe-target cells and thus to accurately determine the relative abundance of inhomogeneously distributed probe-target cells in activated sludge samples (8). All acquired pictures corresponded to optical sections of 1 µm thickness obtained by adjusting the pinhole diameter of the CLSM accordingly. They were recorded as 8-bit images of 512 by 512 pixels with a resolution of 1.6 by 1.6 pixels per µm. For each sample analyzed, detector gain, amplification offset, and amplification gain settings were selected which allowed detection of all probe-labeled cells with an intensity between 20 and 256. Special attention was paid to optimize microscopic parameter settings so that the images of those cells detected by the specific probes were congruent with their counterparts in the picture with the EUB338 probe mix-stained cells (Fig. 1b, c, e). The cell area quantification (see below) relies on this congruency, because it is assumed that for each quantified cell the same area is measured with the specific and with the universal probes. The images were then exported as TIFF files by the image acquisition software delivered with the microscope (Zeiss LSM 5, version 2.01). These files were analyzed with the image processing software (Zeiss Kontron KS400, version 3.0) to measure the combined areas of stained cells within each image as described by Schmid *et al.* (31). The area fraction of specifically stained cells was calculated as percentage of the total area of bacteria stained by the EUB338 probe mix in the same optical section.

Figure 1. Principle of the cell quantification method developed in this study. See text for an explanation of the steps 1-7. **A.** Microscopic picture of activated sludge from the Munich II wastewater treatment plant. **B, C.** Aliquots of the same activated sludge after spiking with 10^8 (B) and 10^9 (C) *E. coli* cells per ml. FISH was performed with probe GAM42a (red) and the EUB338 probe mix (green). The images containing the fluorescence conferred by the probes were superimposed, and *E. coli* cells appear yellow due to color blending. **D.** Calibration curve generated from corrected cell area fractions of *E. coli* in spiked sludge aliquots. **E.** The same microscopic field as in (A) showing simultaneous FISH using probes NEU and Nso1225 (red) and the EUB338 probe mix (green). Cells of ammonia-oxidizers appear yellow due to color blending. **F.** Graph showing the use of the calibration curve (D) for converting the measured area fraction of an autochthonous population to the "equivalent *E. coli* concentration". **G.** Highly magnified optical section through a cell aggregate of ammonia-oxidizing bacteria in activated sludge stained by FISH using probes NEU and Nso1225 (red). **H.** Highly magnified optical section through *E. coli* cells from a pure culture stained by FISH using probe GAM42a (red).


Environmental sample
A
20 µm
Spiking with E. coli
1
FISH
400x microscopic magnification
5
FISH
5,000x (microscopic magnification + electronic zoom)
B
20 µm
10^8 E. coli cells per ml added
C
20 µm
10^9 E. coli cells per ml added
2
Digital image analysis: corrected area fractions of E. coli in spiked sample aliquots
3
FISH
400x microscopic magnification
E
20 µm
4
Digital image analysis: area fraction of autochthonous population
G
2 µm
Autochthonous population
H
2 µm
E. coli (pure culture)
6
Digital image analysis: cell size ratio
D
F
Equivalent E. coli concentration (cells / ml)
Area fraction (%)
0.1
1
10
100
1000
$C_{eq} \cdot f = C$
7
Calibration curve

Determination of cell density

The cell concentrations of probe-defined indigenous bacterial populations in a sample were calculated using a seven-step procedure. Firstly, aliquots of the sample (e. g. activated sludge, Fig. 1a) were spiked with *E. coli* (10^6-10^9 cells·ml^{-1}) as described above (Fig. 1, step 1), and the spiked aliquots were stained by FISH using probe GAM42a and the EUB338 probe mix (Fig. 1b, c). The area fraction of the *E. coli* cells in each spiked aliquot was determined by digital image analysis (Fig. 1, step 2). For activated sludge samples, the area fraction of the inherent γ-*Proteobacteria* was measured in sludge aliquots without addition of *E. coli* cells. The area fraction of these indigenous cells was subsequently subtracted from the area fraction measured with probe GAM42a in the spiked aliquots to obtain the area occupied by *E. coli*. Since spiking the samples with *E. coli* did not only increase the area fraction of the cells stained by probe GAM42a, but also the total area of all bacteria, the measured *E. coli* cell area fraction must be corrected to remain directly proportional to the number of added *E. coli* cells. The corrected area fraction is calculated by the formula

$$A_{ec}^{*} = \frac{100 \cdot A_{ec}}{100 - A_{ec}} \qquad (1)$$

(where A_{ec} is the measured and A_{ec}^{*} is the corrected *E. coli* area fraction in percent). The corrected area fractions were then plotted in a double-logarithmic graph against the *E. coli* concentration, and a regression line was calculated based on these data points (Fig. 1d). The double-logarithmic transformation was necessary to meet a requirement for linear regression, i.e. an equal variance for all measurements. As the accuracy of both the determined area fractions and the amount of *E.coli* cells added was not absolute but relative, i.e. a fairly constant percentage of the value obtained, the logarithmic transformation was required. The regression line was used to calculate the "equivalent *E. coli* concentrations" from the area fractions of specifically labeled bacterial populations (e.g. ammonia-oxidizers stained within activated sludge by FISH, Fig. 1 step 3 and Fig. 1e) in un-spiked aliquots of the samples by applying the following equation (Fig. 1, step 4 and Fig. 1f):

$$C_{eq} = A^{m} \cdot 10^{b} \qquad (2)$$

(where C_{eq} is the "equivalent *E. coli* concentration" of the bacterial population in cells per ml, A is the measured area fraction of this population, m is the slope and b is the ordinate-intercept of the regression line).

Finally, C_{eq} was converted to the real concentration of the bacterial population by taking into consideration differences in size between *E. coli* and the probe-target population. This conversion was accomplished by measuring the average area of single *E. coli* cells and of single cells belonging to the probe-target population of interest. For this purpose, images that contained single cells of *E. coli* and of the probe-target population were acquired at a high magnification (5,000x) with a resolution of 55.6 by 55.6 pixels per µm (Fig. 1 step 5 and Fig. 1g, h). Then a conversion factor was calculated as the ratio of the average cell areas (Fig. 1 step 6):

$$f = \frac{\overline{A}_{ec}}{\overline{A}} \qquad (3)$$

(where f is the conversion factor, $\overline{A}$ is the average single cell area of the population whose concentration was to be determined, and $\overline{A}_{ec}$ is the average single cell area of *E. coli*). Eventually, C_{eq} was converted to the real concentration by multiplication with the conversion factor (Fig. 1 step 7):

$$C = f \cdot C_{eq} \qquad (4)$$

(where C is the concentration of the bacterial population in cells per ml).

Estimation of *in situ* substrate turnover rates

Average substrate turnover rates of ammonia-oxidizing bacteria were estimated based on the measured cell concentrations. The total number of ammonia-oxidizing cells in the aerated nitrifying basin of the municipal Munich II wastewater treatment plant was calculated by multiplying the number of ammonia-oxidizing cells per ml of activated sludge with the

reactor volume. The amount of ammonia-nitrogen converted to nitrite (in mg per hour) was estimated according to the formula

$$NH_{4_t}^{+} = (NH_{4_i}^{+} - NH_{4_e}^{+}) \cdot r \cdot 0.9 \qquad (5)$$

(where $NH_{4_t}^{+}$ is the transformed ammonia-nitrogen in $mg \cdot h^{-1}$, $NH_{4_i}^{+}$ is the ammonia-nitrogen concentration in the influent in $mg \cdot m^{-3}$, $NH_{4_e}^{+}$ is the ammonia-nitrogen concentration in the effluent in $mg \cdot m^{-3}$, r is the reactor influent rate (7,858 $m^3 \cdot h^{-1}$), and 0.9 is a correction factor).

The estimated correction factor takes into account that not all ammonia is removed from the sewage via autotrophic nitrification but also via adsorption (27) and assimilation (Activated Sludge Models 1-3 (12-14)). The estimated amount of ammonia oxidized autotrophically was converted from $mg \cdot h^{-1}$ to $fmol \cdot h^{-1}$ and was divided by the total number of ammonia-oxidizer cells in the reactor to obtain the substrate turnover rate in fmol of ammonia transformed to nitrite per hour and per cell.

Results

Preparation of the samples and spiking with *E. coli*

A relatively homogeneous distribution of the added *E. coli* cells within a spiked sample is critical for obtaining an accurate calibration curve. This is particularly important for the measurements of sludge samples which were amended with relatively low numbers of *E. coli*. Therefore, the area fractions of *E. coli* cells added to activated sludge were compared after vigorously vortexing the sludge for 1 min or after homogenizing it with an Ultra-Turrax blender (IKA Labortechnik, Staufen, Germany) treatment (1 min). The sludge was spiked with *E. coli* either before or after these pretreatments. The area fraction of *E. coli* was determined for each sample by FISH with probe GAM42a and the EUB338 probe mix and digital image analysis using the method previously published by Schmid *et al.* (31). The area fractions were compared to the values measured with spiked sludge that had not been vortexed or homogenized. Neither the kind of pretreatment, nor the order of sludge preparation and spiking affected the measured area fractions and their standard deviations

(Table 2). Consequently, *E. coli* cells were simply added to the fixed activated sludge samples without additional pretreatments in all following experiments.

Table 2. Effect of different activated sludge homogenization procedures on area fraction measurements of *E. coli* cells, which were added (10^7 cells ml^{-1}) artificially to activated sludge from the Munich II wastewater treatment plant. It should be noted that the *E. coli* area fractions depicted in this table were not corrected according to equation 1.

Homogenization procedure	*E.coli* cells added before / after the homogenization	Measured cell area fraction of *E. coli*
none	--	2.7±0.9%
1 min vortex	after	2.2±0.8%
1 min blender	before	2.1±0.7%
1 min blender	after	2.5±0.8%

Evaluation of the quantification protocol using artificial mixtures of pure cultures

The developed quantification approach was first tested with bacterial pure cultures. For this purpose, cultures of *Gluconobacter asaii* (α-subclass of *Proteobacteria*) and *Comamonas testosteroni* (β-subclass of *Proteobacteria*) were mixed after their cell concentrations had been determined with a Neubauer cell counting chamber. The final cell concentrations in the mixture were $3.7 \cdot 10^7 \pm 0.5 \cdot 10^7$ cells·ml^{-1} for *G. asaii* and $6.3 \cdot 10^7 \pm 0.2 \cdot 10^7$ cells·ml^{-1} for *C. testosteroni* (all the confidence limits indicate 95% confidence intervals). Aliquots of this mixture were supplemented with increasing concentrations of *E. coli* cells, and a regression line was generated from a graph depicting the relative cell areas of *E. coli* in the spiked aliquots versus the amount of *E. coli* cells added (Fig. 2a). This regression line should have a slope of approximately 1 as the corrected area fraction of *E. coli* cells is expected to be directly proportional to the amount of *E.coli* cells added. The slope in this and all other determined calibration curves (see also below) was slightly higher than 1 (e.g. 1.25 in Fig. 2a). This implies that small cell additions had a large effect on the area fraction whereas large additions only had a more moderate effect. Subsequently, the un-spiked mixture was hybridized with the EUB338 probe mix and with probe ALF1b or BET42a. It should be noted that the intensities of the fluorescent signals varied considerably among the individual cells of either species, *G. asaii* and *C. testosteroni*.

The cell area fractions of *G. asaii* and *C. testosteroni* were measured and the calibration curve and area correction factor were used to convert the areas to cell concentrations as described above. Table 3 shows the results of this experiment. The concentration determined for *G. asaii* deviates by $0.4 \cdot 10^7$ cells·ml^{-1} (or 10.8%) from the Neubauer cell chamber count, while the difference between the two quantification methods amounts to $0.8 \cdot 10^7$ cells·ml^{-1} (or 12.7%) for *C. testosteroni*.

Table 3. Quantification of *C. testosteroni* and *G. asaii* in a mixture of both pure cultures. Confidence limits indicate 95% confidence intervals.

Species	Area fraction	Equivalent *E. coli* concentration (cells·ml^{-1})	Cell area ratio *E. coli* : species	Cell concentration of the species (cells·ml^{-1})	Neubauer chamber counts (cells·ml^{-1})
C. testosteroni	50.7±3.3%	$2.6 \cdot 10^7 \pm 0.2 \cdot 10^7$	2.13	$5.5 \cdot 10^7 \pm 0.5 \cdot 10^7$	$6.3 \cdot 10^7 \pm 0.2 \cdot 10^7$
G. asaii	51.6±3.5%	$2.6 \cdot 10^7 \pm 0.3 \cdot 10^7$	1.55	$4.1 \cdot 10^7 \pm 0.4 \cdot 10^7$	$3.7 \cdot 10^7 \pm 0.5 \cdot 10^7$

Quantification of ammonia-oxidizing bacteria in activated sludge

The number of autochthonous ammonia-oxidizing bacteria was determined in a nitrifying activated sludge from the Munich II wastewater treatment plant. An equimolar mixture of probes Nso1225 and NEU was used for the *in situ* detection of ammonia-oxidizers of the β-subclass of *Proteobacteria*. Probe Nso1225 targets all recognized ammonia-oxidizers of the β-subclass with the exception of *Nitrosococcus mobilis* (28). The single central mismatch of *N. mobilis* is discriminative under stringent conditions, so probe NEU was used in addition. This probe targets *Nitrosococcus mobilis* and other not yet described ammonia-oxidizers from activated sludge which are also not detected by probe Nso1225 (unpublished results). A first inspection of the analyzed nitrifying activated sludge by FISH with probes NEU and Nso1225 confirmed the occurrence of high amounts of ammonia-oxidizing bacteria of the β-subclass of *Proteobacteria*. Most ammonia-oxidizers were relatively small and formed spherical, tightly packed cell clusters, but others were slightly larger and formed looser aggregates with narrow intercellular cavities.

The cell concentration of the ammonia-oxidizers was determined and confirmed in two experiments. First, their area fraction was measured after FISH with both probes

NEU and Nso1225 and the EUB338 probe mix. The cell concentration of the autochthonous ammonia-oxidizers was then calculated based on a calibration curve that had been generated by spiking of the sludge with *E. coli* (Fig. 2b). In the second experiment, $1.7 \cdot 10^8 \pm 0.3 \cdot 10^8$ *N. europaea* cells per ml were added to the activated sludge. The concentration of the ammonia-oxidizers (consisting of the autochthonous and the added ammonia-oxidizers) in the modified sample was measured by the same procedure as in the original sludge, but a new calibration curve was generated after aliquots of the modified sludge had been spiked with *E. coli* (Fig. 2c). Finally, the concentration of the autochthonous ammonia-oxidizers in the original sludge was subtracted from the concentration of the ammonia-oxidizers in the sludge supplemented with the additional *N. europaea* cells.

For the autochthonous ammonia-oxidizers, a cell area fraction of 8.4±1.4% was measured, which corresponds to a concentration of $9.8 \cdot 10^7 \pm 1.9 \cdot 10^7$ cells per ml of activated sludge. Following the addition of $1.7 \cdot 10^8 \pm 0.3 \cdot 10^8$ *N. europaea* cells per ml, the area fraction of the ammonia-oxidizers increased slightly to 9.4±1.4%. It should be noted that different image acquisition parameters were used in the two experiments making it impossible to compare the area fractions directly. This was necessary since the added ammonia-oxidizers showed a weaker fluorescence after FISH than the autochthonous ammonia-oxidizers. Furthermore, the added *N. europaea* cells affect the calibration curve so that the small change in area fraction corresponds to a large difference in cell numbers when using the appropriate new calibration curve. The resulting absolute cell concentrations, however, are comparable. The concentration of ammonia-oxidizers after amendment was $2.3 \cdot 10^8 \pm 0.3 \cdot 10^8$ cells·ml^{-1}. The difference between this value and the concentration of the autochthonous ammonia-oxidizers amounts to $1.3 \cdot 10^8$ cells·ml^{-1} which should be equal to the $1.7 \cdot 10^8$ added *N. europaea* cells per ml. The deviation between the two values, $4 \cdot 10^7$ cells·ml^{-1}, is 22.4% of the cell addition and thus about twice as high as the differences between the cell concentrations measured by the newly developed quantification method and obtained by using the Neubauer chamber for the pure culture mixtures (see above).

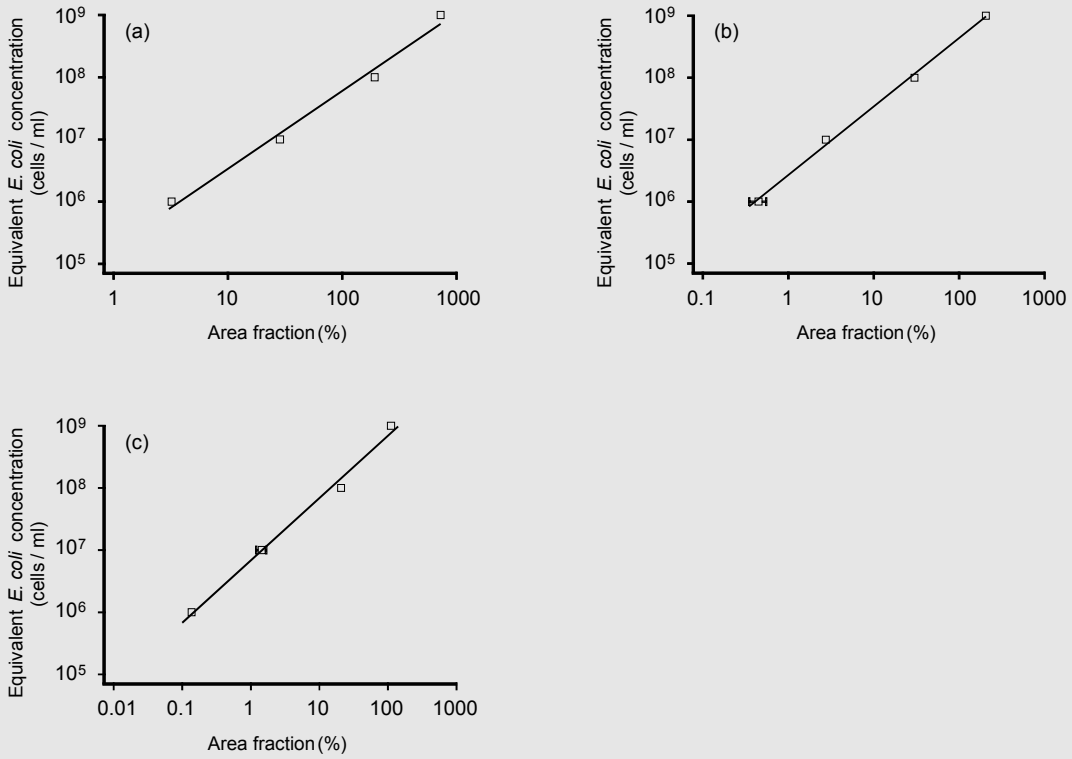


Figure 2. Calibration curves used to convert cell area fractions to equivalent *E. coli* concentrations for the determination of cell concentrations in pure culture mixtures of *Comamonas testosteroni* and *Gluconobacter asaii* (A), in activated sludge from the Munich II wastewater treatment plant (B), and in the same activated sludge after addition of $1.7 \cdot 10^8$ *Nitrosomonas europaea* cells per ml (C). The general linear equation of these curves is $\lg C_{eq} = m \cdot \lg A + b$ (where C_{eq} is the equivalent *E. coli* concentration, *A* is the cell area fraction, *m* is the slope and *b* is the ordinate-intercept of the line). The values of m are 1.253317 (A), 1.105818 (B), and 1.004006 (C), respectively. The values of b are 5.27404 (A), 6.429176 (B), and 6.83411 (C), respectively. Error bars which are smaller than the marker symbols are not shown.

Estimated activity of ammonia-oxidizing bacteria in activated sludge

The average rate of ammonia oxidation per autochthonous ammonia-oxidizer cell within the activated sludge was calculated based on the determined concentration of autochthonous ammonia-oxidizers. As described above, $9.8 \cdot 10^7 \pm 1.9 \cdot 10^7$ ammonia-oxidizer cells·ml^{-1} were found in the activated sludge. Thus, with a total reactor volume of 27,144 m^3 the total amount of ammonia-oxidizers in the reactor was $2.7 \cdot 10^{18} \pm 0.5 \cdot 10^{18}$ cells. During the last two weeks before sampling, the amount of NH_4^+-N was in the range of 10-16 mg·l^{-1} in the influent and in the range of 0.05-0.3 mg·l^{-1} in the effluent of the plant, respectively (Fig. 3). The average

concentrations of NH_4^+-N in the influent (12.1 mg·l^{-1}) and the effluent (0.08 mg·l^{-1}) of the plant during the last six days before sampling were used for the activity estimation. Thus, with a flow rate of 7,858 m^3·h^{-1}, $9.5 \cdot 10^7$ mg NH_4^+-N·h^{-1} were transformed in the basin. Assuming that 10% of the ammonia was not removed by autotrophic oxidation, the ammonia-oxidizers oxidized $8.5 \cdot 10^7$ mg NH_4^+-N·h^{-1} which equals to $6.1 \cdot 10^{18}$ fmol NH_4^+·h^{-1}. Consequently, each ammonia-oxidizer cell converted 2.3±0.4 fmol NH_4^+ to NO_2^- per hour if equal activity of all ammonia-oxidizer cells is assumed.

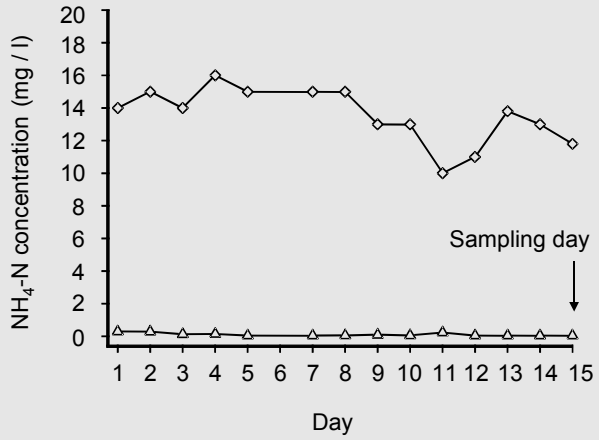


Figure 3. Concentrations of ammonia nitrogen in the influent (◊) and the effluent (Δ) of the nitrification basin of the Munich II wastewater treatment plant measured during the last two weeks before sampling.

Discussion

Cell quantification methods suitable for microbial ecology should provide precise results for environmental samples containing bacteria which are not homogeneously distributed. Furthermore, they should be independent from cultivation as most bacteria in natural or engineered systems have hitherto not been isolated (e.g. 5, 33). A generally applicable method should allow both specific enumeration of single species as well as of higher phylogenetic taxa (e.g. genera or subclasses). New protocols must be tested with regard to these requirements by use of suitable model systems. In this study, mixtures of pure cultures and an activated sludge sample were used, because these model systems have different advantages when evaluating a new quantification method. The selected pure cultures consisted of uniformly shaped cells, which can easily be counted in a counting chamber to verify the results obtained with the new quantification protocol. On the contrary, activated sludge

contains numerous different cultivated and uncultivated prokaryotic species (2, 7, 32) with different morphologies and abundance and thus represents a challenge for any quantification method.

Applicability and accuracy of the newly developed quantification method

The new quantification method was successfully applied to measure cell concentrations of specific populations in bacterial pure culture mixtures as well as in activated sludge. The obtained results were not expressed as relative abundance based on a reference value such as total cell counts, but as absolute cell numbers per volumetric unit. Since the developed quantification procedure is based on FISH with rRNA-directed oligonucleotide probes, it can be used in any environment that is amenable to FISH analysis. It makes no difference whether the quantified organisms grow as single cells or in dense aggregates as long as individual cells can be resolved microscopically. However, the accuracy of the results obtained with the developed method depends on a uniform cell size of the target population (see below). Size variations within a target population can be caused by polymorphism of a single species or if probes with a broader specificity were applied for detection of morphologically different bacterial taxa. It should also be noted that the accuracy of the developed method depends on a relatively homogeneous distribution of reference cells (used for spiking) within the environmental sample. For the analyzed activated sludge sample this was easily achieved by adding the *E. coli* cells to the sample followed by a short mixing step. Since composition and density of aggregates or flocs might vary between different environments, special pretreatment (e.g. homogenization) of other samples may be necessary to ensure optimal dispersal of the cells used for spiking.

The cell concentrations measured with the newly developed method deviated in the mixed pure culture experiments by approximately ±10-13% and in the experiments with activated sludge by approximately ±22% from the Neubauer chamber counts. In addition to the measurement error of the Neubauer chamber, several difficulties with the area measurement of FISH-stained cells could have caused these discrepancies. First of all, the intensity of the FISH signal is a function of the ribosome content of the target cells. Although most cells in actively growing pure cultures contain high ribosome numbers we observed that a fraction of the FISH-stained *C. testosteroni* and *G. asaii* cells emitted less fluorescence than the majority of the labeled cells. Such differences of the fluorescent signal intensity were even more

pronounced between different bacterial populations in the activated sludge sample. The presence of very bright and relatively dark cells in the same sample makes it difficult to find appropriate microscope parameter settings and intensity thresholds during image analysis to differentiate between cells and background. Under such conditions, either the areas of the bright cells are overestimated when the threshold is too low, or the darker cells are not included in the analysis when the threshold is too high. Such errors may still be higher in samples from oligotrophic environments, where the growth rates and ribosome content of indigenous bacterial populations may be more pronounced than in activated sludge.

Problems with fluorescence intensities are also responsible for the upper limit of cell concentrations that can be quantified by the newly developed method. Very high concentrations of probe-target cells (e.g. 10^8 cells per ml) require that the sample is spiked with *E. coli* concentrations between 10^7 and 10^9 cells per ml to obtain a calibration curve that spans at least one order of magnitude above and below the cell concentration to be measured. After addition of 10^9 *E. coli* cells per ml, however, the *E. coli* cells formed thick layers of stacked cells on the microscope slides. Following FISH with the EUB338 probe mix, the local fluorescence intensity within these layers of *E. coli* cells was far higher than the fluorescence intensities observed for most aggregates of autochthonous bacteria. As a consequence, the CLSM detector collected too much light at the locations of the *E. coli* layers, and the *E. coli* cells appeared too large in the images with the EUB338 probe mix-stained cells. The sensitivity of the detector could not be reduced to overcome this problem, because then the darker autochthonous bacteria would not have been detected anymore. This problem hampered the precise determination of area fractions for high *E. coli* cell densities and affected in consequence the precision of the calibration curves. Thick *E. coli* cell layers were not observed after spiking with lower amounts of *E. coli* than 10^9 cells per ml. The quantification accuracy can thus be expected to be higher for lower concentrations of probe-target cells that do not require spiking of the sample with 10^9 *E. coli* cells per ml to obtain a suitable calibration curve.

Furthermore, the conversion of the "equivalent *E. coli* concentration" to the real concentration of the quantified population is a possible source of measurement error. The ratio of the average cell areas is used as conversion factor, but even in pure cultures cell size, and thereby the cell area, may vary considerably. This problem could have contributed to the observed differences between Neubauer chamber counts and the counts inferred from the novel

quantification method. In complex systems like activated sludge cell size variation of probe-target bacteria is frequently observed. Therefore, application of the developed quantification method is recommended for quantification of probe-defined groups of microorganisms that do not show pronounced differences in size, like the two populations of ammonia-oxidizing bacteria detected in this study.

Despite of these possible sources of error, the novel FISH-based quantification method constitutes a straightforward and precise method to determine the absolute numbers of microorganisms in different environments and is especially useful for samples containing biofilms or aggregates. The accuracy of the method is demonstrated by the highly similar cell concentrations obtained using the well-established Neubauer chamber counts and the novel FISH-based quantification method for pure culture mixtures as well as for an activated sludge which was amended with a defined number of *Nitrosomonas europaea* cells. The utility of the developed quantification method to enumerate bacteria in samples where cells are not homogeneously distributed was illustrated by quantification of autochthonous ammonia-oxidizing bacteria in a nitrifying activated sludge. Based on the obtained absolute numbers of ammonia-oxidizing bacteria their average activity in the municipal activated sludge sample was estimated to be 2.3±0.4 fmol $NH_4^+ \cdot cell^{-1} \cdot h^{-1}$, a value which is within the range of per-cell activities measured with pure cultures of *N. europaea* (1.24-23 fmol $NH_4^+ \cdot cell^{-1} \cdot h^{-1}$, (20)). This consistency with pure culture values supports the accuracy of the developed quantification method. Compared to manual counting of probe-labeled cells by microscopy (e.g. 21, 24, 33, 34), the new semi-automatic method is less tedious, not negatively affected by aggregates or cell clusters, and its measured standard error is lower.

Acknowledgments

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Appendix 3

In situ Characterization of *Nitrospira*-like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants

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In situ Characterization of *Nitrospira*-like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants

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Uncultivated *Nitrospira*-like bacteria in different biofilm and activated sludge samples were investigated by cultivation-independent, molecular approaches. Initially, the phylogenetic affiliation of *Nitrospira*-like bacteria in a nitrifying biofilm was determined by 16S rDNA sequence analysis. Subsequently, a phylogenetic consensus tree of the *Nitrospira* phylum including all publicly available sequences was constructed. This analysis revealed that the genus *Nitrospira* consists of at least four distinct sublineages. Based on these data, two 16S rRNA-directed oligonucleotide probes specific for the phylum and genus *Nitrospira*, respectively, were developed and evaluated for their application for fluorescence *in situ* hybridization (FISH). The probes were used to investigate the *in situ* architecture of cell aggregates of the *Nitrospira*-like nitrite-oxidizers in wastewater treatment plants by FISH, confocal laser scanning microscopy and computer-aided 3D visualization. Cavities and a network of cell-free channels inside the *Nitrospira* microcolonies were detected, which were water permeable as demonstrated by fluorescein-staining. The uptake of different carbon sources by *Nitrospira*-like bacteria within their natural habitat under different incubation conditions was studied by combined FISH and microautoradiography. Under aerobic conditions, the *Nitrospira*-like bacteria in bioreactor samples took up CO_2 and pyruvate but not acetate, butyrate, and propionate suggesting the capability of these bacteria to grow mixotrophically in the presence of pyruvate. In contrast, no uptake of any of the tested carbon sources could be observed for the *Nitrospira*-like bacteria under anoxic or anaerobic conditions.

Keywords: *Nitrospira*, FISH, oligonucleotide probes, microautoradiography, CO_2-fixation, 3D visualization

Introduction

Nitrification, the oxidation of ammonia to nitrate catalyzed by bacteria, is a key part of global nitrogen cycling (37). Two distinct, physiologically defined groups of bacteria are involved in this process. In the first step of nitrification, chemolithoautotrophic ammonia-oxidizers transform ammonia to nitrite, which is subsequently oxidized to nitrate by the nitrite-oxidizing bacteria (8). All isolated chemolithoautotrophic, nitrite-oxidizing bacteria belong to one of the four different genera (7) *Nitrobacter* (alpha-subclass of *Proteobacteria*), *Nitrococcus* (gamma-subclass of *Proteobacteria*), *Nitrospina* (delta-subclass of *Proteobacteria*), and *Nitrospira* (phylum *Nitrospira*). While species of the genus *Nitrobacter* have been isolated from a variety of environments including soil and fresh water, it was long assumed that the other three genera were confined to marine environments (7). In recent studies, however, bacteria related to the genus *Nitrospira* were also found to occur in different non-marine natural and engineered habitats. While the first described species of this genus, *Nitrospira marina*, was isolated from ocean water (49) the second isolated species, *N. moscoviensis*, was cultured from an iron pipe of a heating system in Moscow (16). These two species are the only cultivated representatives of the genus *Nitrospira*, but numerous related bacteria have recently been detected by comparative analysis of 16S rRNA sequences obtained from nitrifying bioreactors (11, 22), rhizosphere (30), a freshwater aquarium filter (21), groundwater contaminated with livestock wastewater (12), deltaic sediment (45), and deep sea sediments (25). These published sequences indicate a considerable phylogenetic diversity within the genus *Nitrospira*, however, a thorough phylogenetic analysis of the *Nitrospira* phylum considering all these sequences has not yet been performed. Furthermore, the retrieval of *Nitrospira*-related sequences from the above mentioned environments demonstrates that these bacteria are widely distributed in nature and probably contribute significantly to global nitrite oxidation: However, the lack of pure cultures of *Nitrospira*-related bacteria from soil, water, sediment, and wastewater treatment plants restricts our knowledge on their physiology and genetics.

In contrast to textbook knowledge (5, 19) *Nitrospira*-like bacteria, but not *Nitrobacter* spp., are the dominant nitrite-oxidizers both in most full-scale wastewater treatment plants and in laboratory-scale reactors (22, 36, 43, 48). Based on FISH combined with microelectrode measurements it has been suggested that *Nitrospira*-like nitrite-oxidizers represent K-

strategists adapted to low nitrite and oxygen concentrations, while *Nitrobacter* as an r-strategist thrives if nitrite and oxygen are present in higher concentrations (42). Since many wastewater treatment plants suffer from repeated breakdowns of nitrification performance, more insight into the physiology of *Nitrospira*-like bacteria is required to find measures to stabilize this important step of nutrient removal in modern biological sewage treatment.

In this study we investigated structural and functional features of *Nitrospira*-like bacteria in nitrifying biofilms and activated sludges from different wastewater treatment plants. Based on an in-depth analysis of the phylogeny of the *Nitrospira*-phylum, 16S rRNA-directed oligonucleotide probes for the phylum and genus *Nitrospira* were developed. These probes were used to detect *Nitrospira*-like bacteria *in situ* in wastewater treatment plants and to study the morphology of their microcolonies using confocal laser scanning microscopy and digital image analysis. Furthermore, the uptake of different carbon sources by *Nitrospira*-like bacteria in the bioreactor samples was examined under aerobic and anaerobic conditions by combining FISH and microautoradiography (MAR) (24).

A preliminary part of this study has been presented at the IAWQ conference on biofilm systems (New York, October 17-20, 1999) and at the IWA 2nd international symposium on sequencing batch reactor technology (Narbonne, July 10-12, 2000).

Materials and Methods

Cultivation of reference organisms

All reference strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *Bacillus stearothermophilus* (strain DSM 22) was grown overnight in HD medium (1% (w/v) tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose, 0.7% (w/v) NaCl, pH 7.4) at 55 °C under agitation. According to the instructions of the DSMZ, *Leptospirillum ferrooxidans* (strain DSM 2705) was cultivated in slanted tubes for 14 days at 30°C in a 20:1 mixture of "solution A" (0.01 % (w/v) K_2HPO_4, 0.012% (w/v) $MgSO_4$, 0.2% (w/v) $(NH_4)_2SO_4$, 0.01% (w/v) KCl) and "solution B" (21.9% (w/v) $FeSO_4$ dissolved in 0.1 N H_2SO_4). Paraformaldehyde-fixed

Nitrospira moscoviensis cells were kindly provided by Eberhard Bock and Gabriele Timmermann (University Hamburg, Germany).

Bioreactor data, biofilm and activated sludge sampling, and cell fixation

Samples of nitrifying biofilm were retrieved from an aerated sequencing batch biofilm reactor (SBBR 1) of a pilot wastewater treatment plant near Ingolstadt, Germany. Control samples were obained from a second, continuously operated biofilm reactor (Biofor 2) located on the same plant. The biofilm grew in both reactors on Biolite® expanded clay beads (grain size 4-8 mm) forming a fixed bed with an average volume of 10 m^3. Reactor SBBR 1 received reject water from sludge dewatering by the municipal wastewater treatment plant at Ingolstadt with NH_4-N concentrations of 300-500 $mg \cdot l^{-1}$, an average total chemical demand of oxygen (COD) of 300 $mg \cdot l^{-1}$, and an average conductivity of 5,000-6,000 $\mu S \cdot cm^{-1}$. At the end of each cycle, 40-50 $mg \cdot l^{-1}$ of NH_4-N and up to 70 $mg \cdot l^{-1}$ of NO_2-N were detected in the outlet of the reactor. The cycle time was 4 to 8 h, and the fixed bed was back-washed every 10-48 h. Reactor Biofor 2 received municipal wastewater with an average NH_4-N concentration of 13 $mg \cdot l^{-1}$, an average COD of 191 $mg \cdot l^{-1}$, and an average conductivity of 500-1000 $\mu S \cdot cm^{-1}$. The amount of suspended solids in the municipal wastewater was reduced by a 7 m^3 settler and a rotating sieve with 1.5 mm gap size to protect the fixed bed from clogging. The outlet of the reactor contained between 0.3 and 0.8 $mg \cdot l^{-1}$ of NH_4-N and less than 1 $mg \cdot l^{-1}$ of NO_2-N. The flowrate in reactor Biofor 2 was 8-12 $m^3 \cdot h^{-1}$ with an up-flow water velocity of 5.5-9.5 $m \cdot h^{-1}$ and an up-flow air velocity of 5-20 $m \cdot h^{-1}$. The fixed bed was washed every 10-48 h. Activated sludge samples were taken from the nitrification stage of the Aalborg West (AAV) wastewater treatment plant (Aalborg, Denmark; 27,5000 population equivalents). In addition, control samples were retrieved from the nitrification stage of the Munich II wastewater treatment plant (Munich, Germany; one million population equivalents). The average NH_4-N concentration in the inlet of the nitrification stage of the AAV plant was 25 $mg \cdot l^{-1}$ while the outlet contained less than 1 $mg \cdot l^{-1}$ of NH_4-N and of nitrite. The sludge age was 20 days. The average NH_4-N concentration was 12 $mg \cdot l^{-1}$ in the inlet of the nitrification stage of the Munich II plant, and 0.08 $mg \cdot l^{-1}$ in the outlet. Nitrite concentrations in the outlet of the nitrification stage are not available for this plant. The sludge age was 7-10 days.

Biofilm and activated sludge samples were fixed for 5 h at 4°C in 3% paraformaldehyde as described by Amann (2) immediately after sampling (the biofilm was first detached from the

expanded clay beads by gentle swirling). Sample aliquots used for DNA extraction (see below) were not fixed. Instead, these aliquots were centrifuged (10 min, 4,550 x g), the supernatant was discarded, and the biomass was stored at -20°C. Bacterial pure cultures were harvested by centrifugation (10 min, 10,000 x g), resuspended in PBS and centrifuged again (10 min, 10,000 x g). The supernatant was removed prior to fixation of the cells in 3% paraformaldehyde (see above). *Bacillus stearothermophilus* cells were not fixed with paraformaldehyde. These cells were harvested and washed with PBS like the other pure cultures, but were resuspended in a 1:1 mixture of PBS and 96% (v/v) ethanol immediately after the washing step (40). Fixed samples and fixed pure cultures were stored in PBS:EtOH (1:1) at –20°C.

PCR amplification, cloning, sequencing, and phylogenetic analysis of 16S rRNA genes

DNA was extracted from frozen biofilm samples according to the protocol described by Zhou *et al.* (51). Almost complete (1,497-1,533 nucleotides) bacterial 16S rRNA genes were amplified by PCR, cloned, and sequenced as detailed by Juretschko *et al.* (22). The obtained 16S rDNA sequences were added to the ARB 16S rRNA sequence database of the Technische Universität München by use of the ARB program package (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/). The sequences were aligned by the ARB_EDIT module of the program, and the alignments were refined by visual inspection. Nucleic acid similarities were calculated by using the respective tool of the ARB program. Phylogenetic trees were computed by application of the ARB neighbour joining and maximum parsimony tools, and by maximum likelihood analysis on different data sets. Treeing analyses were performed with and without application of a 50% conservation filter for the *Nitrospira* phylum. This filter (generated by the respective function of the ARB program) was based on all sequences affiliated with the phylum *Nitrospira* that were longer than 1,400 nucleotides and was used to exclude highly variable alignment columns which are not conserved in at least 50% of the *Nitrospira*-phylum sequences. For calculating consensus trees, all sequences longer than 1,300 nucleotides were first processed by the maximum likelihood method to determine the basic topology of the tree. The shorter sequences were added subsequently by use of the ARB_PARSIMONY function of the ARB program without changing the tree topology according to the recommendations given by Ludwig *et al.* (26). Bootstrap values were determined by 100 iterations of maximum parsimony bootstrapping analysis without application of a conservation filter. Neighbour joining trees were calculated based on different

sequence sets to verify the results obtained by the other treeing methods. Different sets of outgroup sequences, which represented members of several other phyla of the domain *Bacteria* and which were longer than 1,500 nucleotides, were used in all treeing calculations. Checks for chimeric sequences were performed by independent phylogenetic analysis of the first 513 5' base positions, the middle 513 base positions, and the last 513 3' base positions of the sequences.

Incubation of activated sludge and biofilm with radioactive substrates

Substrate uptake experiments were performed with living activated sludge and biofilm samples. The radioactively labeled substrates used were ^{3}H-acetate, ^{14}C-pyruvate (Amersham, Little Chalfont, U.K.), ^{14}C-butyrate, ^{14}C-propionate, and ^{14}C-bicarbonate (NEN Life Science, MA, U.S.A.). Fresh activated sludge and biofilm samples were harvested the day before the experiments were performed, kept at 4°C and brought to the laboratory. The dry matter content (suspended solids, SS) of activated sludge and biofilm was determined as described by Lee *et al.* (24), and the biomass was diluted to a concentration of 1 g SS·l^{-1} with sterile-filtered supernatant from the respective bioreactors. For incubation with all substrates except for bicarbonate, 3 ml of diluted activated sludge or biofilm were transferred to 9 ml glass serum vials. For incubation with bicarbonate, 5 ml of diluted sample were transferred to 25 ml serum vials. The incubation conditions applied were i) aerobic, ii) anoxic (i. e. anaerobic in the presence of 1 mM nitrate), or iii) anaerobic. The nitrate concentrations in the samples were estimated by use of a nitrate test kit (Merckoquant Nitrate-test, Merck, Darmstadt, Germany) prior to the anaerobic incubations to ensure that these experiments were not influenced by nitrate present in the sludge or biofilm liquid. No nitrate was detected in the samples from the AAV plant and from reactor Biofor 2, but the biofilm from reactor SBBR1 contained approximately 500 mg·l^{-1} of nitrate. This biofilm was therefore centrifuged (10 min, 5,000 x g), the supernatant containing nitrate was discarded, and the biofilm was resuspended in sterile-filtered, nitrate-free biofilm liquid. This biofilm liquid had previously been obtained by anaerobic overnight incubation of an aliquot of the same biofilm sample. Following this pretreatment, no nitrate was detected in the biofilm liquid by the nitrate test kit. All serum vials for anoxic and anaerobic incubations were closed with thick gas-tight butyl rubber stoppers and were flushed with pure nitrogen gas prior to the incubation, and all following steps were performed using strict anaerobic techniques. Unlabeled organic substrates were added to a final concentration of 1 mM. This concentration was estimated to be sufficient to

avoid starvation of the bacteria in the incubated samples due to substrate depletion. The respective labeled substrates were then added to a final activity of 10 µCi (specific activities were 30, 60, 58, 10, and 5 mCi·mmol^{-1} for ^{14}C-pyruvate, ^{14}C-propionate, ^{3}H-acetate, ^{14}C-butyrate, and ^{14}C-bicarbonate, respectively). Aerobic incubations were vigorously shaken for 3 h, while anoxic and anaerobic incubations were incubated for 4 h without agitation. All samples containing ^{14}C-bicarbonate, however, were incubated for 5 h to compensate for the presumably lower CO_2 fixation rates. Moreover, 1 mM NH_4Cl was added to these samples as substrate for the indigenous ammonia-oxidizers. Nitrite resulting from their activity could serve as energy source for the nitrite-oxidizers during the incubations with bicarbonate. The incubated samples were centrifuged (10 min, 5,000 x g) and fixed in 3% (w/v) paraformaldehyde as described above. The samples were then resuspended in a 1:1 mixture of PBS and 96% (v/v) ethanol and stored at –20°C.

The amounts of organic substrates taken up by the biomass were determined to confirm that the substrates were not depleted during the incubations. Aliquots (0.5 ml) of the incubated samples were taken before and after the experiments. Aliquots of anaerobic incubations were taken under strictly anaerobic conditions. The aliquots were immediately cooled on ice, centrifuged (5 min, 10,000 x g), filtered (0.2 µm sterile filters, Millipore), and the supernatant was frozen for later analysis. Amounts of pyruvate, acetate, propionate and butyrate in the supernatant of the respective incubations were measured by HPLC on a Dionex ion-chromatograph with a suppressed conductivity detector, 1 mM NaOH as mobile phase and an IonPac AS11-HC column. The uptake of ^{14}C-bicarbonate was estimated by measuring the amount of ^{14}C-bicarbonate bound by the biomass after incubation. The radioactive bicarbonate in the biomass was quantified by liquid scintillation counting (Tri-Carb Analyzer 1600TR, Packard Instrument, CT, U.S.A.) of an aliquot. Unbound CO_2 had been removed from these aliquots prior the measurement by lowering the pH to less than 1 with 1 N HCl and thoroughly flushing with N_2 for 30 minutes. Aliquots of pasteurized biofilm and sludge were incubated with the respective radioactive substrates to test for adsorption and precipitation phenomena in all experiments.

16S rRNA-directed oligonucleotide probes

The program ARB and the current version of the ARB 16S rRNA sequence database (approx. 15,000 entries) were used to develop new 16S-rRNA directed oligonucleotide probes

(Table 1). Groups of specified probe target sequences were scanned for unique sequence stretches by the "probe design" tool of the program, and the identified candidate probe sequences were matched against all other sequences in the database with the "probe match" tool. The probes used for *in situ* hybridization were 5'-labeled with the dye FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimide ester) or with one of the sulfoindocyanine dyes Cy3 and Cy5. Labeled probes and unlabeled competitor oligonucleotides were obtained from Thermo Hybaid (Interactiva Division, Ulm, Germany) or MWG (Ebersberg, Germany). The following oligonucleotide probes were used in addition to the probes developed in this study: (i) NEU, specific for halophilic and halotolerant *Nitrosomonas* spp. and *Nitrosococcus mobilis* (47); (ii) Nso1225, specific for ammonia-oxidizers in the beta subclass of *Proteobacteria* (33) except for *N. mobilis* (38); (iii) NIT3, complementary to a sequence region of all *Nitrobacter* species (48); and (iv) the EUB338 probe mix consisting of probe EUB338 (3), EUB338-II and EUB338-III (14) covering the domain *Bacteria*. All probes developed in this study were named conforming to the standard introduced by Alm *et al.* (1) while names of previously published probes were left unchanged to avoid confusion.

Table 1. 16S rRNA-targeted oligonucleotide probes developed in this study. The names, sequences and target sites of the probes as well as the formamide concentrations in the hybridization buffer and the salt concentrations in the wash buffers required for specific *in situ* hybridization are specified.

Probe	Sequence (5'-3')	Target site[a] (16S rRNA position)	% Formamide/ mM NaCl
S-G-Ntspa-0662-a-A-18	GGAATTCCGCGCTCCTCT	662-679	35 / 80
Comp-Ntspa-0662	GGAATTCCGCTCTCCTCT	662-679	--[b]
S-*-Ntspa-0712-a-A-21	CGCCTTCGCCACCGGCCTTCC	712-732	50 / 28
Comp-Ntspa-0712	CGCCTTCGCCACCGGTGTTCC	712-732	--[c]

Fluorescence *in situ* hybridization and microautoradiography

For determining oligonucleotide probe dissociation profiles, 5 µl of fixed reference cells from pure cultures were spotted onto microscope slides (Paul Marienfeld, Bad Mergentheim, Germany) and were dried for 10 min at 46°C. Thereupon, *in situ* hybridization was performed

as detailed by Manz *et al.* (29). Different concentrations of formamide in the hybridization buffers and of sodium chloride in the washing buffers were used to measure probe binding at increasing hybridization stringency.

A modified FISH protocol was used to preserve the three-dimensional structure of bacterial aggregates in biofilm and activated sludge flocs. Silicon tube segments with a diameter of 5 mm and a length of 5-8 mm were glued onto microscope cover slips by using bicomponent glue. These "hybridization chambers" were filled with 20 µl of the biofilm or activated sludge samples. After sedimentation (by gravity) of the biomass approximately 10 µl of the supernatant was removed and replaced by 20 µl of 1% (w/v) molten agarose (GibcoBRL ultraPure agarose, Life Technologies, Paisley, Scotland) at a temperature of approximately 37°C. After solidification of the agarose the embedded samples were dehydrated by dipping the slips successively into 50, 80 and 96% (v/v) ethanol for 3 min each. Subsequently, FISH was performed as described previously (29), but 20 µl of hybridization buffer and increased amounts of probes (60 ng of probes labeled with Cy3 or Cy5 and 100 ng of probes labeled with FLUOS) were applied. Following the hybridization and washing steps the slips were immersed for 10 sec in ice-cold, de-ionized water to remove buffer salts from the slip surface.

Fixed biofilm or activated sludge samples, which had been incubated with radioactive substrates, were embedded in cryo-embedding compound (cat. no. 350100, Microm, Walldorf, Germany) and sliced with a microtome (model HM 505E, Microm, Walldorf, Germany). Sections with a thickness of 5-10 µm were applied onto microscope cover slips, and FISH was performed with suitable oligonucleotide probes. The steps of the MAR protocol were performed as described by Lee *et al.* (24). Different exposure times (2, 5, and 7 days) before the development of the radiographic film emulsion were tested for all samples incubated with the different substrates (shorter exposure resulted in weaker signals, while prolonged exposure caused increased background noise). Microscope slips with developed film were stored at 4°C till microscopic analysis.

Microscopy and digital image analysis

All samples hybridized with oligonucleotide probes were embedded in Citifluor (Citifluor, Canterbury, U. K.) prior to microscopic observation. Alternatively, biofilm was embedded in a 1:1 mixture of Citifluor and a 0.01% (w/v) fluorescein solution for negative staining.

Fluorescence signals were recorded using a LSM 510 confocal laser scanning microscope (CLSM; Zeiss, Oberkochen, Germany) equipped with two HeNe lasers (543 and 633 nm, respectively) for detection of Cy3 and Cy5, and one Ar ion laser (450-514 nm) for detection of FLUOS. Probe dissociation profiles were obtained by quantification of the average fluorescence emitted by probe-stained cells as described by Daims *et al.* (14). Diameters of cell aggregates were determined by using the measurement tools of the software delivered with the CLSM (LSM 510, version 2.01). Three-dimensional reconstructions of *Nitrospira* cell aggregates were generated from stacked optical sections through biofilm samples. The optical sections with a thickness of 0.5-0.7 µm were acquired with the CLSM and saved as monochrome, 8-bit TIFF files. These images were imported in consecutive order by a 3D image analysis and visualization program currently being developed (Daims, unpublished data). During this step, the intensities of the image pixels were stored in a three-dimensional voxel array in computer memory. Thereupon, background noise was reduced by three-dimensional median filtering, and the pixel intensity data were analyzed to differentiate between cell material and voids within cell clusters. Isosurfaces (i. e. surfaces connecting points of the same intensity level) were extracted from the intensity array by a modified implementation of the *Marching Tetrahedra* algorithm (18) to display the surfaces of cell clusters. Prior to the final rendering of the cell aggregates, appropriate intensity thresholds for these isosurfaces had to be determined to visualize the data correctly. This was achieved by displaying all existing interfaces between the cell aggregates, the surrounding medium, and internal voids as semi-transparent layers in one image. These interfaces were detected without the need of specifying intensity thresholds by a modification of the technique described by Csebfalvi and Gröller (13). Isosurfaces obtained with different, user-specified intensity thresholds were visually compared to these interfaces, and the most suitable thresholds were selected for drawing the isosurfaces in the final images. Subsequently, all voxels contributing to voids within cell clusters were rendered as semi-transparent, colored cubes to visualize the cavities and channels in the cell aggregates.

Results

Phylogenetic affiliation of *Nitrospira*-like bacteria in the sequencing batch biofilm reactor

Biofilm was taken from the nitrifying sequencing batch biofilm reactor SBBR 1, total DNA was extracted, and a 16S rDNA clone library was established. Among the 129 cloned and phylogenetically analyzed 16S rDNA sequences, six almost identical sequences shared high overall similarities (above 97%) with 16S rRNA sequences of *Nitrospira*-like bacteria obtained from nitrifying bioreactors in other studies (Fig. 1). None of the 129 analyzed 16S rRNA sequences retrieved from reactor SBBR 1 grouped with the other two main lineages of the phylum *Nitrospira* (the genus *Leptospirillum* and the *Thermodesulfovibrio* / "*Magnetobacterium*" lineage).

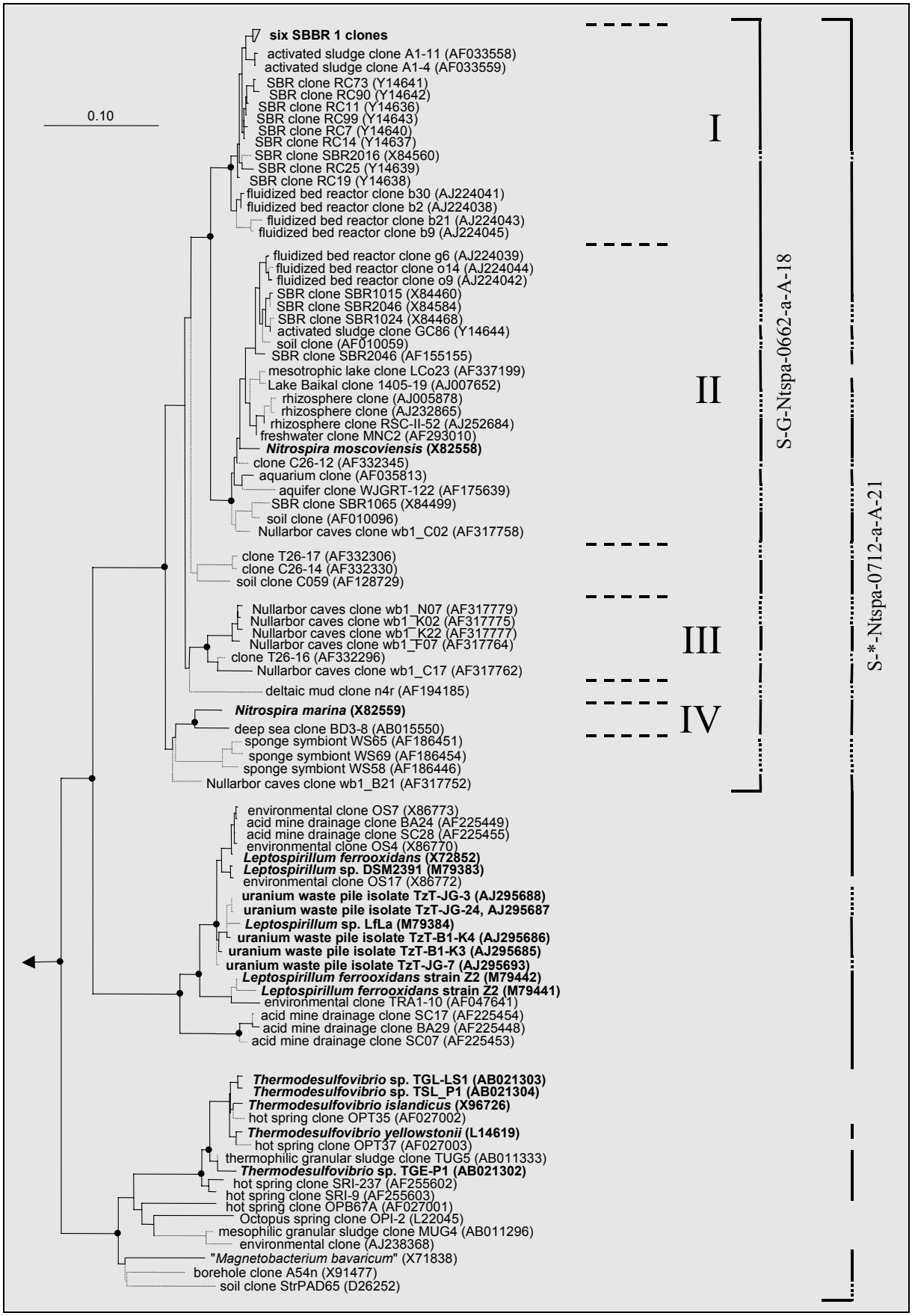
16S rRNA-directed oligonucleotide probes for *in situ* detection of the genus and the phylum *Nitrospira*

In this study, two 16S rRNA directed oligonucleotide probes were developed for *in situ* identification of members of the genus and phylum *Nitrospira*, respectively. The sequences and binding positions of these probes and the recommended buffer compositions for specific *in situ* hybridization are summarized in Table 1. The target organisms of each probe are indicated by brackets in Fig. 1.

Probe S-G-Ntspa-0662-a-A-18 targets the two described species *N. moscoviensis* and *N. marina* as well as all environmental 16S rRNA sequences clustering with the genus *Nitrospira* line of descent for which the probe target site has been sequenced (Fig. 1). The 16S rRNA sequences of these organisms are fully complementary to probe S-G-Ntspa-0662-a-A-18 at the probe binding site (*E. coli* position 662-679), while all members of the other main lineages in the phylum *Nitrospira* possess at least one central mismatch to this probe at its binding site (Fig. 2 a). Several additional non-target bacteria, for example *Bacillus stearothermophilus*, have a single C→A transversion at *E. coli* position 669 (Fig. 2 a). The probe dissociation profile of probe S-G-Ntspa-0662-a-A-18 indicates that this single

mismatch does not prevent binding of the probe to the 16S rRNA of these non-target organisms (Fig. 3 a). Fluorescence emitted by probe-stained cells of the target species *N. moscoviensis* was clearly visible after FISH with up to 55% formamide in the hybridization buffer. However, cells of the non-target organism *B. stearothermophilus* were also stained by the probe and appeared even brighter. The probe-conferred fluorescence of the *Bacillus* cells was probably stronger, because these heterotrophic cells contained more ribosomes than the slow-growing, autotrophic cells of *N. moscoviensis*. Therefore, a competitor oligonucleotide (named Comp-Ntspa-0662) was designed which is fully complementary to the 16S rRNA of all non-target organisms with the same sequence as *B. stearothermophilus* between *E. coli* positions 662 and 679. This competitor suppressed completely the hybridization of probe S-G-Ntspa-0662-a-A-18 with *B. stearothermophilus* if applied in equimolar amounts together with the probe in a hybridization buffer containing at least 35% formamide (Fig. 3 a). Under these conditions, probe S-G-Ntspa-0662-a-A-18 also did not bind to *Leptospirillum ferrooxidans* and *Thermodesulfovibrio yellowstonii* (data not shown). The bacterial probe EUB338 stained *N. moscoviensis* as well as *B. stearothermophilus* at formamide concentrations between 0 and 60% without any visible decrease of the fluorescence signal intensity (data not shown).

Fig. 1. Phylogenetic tree of the phylum *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum likelihood analysis of all sequences longer than 1,300 nucleotides. Shorter sequences were successively added by use of the ARB_PARSIMONY module of the ARB program without changing the overall tree topology. Branches leading to sequences shorter than 1,000 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high parsimony bootstrap support above 90% based on 100 iterations. The scale bar indicates 0.1 estimated changes per nucleotide. Sequences of *Nitrospira*-like bacteria retrieved in this study from reactor SBBR 1 and sequences that belong to isolated strains are printed bold. The four sublineages of the genus *Nitrospira* are delimited by horizontal dashed lines and marked by the numbers I to IV. The brackets illustrate the coverage of the 16S rRNA-targeted oligonucleotide probes developed in this study. Dotted bracket segments indicate that the corresponding partial sequences do not include the probe target site. Brackets are interrupted where sequences are not targeted by the respective probe.


0.10
six SBBR 1 clones
activated sludge clone A1-11 (AF033558)
activated sludge clone A1-4 (AF033559)
SBR clone RC73 (Y14641)
SBR clone RC90 (Y14642)
SBR clone RC11 (Y14636)
SBR clone RC99 (Y14643)
SBR clone RC7 (Y14640)
SBR clone RC14 (Y14637)
SBR clone SBR2016 (X84560)
SBR clone RC25 (Y14639)
SBR clone RC19 (Y14638)
fluidized bed reactor clone b30 (AJ224041)
fluidized bed reactor clone b2 (AJ224038)
fluidized bed reactor clone b21 (AJ224043)
fluidized bed reactor clone b9 (AJ224045)
fluidized bed reactor clone g6 (AJ224039)
fluidized bed reactor clone o14 (AJ224044)
fluidized bed reactor clone o9 (AJ224042)
SBR clone SBR1015 (X84460)
SBR clone SBR2046 (X84584)
SBR clone SBR1024 (X84468)
activated sludge clone GC86 (Y14644)
soil clone (AF010059)
SBR clone SBR2046 (AF155155)
mesotrophic lake clone LCo23 (AF337199)
Lake Baikal clone 1405-19 (AJ007652)
rhizosphere clone (AJ005878)
rhizosphere clone (AJ232865)
rhizosphere clone RSC-II-52 (AJ252684)
freshwater clone MNC2 (AF293010)
***Nitrospira moscoviensis* (X82558)**
clone C26-12 (AF332345)
aquarium clone (AF035813)
aquifer clone WJGRT-122 (AF175639)
SBR clone SBR1065 (X84499)
soil clone (AF010096)
Nullarbor caves clone wb1_C02 (AF317758)
clone T26-17 (AF332306)
clone C26-14 (AF332330)
soil clone C059 (AF128729)
Nullarbor caves clone wb1_N07 (AF317779)
Nullarbor caves clone wb1_K02 (AF317775)
Nullarbor caves clone wb1_K22 (AF317777)
Nullarbor caves clone wb1_F07 (AF317764)
clone T26-16 (AF332296)
Nullarbor caves clone wb1_C17 (AF317762)
deltaic mud clone n4r (AF194185)
***Nitrospira marina* (X82559)**
deep sea clone BD3-8 (AB015550)
sponge symbiont WS65 (AF186451)
sponge symbiont WS69 (AF186454)
sponge symbiont WS58 (AF186446)
Nullarbor caves clone wb1_B21 (AF317752)
environmental clone OS7 (X86773)
acid mine drainage clone BA24 (AF225449)
acid mine drainage clone SC28 (AF225455)
environmental clone OS4 (X86770)
***Leptospirillum ferrooxidans* (X72852)**
***Leptospirillum* sp. DSM2391 (M79383)**
environmental clone OS17 (X86772)
uranium waste pile isolate TzT-JG-3 (AJ295688)
uranium waste pile isolate TzT-JG-24, AJ295687
***Leptospirillum* sp. LfLa (M79384)**
uranium waste pile isolate TzT-B1-K4 (AJ295686)
uranium waste pile isolate TzT-B1-K3 (AJ295685)
uranium waste pile isolate TzT-JG-7 (AJ295693)
***Leptospirillum ferrooxidans* strain Z2 (M79442)**
***Leptospirillum ferrooxidans* strain Z2 (M79441)**
environmental clone TRA1-10 (AF047641)
acid mine drainage clone SC17 (AF225454)
acid mine drainage clone BA29 (AF225448)
acid mine drainage clone SC07 (AF225453)
***Thermodesulfovibrio* sp. TGL-LS1 (AB021303)**
***Thermodesulfovibrio* sp. TSL_P1 (AB021304)**
***Thermodesulfovibrio islandicus* (X96726)**
hot spring clone OPT35 (AF027002)
***Thermodesulfovibrio yellowstonii* (L14619)**
hot spring clone OPT37 (AF027003)
thermophilic granular sludge clone TUG5 (AB011333)
***Thermodesulfovibrio* sp. TGE-P1 (AB021302)**
hot spring clone SRI-237 (AF255602)
hot spring clone SRI-9 (AF255603)
hot spring clone OPB67A (AF027001)
Octopus spring clone OPI-2 (L22045)
mesophilic granular sludge clone MUG4 (AB011296)
environmental clone (AJ238368)
"*Magnetobacterium bavaricum*" (X71838)
borehole clone A54n (X91477)
soil clone StrPAD65 (D26252)
I
II
III
IV
S-G-Ntspa-0662-a-A-18
S-*-Ntspa-0712-a-A-21

Probe S-*-Ntspa-0712-a-A-21 is fully complementary to the region between *E. coli* positions 712 and 732 on the 16S rRNA of all members of the phylum *Nitrospira* with the following exceptions (Fig. 2 b): *Thermodesulfovibrio islandicus* has three mismatches at the probe target site. *Thermodesulfovibrio* sp. TGL-LS1, *Thermodesulfovibrio* sp. TSL-P1 and hot spring clone OPB67A have one mismatch at position 720. Octopus spring clone OPI-2 has the same mismatch at position 720 and one additional G→A transition at position 712. The environmental sequences OPT35, OPT37, MUG4, and AJ238368 have other mismatches in the probe binding region (Fig. 2 b). Within the genus *Leptospirillum*, only the environmental sequence OS17 has two mismatches at positions 716 and 724 (Fig. 2 b). Among the members of the genus *Nitrospira*, the mesotrophic lake clone LCo23 (AF337199) has one G→A transition at position 720, and *N. marina* has one G→A transition at position 712 (Fig. 2 b). We assume that this single mismatch does not prevent binding of probe S-*-Ntspa-0712-a-A-21 to *N. marina*, because it is located marginally at the 5'-end of the probe target region. The target species *N. moscoviensis* and *L. ferrooxidans* were stained by probe S-*-Ntspa-0712-a-A-21 with up to 70% formamide in the hybridization buffer (Fig. 3 b). Probably due to the length (21 nucleotides) and high G+C content of this probe (76.2%), no clear dissociation of the probe from the target organisms was observed even at the highest formamide concentration tested. In addition, the competitor Comp-Ntspa-0712 (see below), which was applied together with probe S-*-Ntspa-0712-a-A-21 in these experiments, did not prevent hybridization of the probe to the target organisms. Fig. 3 b contains also the dissociation profile of probe S-*-Ntspa-0712-a-A-21 with *Desulfovibrio desulfuricans*. This species represents a group of non-target organisms with two mismatches in the binding region of the probe (Fig. 2 b). These mismatches did not prevent hybridization of the probe to *D. desulfuricans*, but instead this species was stained as efficiently as *N. moscoviensis* with up to 60% formamide in the hybridization buffer, and weaker but detectable fluorescence signals were still observed when more formamide was applied (Fig. 3 b). Therefore, a competitor oligonucleotide (named Comp-Ntspa-0712) complementary to the 16S rRNA of all organisms with the same sequence as *D. desulfuricans* between *E. coli* positions 712 and 732 was designed to improve the specificity of probe S-*-Ntspa-0712-a-A-21. If Comp-Ntspa-0712 was added in equimolar concentrations to the hybridization buffer, probe S-*-Ntspa-0712-a-A-21 did not stain *D. desulfuricans* even under conditions of low stringency (Fig. 3 b).

A Probe S-G-Ntspa-0662-a-A-18

Probe target site sequence	5' AGAGGAGCGCGGAAUUCC 3'
Nitrospira moscoviensis	5' ---------- 3'
Nitrospira marina	5' ---------- 3'
SBBR 1 clone B28	5' ---------- 3'
Leptospirillum ferrooxidans	5' -----GAA--------U- 3'
Thermodesulfovibrio yellowstonii	5' -------G----N----- 3'
"*Magnetobacterium bavaricum*"	5' --G---UG---------- 3'
Bacillus stearothermophilus	5' -------A---------- 3'
Bacillus denitrificans	5' -------A---------- 3'
Rhodospirillum salinarum	5' -------A---------- 3'

B Probe S-*-Ntspa-0712-a-A-21

Probe target site sequence	5' GGAAGGCCGGUGGCGAAGGCG 3'
Nitrospira moscoviensis	5' ---------- 3'
Nitrospira marina	5' A---------- 3'
mesotrophic lake clone LCo23	5' --------A---------- 3'
SBBR 1 clone B28	5' ---------- 3'
Leptospirillum ferrooxidans	5' ---------- 3'
environmental clone OS17	5' ----A-------U-------- 3'
Thermodesulfovibrio yellowstonii	5' ---------- 3'
Thermodesulfovibrio islandicus	5' -----A--U---U-------- 3'
Thermodesulfovibrio sp. TGL-LS1	5' --------U---------- 3'
Thermodesulfovibrio sp. TSL-P1	5' --------U---------- 3'
hot spring clones OPT35, OPT37	5' ----A---U--------A--- 3'
granular sludge clone MUG4	5' ------U-U---------- 3'
environmental clone AJ238368	5' ------U-U---------- 3'
"*Magnetobacterium bavaricum*"	5' ---------- 3'
Desulfovibrio desulfuricans	5' ----CA---------- 3'
Saccharopolyspora hirsuta	5' ----CA---------- 3'

Fig. 2. Target site sequences and corresponding 16S rRNA sequence regions of target and non-target organisms for (A) probe S-G-Ntspa-0662-a-A-18 and (B) probe S-*-Ntspa-0712-a-A-21. Hyphens represent identical nucleotides. Mismatches between the rRNA sequences of organisms and the probe target site sequence are indicated by capitals.

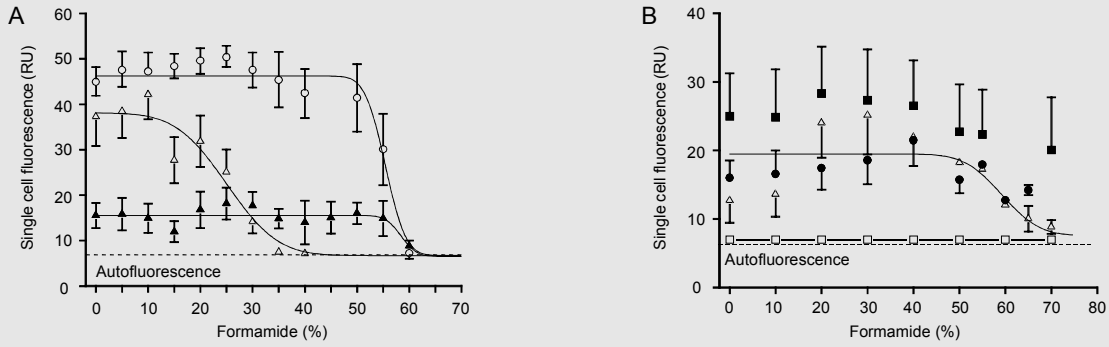


Fig. 3. Probe dissociation profiles of the oligonucleotide probes developed in this study with reference organisms under increasingly stringent hybridization and washing conditions. For each data point the mean fluorescence intensity of at least 100 cells was determined. Regression curves were calculated by the plotting software based on a sigmoidal curve fit model. Error bars indicate standard deviations. Error bars which are smaller than the marker symbols are not shown. **(A)** Hybridization of the target organism *Nitrospira moscoviensis* with probe S-G-Ntspa-0662-a-A-18 in the presence of Comp-Ntspa-0662 (▲). Hybridization of the non-target bacterium *Bacillus stearothermophilus* with probe S-G-Ntspa-0662-a-A-18 without (○) and with (Δ) competitor Comp-Ntspa-0662. **(B)** Hybridization of the target organisms *N. moscoviensis* (●) and *L. ferrooxidans* (■) with probe S-*-Ntspa-0712-a-A-21 in the presence of competitor Comp-Ntspa-0712. Hybridization of the non-target bacterium *Desulfovibrio desulfuricans* with probe S-*-Ntspa-0712-a-A-21 without (Δ) and with (□) competitor Comp-Ntspa-0712. The regression curve refers to the data points obtained for *D. desulfuricans* without addition of the competitor.

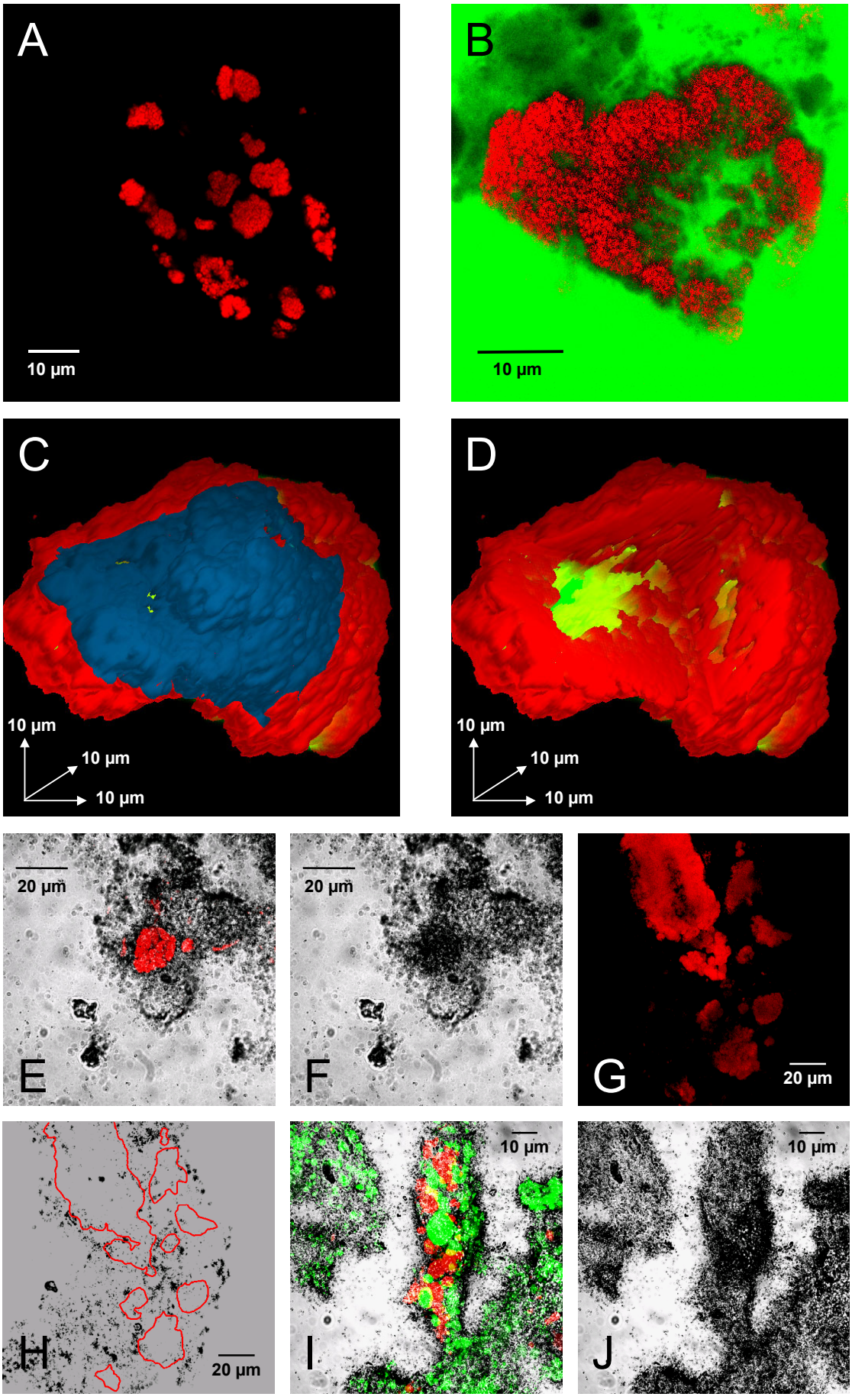
In situ detection and morphology of *Nitrospira* microcolonies in biofilm and activated sludge

Activated sludge and biofilm from four different nitrifying bioreactors were screened for *Nitrospira*-related bacteria by FISH with the newly developed oligonucleotide probes. *Nitrospira*-related 16S rRNA sequences had been obtained in this study from the reactor SBBR 1 (see above). The presence of *Nitrospira*-like organisms in this reactor was confirmed by FISH with probes S-G-Ntspa-0662-a-A-18 and S-*-Ntspa-0712-a-A-21 (Fig. 4 b). Both probes exclusively stained the same cell aggregates with average diameters of 12.4 ± 7.0 µm (the diameters of 50-100 probe-stained aggregates were determined in each sample and mean diameters with standard errors are specified). Similar results were obtained for an additional biofilm reactor (Biofor 2), where both probes also stained exclusively the same cell aggregates, which had an average diameter of 11.4 ± 7.6 µm. The smallest and largest measured diameters of the *Nitrospira* cell clusters were 4.9 respective 38.1 µm in reactor SBBR 1 and 1.6 respective 31.7 µm in reactor Biofor 2. Some of the *Nitrospira* microcolonies in these biofilms had a spherical appearance, but most of them were shaped more irregularly. Their most remarkable feature, however, were cavities and channels within the cell colonies. Negative staining of biofilm from reactor SBBR 1 with fluorescein after FISH with probe S-G-Ntspa-0662-a-A-18 revealed that the fluorescein, which did not cross the boundaries of the *Nitrospira* cells, could penetrate these voids (Fig. 4 b). The absence of bacterial cells from the cavities was confirmed by additional hybridization with the EUB338 probe mix (data not shown). The impression that the *Nitrospira* microcolonies could be interlaced by a network of microscopic channels was verified by three-dimensional reconstruction of a probe-stained *Nitrospira* microcolony (Fig. 4 c,d). *Nitrospira* microcolonies with this intricate morphology occurred frequently in the biofilms, but were rare in both activated sludge samples. *Nitrospira* aggregates detectable with both probes (S-G-Ntspa-0662-a-A-18 and S-*-Ntspa-0712-a-A-21) were abundant in these sludges, but compared to the biofilm samples the cells were packed more tightly and the aggregates were significantly smaller and had a more spherical shape (Fig. 4 a). The average diameter of a *Nitrospira* microcolony in the AAV plant was 2.8 ± 1.6 µm with a minimum of 0.9 µm and a maximum of 7.5 µm. In the activated sludge sample from the Munich II plant, their average diameter was 3.9 ± 2.3 µm with a minimum of 1.0 µm and a maximum of 13.6 µm.

All samples were also screened by FISH with a set of probes targeting nitrifying bacteria other than *Nitrospira*. *Nitrobacter* spp. were neither detected in the biofilm from reactor Biofor 2 nor in the two activated sludge samples using probe NIT3. In reactor SBBR 1, however, probe-stained *Nitrobacter* cells occurred frequently, but were less abundant than *Nitrospira*-like bacteria (data not shown). Furthermore, ammonia-oxidizers from the beta-subclass of *Proteobacteria* were detected frequently in all samples using probes NEU and Nso1225 (Fig. 4 i).

Uptake of carbon sources by *Nitrospira*-like bacteria in wastewater treatment plants

The uptake of organic substrates by *Nitrospira*-like bacteria in wastewater treatment plants was studied by incubation of living nitrifying biofilm and activated sludge with radioactive carbon sources followed by FISH and microautoradiography. Biofilm samples from reactors SBBR 1 and Biofor 2, and activated sludge from the AAV plant were incubated with different carbon sources as described in the Materials and Methods section. The liquid scintillation counts and HPLC measurements performed before and after all incubations confirmed that the substrates were not depleted during the incubation time (data not shown). Following incubation, paraformaldehyde-fixation and cryo-sectioning, the samples were hybridized with the *Nitrospira*-specific probes developed in this study, and the EUB338 probe mix. After completion of the FISH-MAR procedure the samples were screened for probe-stained microcolonies of *Nitrospira*-like bacteria. Silver grain formation above these aggregates indicated that the cells took up the respective substrates during the incubation period. All substrate uptake experiments were qualitative except for one experiment with bicarbonate and activated sludge from the AAV plant, which was evaluated quantitatively by counting the MAR-positive and MAR-negative *Nitrospira* microcolonies (see below). Under aerobic incubation conditions, most detected *Nitrospira*-colonies in the biofilm sample from reactor SBBR 1 took up ^{14}C-bicarbonate (Fig. 4 e,f). No uptake of acetate, propionate and butyrate by *Nitrospira*-like bacteria was observed after aerobic incubation (Fig. 4 g,h). Most of the *Nitrospira* colonies in the biofilm, however, were clearly MAR-positive after aerobic incubation with ^{14}C-labeled pyruvate (Fig. 4 i,j). Uptake of pyruvate by the remaining *Nitrospira* colonies was uncertain, because only weak silver grain formation was observed above these aggregates. Similar results as for reactor SBBR 1 were obtained with the biofilm from reactor Biofor 2 (data not shown). *Nitrospira*-like bacteria did not take up any substrate tested in all three samples under anoxic or anaerobic conditions.


A
10 µm
B
10 µm
C
10 µm
10 µm
10 µm
D
10 µm
10 µm
10 µm
20 µm
E
20 µm
F
G
20 µm
H
20 µm
10 µm
I
10 µm
J

Other bacteria in the samples, which were stained by the EUB338 probe mix, took up the different substrates under the anoxic incubation conditions and, to a much lesser extent, under the anaerobic incubation conditions (data not shown). These bacteria were not identified by FISH with more specific probes, but served as positive control for the incubation experiments and the FISH-MAR procedure. In contrast, no MAR-positive cells were detected in the control experiments performed with pasteurized biofilm (data not shown). Therefore, adsorption of labeled substrates to cells or other organic components did not account for the MAR signals observed with living biofilm and activated sludge.

An additional experiment was performed to determine the fraction of detectable *Nitrospira* microcolonies taking up bicarbonate in activated sludge from the AAV wastewater treatment plant. The sludge was incubated with ^{14}C-bicarbonate under aerobic conditions, and the

Fig. 4. *In situ* analyses of *Nitrospira*–like bacteria within activated sludge and biofilms. **(A)** *Nitrospira* cell aggregates detected in activated sludge by FISH with probe S-G-Ntspa-0662-a-A-18 (red). **(B)** *Nitrospira* cell aggregate detected in biofilm from reactor SBBR 1 by FISH with probe S-G-Ntspa-0662-a-A-18 (red). The biofilm was also stained with fluorescein (green). **(C, D)**. Three-dimensional reconstruction of a *Nitrospira* cell aggregate from reactor SBBR 1 stained by FISH with probe S-G-Ntspa-0662-a-A-18. *Nitrospira* cells are colored red; the blue part of the microcolony in (C) was digitally removed to allow insight into the aggregate (D). Voids within the aggregate are marked in green. **(E, F)** Uptake of bicarbonate by *Nitrospira*-like bacteria in biofilm from reactor SBBR 1 under aerobic incubation conditions. (E) shows *Nitrospira* cells stained by probe S-G-Ntspa-0662-a-A-18 (red) combined with the micrograph of the radiographic film at the same position. (F) shows only the film to visualize the MAR signal at the position of the *Nitrospira* cells. Other MAR signals were caused by CO_2-fixing bacteria, which were not detected by the *Nitrospira*-specific probe. **(G, H)** No uptake of acetate by *Nitrospira*-like bacteria in biofilm from reactor SBBR 1 under aerobic incubation conditions. (G) shows *Nitrospira* cells stained by probe S-G-Ntspa-0662-a-A-18 (red). (H) shows the micrograph of the radiographic film at the same position. The localization of the *Nitrospira* microcolonies in (G) is indicated by red borderlines in (H). **(I, J)** Uptake of pyruvate by *Nitrospira*-like bacteria (stained by probe S-G-Ntspa-0662-a-A-18, red) and by ammonia-oxidizers (stained by probes NEU and Nso1225, green) in biofilm from reactor SBBR 1 under aerobic incubation conditions. The fluorescence recorded in a stack of images by the CLSM has been combined by orthographic projection in (I). Stacked cells of *Nitrospira*-like bacteria and ammonia-oxidizers appear therefore in yellow. The image stack was acquired to ensure that all nitrifiers which contributed to the MAR signal are visible in the final image.

subsequent steps of the FISH-MAR protocol were performed. Different microscope slips covered with the radiographic film emulsion were exposed for five, six or ten days. Following film development, 200 *Nitrospira* microcolonies were investigated for CO_2-fixation for each exposure time in several microscope fields per slip to obtain the fraction of colonies with CO_2 uptake activity. After five days of exposure 56% of the counted *Nitrospira* colonies were MAR-positive. These colonies usually had diameters above 3.2 µm, while most smaller colonies were MAR-negative. Similar results were obtained after six days of exposure, when 51% of all counted colonies were MAR-positive with the same correlation of colony size and MAR signals. After ten days of exposure, however, the fraction of clearly MAR-positive colonies had increased to 77%. The remaining cell aggregates were very small (diameters below 2.0 µm), and silver grain formation above these colonies possibly indicating bicarbonate uptake could not be distinguished from side effects due to background radiation or radiation emitted by adjacent larger colonies.

Discussion

Phylogenetic trees containing all publicly available 16S rRNA sequences related to the phylum *Nitrospira* were calculated using maximum parsimony and maximum likelihood treeing methods on different data sets (see Materials and Methods). The resultant consensus tree of the phylum is shown in Fig. 1 and is almost identical in topology to the respective neighbour joining tree (data not shown). Consistent with the original definition of the phylum *Nitrospira* proposed by Ehrich *et al.* (16), the phylum currently consists of three main, monophyletic lineages which are supported by all treeing methods and high bootstrap values. All sequences in the first main lineage are affiliated to *Nitrospira moscoviensis* and *Nitrospira marina*, the sequences in the second main lineage are related to *Leptospirillum ferrooxidans*, and the organisms in the third main lineage are relatives of *Thermodesulfovibrio yellowstonii* and "*Magnetobacterium bavaricum*". Our analyses demonstrate that the line of descent containing both *Nitrospira* species can further be subdivided into at least four monophyletic sublineages, which are supported by all treeing methods and high bootstrap values above 90% (I-IV; Fig. 1). The sequences grouping together in each sublineage share 16S rRNA similarities of at least 94.9%. In contrast, the sequence similarities of sequences that belong to different sublineages are always below 94.0 %. Therefore, it can be postulated based on the

suggestion by Stackebrandt and Goebel (44) that the genus *Nitrospira* contains in addition to *N. marina* and *N. moscoviensis* at least two new candidate species represented by sublineage I and III, respectively. However, valid description of these species has to await the isolation and phenotypic characterization of these organisms. Comparison of sublineage affiliation and sampled environments suggests that each of the sublineages I, III, and IV encompass a specialized groups of nitrite-oxidizers adapted to a certain habitat while sublineage II contains nitrite-oxidizers which can thrive in different systems. One might speculate that the apparently unequal distribution of the sublineages in nature is a direct consequence of evolutionary events or of limited searches that have been made for these organisms.

In detail, sublineage I contains only uncultivated organisms found in nitrifying bioreactors. These bacteria were detected in a laboratory-scale sequencing batch reactor (11), a laboratory-scale fluidized bed reactor (43), and an industrial full-scale activated sludge basin (22). In addition, all six *Nitrospira*-related 16S rRNA sequences obtained from reactor SBBR 1 in this study, group with this sublineage (Fig. 1). Sublineage II contains the cultivated species *N. moscoviensis* as well as 16S rRNA sequences of uncultivated bacteria retrieved from diverse habitats including bioreactors (9, 11, 43), freshwater aquaria (21), soil, rhizosphere (30), and lake water. The topology of the tree implies that these two sublineages emerged from the same ancestor after the divergence of the other two sublineages (Fig. 1). Sequences retrieved from different nitrifying bioreactors occur in sublineages I and II, but no obvious correlation between the types or operational modes of the source reactors and the affiliation of the corresponding sequences to one of the sublineages exists. For example, each sublineage contains organisms found in sequencing batch reactors. Furthermore, the sequences retrieved from the same fluidized bed reactor by Schramm *et al.* (43) are distributed over both sublineages (clones b30, b2, b21, b9 and g6, o14, o9; Fig. 1). Sublineage III consists mainly of sequences found in aquatic samples from Nullarbor caves, Australia (20) (Fig. 1). Sublineage IV hosts the cultivated species *N. marina* and a related organism detected in the deep sea. It should be noted that eight environmentally retrieved 16S rDNA sequences [clones n4r (AF194185), C059 (AF128729), T26-17 (AF332306), C26-14 (AF332330), WS58 (AF186446), WS65 (AF186451), WS69 (AF186454), and wb1_B21 (AF317752)] affiliated with the *Nitrospira*-lineage are possibly not members of the above described four sublineages. These sequences, however, are incomplete and their exact phylogenetic affiliation to the other sublineages of the genus thus cannot be determined.

Based on the available 16S rRNA sequences, both a *Nitrospira*-phylum and a *Nitrospira*-genus specific oligonucleotide probe suitable for FISH were developed. Probe S-*-Ntspa-0712-a-A-21 covers most known members of the phylum *Nitrospira* while all organisms that belong to other phyla in the ARB database (status, May 2001) have nucleotide mismatches to the probe sequence. A probe targeting the phylum *Nitrospira* has to our knowledge not been published before and thus probe S-*-Ntspa-0712-a-A-21 extends the current set of "bacterial group"-specific probes (28, 29, 32, 35, 40), which have found widespread application in microbial ecology (e. g. (4, 46)). Probe S-G-Ntspa-0662-a-A-18 covers the whole genus *Nitrospira* including the four sublineages mentioned above. Non-target organisms without nucleotide mismatches at the probe binding site, or elsewhere on the rRNA, are not known. A similar probe, which targets positions 664-685 on the 16S rRNA of *Nitrospira*-like bacteria, was published in a previous study (21). This probe is also suitable for *in situ* hybridization (36), but it does not cover the *Nitrospira*-like bacteria detected in an industrial activated sludge plant by Juretschko *et al.* (22). Several other 16S rRNA-targeted oligonucleotide probes for the *in situ* detection of *Nitrospira*-like bacteria were published previously (22, 43). These probes, however, were designed to target only certain *Nitrospira*-related sequences retrieved from wastewater treatment plants and do not cover the whole genus *Nitrospira*.

Application of the newly developed *Nitrospira*-specific probes revealed that the microcolony architecture of the target organisms differed significantly between the activated sludge and biofilm samples. Constant turbulence, shearing and the limited sludge age most likely prevent large, morphologically complex cell aggregates of slowly growing nitrite-oxidizers in activated sludge flocs. Accordingly, the *Nitrospira* microcolonies in the activated sludge samples were relatively small, compact, and almost spherical. In contrast, the *Nitrospira* aggregates in the biofilm samples were significantly larger and had a more complex and irregular morphology with internal cavities and a network of cell-free channels. Structurally similar networks formed by channels and cavities between cell aggregates were previously detected by confocal laser scanning microscopy in biofilms of different origin and composition (e.g. (23, 31)). Channels were also found in microbial granules and have been interpreted as facilitating the exchange of nutrients and gases between the surface and deeper regions (27, 39, 50). This idea was strongly supported by de Beer *et al.* (15), who showed that O_2 concentrations inside a biofilm were significantly higher in voids than in cell clusters. The network of channels and voids we observed within cell clusters of *Nitrospira*-like bacteria may have similar effects. Staining with fluorescein demonstrated that the channels are

permeable for low-molecular-weight, water soluble substances and thus could facilitate the diffusion of nitrite, CO_2, O_2 and metabolic waste compounds throughout the aggregates. Furthermore, the voids and channels in *Nitrospira* cell clusters indicate that the modern concept of biofilm architecture as a complex assembly of cell aggregates, organic polymers and cavities may apply also on a smaller scale to single bacterial microcolonies.

The *Nitrospira*-specific probes were used together with a previously published probe for the *in situ* detection of *Nitrobacter* (48) to investigate the community structure of nitrite-oxidizing bacteria in the nitrifying activated sludge and biofilm samples. While *Nitrospira* was present in significant amounts in all analyzed samples, *Nitrobacter* cells were only detected in the sequencing batch biofilm reactor SBBR 1. Reactor SBBR 1 receives reject water from sludge dewatering, which is particularly rich in ammonia and dissolved salts. Due to the batch performance of the reactor, ammonia, nitrite, and nitrate concentrations vary significantly during an operating cycle (Fig. 5). The repeated, pronounced temporal nitrite concentration shifts within SBBR 1 create an ecological niche for nitrite-oxidizers adapted to high nitrite-concentrations which does not occur in continuously operated bioreactors. This niche is obviously filled by *Nitrobacter*, who according to Schramm *et al.* (42) is a putative r-strategist for nitrite and oxygen. In contrast, the *Nitrospira*-like bacteria were postulated to be K-strategists which can grow with lower nitrite (and oxygen) concentrations and can thus co-exist with *Nitrobacter* in SBBR 1.

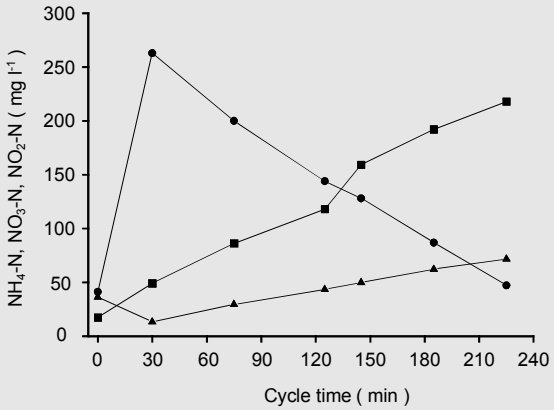


Fig. 5. Concentrations of NH_4-N (●), NO_3-N (■), and NO_2-N (▲) in reactor SBBR 1 during a representative operating cycle of the reactor.

The activity and *in situ* physiology of *Nitrospira*-like organisms in wastewater treatment plants were studied by a recently developed combination of FISH and microautoradiography (MAR) (24). The FISH-MAR experiments with radioactive bicarbonate demonstrated that *Nitrospira*-like bacteria growing in aerated bioreactors fix CO_2. This finding was not unexpected since pure cultures of *N. moscoviensis* and *N. marina* grow autotrophically under laboratory conditions (16, 49), but CO_2 fixation of the recently discovered *Nitrospira*-like bacteria has not been demonstrated previously. Furthermore, CO_2 uptake can be exploited as a method to demonstrate *in situ* activity of nitrifying bacteria. Such a method is urgently required since positive FISH signals were also observed for starved or metabolically inhibited nitrifying bacteria (34, 47). In the analyzed municipal activated sludge more than 75% of all *in situ* detected *Nitrospira* microcolonies were unambiguously active as visualized by CO_2 incorporation. The high proportion of active *Nitrospira* cell aggregates in the activated sludge may result from the permanent removal of inactive, non-proliferating cells due to the continuous reactor operation. In addition, the distribution of oxygen and nutrients is relatively homogeneous in most aerated activated sludge basins. The sludge flocs are also permanently moved, disintegrated and recombined by turbulence and shear forces. Starvation of whole cell aggregates due to their position in a floc or in the basin is thus rather unlikely.

In addition to CO_2 fixation, the majority of the *Nitrospira*-like bacteria in the different samples took up pyruvate suggesting that these bacteria can grow mixotrophically in wastewater treatment plants. This capability might contribute to the competitiveness of the *Nitrospira*-like bacteria in these systems. Growth with pyruvate as carbon source has also been reported for pure cultures of *Nitrobacter* (6) and *Nitrospira marina*, which reached the highest cell densities in nitrite media containing pyruvate (49). In contrast to the aerobic incubation experiments no uptake of organic carbon sources or CO_2 by *Nitrospira*-like bacteria was observed in the absence of oxygen. However, combined FISH and microsensor measurements revealed that high cell numbers of *Nitrospira*-like bacteria can persist in biofilm zones with low oxygen pressure (17, 36, 41). The ability to switch from aerobic respiration to an anaerobic metabolism should be advantageous in such habitats. According to our experiments, *Nitrospira*-like bacteria do seemingly not denitrify with acetate, propionate, butyrate or pyruvate as electron donors, but they might employ other strategies to survive periods of limited oxygen availability. Pure culture experiments indicated that *N. marina* is obligately aerobic (49) while *N. moscoviensis* can oxidize H_2 with nitrate as electron acceptor and CO_2 as sole carbon source (16). Hydrogenase activity was not examined in this study, but

we do not exclude that the *Nitrospira*-like bacteria living in engineered systems could take advantage of this or other alternatives to aerobic nitrite oxidation.

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Appendix 4

Nitrification in Sequencing Biofilm Batch Reactors: Lessons from Molecular Approaches

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Nitrification in Sequencing Biofilm Batch Reactors: Lessons from Molecular Approaches

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The nitrifying microbial diversity and population structure of a sequencing biofilm batch reactor receiving sewage with high ammonia and salt concentrations (SBBR 1) was analyzed by the full-cycle rRNA approach. The diversity of ammonia-oxidizers in this reactor was additionally investigated using comparative sequence analysis of a gene fragment of the ammonia monooxygenase (*amoA*), which represents a key enzyme of all ammonia-oxidizers. Despite of the „extreme" conditions in the reactor, a surprisingly high diversity of ammonia- and nitrite-oxidizers was observed to occur within the biofilm. In addition, molecular evidence for the existence of novel ammonia-oxidizers is presented. Quantification of ammonia- and nitrite-oxidizers in the biofilm by Fluorescent *In situ* Hybridization (FISH) and digital image analysis revealed that ammonia-oxidizers occurred in higher cell numbers and occupied a considerably larger share of the total biovolume than nitrite-oxidizing bacteria. In addition, ammonia oxidation rates per cell were calculated for different WWTPs following the quantification of ammonia-oxidizers by competitive PCR of an *amoA* gene fragment. The morphology of nitrite-oxidizing, unculturable *Nitrospira*-like bacteria was studied using FISH, confocal laser scanning microscopy (CLSM) and three-dimensional visualization. Thereby, a complex network of microchannels and cavities was detected within microcolonies of *Nitrospira*-like bacteria. Microautoradiography combined with FISH was applied to investigate the ability of these organisms to use CO_2 as carbon source and to take up other organic substrates under varying conditions. Implications of the obtained results for fundamental understanding of the microbial ecology of nitrifiers as well as for future improvement of nutrient removal in wastewater treatment plants (WWTPs) are discussed.

Keywords: Nitrification; ammonia monooxygenase; *Nitrospira*; fluorescent *in situ* hybridization; process stability

Introduction

The optimization of biological nitrogen removal from sewage water is among the most challenging tasks in modern wastewater treatment. Despite of their importance, knowledge about the identity and ecology of the microorganisms catalyzing N-removal in WWTPs is still scarce. The lack of fundamental microbiological understanding, which is more or less directly reflected in the frequent failure of N-removal in plants worldwide, hampers knowledge-driven process improvements. Exploitation of new microbial pathways, growth and maintenance of stable and highly active microbial consortia as well as efficient bioaugmentation strategies are achievable goals if we shift our research focus from empirical studies to investigations tackling key fundamental issues of microbial ecology of wastewater treatment.

Here we report on the investigation of the nitrifying bacterial community present in a pilot-scale sequencing biofilm batch reactor (SBBR). Since a vast majority of the *Bacteria*, including important nitrifiers, can still not be isolated and grown as pure cultures, we used molecular approaches like 16S rRNA and *amoA* gene sequence analysis, fluorescent *in situ* hybridization, microautoradiography (MAR), confocal laser scanning microscopy, digital image analysis and competitive PCR to detect, identify and characterize the ammonia- and nitrite-oxidizing bacteria living in the reactor. Based upon these techniques we achieved to set up an inventory of ammonia- and nitrite-oxidizers in the biofilm, to describe their morphologies and community structures, and to determine some of their key physiological treats like the substrate turnover rates of ammonia-oxidizers and the ability of unculturable nitrite-oxidizers to fix CO_2 autotrophically and to utilize other organic carbon sources. As previous studies on the physiology of nitrifiers in WWTPs were solely based on cultivation-dependent approaches, our results provide for the first time insight into the microbiology of these bacteria *in situ* and without the changes and limitations introduced by classical enrichment and isolation procedures. Hence we are able to suggest strategies for an improvement of the stability of nitrifying reactors and for selective bioaugmentation with bacteria well-adapted to the respective operational conditions.

Materials and Methods

Reactor data

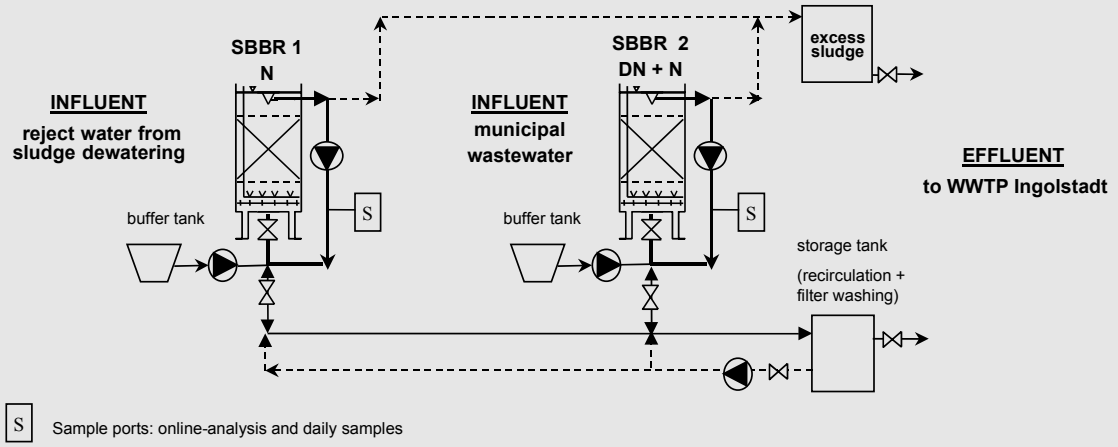

Fig. 1. SBBR reactors at the Ingolstadt pilot wwtp (discontinuously operated, different wastewater sources).

Table 1. Technical data of reactor SBBR 1.

Reactor properties	Values / Units
area	3.8 m^2
total height	4.5 m
support material	Biolite®
grain size	4-8 mm
fixed bed volume (average)	10 m^3
cycle time	5 – 8 hours
washing applied every	10-48 hours

Table 2. Average wastewater composition.

Components [mg L^{-1}]	Reject water from sludge dewatering treated in SBBR 1
SS	30
COD_{total}	300
NH_4-N	400-500
NO_3-N	<0.05
Conductivity [µS cm^{-1}]	5000-6000

Reactor SBBR 1 (Fig. 1) has a total volume of 17 m^3 and contains approx. 10 m^3 of expanded clay, which is submerged in the reactor fluid after completion of the filling phase. The SBBR cycle consists of three phases: filling, mixing (with aeration for nitrification), and drawing. The reactor is back-washed every second day in order to remove excess biomass. The influent is different for the two SBBRs of the pilot WWTP: SBBR 1 receives reject water from sludge dewatering, while SBBR 2 (not covered by this study) receives municipal wastewater. Tables 1 and 2 contain additional technical data and the average composition of the reject water from sludge dewatering.

Biofilm sampling, fluorescent *in situ* hybridization, confocal microscopy, and cell quantification

Sampling of biofilm and subsequent cell fixation were performed as described by Amann *et al*. (1995). The following 16S rRNA-targeted probes were applied in this study: (i) S-*-Ntspa-0712-a-A-21, specific for most members of the phylum *Nitrospira*, and S-G-Ntspa-0662-a-A-18, specific for the genus *Nitrospira* (Daims *et al*., in press); (ii) NEU, targeting most halophilic and halotolerant *Nitrosomonas* species and *Nitrosococcus mobilis* (Wagner *et al*., 1995); (iii) Nso1225 and Nso190, which are complementary to signature sequences of most ammonia-oxidizers in the beta subclass of the *Proteobacteria* (Mobarry *et al*., 1996); (iv) NmV, specific for the *Nitrosococcus mobilis* lineage (Pommerening-Röser *et al*., 1996); (v) NIT3, directed against all known *Nitrobacter* species (Wagner *et al*., 1996); (vii) EUB338, EUB338-II, and EUB338-III, which detect the majority of all known *Bacteria* (Amann *et al*., 1990; Daims *et al*., 1999); (viii) specific probes to detect different strains of *N. mobilis* (unpublished data). *In situ* hybridization with fluorescently labeled probes and image acquisition were performed as detailed by Juretschko *et al*. (1998) and Manz *et al*. (1992). Bacterial populations were quantified by cell area measurement in CLSM images (Morgenroth *et al*., in preparation), and quantification results were expressed as percentage share of the total bacterial cell area, which was determined after FISH with the EUB338 probe mix. Single cell numbers in the CLSM images were calculated by dividing the total cell area occupied by a specified population by the average single cell area of the respective population. The average single cell area was determined previously by manual counting of the cells in CLSM images followed by measurement of their cell area in the same images.

Three-dimensional visualization

Stacks of optical sections acquired by confocal laser scanning microscopy were converted to the IBM Open Visualization Data Explorer (International Business Machines, Inc.) "general array importer" format using a conversion program which was developed in the course of this study at the Department of Microbiology, Technical University Munich. Thereupon, the three-dimensional data were imported and displayed by the Open Visualization Data Explorer software package, version 4.0.4.

Determination of 16S rRNA and *amoA* sequences and phylogenetic analysis

Cloning, sequencing, and phylogenetic analysis of 16S rRNA and *amoA* sequences were performed as specified by Juretschko *et al*. (1998) and Purkhold *et al*. (in preparation). Two different primer pairs were employed for 16S rRNA gene amplification: (i) primers 616F (5'-AGRGTTYGATYMTGGCTCAG-3') and 630R (5'-CAKAAAGGAGGTGATCC-3'), and (ii) primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'; (Lane, 1991)) and 1492R (5'-GGTTACCTTGTTACGACTT-3').

Determination of ammonia turnover rates

As alternative to the cell number determination based on FISH and image analysis, the volumetric cell titers (cells per ml sample or cells per m^3 reactor volume, respectively) of ammonia-oxidizing bacteria were also measured by competitive PCR of an *amoA* sequence fragment (unpublished data). Based on these results, the ammonia turnover rates in the WWTPs were calculated assuming that 80% of the ammonia in the inlet were oxidized to nitrite and 20% were assimilated by the ammonia-oxidizers and other bacteria. The ammonia turnover rates were expressed as pmol of oxidized NH_4^+ per hour and cell.

Combination of FISH and MAR

FISH and MAR were performed in combination as described by Lee *et al*. (1999). Radiolabeled substrates used were ^{14}C-bicarbonate, ^{14}C-propionate, ^{14}C-pyruvate, ^{14}C-butyrate and ^{3}H-acetate.

Biofilm samples were incubated with these substrates under aerobic, anaerobic (fermentative) and anoxic (with nitrate as inorganic electron acceptor) conditions.

Results

Analysis of nitrifying bacterial populations in reactor SBBR 1 based on 16S rRNA sequencing and FISH

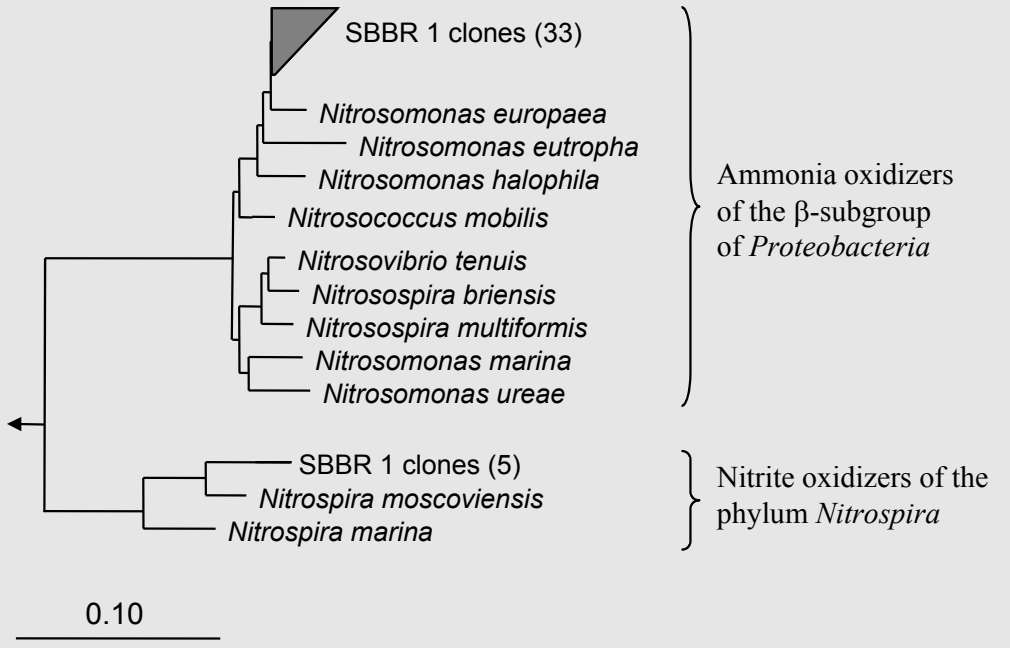

Fig. 2. 16S rRNA sequences of nitrifying bacteria retrieved from reactor SBBR 1. The braces encompass main bacterial lineages and indicate clusters of ammonia- or nitrite-oxidizing bacteria. The tree was calculated by the Neighbor-Joining method with a 50% bacterial conservation filter. The scale bar indicates 0.1 changes per nucleotide.

Among more than 100 full 16S rRNA sequences retrieved from reactor SBBR1, 33 belonged to ammonia-oxidizers within the beta subgroup of the *Proteobacteria*, and five belonged to nitrite-oxidizing *Nitrospira*-like bacteria (Fig. 2). All 16S rRNA sequences of ammonia-oxidizing bacteria were closely related to each other and to the species *Nitrosomonas europaea* and *N. eutropha* (Fig. 2). The identified *Nitrospira*-like bacteria were related to *Nitrospira moscoviensis* and grouped together with 16S rRNA sequences of *Nitrospira*-like bacteria retrieved from other

WWTPs (Burrell *et al*., 1998; Juretschko *et al*., 1998; Schramm *et al*., 1998). The abundance of these organisms in the SBBR 1 biofilm could be confirmed unequivocally by FISH with the two *Nitrospira*-specific oligonucleotide probes. Interestingly, in addition to *Nitrospira*-like bacteria we detected smaller numbers of *Nitrobacter* cells by FISH with the *Nitrobacter*-specific probe NIT3, but despite of the relatively large number of analyzed 16S rRNA sequences, no sequence was related to *Nitrobacter*. Compared to the nitrite-oxidizers, the binding patterns of the probes specific for ammonia-oxidizers were more complex and pointed to the existence of at least five different ammonia-oxidizing populations in the SBBR 1 biofilm: we observed (i) cells stained by probes NEU and Nso1225; (ii) cells stained by probe Nso1225 only; (iii) cells stained by probe NEU only, (iv) cells stained by probes NEU and NmV; and (v) cells stained by all three probes NEU, NmV, and Nso1225. These results indicate that reactor SBBR 1 harbors at least two new, yet undiscovered ammonia-oxidizers, because probe Nso1225 detects all known ammonia-oxidizing beta-*Proteobacteria* including the known target organisms of NEU (halotolerant ammonia-oxidizers) and NmV (*Nitrosococcus mobilis*). Hence, the cells which were hit by probes NEU and/or NmV and not by probe Nso1225 must belong to new *Nitrosomonas* or *Nitrosococcus* strains or species, respectively. Although FISH demonstrated that a high diversity of ammonia-oxidizers exists in reactor SBBR 1, the 33 analyzed 16S rRNA sequences of ammonia-oxidizing bacteria covered only one sequence type with complete sequence analogy to probes NEU and Nso1225 and two mismatches to probe NmV at the respective probe binding sites. In contrast to the FISH results, which indicated that *Nitrosococcus* spp. occur frequently in the reactor (cells hit by both probes NmV and NEU, and partly by Nso1225), no 16S rRNA sequence affiliated to *N. mobilis* was found in the gene bank.

Diversity of ammonia-oxidizing bacteria in SBBR 1 detected by *amoA* sequence evaluation

Consistent with the 16S rRNA sequence analysis, ammonia-oxidizers closely related to *Nitrosomonas europaea* and *N. eutropha* were detected in SBBR 1 based on their *amoA* gene sequences. Furthermore, *amoA* analysis confirmed that bacteria related to *Nitrosococcus mobilis*, which were also detected by FISH with probe NmV (see above), occur in the SBBR 1 biofilm. Interestingly, we identified two different sequence groups which belong clearly to the *N. mobilis* lineage but show different degrees of relationship to the species *N. mobilis*, indicating the possible existence of other, yet unknown *Nitrosococcus* species. The coexistence of two different *N. mobilis* lineages in the SBBR 1 biofilm could also be confirmed by FISH based on different

probe binding patterns (see above). Other ammonia-oxidizing bacteria, e.g. relatives of *Nitrosospira* / *Nitrosovibrio*, were not detected in SBBR 1 by *amoA* analysis.

Morphology and localization of nitrifying bacteria

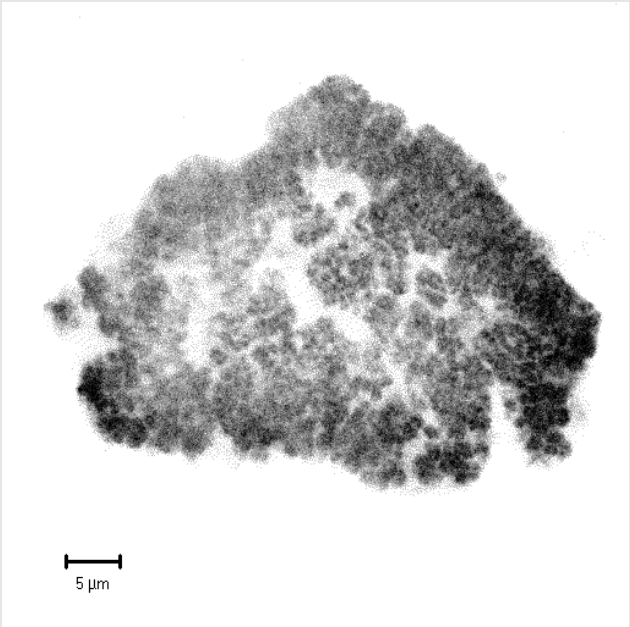

Fig. 3. Optical CLSM section through a *Nitrospira* microcolony showing the interlacing channels and cavities. The thickness of the section is 0.5 µm. The colony was stained by FISH with probe S-G-Ntspa-0662-a-A-18.

In the biofilm from reactor SBBR 1, as well as in all other nitrifying biofilms and activated sludges we examined, ammonia- and nitrite-oxidizing bacteria grew as spherical microcolonies of varying size. Single cells or small aggregates of less than ten cells occurred only occasionally. Within the colonies of ammonia-oxidizing bacteria, the cells were tightly packed and little or no spacing between them was observed. In contrast, *Nitrospira*-like bacteria formed mostly large aggregates interlaced by narrow microchannels and larger, cell-free cavities (Fig. 3). Three-dimensional visualization of these colonies demonstrated that the channel-like structures formed indeed a three-dimensional network, which met the surrounding medium at the colony surface. The accessibility of this network for water and water-soluble substances could be demonstrated by negative fluorescein staining combined with *Nitrospira*-specific FISH (not shown). Simultaneous FISH with probes specific for ammonia- and nitrite-oxidizing bacteria showed that these organisms were frequently located in close vicinity to each other, reflecting their mutualistic relationship (not shown).

Quantification of nitrifying bacteria in reactor SBBR 1

Ammonia- and nitrite-oxidizing bacteria were quantified by digital image analysis as indicated in the Materials and Methods section. As shown in Fig. 4, ammonia-oxidizers occupied a particularly high percentage of the total bacterial cell area in the CLSM images processed (slightly more than 55 %), while the combined cell area of all nitrite-oxidizers (*Nitrospira* and *Nitrobacter*) was much smaller (about 8 %). Among the nitrite-oxidizers, *Nitrospira*-like bacteria were clearly the dominant population. The quantitative difference between ammonia- and nitrite-oxidizers was less drastic in terms of absolute cell numbers: in the 25 analyzed CLSM images we counted about 37,000 cells of ammonia-oxidizers and in total 28,000 cells of nitrite-oxidizers (*Nitrospira* and *Nitrobacter*). Compared with their little share of the total bacterial cell area, the cell numbers of the nitrite-oxidizers were relatively high because of their small cell sizes and the high cell densities in their microcolonies.

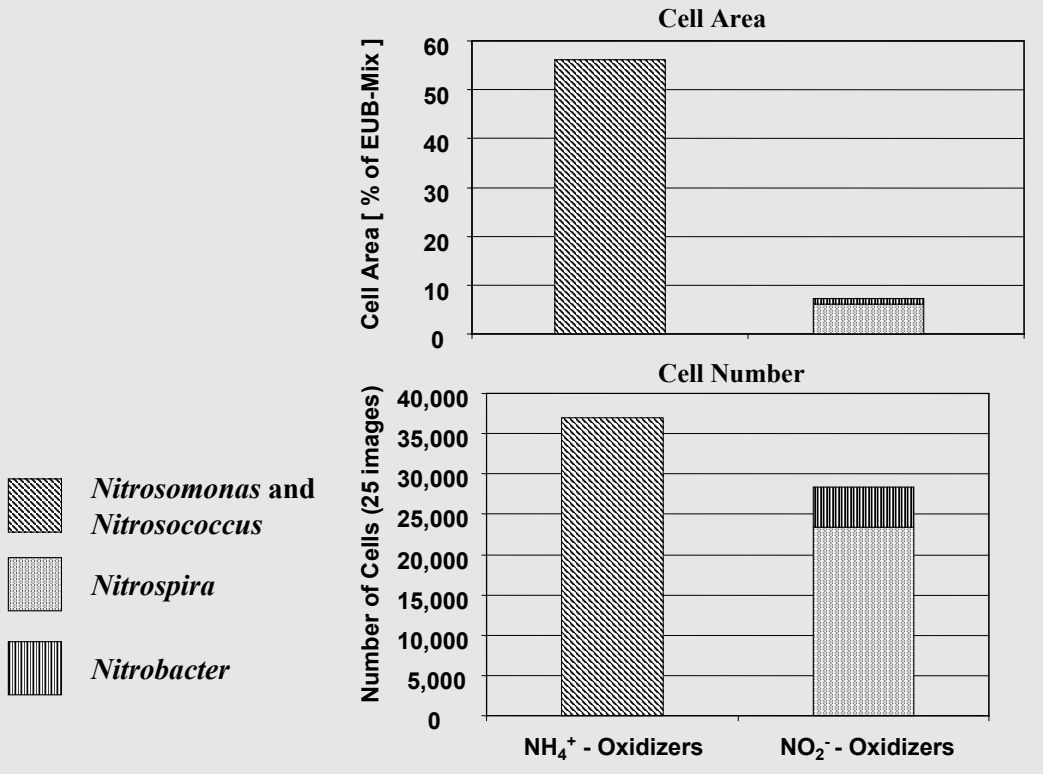

Fig. 4. Quantification of nitrifiers in the biofilm from reactor SBBR 1 by digital image analysis. Cell areas of nitrifying populations were measured in 25 CLSM images and expressed as percentage share of the total area of all bacteria in the same images (upper chart). Absolute cell numbers were calculated based on the measured cell areas as described in the Materials and Methods section (lower chart).

Determination of ammonia turnover rates

The volumetric titers and ammonia turnover rates of ammonia-oxidizing bacteria in different biofilms and activated sludges, among them the biofilm from reactor SBBR 1, were determined as described in the Materials and Methods section. The results are shown in Table 3. The samples were taken from the Ingolstadt pilot plant (SBBR 1 and SBBR 2) and two large municipal WWTPs ("Großlappen" and "Dietersheim").

Table 3. Ammonia turnover rates for different bioreactors.

Bioreactor	turnover rate [pmol NH_4^+ / h · cell]
SBBR 1 (nitrifying biofilm)	0.048
SBBR 2 (denitrifying biofilm)	0.003
Großlappen WWTP, basin II (nitrifying activated sludge)	0.016
Dietersheim WWTP, basin II (nitrifying activated sludge)	0.043

Substrate uptake by *Nitrospira*-like bacteria

The incubation of biofilm samples with different radiolabeled substrates (for details, refer to the Materials and Methods section) followed by microautoradiography and FISH showed that, in presence of oxygen, *Nitrospira*-like bacteria were able to fix CO_2 and to take up small amounts of pyruvate (data not shown). No uptake of CO_2 or pyruvate was observed under anoxic or anaerobic conditions. Furthermore, the additional substrates tested (acetate, propionate and butyrate) were not taken up by *Nitrospira*-like bacteria under any of the conditions applied.

Discussion

Diversity and quantification of nitrifying bacteria

The FISH analysis of nitrifying bacteria in reactor SBBR 1 revealed a notably high diversity of ammonia-oxidizers despite of the "extreme" growth conditions generated by the high ammonia and salt concentrations in the received reject water from sludge dewatering (Table 2). In contrast to these results, we found the ammonia-oxidizing community in an activated sludge exposed to comparably selective conditions to be almost a monoculture of the marine, halophilic species *Nitrosococcus mobilis* (Juretschko *et al*., 1998). This discrepancy could be explained on the basis of the presumably numerous nutrient gradients in the biofilm and its more complex spatial organization, which form a higher variety of ecological niches available for bacteria with different growth requirements. The results of the FISH analysis were confirmed by *amoA* sequence evaluation, which pointed as well at the co-existence of several different organisms affiliated to *N. europaea*, *N. eutropha* and *N. mobilis* in SBBR 1. Interestingly, both approaches indicated independently that novel ammonia-oxidizers related to *N. mobilis* live in the reactor and, moreover, FISH demonstrated the occurrence of additional, presumably unknown ammonia-oxidizers displaying unusual probe binding patterns. The lack of diversity among the analyzed 16S rRNA sequences seems to contradict the results of FISH and *amoA* sequencing, but previous attempts to amplificate *Nitrosomonas* and *Nitrosococcus* 16S rRNA from environmental samples by PCR were also hampered, probably by incomplete cell lysis during DNA extraction or by PCR biases (Juretschko *et al*., 1998). Similar effects might have caused the differences between 16S rRNA and FISH analyses of nitrite-oxidizing bacteria, as we retrieved only 16S rRNA sequences affiliated to *Nitrospira*-like bacteria although FISH revealed that these organisms co-exist with *Nitrobacter* in SBBR 1. The occurrence of both nitrite-oxidizers above the detection limit of FISH (approx. 10^3 cells per ml sample) in the same biofilm was a surprising observation, because in most reactors examined, only *Nitrospira*-like bacteria were detectable by FISH. *Nitrobacter*, on the other hand, could be isolated from WWTP samples, but was less abundant than *Nitrospira* and could hence not be detected *in situ* (Wagner *et al*., 1996).

Although both *Nitrobacter* and *Nitrospira*-like bacteria were detected in SBBR 1, the quantification of their populations yielded much higher cell numbers for *Nitrospira* than for *Nitrobacter* (Fig. 4). This difference was even more pronounced for the total cell areas, which

are an approximation of the biovolume and hence of the biochemical reaction space occupied by each population. The quantitative dominance of *Nitrospira*-like bacteria over *Nitrobacter*, which corresponds to the results of other studies (Burrell *et al*., 1998; Juretschko *et al*., 1998; Schramm *et al*., 1998; Schramm *et al*., 1999), indicates that the former are more successful under the growth conditions in most WWTPs and should hence be regarded as the actual key organisms of nitrite oxidation in wastewater treatment. The factors, however, which select for *Nitrospira* or *Nitrobacter* are still unknown and deserve further investigation since detailed knowledge about this topic would help to improve the growth conditions for the most efficient nitrite-oxidizers and hence to increase both stability and performance of nitrifying bioreactors.

Although pure culture studies with *Nitrosomonas* and *Nitrobacter* demonstrated that *Nitrosomonas* strains grow slower than *Nitrobacter* below a temperature of 30°C (Novak and Svardal, 1989), we surprisingly found higher cell numbers of ammonia-oxidizers than of nitrite-oxidizers in SBBR 1, and the ammonia-oxidizers occupied a larger share of the total biovolume (as approximated by the cell areas, Fig. 4). Even though the conditions in SBBR 1 might support higher growth rates of ammonia-oxidizers than those measured in the above-mentioned studies, it should be considered that metabolically inactive cells are not washed as rapidly out of biofilm systems as out of activated sludge, but can still be stained by FISH due to their persistently high ribosome content. Since we were able to demonstrate that the decay rates of ammonia-oxidizers are lower than those of *Nitrospira*-like bacteria (Morgenroth *et al*., in preparation), it remains possible that the surplus ammonia-oxidizers in SBBR 1 were partly inactive and did not contribute to the nitrification process anymore. However, further examination and comparison with other systems is required as the growth rates of nitrifying bacteria under realistic conditions, i.e. the conditions present in the reactors, are fundamental parameters for the operation of nitrifying WWTPs.

Determination of volumetric cell titers and average activities of ammonia-oxidizing bacteria

The application of competitive PCR to measure the volumetric cell titers of ammonia-oxidizers in activated sludges and biofilms enabled us to determine and compare the average ammonia turnover rates per cell in different reactors. Interestingly, the results (Table 3) were of a similar magnitude as ammonia turnover rates measured with pure cultures (0.0018-0.023 pmol NH_4^+ per hour and cell, (Belser, 1979)). The applicability of our approach is further supported by the very

low turnover rate of 0.003 pmol NH_4^+ per hour and cell measured for the denitrifying reactor SBBR 2, which was used as negative control. These results demonstrate that the competitive PCR constitutes a reproducible and straightforward technique, which complements FISH as a suitable method for the reliable and rapid quantification of ammonia-oxidizing bacteria. Once established, it can be used to monitor the population sizes of ammonia-oxidizers even within the frequently observed, short-termed changes of environmental conditions in bioreactors and to deduce basic microbiological characteristics like substrate turnover rates, which are also of substantial technical importance.

Morphology and physiological traits of *Nitrospira*-like bacteria

Nitrospira-like bacteria in the SBBR 1 biofilm formed large and morphologically conspicuous aggregates interlaced by a network of microchannels and cavities, which met the surrounding medium at the surface of the colonies. Our experiments with fluorescein solutions showed that this network, which might contain extracellular polymeric substances or be really "empty", is accessible for water and water-soluble substances. Therefore it might facilitate the diffusion of nutrients and oxygen towards the cells located in the inner parts of the microcolonies, whereby the metabolic activity and nitrite-oxidating performance of these cells would be increased.

The microautoradiographic experiments provided first insights into the physiology of the unculturable *Nitrospira*-like bacteria living in WWTPs. Their proposed ability to use CO_2 as carbon source could be confirmed, but the availability of oxygen as terminal electron acceptor was obligatory for autotrophy. Among the other substrates tested, only pyruvate was taken up, and likewise under aerobic conditions only. It is, however, possible that the uptake of the other organic substrates was inhibited by the concurrent availability of nitrite as source of energy. Although no nitrite was added to the incubation medium, the ammonia-oxidizers present in the biofilm sample could have produced nitrite in sufficient amounts to prevent heterotrophic metabolism of the *Nitrospira*-like bacteria. Therefore, our results reflect the substrate uptake of *Nitrospira*-like bacteria in SBBR 1, because the incubation conditions resembled those in the bioreactor, while further FISH/MAR-experiments will be required for a comprehensive physiological characterization of *Nitrospira*-like bacteria.

Conclusions

The powerful new technical approaches in molecular microbial ecology permit a new level of insight into the population structure and function of complex microbial communities which are present and active in WWTPs (e. g. Wagner *et al*., 1993; Wagner *et al*., 1994a; Wagner *et al*., 1994b; Wagner *et al*., 1995; Lee *et al*., 1999). Here we used these techniques to investigate the diversity, population structure and physiology of nitrifying bacteria in a sequencing biofilm batch reactor. We believe that the importance of the diversity within certain bacterial groups catalyzing critical steps in nutrient removal for process stability has been previously not adequately addressed by microbiologists and engineers. For illustration, please imagine two reactors with equal numbers of active ammonia-oxidizing bacteria but significant differences in the diversity within this bacterial group. Both reactors will most likely behave very similar under optimal conditions, thus no differences in sum parameter measurements (e.g. oxygen uptake rate) will be observable. However, if the microbial communities are confronted by unfavorable conditions (e.g. toxic sewage compounds, pH and temperature swings, grazing, phage attack, etc.) , the stability of the ammonia-oxidation process should, at least if the rules of macroecology apply, be higher in the reactor harboring the more diverse ammonia-oxidizing community. If this holds true in future experimental investigations and if we learn how to increase the diversity of such bacterial groups by changes in the process regime, the design of more robust and reliable nutrient removal plants might become possible. Understanding the diversity of key WWTP microbes should also lead to more efficient bioaugmentation strategies. Bioaugmentation as performed today does in most cases totally ignore the microbial community structure of the respective WWTP. Consequently, microorganisms are added which will not find an adequate niche in the system and thus rapidly disappear either by wash-out or by grazing. Addition of the „right“ microbes (those microbes which do occur in the plant with optimal performance) in a grazing-protected format should greatly enhance the success of future bioaugmentation attempts. As detailed microbiological knowledge is required to select suitable bacteria for bioaugmentation and to enhance the microbial diversity in treatment plants, the tools offered by molecular biology are indispensable. They enable us to understand the biology of important bacterial "major players" and to define the range of environmental conditions that allow certain species or communities to grow and perform substrate conversions at optimal rates.

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Appendix 5

Activated Sludge – Molecular Techniques for Determining Community Composition

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Activated Sludge – Molecular Techniques for Determining Community Composition

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Introduction

Wastewater treatment is one of the most important biotechnological processes which is used worldwide to treat polluted sewage and to ameliorate anthropogenically induced damage to the environment. Dependent on the treatment goals different types of sewage treatment plants are used. This chapter focuses on the microbiology of the activated sludge process, which is most commonly used and in which the microbial biomass is aerated and kept in suspension during the treatment process. In addition we also cover activated sludge and biofilm nutrient removal plants in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and/or biologically enhanced phosphorus removal. It is common knowledge that in all types of wastewater treatment plants prokaryotic microorganisms dominate and represent the “causative agent” responsible for the observed conversions. On the other hand, certain microorganisms cause the most frequently encountered problems in wastewater treatment (Kämpfer and Wagner, this volume) Thus, the efficiency and robustness of a wastewater treatment plant mainly depends on the composition and activity of the microbial communities present in its different stages. Although biological wastewater treatment has been intentionally used for more than a century, due to methodological limitations knowledge on the microbiology of this process was scarce till the end of the ‘80s. Consequently, these microbial communities were considered generally as a "black box", and progress in the design and control of wastewater treatment plants was derived mainly from empirical research in

civil engineering. Only after the introduction of molecular techniques in microbial ecology during the last decade, it became possible to determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different process types. It is the aim of this chapter to review these new insights and to provide some guidance on how this knowledge could be extended by implementing newly developed methods and be used for future improvement of wastewater treatment.

Methods for microbial diversity analysis in wwtps

This chapter briefly summarizes established methods for microbial community analysis. It is not the goal to provide an encompassing overview on technical details of each method but rather to discuss the advantages and limitations of each approach for its use in wastewater treatment microbiology.

Light microscopy and cultivation

Traditionally, two approaches to investigate the microorganisms in wwtps were applied. Most frequently, the obtained samples were analyzed by standard light microscopy to get an overview on the abundance of floc forming and filamentous bacteria. Due to the importance of the latter group of organisms for sludge bulking and foaming, keys were developed for a provisional identification of filamentous bacteria using (i) their reaction to Gram- and Neisser-staining and (ii) morphological characteristics (1, 2). However, recent molecular approaches revealed that microorganisms affiliated with different bacterial domains are identified as the same filament type using these keys (Kämpfer and Wagner, this volume). Furthermore, polymorphism of certain filaments has been described (Kämpfer and Wagner, this volume, and (3)) which further complicates morphology-based identification.

The second approach is based on cultivation and isolation of bacteria from wwtps. The number of active bacteria is estimated by most probable number (MPN) techniques and/or total plate counts. After isolation, bacteria are identified using either physiological parameters or chemotaxonomic markers like cellular fatty acids or respiratory quinone profiles. The latter two biomarkers can also be used directly for profiling activated sludge microbial communities

(4-6) but provide only a relatively low resolution information. Numerous bacteria were isolated and identified from wwtps by using cultivation-based techniques, leading to the perception that for example pseudomonads, enterobacteriaceae, and acinetobacters are key components of the microbial flora of these systems. After the introduction of molecular techniques for community analyses it became obvious that only less than 16% of the microorganisms can be isolated from activated sludge by standard cultivation and that those bacteria forming colonies on the respective media are generally of minor numerical importance *in situ* (7-10). Therefore, cultivation offers only a very biased and incomplete view on the bacterial diversity in wwtps. On the other hand, directed cultivation of *in situ* important microorganisms is still a prerequisite for their encompassing physiological and genetical analysis.

Immunofluorescence

A more direct identification and quantification of bacteria in wwtps is offered by the use of fluorescent antibodies (FA). However, the production of these antibodies generally still requires the prior isolation of the target organism restricting the method to culturable bacteria. Once available, the FA-technique can be used to identify and quantify defined microorganisms within activated sludge or biofilms. For example *Sphaerotilus natans*, *Acinetobacter* sp. (11), and *Thiothrix* sp. were detected by this technique (12). The specificity of antibodies can not be adjusted and is usually below the species level. This is an advantage if for example the fate of specific strains should be monitored. On the other hand, the high serological diversity of many bacterial genera and even species (13, 14) would require the simultaneous use of a huge number of FA for their detection thus rendering the method cumbersome. Furthermore, FA are relatively large molecules and thus do not easily penetrate through dense extracelullar polymeric substances of flocs and biofilms. This problem can be avoided if the FA-technique is applied to cryosections. Another difficulty of the FA-technique is that it often suffers from a high background fluorescence caused by unspecific binding of the FA to for example detritus (14, 15) and filament sheaths. In addition, the expression of the detected epitopes on the cell surface can vary with changing environmental conditions, a fact which can cause false-negative results. Despite these limitations, the FA-technique has the unique advantage that detection does not require the killing of the cells. Thus, antibodies can be used to enrich the target cells for example via flow cytometry, coated microtiter plates, or magnetic beads for subsequent cultivation.

The 16S rDNA approach

The comparative sequence analysis of environmentally retrieved 16S rDNA sequences has become the gold standard for cultivation-independent assessment of bacterial diversity in natural and engineered systems (16). Current 16S rDNA databases contain more than 20,000 entries (17) and thus provide a high-resolution framework for the assignment of those sequences obtained in 16S rDNA libraries from environmental diversity surveys (18). The approach consists of DNA extraction, subsequent PCR amplification of (a fragment of) the 16S rDNA gene using primers targeting regions conserved in the bacterial domain, cloning, and sequencing. The obtained sequences are analyzed together with adequate reference sequences to infer their phylogenetic affiliation. A meaningful phylogenetic analysis requires the use of almost full-length 16S rDNA sequences and of different treeing methods applied to different data sets (19).

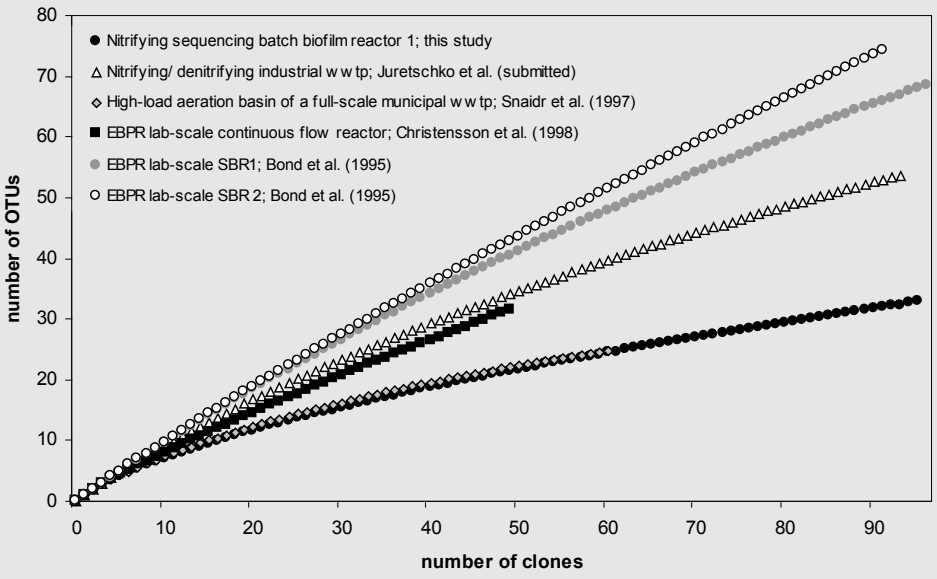


Fig. 1. Rarefaction analyses of 16S rDNA inventories of different wwtps. A 97% similarity threshold was used for assignment to an operational taxonomic unit. The studies of Liu *et al.* (25) and Dabert *et al.* (26) were not considered since only one sequence per operational taxonomic unit has been deposited in public databases.

Table 1. Summary of 16S rRNA-based diversity surveys of wastewater treatment plants and reactors. EBPR = enhanced biological phosphorous removal. SBR = sequencing batch reactor. SBBR = sequencing biofilm batch reactor. Clones were assigned to different OTUs (operational taxonomic units) if they shared less than 97% 16S rDNA sequence similarity with each other.

wwt plants and reactors	No. of Clones sequenced	No. of OTUs	Coverage C [j] [%]	*Proteobacteria* [h]				Bacterial divisions [i]											
				α	β	γ	δ	ε	*Cytophagales*	*Acidobacterium*	Low-G+C gram positives	High-G+C gram positives	*Nitrospira*	*Verrucomicrobia*	*Planctomycetes*	Green sulfur	Green non sulfur	*Fibrobacter*	*Fusobacteria*	OP11	Unaffiliated
high-load aeration basin of a full-scale municipal plant [a]	62	20	81	3	52	18		15			10			2			2				
nitrifying / denitrifying industrial plant [b]	94	53	64	26	31		2			5	1	1	2	3	12	1	16				
nitrifying SBBR1 [c]	96	33	78	5	51	22	4	1	2	5		1	8								
EBPR lab-scale SBR1 + sodium acetate [d]	97	69	48	13	33	8	3	2	5	3	1	4	3	1	13		4		3		3
EBPR lab-scale SBR2 unsupplemented [d]	92	75	28	17	25	10	1	7	13	7		2	2	1	9		3		2		
EBPR lab-scale continuous flow reactor [e]	51	30	51	16	8	8	4	4	6	2		37			8	2	6				
EBPR lab-scale SBR [f]	92	50	46	4	17	5	3		39	9			4	3	4	3			1		
EBPR lab-scale reactor [g]	150	16	93	5	14	7	1		50		5	9						9			

[a] Snaidr *et al.* (1997); [b] Juretschko *et al.* (submitted); [c] this study; [d] Bond *et al.* (1995); [e] Christensson *et al.* (1998); [f] Dabert *et al.* (2001); [g] Lui *et al.* (2001); [h] *Proteobacteria* are present at the subdivision level due to the extensive representation of this division; [i] Relative incidence of division-level representatives in respective studies; [j] Coverage was calculated according to the formula $C = 1 - (n1 \times N^{-1}) \times 100\ \%$, n1 = No. of OTUs consisting of only one sequence, N = No. of all sequences in the 16S rDNA clone library.

Due to development of pre-manufactured kits (e.g. for cloning) and automated sequencers the analyses of relatively high clone numbers per library has become possible. It is however important, but rarely done in published studies, to determine whether the number of analyzed clones does sufficiently well represent the diversity in the established library. For this purpose the clones should be grouped into operational taxonomic units (OTUs) according to their 16S rDNA similarities with each other. We recommend to use a 97 % similarity threshold for OTU assignment, since it has been demonstrated that two organisms with a lower 16S rDNA similarity do also have DNA-DNA similarities of below 70% and thus represent different genospecies (20, 21). However, organisms with highly similar or even identical 16S rDNA sequence might nevertheless represent two different species (20) and thus the OTU concept leads to underestimation of the actual species richness. Once the clones have been assigned to OTUs, rarefaction analyses (22, 23) or coverage estimates (24) should be performed to figure out whether the analyzed clone number represents a sufficient sample size. Figure 1 shows rarefaction analyses of 16S rDNA surveys of wwtps, the respective coverage estimates are depicted in Table 1.

The rarefaction analyses and coverage estimates show that most studies did not adequately harvest the diversity of their 16S rDNA libraries. This is understandable, since the number of identical or highly similar clone sequences increases with the number of sequenced clones and thus extensive clone sequencing creates highly redundant information. Therefore, screening of the 16S rDNA clones with fingerprinting techniques (see below) for selection of different clones for sequence analyses is recommended.

It is however important to realize that even if the complete diversity of an environmental 16S rDNA clone library is harvested, the obtained species inventory might not represent the naturally occurring diversity. In other words, not all and sometimes even not all numerically important bacterial populations of an environmental sample will be found in a respective 16S rDNA library. This failure might be caused by (i) inefficient DNA extraction (27) (e.g. certain Gram-positive bacteria are difficult to lyse), (ii) inadequate coverage of the selected PCR primers, (iii) kinetic and stochastic biases introduced by the PCR amplification (28-30), and (iv) cloning biases. For example the high coverage but low overall OTU number obtained in the 16S rDNA library analysis of activated sludge from a municipal high load wastewater treatment basin (31) does not necessarily reflect low species richness but was more likely caused by the omission of a dedicated DNA extraction protocol prior to PCR amplification.

For establishing a most representative 16S rDNA library, one might consider to (i) use different DNA extraction techniques and to combine the isolated nucleic acids prior to PCR amplification, (ii) to use more than one primer set for amplification, and (iii) to use different vectors for cloning. Regarding the PCR conditions best suited for a general diversity survey, extremely stringent annealing conditions should be avoided in order to allow binding of the primers to organisms with nucleotide exchanges in the target position. This procedure might lead to the amplification of undesired PCR products and the necessity to purify the expected PCR amplificate by agarose gel electrophoresis and band excision prior to cloning. Furthermore, the PCR cycle number has a profound influence on the composition of the PCR amplificate if complex template mixtures are used (29). Highly abundant and less abundant organisms are more likely to be represented in comparable numbers in the PCR product if high cycle numbers are used (an effect caused by template reannealing at high concentrations, 29). In contrast, the 16S rDNA composition of PCR products generated with low cycle numbers more accurately represents the composition of the 16S rDNA sequences in the isolated nucleic acids.

On the other hand the 16S rDNA approach can also indicate or even create artificial diversity. Small sequence differences between multiple rRNA operons of a single bacterium, the so-called microheterogeneities, for example (32), might be interpreted after amplification and cloning as microdiversity which, however, does not exist on the organism level. Furthermore, the formation of chimeric sequences via *in vitro* recombinations (33, 34) and the introduction of point mutations via the thermostable DNA polymerase (35, 36) leads to the retrieval of sequences which differ from their natural counterparts. While chimeric sequences composed of distantly related sequence fragments can be recognized easily by standard software tools (37) or comparative phylogenetic analyses of different sequence regions (27), it is virtually impossible to identify chimera between closely related organisms. Polymerase-induced mutations can only be detected with a certain probability in highly conserved sequence regions by database comparison.

In essence, the 16S rDNA approach described above is an essential tool for microbial diversity analyses in wwtps. However, due to the numerous biases inherent to this approach quantitative data on the microbial community composition can only be obtained if it is combined with quantitative dot blot or *in situ* hybridization techniques, which are described below.

16S rDNA-based fingerprinting techniques

16S rDNA-based fingerprinting techniques nicely supplement the 16S rDNA approach described above. The common principle of these methods is to separate PCR products of the same length but different sequence to visualize the diversity within the amplificate by a banding pattern. Many of these techniques were invented to analyze mutations in medical research and were later on adapted to environmental microbiology. The most frequently applied fingerprinting technique in wastewater microbiology is the denaturing gradient gel electrophoresis (DGGE) (6, 38-40), but also terminal restriction fragment length polymorphism (T-RFLP) (41), gelretardation (42), and single strand conformation polymorphism (SSCP) (43, 44) have successfully been used. The main advantage of the fingerprinting techniques is that high sample numbers can be processed in a relatively short time to gain an overview on the dynamics of complex microbial communities in wwtps. DGGE and gelretardation bands of interest can be excised, cloned and sequenced for subsequent identification. DGGE offers a higher resolution compared to gelretardation but is, in contrast to the latter method, limited to fragments smaller than 500 nucleotides which are not well-suited for phylogenetic analyses. The organism represented by a T-RFLP or SSCP band can only be identified if a 16S rDNA clone library is established in addition and if the obtained clones are sequenced for identification and are used also as reference in the respective fingerprinting protocol. In general, the fingerprinting techniques are affected by the same DNA-extraction and PCR-amplification biases as the 16S rDNA approach and thus can not provide quantitative data.

Hybridization techniques using rRNA-targeted probes

Dot blot and *in situ* hybridization techniques using rRNA-targeted oligonucleotide probes allow, in contrast to all other methods discussed in this chapter, to quantitatively determine the composition of complex microbial communities. The specificity of the probes can be adjusted to different phylogenetic levels. Specialized software tools have been developed to assist in probe design and evaluation (17). After design, for each probe optimal hybridization conditions have to be carefully determined using target and non-target microorganisms. This evaluation requires different procedures for the dot blot (45) and *in situ* format (46, 47). Today, a considerable number of ready-to-use domain-, division-, genus-, and species-specific probes is available for identification of the respective target microorganisms within their

natural habitat, for a review see Amann *et al*. (16, 48). For high resolution analyses of a certain environmental sample additional probes must be designed for cloned sequences obtained with the 16S rDNA approach. Application of these probes in the dot blot or *in situ* format allows to detect and quantify the corresponding microorganisms within the habitat for which the 16S rDNA library was established. The complete procedure including the 16S rDNA gene library analysis, clone-specific probe design, and quantitative hybridization experiments is referred to as the full-cycle rRNA approach (16).

In the dot blot format, total RNA is extracted from the environment and is immobilized on a membrane together with dilution series of RNA of reference species. Reference rRNA of uncultured microorganisms can be obtained by *in vitro* transcription of the respective cloned 16S rDNA sequences. Subsequently, the membrane is hybridized with a radioactively labeled probe and after a stringent wash step the amount of target rRNA is quantified with a Phospho-Imager. Results are expressed as ng of target rRNA per weigth or volume of the environmental sample. Alternatively, the membrane can be re-hybridized with a bacterial or universal probe and the amount of population-specific rRNA detected with the specific probe is expressed as fraction of the total (bacterial) rRNA. For details of the method the reader is referred to Raskin *et al*. (49). Quantitative dot blot hybridization measures the abundance of the target population via its rRNA content. Since the rRNA content of a microbial cell varies dependent on its activity and physiological history (50), and on the prevailing environmental conditions, the obtained data can not be translated to cell numbers. Furthermore, the method might be biased by varying efficiency of RNA extraction from different microbial populations and degradation of the rRNA (which might affect different probe target sites in a different way) during the procedure. Quantitative dot blot analyses have been used in wastewater microbiology (45, 51-53) but are more widely applied on environments like soil and microbial mats (54, 55), which are due to their high background fluorescence not well-suited for fluorescence *in situ* hybridization (FISH)

In situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes is perfectly suited to identify the target organisms within activated sludge flocs or biofilms by specific staining. For this purpose, the environmental samples are pre-treated by addition of chemical fixatives in order to kill the cells, to preserve their morphology, and to make them accessible for oligonucleotide probes. For Gram-negative bacteria a paraformaldehyde-based fixative fulfills these requirements and allows to store the samples at –20 °C for several years (16). However, a fixation protocol permeabilizing all Gram-positive bacteria still has to be

developed. Many but not all Gram-positive microorganisms can efficiently be permeabilized for FISH by addition of 50% EtOH (56). Others, for example the mycolata containing mycolic acids in their cell walls, require additional or alternative pre-treatments including the use of cell wall lytic enzymes e.g. (57-59). Fixed samples are hybridized with probes labeled with fluorescent dyes. For environmental studies, the dyes FLUOS (5,(6)-carboxyfluorescein-N-hydroxysuccimide ester: green fluorescence), Cy3 (orange fluorescence), or Cy5 (infrared fluorescence) are well-suited and allow to detect specifically the target cells of up to three simultaneously applied probes (60). If more than one probe is used in a hybridization experiment and if the probes require different conditions to ensure specificity, subsequent hybridization reactions from high to low stringency have to be performed (3). In contrast to dot blot hybridization, simultaneous binding of multiple probes to the same target cells can be proven by FISH through microscopic observation of the hybridized cells and the use of different probe labels. Therefore, sets of probes with hierarchical or identical specificity can be used to increase the reliability of the identification.

For many years, quantitative FISH data on the bacterial community structure of activated sludge or biofilms required microscopic counting of stained cells, a time-consuming procedure which is relatively inaccurate in samples containing densely clustered cells. Generally, the abundance of a certain population of interest is expressed as percentage of all cells which can be stained with either bacterial probes e.g. (7, 47) or a DNA-binding dye like DAPI (61). The combination of FISH and confocal laser scanning microscopy (62) did not only dramatically improve the image quality by exclusion of blurring out-of-focus fluorescence, but allowed also the development of semiautomatic quantification protocols by use of digital image analyses (42, 63). These methods measure the specifically stained biovolume of a target population and refer it to the volume of those microorganisms stained by the bacterial probe set or a DNA-binding dye. While using the manual technique only a few hundred or thousand cells are counted, the semi-automated protocols detect more than 100,000 cells per measurement and thus are much more reliable and reproducible. However, it is important to realize that numbers obtained by this technique or its manual counterpart can not directly be used to compare the abundance of detected bacterial populations in different samples, because differences in biomass between the samples are not considered. Thus for inter-sample comparison, the respective numbers need to be normalized by taking into account biomass differences estimated for example via volatile suspended solids (VSS) determinations (64) or total DAPI counts per sample volume on a membrane filter, e.g. (8).

Confocal laser scanning microscopic analyses of FISH results may also be used to investigate the spatial distribution of microorganisms in activated sludge flocs and biofilms and to study co-localization of microbial populations, e.g. (27, 62, 65). However, such analysis can only be performed with high accuracy, if the architecture of the flocs or biofilms is preserved by embedding (for embedding protocols, see (66) or cryosectioning, e.g. (65, 67)) and is thus not destroyed during fixation and hybridization.

One potential disadvantage of FISH is its relatively high detection limit of approx. 10^3-10^4 target cells ml^{-1}. Furthermore, only cells with a ribosome content of more than about 1000 copies can be detected by probes without additional signal amplification (68). Since microorganisms with a low cellular ribosome content are probably not active this requirement does not hamper the *in situ* analysis of physiologically active cells in wwtps. This is also reflected by the fact that about 90% of all DAPI-positive cells can be detected with the bacterial probe set in a typical wwtp (47). On the other hand, it is frequently assumed that a high cellular ribosome content of a microbial cell leading to a bright FISH signal is an indicator for its physiological activity at the time of sampling. This interpretation can be misleading since for example nitrifying bacteria maintain high ribosome levels even after complete inhibition for several days (14) or starvation for a month (69). More direct *in situ* analyses of the activity may be achieved by the use of oligonucleotide probes targeting the 16S/23S intergenic spacer region as demonstrated for *Acinetobacter* sp. (70) and the anaerobic ammonium-oxidizer Candidatus (71).

In summary, a variety of molecular methods is available for investigating microbial diversity in wastewater treatment plants. In particular, the full-cycle rRNA approach allows to precisely measure the composition and dynamics of microbial communities in these systems. The following chapter provides and overview on the current status of knowledge which has been accumulated by application of these research approaches in wastewater microbiology.

General microbial diversity in wastewater treatment plants

FISH with group-specific probes

Group-specific rRNA-targeted probes (Table 2) have been widely applied in the so-called top-to bottom approach for the investigation of activated sludge to obtain a fast but relatively low-resolution analysis of the respective microbial communities (7, 8, 10, 25). These studies showed the dominance of *Proteobacteria* and revealed that the beta-subclass is the most abundant subclass in these systems. Alpha- and gamma-subclass *Proteobacteria* are found in lower but generally still considerable numbers. Subsequently, the widespread distribution of members of the *Cytophaga-Flavobacterium* phylum (8, 72), the *Planctomycetales* (73), and the High-GC gram-positive bacteria (8, 25) in activated sludge was demonstrated. In addition, application of group- and genus specific probes showed that the numerically important bacterial groups in activated sludge are dramatically underrepresented after cultivation and that certain bacterial groups with a low *in situ* abundance are best adapted to laboratory cultivation and thus are isolated most frequently. For example, gamma subclass *Proteobacteria* generally occur in relatively low numbers in activated sludge but clearly dominate the heterotrophic flora obtainable on nutrient rich agar plates (7-9).

16S rDNA based diversity surveys

The most detailed knowledge on species richness of microbial communities in wastewater treatment plants can be obtained by phylogenetic inventories via 16S rDNA clone libraries established using bacterial or universal primers. However, probably due to the tediousness of the method, such surveys have only been published for six treatment systems, including five laboratory scale reactors (25, 26, 74, 75), and one high load basin of a full-scale municipal wastewater treatment plant (31). In Table 1, the results of these surveys are summarized. In addition to published results we have included in this table two yet unpublished studies from our laboratory dealing with a pilot scale nitrifying sequencing batch biofilm reactor (76) and an intermittently aerated nitrifying/denitrifying industrial activated sludge plant connected to a rendering plant (77). It is obvious that the number of plants for which 16S rDNA libraries have been established is yet too low to provide an encompassing overview of the entire microbial diversity of such systems. While several SBR reactors with enhanced biological

phosphorus removal have been investigated, for the other plant types only single surveys were performed. Nevertheless some general conclusions can be drawn already. Of the 36 divisions recognized for the bacterial domain (18), members of 13 divisions were detected in the surveys indicating considerable microbial diversity in wastewater treatment plants. Consistent with the FISH results mentioned above, *Proteobacteria* are abundant in each library and represent more than 50% of the clones in five of the eight surveys. With one exception, beta-*Proteobacteria* are the most frequently retrieved members of this division. Apart from the *Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* phylum, the Green-Non-Sulfur bacteria and the *Planctomycetes* were detected in significant numbers. One library of a plant designed for enhanced biological phosphorus removal in a continuous flow system is dominated by high G+C Gram positive bacteria, consistent with the proposed importance of this group for phosphorus removal (see below) (8).

Table 2. Coverage of the main prokaryotic lines of descent by group-specific, rRNA-targeted oligonucleotide probes. Sensitivity and specificity of the probes were determined by using the Arb "probe match" module on current Arb databases of almost complete 16S and 23S rRNA sequences.

Probe	Sensitivity[a] (%)	Specificity[b]	Reference	Target group and total coverage (%)[c]
ALF1b	37.6	1,105	(46)	α-*Proteobacteria* 84.7
ALF968	76.6	132	(78)	
BET42a	92.6	22 [d]	(46)	β-*Proteobacteria* 92.6
GAM42a	90.8	45 [d]	(46)	γ-*Proteobacteria* 90.8
LGC A	41.1	9	(59)	Low G+C gram-positive bacteria 52.1
LGC B	29.1	7	(59)	
LGC C	10.9	0	(59)	
CYA762	53.7	38	(68)	*Cyanobacteria* 92.2
CYA664	35.3	0	(68)	
CYA361	86.9	8	(68)	
CIV/V1342	6.2	5	(68)	
DHP1006	100	0	(79)	*Synergistes* 100
TM7905	100	2	(80)	TM7 100
GSB532	75	0	(81)	Green sulfur bacteria 75

Probe	Sensitivity[a] (%)	Specificity[b]	Reference	Target group and total coverage (%)[c]
CFB286	49.3	0	(82)	*Cytophagales* 90.5
CFB563	27.2	0	(82)	
CFB719	31.1	4	(82)	
CFB972	27.2	96	(82)	
CFB1082	39.2	2	(82)	
CF319	41.9	28	(72)	
BAC303	33.1	0	(72)	
Fibro	100	0	(83)	*Fibrobacter* 100
Pla46	92.6	6	(84)	*Planctomycetales* 95.1
Pla886	82.7	2,561 [d]	(84)	
EUB338-II	75.3	1 [d,e]	(47)	
EUB338-III	70	31 [d,e]	(47)	*Verrucomicrobiales* 70
Ntspa712	85	0 [d]	(76)	*Nitrospira* 85
IRog1	38.4	1	(85)	*Acidobacterium*/*Holophaga* 44.2
IRog2	44.2	0	(85)	
HGC	82.5	10	(56)	High G+C gram-positive bacteria 82.5
EUB338	90.4	0	(86)	domain *Bacteria* 91.8
EUB338-II	0.8	0	(47)	
EUB338-III	0.6	0	(47)	
CREN499	30.6	0	(87)	*Crenarchaeota* 30.6
EURY498	50	2	(87)	*Euryarchaeota* 50
Arch915	88.3	0	(48)	*Archaea* 88.3
				domain *Archaea* 89.8

[a] The fraction of the sequences within the target group which have not more than 0.4 weighted mismatches to the probe sequence.

[b] The number of non-target sequences which have up to 0.4 weighted mismatches to the probe sequence.

[c] Total coverage of the target group by a combination of all listed probes which are specific for this group.

[d] The probe specificity is improved by a competitor oligonucleotide as detailed in the publication cited.

[e] The probe targets parts of the specified group and additional bacteria, because it belongs to the *Bacteria*-specific probe set.

Fluorescence i*n situ* hybridization and the full-cycle rRNA approach

Till today the microbial community structures of activated sludges from two wastewater treatment plants have been investigated using the full-cycle rRNA approach. Snaidr and coworkers analyzed a high-load aeration basin of a large municipal wastewater treatment plant (31, 60) while Juretschko and colleagues studied an intermittently aerated industrial wastewater treatment plant designed for simultaneous nitrification and denitrification (27, 77). The results of the respective 16S rDNA clone libraries are depicted in Table 1. Snaidr and co-workers designed probes for a few selected clones and showed that a high microdiversity of bacteria of the beta1 group of the beta subclass of *Proteobacteria* was present in the municipal activated sludge. Furthermore, *Sphingomonas*- and *Arcobacter*-related populations were detected with a relative abundance of 3 and 4% of all cells, respectively.

The composition of the microbial community in the industrial plant was investigated in more detail using semi-automatic quantitative FISH. Hybridization with group-specific probes demonstrated that the beta-subclass of *Proteobacteria* made up almost half of the total biovolume of those bacteria detectable with the bacterial probe set (Figure 2A). Other *in situ* important groups were the alpha-subclass of *Proteobacteria*, the *Nitrospira*-phylum, the *Planctomycetes*, and the Green-non-sulfur bacteria. Figure 2A also depicts differences between the *in situ* abundance of the groups and their representation in the clone library.

The composition of the beta-subclass of *Proteobacteria*, the numerically most important group within this wwtp, was further analyzed using OTU-specific probes for quantitative FISH (Figure 2B). Bacteria related to *Zoogloea ramigera* and the *Azoarcus*/*Thauera* cluster were the *in situ* most abundant members of this subclass and accounted for 36% and 34% of the biovolume of the beta-*Proteobacteria*. In addition, significant numbers of the ammonia-oxidizer *Nitrosococcus mobilis* (which was not present in the clone library), *Ideonella dechloratans*-like bacteria, and *Brachomonas denitrificans*-related microorganisms were recorded. In total only 6% of the bacteria of the beta-*Proteobacteria* detectable *in situ* could not be assigned to a specific genus.

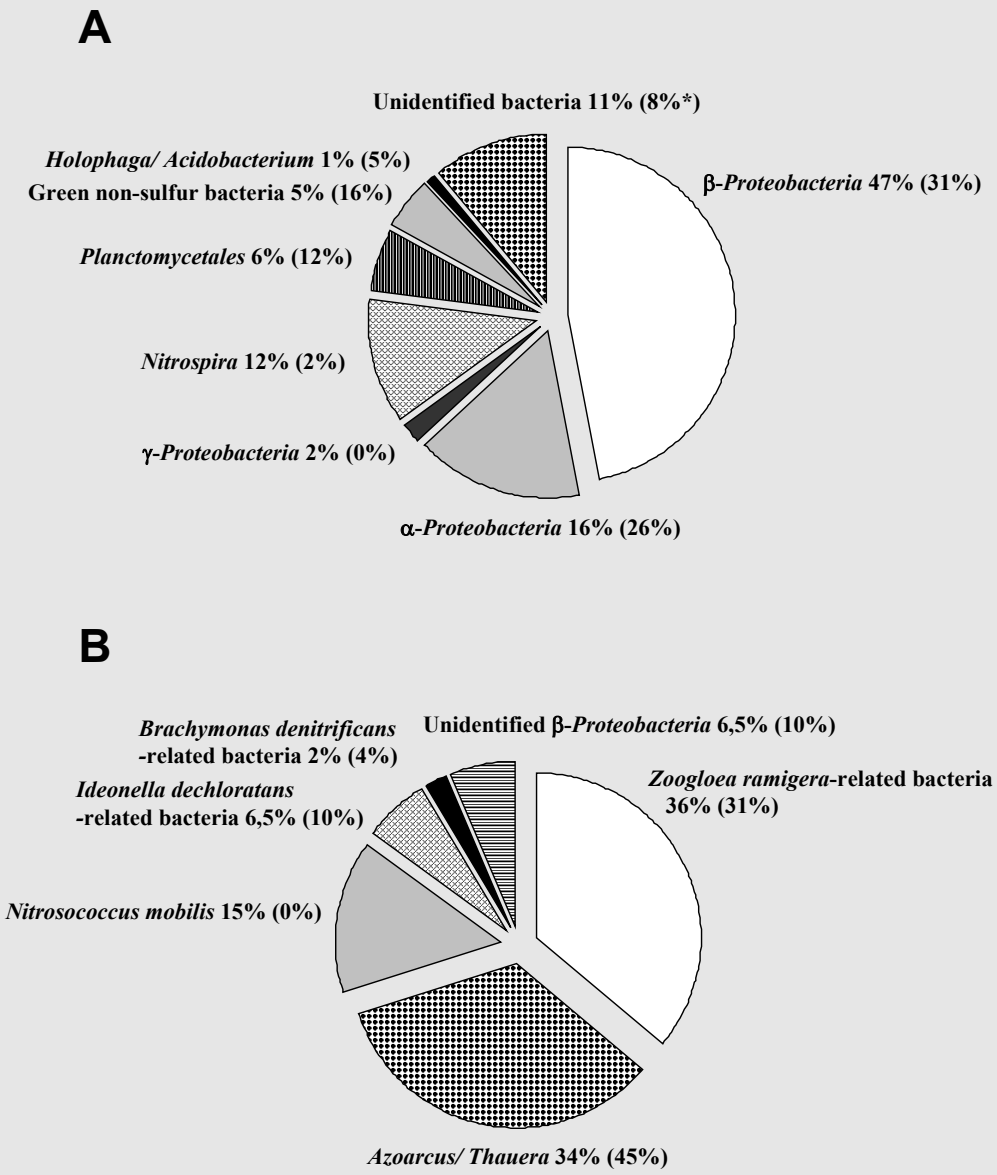


Fig. 2. Microbial community structure of an industrial nitrifying/denitrifying activated sludge analyzed by FISH using group-specific (panel A) or beta-subclass OTU-specific (panel B) probes. The biovolume of the specifically stained cells was determined using confocal laser scanning microscopy and digital image analyses and expressed as percentage of the biovolume of those cells detectable by *in situ* hybridization with the bacterial probe set. For panel B the biovolume of the beta-subclass *Proteobacteria* was set to 100% and the other groups shown in this panel were scaled accordingly. Numbers in brackets show the abundance of the respective target organisms in the 16S rDNA clone library established for the same sample. * sequences not affiliated with the depicted groups.

Functional groups of microorganisms in wwtps

From an applied perspective, the identification and characterization of those bacteria responsible for specific transformations of sewage compounds are of primary importance. In particular, the organisms responsible for nitrogen and phosphorus removal are of interest. However, 16S rDNA sequence-based identification of a microorganism does generally not allow to infer its functional properties. Phylogenetically closely related microorganisms may possess different metabolic potentials. For example, certain obviously non-photosynthetic bacteria which have been implicated with enhanced biological phosphorus removal (EBPR) are closely related to the phototrophic beta-subclass *Proteobacterium Rhodocyclus purpureus* (Hesselmann *et al.*, 1999; see also below), which is not capable to perform the respective phosphorus transformations. On the other hand, several physiological traits important for wwtps like for example denitrification are found dispersed in many different phylogenetic lineages. Only for a few physiological groups an unambiguous linkage between phylogeny and physiology exists. One example is the lithoautotrophic ammonia-oxidizers which form two monophyletic clusters in the beta- and gamma-subclasses of *Proteobacteria*, respectively (88). All known organisms affiliated with these clusters indeed gain their energy by oxidation of ammonia to nitrite.

One way to identify microorganisms capable to perform a certain function in a wwtp is to use lab-scale reactors, inoculated with activated sludge or biofilm material, which select for the respective functional microbial group. Using this approach enrichments of yet uncultured nitrite oxidizers (89), anaerobic ammonium oxidizers (90), and bacteria catalyzing EBPR (91, 92) were obtained. Although these studies provided important new insights, enrichment in reactors might favor the growth of organisms not important in a full-scale wwtp by inducing biases comparable to those caused by cultivation approaches. This effect is probably pronounced in particular if the reactor is fed with artificial sewage or if the sewage is amended with nutrients like acetate. Therefore, the identification of organisms catalyzing a certain function in a reactor enrichment always has to be complemented with investigations on the *in situ* abundance and function of these bacteria in full-scale wwtps.

A major current research challenge are the development and validation of techniques for *in situ* analyses of the function of microorganisms within their environment. For example,

selected genes coding for key enzymes of certain metabolic pathways (often referred to as "functional genes") can be used both as phylogenetic and functional markers for the respective organisms. Using this approach, specific primers are used for PCR amplification of a fragment of the respective gene from a wwtp sample. After cloning and sequencing, the affiliation of the environmental gene fragments is determined by comparative sequence analyses with reference cultures. Although this approach also suffers from DNA extraction, PCR, and cloning biases (see above), it combines, in contrast to the 16S rDNA approach, identification with assignment of a metabolic potential. In wastewater microbiology, the *dsrAB* genes coding for the dissimilatory sulfite reductase of sulfate-reducers (93) and the *amoA* gene encoding the active-site subunit of the ammonia-monooxygenase of ammonia-oxidizers (94, 95) have been used to investigate the diversity of both functional groups (88, 96). However, the mere detection of a functional gene does not prove that this gene was transcribed, translated, and functionally active in the respective organism.

Another way to link *in situ* identification of microorganisms in wwtps with a specific function is to combine FISH and microelectrodes (97). For example zones of nitrification or denitrification in a biofilm can be determined using the appropriate microelectrodes. Equipped with this information, the microorganisms living in the respective zones can be identified using FISH (65, 67, 98, 99). If quantitative FISH data are available, this sophisticated approach can even be used to estimate substrate conversion rates per cell and K_S values (65). Nevertheless, the combination of FISH and microelectrodes has several significant limitations. Microelectrodes are not available for all important substances (for example a microelectrode suitable for phosphate determinations has not yet been developed), and those that exist do not function under all environmental conditions. Microelectrode measurements are one-dimensional and thus are heavily influenced by spatial heterogeneity of the sample. This is particularly important since it is currently not possible to detect the injection channel of the microelectrode in a subsequent FISH analyses in order to investigate the microbial communities surrounding this site. Therefore, FISH-microelectrode measurements are much easier to interpret in biofilms with a layered distribution of microorganisms than in heterogeneous and structurally dynamic activated sludge flocs. Furthermore, the spatial resolution of microelectrodes of 10-50 μm is clearly above the single cell level and does not allow to directly investigate the function of single microbial cells in an environment.

The *in situ* physiology of microorganisms in wwtps can be investigated using a combination of FISH and microautoradiography (100, 101). A small volume of the native sample is incubated with radioactively labeled substrates under suitable environmental conditions. After incubation the samples are fixed, and substrate not incorporated by the cells is removed by a series of wash steps. Subsequently, samples are cryosectioned and transferred to a cover slip for FISH analyses. Finally the samples are covered with an autoradiographic emulsion, developed, and viewed with an inverse confocal laser scanning microscope. Cells are identified by their probe-conferred fluorescence and silver grain formation on top of the cells (in the emulsion) indicates physiological activity and substrate uptake under the selected environmental conditions. If combined with an appropriate study design, most functionally important groups of microorganisms can be identified and quantified using the combination of FISH and microautoradiography (100). It should be stressed that radioactive labeling of cells in the FISH-microautoradiography procedure is unlikely to be caused by simple substrate uptake since the cells are permeabilized for oligonucleotide penetration and therefore unbound substrate is removed during the multiple wash steps. Consequently, microautoradiographic detection requires incorporation of the substrate into a macromolecule by the enzymatic apparatus of the cell. The major disadvantages of FISH-microautoradiography are that it is relatively expensive and time-consuming, and that radioactive derivatives of many substances are not easily available. Furthermore, the spatial resolution of microautoradiography depends on the isotope used for substrate labeling. While tritium-labeled substrates often allow single cell resolution in cryosections, lower resolution on a cell cluster level is achieved using ^{14}C or ^{32}P-labelled substrates.

In the following sections the current knowledge on the diversity of important physiological groups of bacteria in wwtps is reviewed.

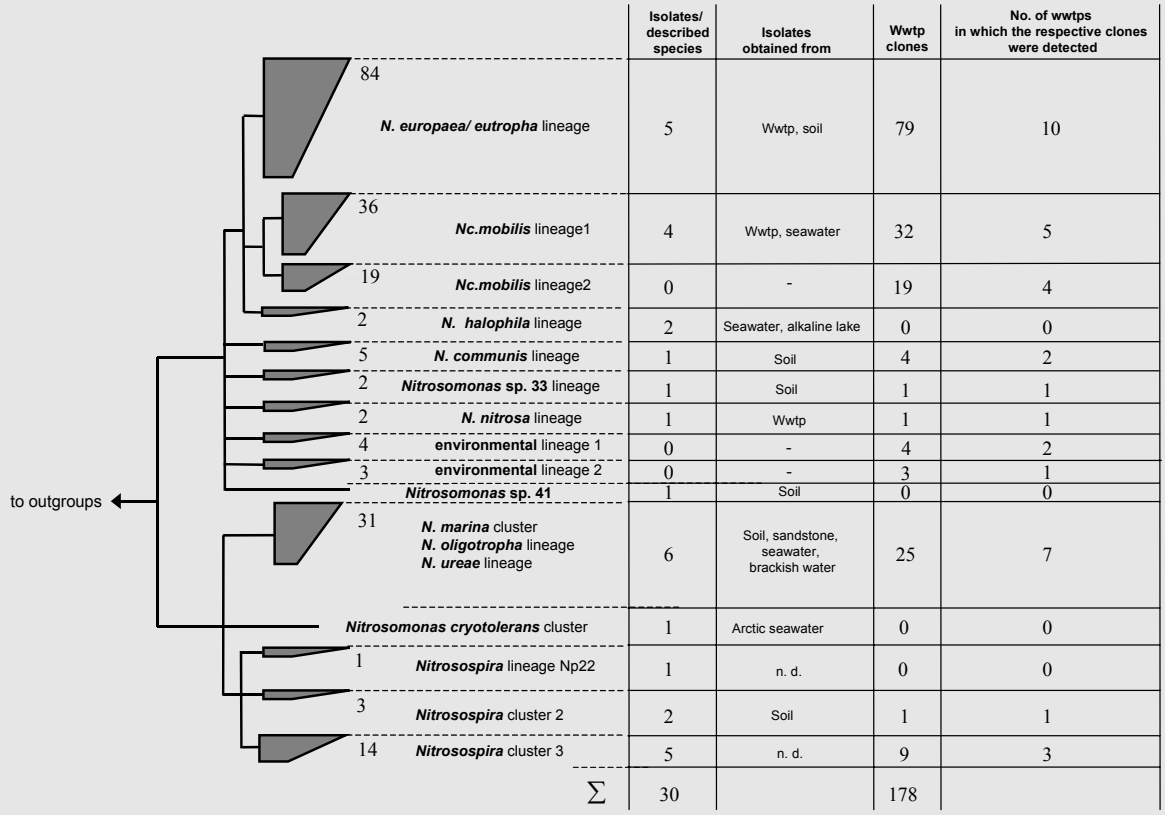
Nitrifying bacteria

The nitrifying bacteria encompass two groups of microorganisms, the ammonia- and the nitrite-oxidizing bacteria, which catalyze the oxidation of ammonia to nitrite and of nitrite to nitrate, respectively. Since most of the nitrogen in the influent of a wwtp is present either in form of urea (which is hydrolyzed to ammonia) or ammonium/ammonia, the nitrifying bacteria play a central role in nitrogen removal in wwtps. It is important to lower the ammonia concentrations in the effluent of wwtps since this compound is toxic to aquatic life

and promotes eutrophication in the receiving waters. Nitrifying bacteria are extremely slow-growing microorganisms and are recalcitrant to cultivation attempts. Due to the sensitivity of nitrifying bacteria to disturbances like pH- and temperature shifts, breakdown of the nitrification process is frequently reported from municipal and especially industrial wwtps.

According to microbiology and civil engineering textbooks the model ammonia-oxidizer is *Nitrosomonas europaea*. However, FISH analyses in nitrifying activated sludge and biofilms showed that other ammonia-oxidizers are more important. In an industrial nitrifying/denitrifying plant the dominant ammonia-oxidizer was *Nitrosococcus mobilis*, a bacterium which was previously considered to occur in brackish water only (27). Subsequently, *N. mobilis* was also detected in significant numbers in a nitrifying sequencing batch biofilm reactor (76). In contrast, *Nitrosospira*-related ammonia-oxidizers were found to be dominant *in situ* in a laboratory scale fluidized bed reactor (102). Although *Nitrosospira* was also reported in a PCR-based study as important ammonia-oxidizer genus in wwtps (103), this finding could not be confirmed by FISH analyses of various wwtps and by a large *amoA*-based ammonia-oxidizer diversity survey in wwtps (Purkhold *et al.*, 2000). Today it is generally accepted that nitrosomonads (including *Nitrosococcus mobilis*) and not nitrosospiras (encompassing the genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*) are important for ammonia oxidation in wwtps. This perception is also reflected in Figure 3 which shows the affiliation of 178 *amoA* clones retrieved from various nitrifying wwtps. Only 10 *amoA* clones from these systems cluster with the nitrosospiras while the remaining 168 clones are affiliated with the nitrosomonads. Figure 3 also shows that almost all recognized lineages of beta-subclass ammonia-oxidizers can be found in wwtps. Numerically, the *Nitrosomonas europaea/Nitrosomonas eutropha*-lineage, the *Nitrosococcus mobilis*-lineage, and the *Nitrosomonas marina*/*Nitrosomonas oligotropha*/*Nitrosomonas urea* cluster are most frequently detected.

In conclusion, wwtps harbor a diversity of ammonia-oxidizers of the beta subclass of *Proteobacteria*, which was enormously underestimated previously. Most of these ammonia-oxidizers are, based on comparative *amoA* sequence analyses, relatively close relatives of described ammonia-oxidizer species. Interestingly, quantitative FISH results indicate that some nitrifying wwtps are dominated by a single ammonia-oxidizer species (27) while other plants harbor at least five different co-existing ammonia-oxidizer populations which are present in significant numbers (76).



	Isolates/ described species	Isolates obtained from	Wwtp clones	No. of wwtps in which the respective clones were detected
84 ***N. europaea/ eutropha*** lineage	5	Wwtp, soil	79	10
36 ***Nc.mobilis*** lineage1	4	Wwtp, seawater	32	5
19 ***Nc.mobilis*** lineage2	0	-	19	4
2 ***N. halophila*** lineage	2	Seawater, alkaline lake	0	0
5 ***N. communis*** lineage	1	Soil	4	2
2 ***Nitrosomonas*** **sp. 33** lineage	1	Soil	1	1
2 ***N. nitrosa*** lineage	1	Wwtp	1	1
4 **environmental** lineage 1	0	-	4	2
3 **environmental** lineage 2	0	-	3	1
Nitrosomonas **sp. 41**	1	Soil	0	0
31 ***N. marina*** cluster ***N. oligotropha*** lineage ***N. ureae*** lineage	6	Soil, sandstone, seawater, brackish water	25	7
Nitrosomonas cryotolerans cluster	1	Arctic seawater	0	0
1 ***Nitrosospira*** lineage Np22	1	n. d.	0	0
3 ***Nitrosospira*** cluster 2	2	Soil	1	1
14 ***Nitrosospira*** cluster 3	5	n. d.	9	3
Σ	30		178	

Fig. 3. Diversity of *amoA* clones retrieved from different nitrifying wwtps. A schematic AmoA–based phylogenetic classification of the beta-subclass ammonia oxidizing bacteria is shown. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The cluster designations were adopted from those of Purkhold *et al.* (88). The height of each tetragon represents the number of sequences in the cluster. Gamma-subclass ammonia-oxidizers are not included since they could up to know neither be detected by FISH nor by *amoA*-analyses in nitrifying wwtps.

Traditionally, members of the genera *Nitrobacter* were considered as the most important nitrite-oxidizers in wwtps (104). Therefore, the finding that *Nitrobacter* could not be detected by FISH with specific 16S rRNA-targeted probes in various nitrifying wwtps came as a surprise (105). Using the full cycle rRNA approach the occurrence of novel, yet uncultured *Nitrospira*-like nitrite-oxidizing bacteria in nitrifying wwtps could be demonstrated (27, 76, 99, 106, 107). The importance of these microorganisms for nitrite-oxidation in wwtps was also confirmed by reactor enrichment studies (89). Today, four different phylogenetic lineages, two of them containing 16S rDNA clones of wwtps, within the genus *Nitrospira* have been recognized (Figure 4) and phylum- as well as genus-specific probes suitable for FISH are available (108). Combination of FISH and microautoradiography showed that the

Nitrospira-like nitrite-oxidizers in activated sludge fix CO_2 and can also grow mixotrophically using pyruvate but not acetate, butyrate, and propionate (108).

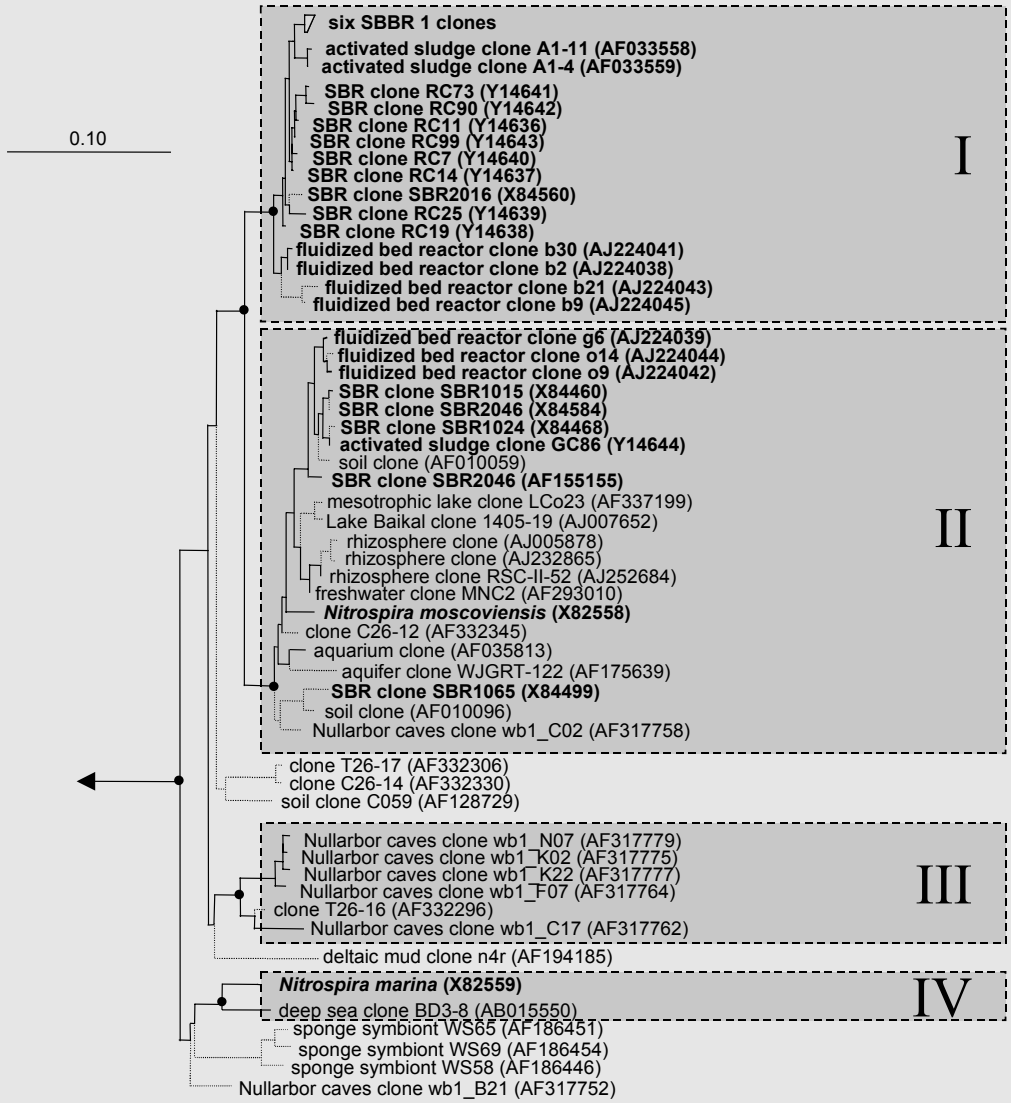


Fig. 4. Phylogenetic tree of the genus *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum likelihood analysis of all sequences longer than 1,300 nucleotides. Shorter sequences were successively added without changing the overall tree topology. Branches leading to sequences shorter than 1,000 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high parsimony bootstrap support above 90% based on 100 iterations. The scale bar indicates 0.1 estimated changes per nucleotide. Clones from wwtps and reactors as well as sequences that belong to isolated strains are depicted in bold. The four sublineages of the genus *Nitrospira* are boxed in gray and marked by the numbers I to IV. Figure modified from (108).

It has been postulated that the predominance of *Nitrospira*-like bacteria over *Nitrobacter* in most wwtps is a reflection of their different survival strategies. While *Nitrospira*-like nitrite-oxidizers are, according to data extracted from microelectrode-FISH analyses, K-strategists and thus may possess a low μ_{max} but are well-adapted to low nitrite and oxygen concentrations, *Nitrobacter* was postulated to be a relatively fast-growing r-strategist with low affinities to nitrite and oxygen (65). Since nitrite-concentrations in most reactors from wwtps are low *Nitrospiras* will outcompete *Nitrobacter* in these systems. In plants with temporally or spatially elevated nitrite concentrations, for example in nitrifying sequencing batch reactors, both nitrite-oxidizers should be able to co-exist. Consistent with this hypothesis co-occurrence of *Nitrobacter* and *Nitrospira*-like bacteria has been observed by FISH in a nitrifying sequencing batch biofilm reactor (108).

Denitrifying bacteria

Denitrification, the anaerobic respiration with nitrite or nitrate as electron acceptor, is used in wastewater to convert the product(s) of nitrification into gaseous nitrogen compounds (mainly dinitrogen) and thus to remove them from the sewage. Most attempts to identify and enumerate denitrifiers in activated sludge were based on cultivation-dependent approaches. Members of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus*, and *Hyphomicrobium* were isolated as part of the denitrifying microbial flora from wwtps (109-113). The latter genus was also detected microscopically by its typical cell morphology in denitrifying activated sludge (114, 115). However, little is known about whether the above listed bacterial genera are representative for the *in situ* active denitrifiers in wwtps. Neef and collegues detected using specific FISH probes significant numbers of *Paracoccus* spp. and *Hyphomicrobium* spp. in a denitrifying sand filter supplemented with methanol as reduced carbon compound for nitrate reduction. But both genera were present in numbers below 0.1% of the total cell counts in a non-denitrifying sand filter run in parallel without addition of methanol indirectly suggesting an active participation of both genera in the denitrification process (73). Molecular studies of the community composition of denitrifying bacteria are difficult to perform since the denitrifying phenotype can not be inferred from the phylogeny of a microorganism. The capability to denitrify is found in many bacterial divisions and is not confined to defined phylogenetic clusters within these divisions. However, the combination of FISH and microautoradiography (100) allows to identify denitrifiers *in situ*. For this purpose, two types of experiments need to be performed. In the first experiment the wwtp sample is

incubated under anaerobic condition in absence of nitrate or nitrite with radioactively labeled substrates which are typically used as electron donors for denitrification. In the second experiment the sample is incubated with the same labeled substrates under anaerobic conditions but in the presence of nitrate or nitrite. Bacterial species, identified by FISH, which take up substrate under anaerobic conditions exclusively in the presence of nitrate or nitrite are most likely denitrifiers. The use of this technique in combination with the full-cycle rRNA approach revealed that novel, still uncultured *Azoarcus*-related bacteria are important denitrifiers in an industrial nitrifying/denitrifying wwtp (116).

Anaerobic ammonium oxidizing bacteria

Unexpected nitrogen losses are frequently observed in wastewater treatment plants. In these plants ammonia was obviously converted to gaseous nitrogen compounds in the absence of organic electron donors excluding the activity of conventional denitrifiers. Using the full-cycle rRNA approach we could demonstrate that novel planctomycetes distantly related to the recently described anaerobic ammonium oxidizer Candidatus "Brocadia anammoxidans" (90, 117) did occur in high numbers in these systems (42, 117) and were thus probably responsible for the nitrogen removal. Consequently, the new candidatus genus "Kuenenia stuttgartiensis" was proposed (42). Using specific 16S and 23S rRNA-targeting probes for both anaerobic ammonium oxidizers (42, 71) a predominance of *Candidatus* "Kuenenia stuttgartiensis" over *Candidatus* "Brocadia anammoxidans" in wwtps was observed.

The anaerobic ammonium oxidizers are a perfect example that completely unexpected organisms are hidden in wwtps. Both anaerobic ammonium oxidizers form a monophyletic deep-branching lineage within the Planctomycetales (Figure 5) and oxidize ammonium anaerobically with nitrite to dinitrogen gas (42, 90, 118). Anaerobic ammonium oxidation in contrast to classical nitrification and denitrification allows to remove ammonium in a one-step process saving space, energy, and money since no aeration or addition of organic carbon for denitrification are required. Anaerobic ammonium oxidation is a very promising method for the treatment of sewage with a high ammonium and low organic carbon content. However, this process needs a mixture of ammonium and nitrite and thus must be coupled to partial nitrification for nitrite production (42, 117, 119). Furthermore, establishment of anaerobic ammonium oxidizing activity in a wwtp takes a very long time and once established the process is sensitive to perturbation.

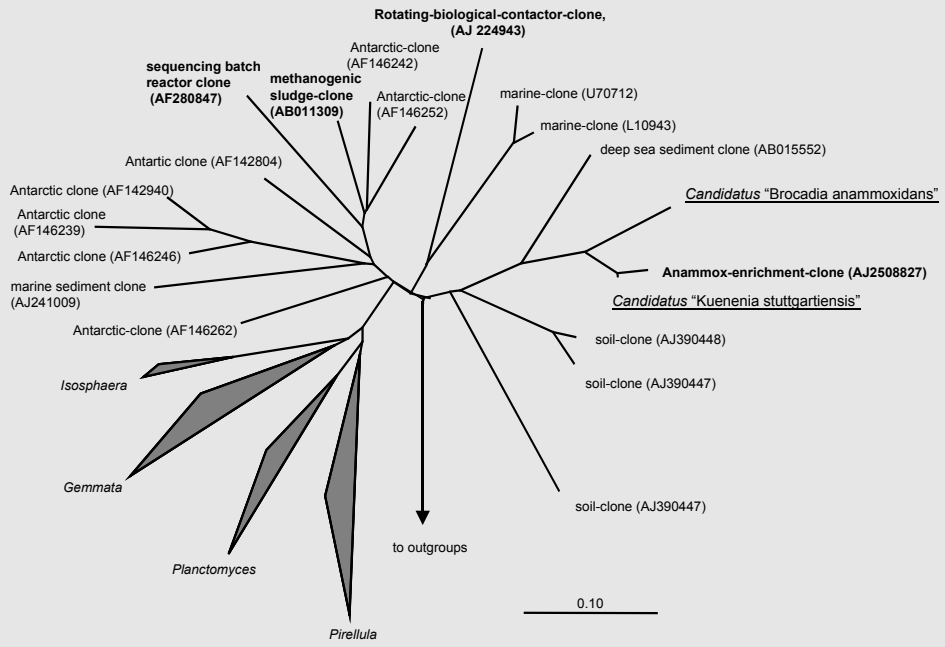


Fig. 5. Phylogenetic 16S rDNA tree showing the deep-branching of the anaerobic ammonium oxidizers within the *Planctomycetales*. In addition to the described anaerobic ammonium oxidizers *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" several environemental clone sequences, including sequences from wwtps depicted in bold, are unaffiliated with cultured members of the division. The physiology of the organisms represented by these sequences still needs to be clarified. Figure modified from Schmid *et al.* (71).

Bacteria important for enhanced biological phosphorus removal

Phosphorus removal from wastewater is important to prevent eutrophication and is therefore an integral part of modern municipal and industrial nutrient removal wwtps. Phosphorus can be precipitated by the addition of iron or aluminum salts and subsequently be removed with the excess sludge. Chemical precipitation is a very reliable method for phosphorus removal but increases significantly the sludge production and thus creates additional costs. Furthermore, the use of chemical precipitants often introduces heavy metal contamination into the sewage and increases the salt concentration of the effluent. Alternatively, phosphorus removal can be achieved by microbiological mechanisms in a process called enhanced biological phosphorus removal (EBPR), for reviews see (120-123). This process is characterized by cycling the activated sludge through alternating anaerobic and aerobic conditions. In the anaerobic stage the bacteria responsible for EBPR are supposed to gain energy from polyphosphate hydrolysis accompanied by subsequent P_i release for uptake of

short-chain fatty acids and their storage in form of polyhydroxyalkanoates (PHA). Two different models were postulated for the production of the reducing equivalents for this anaerobic metabolism (124, 125) In the subsequent aerobic stage, the polyphosphate accumulating organisms (PAOs) possess a selective advantage compared to other microorganisms which were not able to take up fatty acids under the preceding anaerobic conditions by utilizing the stored PHA in an otherwise carbon-poor environment. In parallel, PAOs restore their polyphosphate pools by aerobic uptake of available phosphate from the wastewater. After sedimentation in the secondary clarifier, a part of the biomass is recycled to the anerobic stage and mixed with new wastewater while the excess sludge containing the intracellular polyphosphates is removed from the system.

In contrast to chemical precipitation, EBPR plants have been frequently reported to fail. This raised interest in the microbiology of the process. Traditionally, based on cultivation experiments gamma-subclass *Proteobacteria* of the genus *Acinetobacter* were believed to be the only PAOs (126-128). However, today it has become apparent that *Acinetobacter* can accumulate polyphosphate but does not possess the above described PAO metabolism, e.g. (121). Furthermore, cultivation-independent methods like fluorescent antibody staining (11), respiratory quinone profiles (4), and FISH with a genus specific probe (8, 9) demonstrated that the relative abundance of *Acinetobacter* in EBPR systems was dramatically overestimated due to cultivation biases further confirming that *Acinetobacter* is not of importance for EBPR

Several other bacteria isolated from EBPR reactors have been suggested as PAO candidates. *Microlunatus phosphovorus*, a high GC Gram-positive bacterium accumulates aerobically polyphosphate and consumes it under anaerobic conditions but fails to take up acetate or accumulate PHA under anaerobic conditions (129, 130). FISH with a probe specific for *Microlunatus phosphovorus* demonstrated the presence of this organism in an EBPR plant (2.7% of the total bacteria) (131) but no direct indications for the importance of this bacterium for EBPR are available Furthermore, *Lampropedia* spp. were shown to possess the basic metabolic features of a PAO (132) but their acetate-uptake-phosphate-release ratio was much lower that EBPR models predict, and no additional data regarding the abundance or activity of these morphologically conspicuous bacteria in EBPR systems have been published.

Compared to these cultivation-based attempts, the hunt for PAOs was more successful using molecular tools for analyses of EBPR systems. Beta-subclass *Proteobacteria* and high GC Gram-positive bacteria (actinobacteria) increased in number after addition of acetate to the raw sewage of a EBPR-full-scale wwtp suggesting that these groups benefit from the enhanced availability of short chain fatty acids in the anaerobic basin and thus represent candidates for PAO (8). Additional support for the importance of both groups for EBPR stems from FISH experiments in an efficient EBPR laboratory scale sequencing batch reactor (133) and respiratory quinone profiling in a lab-scale EBPR system (6). Recently, high GC Gram-positive bacteria related to *Terrabacter tumescens*, *Tetrasphaera japonica*, and *Tetrasphaera austrialensis* were reported to be abundant in EBPR systems and thus might be important for EBPR (25, 75, 92). While the function of actinobacteria as PAOs still has to be proven, evidence is available that beta2-subclass *Proteobacteria* related to *Rhodocyclus* and *Propionibacter* are important PAOs in many EBPR systems. These bacteria, for which the name Candidatus 'Accumulibacter phosphatis' was suggested (91) were detected in high numbers in EBPR systems (26, 74, 91, 92, 134). Furthermore, phosphorus accumulation by these bacteria in the aerobic phase was demonstrated by sequential FISH and polyphosphate staining (25, 92). In addition, acetate uptake in the anaerobic phase and phosphorus uptake under aerobic conditions could be demonstrated for Candidatus 'Accumulibacter phosphatis' using FISH and microautoradiography (134).

A potential reason for the failure of EBBR plants is the presence of bacteria which use previously stored compounds such as glycogen (also referred to as glycogen accumulating organisms, GAOs) to compete with the PAOs for substrate uptake under anaerobic conditions (41, 135, 136). Cech and Hartmann (135) described Gram-negative cocci in clumps and packages of tetrades in activated sludge and called these morphotypes G-bacteria, since their numbers increased after glucose addition. Mino *et al*. (122) suggested that bacteria with a similar morphology are GAOs. However, recent molecular and physiological analyses of various G-bacteria isolated from wwtps revealed a high phylogenetic diversity of this morphotype and provided no support for their role as GAOs, for a review see (137). Molecular community analyses of deteriorated EBPR reactors revealed the predominance of a novel bacterial group within the gamma-subclass of *Proteobacteria* (40). The phylogenetic affiliation of this deeply branching group can not unambiguously be resolved. These bacteria are good GAO candidates since they occur in deteriorated EBPR systems, probably accumulate PHA, and store little or no polyphosphates (25, 40).

Conclusions and future perspectives

During the last decade, the application of molecular tools in wastewater microbiology has revolutionized our view on the microbial ecology of these systems. Different groups of still not culturable bacteria were identified and shown to be responsible for nitrite oxidation, denitrification, and enhanced biological phosphorus removal. Surprisingly, the model organisms described in text books for these processes and for ammonia oxidation were shown to be generally not of importance for wastewater treatment. Furthermore, a new process, anaerobic ammonium oxidation, was detected in wwtps and the responsible (still not cultured) microorganisms were identified. It is important to note that significant diversity exists in each of these functional groups of bacteria. In the next research phase in wastewater microbiology, more detailed knowledge on the biology of the above mentioned non-cultured bacteria needs to be gained. Therefore, an increased effort on the development of suitable cultivation strategies for these bacteria is needed. In parallel, the use of techniques referred to as "environmental genomics" should allow to investigate the genome composition of these bacteria without the need of cultivation.

While researchers interested in microbial ecology will probably find the results summarized in this chapter interesting and important, engineers might ask what practical value can be extracted from the accumulated knowledge. Regarding application, the most obvious benefit of the progress described is that it provides a basis for a more knowledge-driven treatment of wwtp failures. One strategy to improve a particular aspect of process performance in a wwtp, for example during start-up or after its breakdown, is the addition of specialized microorganisms or activated sludge from another wwtp (138). This operational tool which is called bioaugmentation does however frequently fail (for example (63) and references therein). Such failure is typically caused by addition of the "wrong" microorganisms, for example the model organisms for nitrification, which can not compete successfully with the autochthonous bacteria in the plant and are thus eliminated or washed-out. Therefore, it is important to identify those microorganisms responsible for nitrogen or phosphorus removal in a functioning wwtp. If problems arise these results can be used as guidance to select the appropriate bacterial additive (for culturable microorganisms) or a well-suited activated sludge from a neighboring wwtp containing a comparable microbial flora. Once the

appropriate bacteria have been selected they need to be protected for example by polymer embedding from grazing by protozoa (63).

Compared to curing failure of a certain process in a wwtp by bioaugmentation, protecting the plant from process deterioration is a more sustainable strategy. For this purpose we need to understand the links between the diversity of a functionally important bacterial group and the stability of the catalyzed process. Preliminary observations indicate that plants with low functional redundancy due to the presence of a low diversity of bacteria of a certain functional group are more sensitive to failure of the respective process than plants harboring a high diversity of the same bacterial group. If increase in diversity can indeed be proven to cause process stabilization then it will be important to learn how plant design and process parameter control can be optimized to increase the diversity of functionally important bacterial groups. To answer these ecologically and economically important questions it will be necessary to determine the microbial community composition of a large number of different samples obtained from tightly controlled reactor studies as well as full scale wwtps. Due to the tediousness of many of the established molecular methods this kind of research will greatly benefit from the implementation of modern high throughput techniques like DNA microarrays for measuring microbial community composition in complex samples (139).

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Appendix 6

Development of a Visualization and Image Analysis Software Tool

In appendix 3 of this thesis, a modified FISH protocol was developed that preserves the three-dimensional structure of a sample during the dehydration, hybridization, and washing steps. After application of this FISH protocol, morphological properties and the spatial arrangement of microorganisms can be studied by use of a CLSM as described in section 1.4 of the general introduction. Single optical sections contain only two-dimensional information, which may be insufficient to understand three-dimensional structures. This limitation is overcome if the 3D-information in serial image stacks can be visualized by suitable computer software. In many cases, image stacks acquired by a CLSM contain also quantitative data like surface areas or biovolumes. The computer-aided extraction of such quantitative features from images is referred to as "image analysis" rather than "visualization".

The development of a software tool for the 3D-visualization and analysis of image stacks was started in the course of this dissertation. The aim of this project is to write a convenient, flexible, and expandable program that offers most of the visualization and image analysis functions needed in microbial ecology. In contrast to many commercial applications, this program should not be overloaded with features and should run on low-cost computer hardware. The current program version offers already several different methods to visualize image stacks and can quantify probe-stained bacteria by automated cell area measurement. The program has been named "Stackwizard" and was already used to study the morphology of microcolonies formed by *Nitrospira*-like bacteria (appendix 3). The following sections describe briefly the most important features of the program.

Visualization of three-dimensional datasets

Each serial image stack is imported by "Stackwizard" as a set of TIFF-images. The imported stacks are stored in computer memory as a 3D-array of scalar values in the range between 0 and 255. These values encode the intensities (i.e. the brightness) of the pixels in the images. A value of zero means that a pixel is black, while the value 255 represents the highest possible intensity. Each value is localized by its x, y, and z coordinates in the 3D-array. The visualization of this 3D-array on screen requires (i) a selection of the relevant data (e. g. points that belong to surfaces of cells), and (ii) a projection that converts 3D-coordinates to 2D-coordinates (because the computer screen is two-dimensional). "Stackwizard" offers two different, basic approaches to visualization: "isosurface rendering" and "volume rendering".

The next paragraphs provide an overview of these rendering modes and of the underlying algorithms.

Isosurface rendering

In many situations, for example in co-localization studies, only the surfaces of cells or cell aggregates must be visualized. "Stackwizard" contains an algorithm that extracts surfaces from 3D-image data and renders 2D-projections of these 3D-surfaces. The surfaces are defined as *isosurfaces*, which contain points of the same intensity. This intensity works like a threshold that defines which points are inside and which are outside of an object. The isosurface is interpolated in regions where only points with intensities above and below the threshold exist. This principle is illustrated by a two-dimensional diagram in Fig. 1. The threshold is supplied by the user, who must find a correct threshold that defines the real surface of an object. Since this can be difficult, "Stackwizard" offers additional features which help finding the most suitable thresholds for isosurface rendering (see below).

The isosurfaces are rendered by an implementation of the *Marching Tetrahedra* algorithm (Guéziec and Hummel, 1995). This algorithm approximates 3D-surfaces by drawing them as meshes of connected triangles. Thus, the algorithm must determine the 3D-coordinates of the three vertices of each individual triangle in order to draw the complete surface. The triangle vertices are found by the following procedure: (1) The whole image stack is treated like a large box that contains the intensity data (Fig. 2a). A small virtual cuboid moves through this

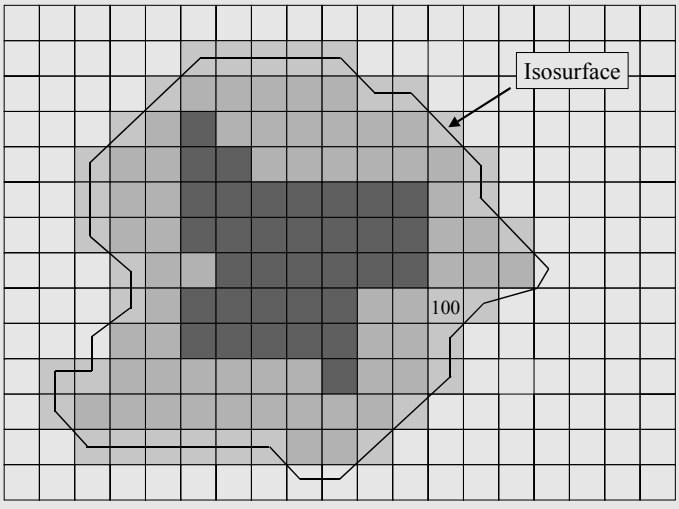


Fig. 1. Two-dimensional illustration of an isosurface. The surface connects all points with an intensity of 100 (the threshold). These points form the surface of an object. The surface is interpolated at positions where the image data contain only points with intensities different from 100. Darker fields depict points of higher intensities.

box in all three spatial directions as indicated by the arrows in Fig. 2a. (2) At each position, the small cuboid is subdivided into six tetrahedra. One of these tetrahedra is depicted in Fig. 2b. Each tetrahedron has four vertices. (3) The program determines which vertices of a tetrahedron are located inside, and which are located outside of an object (e.g. a cell aggregate). This is done by retrieving the intensity value at the 3D-position of each tetrahedron vertex from the image stack and by comparing this value to the user-supplied threshold. If the value is greater than or equal to the threshold, then the vertex is located inside of an object or exactly on its surface, respectively. Otherwise it is located outside (Fig. 2c). The program then draws triangles within the tetrahedron. These triangles separate all tetrahedron vertices which are inside of an object from those which are outside (Fig. 2c). Since the tetrahedron has four vertices, in total 16 different cases must be distinguished to draw all triangles required to separate "inside" from "outside" for every possible combination. (4) Step 3 is repeated for all six tetrahedra in the small cuboid. (5) The small cuboid moves to the next position in the large box of image data, and the steps 2-4 are repeated. (6) Step 5 is repeated until the cuboid has moved through the whole image stack in all directions. The accuracy of the approximated surface depends on the size of the moving cuboid. A very small cuboid gives better results, because the tetrahedra are smaller and the image stack is scanned with a higher resolution. Larger cuboids result in less accurate surfaces, which are calculated at higher speed. The surface of a *Nitrospira* cell aggregate, which was displayed by isosurface rendering, is shown in Fig. 4a as an example of this visualization technique.

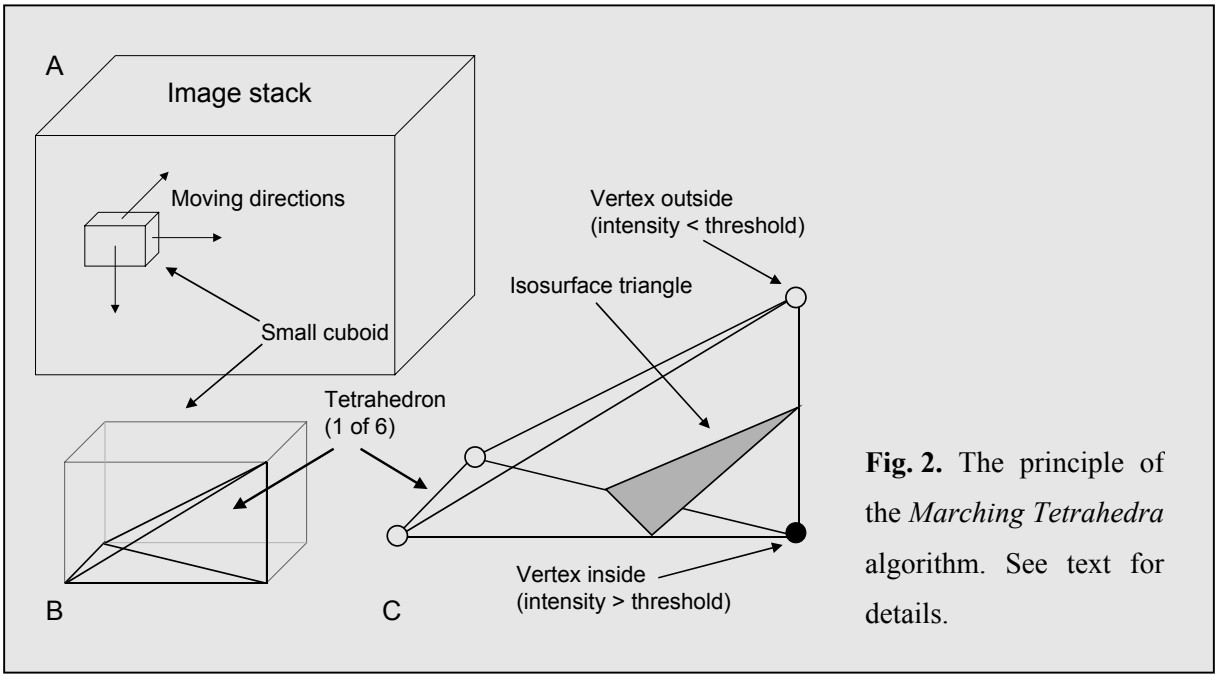


Fig. 2. The principle of the *Marching Tetrahedra* algorithm. See text for details.

Volume rendering

In this mode, the program visualizes complete image stacks and not only surfaces. The image stack data are sampled along viewing vectors, which define the direction of hypothetical light rays from the position of the viewer through the stack to the screen surface (Fig. 3). The 3D-coordinates of the sampled points are converted to 2D-screen coordinates by orthogonal projection. Thereupon, the projected points are drawn to screen according to rules defined by a *transfer function*. This transfer function determines the visual appearance of the projected data. For example, the *Maximum Intensity* transfer function draws only the brightest data points which were found along the viewing vectors. Maximum Intensity projections are useful to depict very dense internal structures like nuclei or regions of high ribosome density, which would otherwise be obscured by surrounding structures like cell surfaces (Fig. 3). Detailed images of bacterial cell aggregates can also be obtained by this technique (Fig. 4b). The *Local Maximum Intensity* projection is a variation, which is more suitable to display the outer surfaces of objects. This transfer function projects the first point whose intensity exceeds a user-defined threshold. If no such point is found along a particular viewing vector, then the point with the highest intensity along this vector is projected instead. This function is well-suited to visualize for example bacterial aggregates in biofilms (Fig. 4c,d). In the volume rendering mode, different viewing perspectives are realized by multiplying the 3D-coordinates of each projected data point with a rotation matrix prior to the projection (Fig. 4c,d). "Stackwizard" offers several transfer functions to satisfy different visualization requirements. In addition to the Maximum Intensity and Local Maximum Intensity projections, a "Bubble Model" transfer function has been implemented that supports the user in finding suitable thresholds for the isosurface rendering mode. This transfer function is based on an algorithm described by Csebfalvi and Gröller (2000). This algorithm displays the inherent surfaces of objects as semi-transparent interfaces, which look similar to the surfaces of soap-bubbles. The result is an image that contains all "natural" external and internal object surfaces, all of which are easily visible because nothing is drawn completely opaque. "Stackwizard" offers the option to mix this transfer function with isosurface rendering in the same image. The user can then decide visually if the threshold used for drawing the isosurface was suitable to extract all inherent object surfaces as isosurfaces. Fig. 4e shows a phase contrast image of ciliate cells found in biofilm. Fig. 5f depicts a colony of ciliate cells that belong to the same species as the cells shown in Fig. 5e. The colony was visualized by using the "Bubble Model" transfer function. This example demonstrates that (when compared to the

phase contrast image) the cell shapes and all inherent surfaces of the cells, including bents in the cell walls, were detected and displayed precisely by the visualization algorithm.

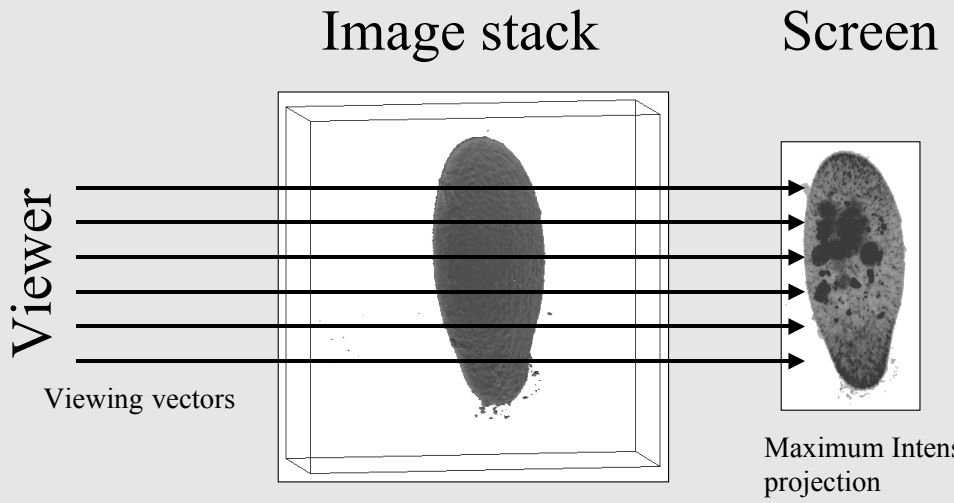
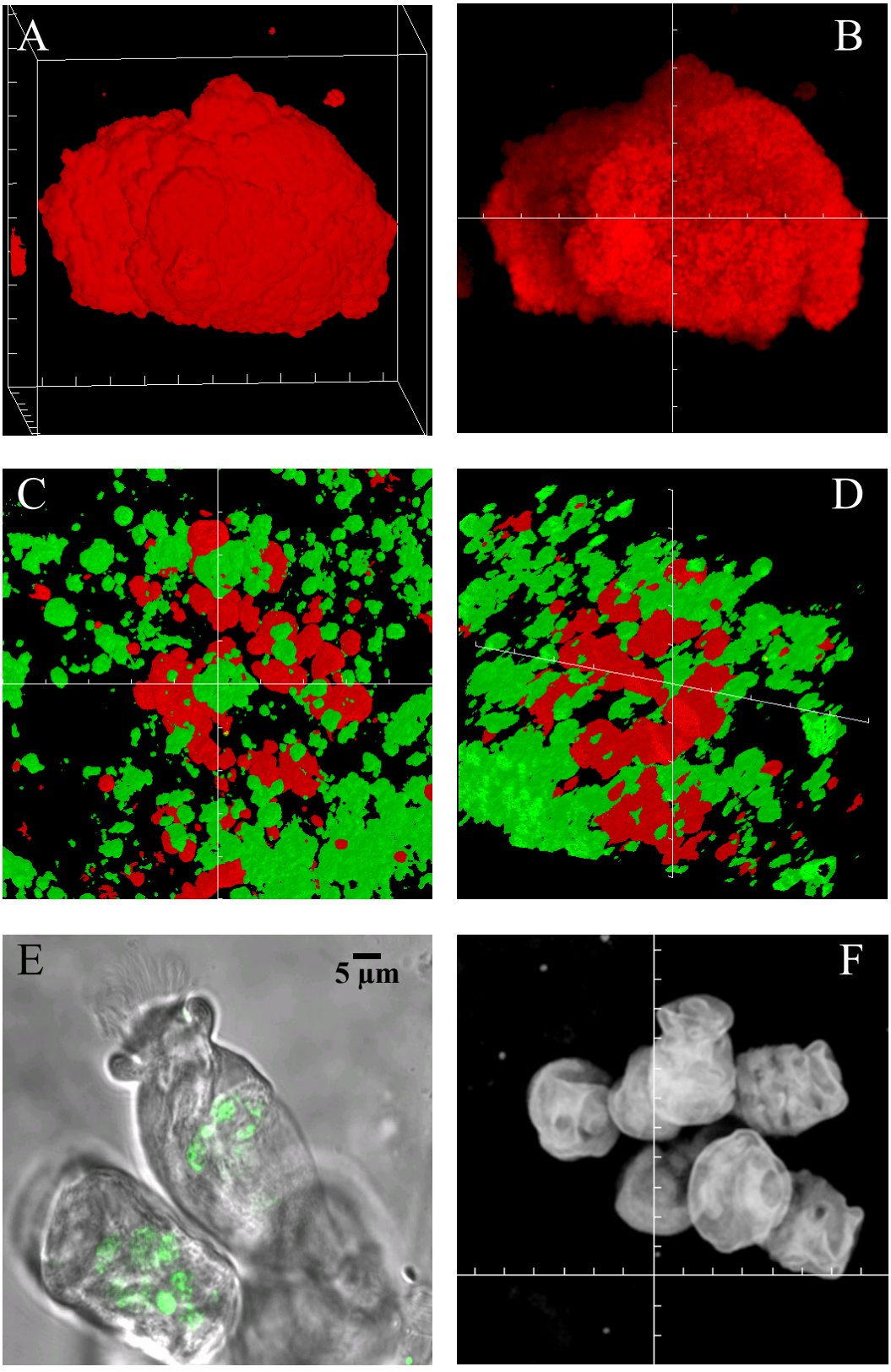


Fig. 3. The principle of volume rendering. Image stack data are sampled in computer memory along virtual viewing vectors and are projected to the screen according to a transfer function. The Maximum Intensity transfer function is illustrated in this example of a projected *Paramecium* cell.

Advantages and disadvantages of the two rendering modes

The exclusive display of surfaces in the isosurface rendering mode can speed up the calculation of the images and facilitates the interpretation of 3D-image data considerably. Although the *Marching Tetrahedra* algorithm works reliably in most cases, the interpolation of an isosurface in regions that lack points of the same intensity as the threshold may sometimes cause incorrect surfaces. Such errors cannot occur in the volume rendering mode, because here no interpolation of geometric features (like surfaces) is performed. On the other hand, the projections obtained by volume rendering can be complex and difficult to interpret, because the complete stack data are visualized. Rendering time is another important issue: if the resolution of one optical section is 512·512 pixels, then a stack of 100 sections contains already $2.62 \cdot 10^7$ points. Since volume rendering displays complete stacks, acceptable rendering times can be achieved on low-cost hardware only with highly optimized algorithms.


A
B
C
D
E
5 µm
F

Therefore, the volume rendering routines implemented in "Stackwizard" perform a pre-processing step of the stack data, which is repeated automatically when necessary (for example after the stack data have been edited by the user). This pre-processing compresses the stack data and eliminates those points that are irrelevant for visualization (e.g. black points with an intensity close to zero). While the pre-processing itself is time-consuming, it accelerates the actual rendering by a factor of ten or more, depending on the original stack data.

Since both rendering modes have certain advantages, "Stackwizard" allows to combine them in one visualization. Therefore, surfaces and internal features of objects can be displayed in the same final image.

Common features of the two rendering modes

"Stackwizard" can visualize arbitrary numbers of stacks in combination in all rendering modes. The colors of the stacks are selected by the user and are blended automatically at positions where stacks overlap spatially (e. g. yellow is displayed where objects of a red and a green stack overlap). All visualizations can be viewed from arbitrary perspectives by rotation around the three axes of the 3D-coordinate system. The 3D-impression can be improved by

Fig. 4. Examples of image stacks visualized by "Stackwizard". **A.** Microcolony of *Nitrospira*-like bacteria displayed by isosurface rendering. The *Nitrospira* cells were stained by FISH with probe S-G-Ntspa-0662-a-A-18 (appendix 3), and a stack of 100 optical sections through the colony was acquired using the CLSM. All axis intervals indicate 5 µm. **B.** The same microcolonly as in (A), but visualized by volume rendering (Maximum Intensity projection). All axis intervals indicate 5 µm. **C, D.** 3D-visualization of a nitrifying biofilm with ammonia-oxidizers stained by FISH with probe NEU (green) and *Nitrospira*-like bacteria stained by probe S-G-Ntspa-0662-a-A-18 (red) created from a stack of 40 optical sections by volume rendering (Local Maximum Intensity projection). All axis intervals indicate 23 µm. The stack was rendered without rotation (C) and with rotation around the x and y axes of the coordinate system (D) to obtain different viewing perspectives. **E.** Phase contrast image of *Epistylis cf. coronata* cells found in a nitrifying biofilm. Bacteria (green) in the gastric vacuoles were labeled by FISH with the EUB338 probe mix (appendix 1). The ciliate cells were preserved during FISH by embedding the biofilm in agarose (appendix 3). This image is provided for comparison with (F). **F.** Colony of *Epistylis cf. coronata* cells visualized by volume rendering with the "Bubble Model" transfer function. The image was calculated from a stack of 150 optical slices. All axis intervals indicate 10 µm.

surface lighting. The lighting model includes ambient and diffuse reflection and up to six independently adjustable light sources. All visualizations can be supplemented with coordinate axes, which are subdivided into user-defined intervals. Image stacks can be modified by removing selected parts of the 3D-data interactively. For example, parts of objects can be clipped in order to uncover internal structures. All visualizations are updated immediately to reflect such modifications. The program includes also functions to remove background noise from images, to smooth surfaces, to display surfaces as wireframes, to combine two different image stacks by boolean operations, and to automatically detect cavities within large objects like cell aggregates.

Cell area quantification

Bacterial populations in environmental samples can efficiently be quantified by FISH with rRNA-targeted oligonucleotide probes, confocal laser scanning microscopy, and digital image analysis ((Bouchez *et al.*, 2000; Schmid *et al.*, 2000), see also appendix 2). The areas of probe-stained cells are measured in the image analysis step. This cell area measurement can be performed by "Stackwizard". For this purpose, two image sets must be imported into the program. In contrast to the image stacks used for 3D-visualization, these image sets need not be serial optical sections. The first set contains the images of the cells stained by a highly specific probe (this is the bacterial population to be quantified). The second set contains the images of the cells stained by the *Bacteria*-specific probe set or by a nucleic acid-staining dye. The images in both sets must have been acquired at exactly the same locations within a sample. Thereupon, the total areas of all objects in the two image sets are measured. The total area of the cells in the first image set is then displayed as fraction of the total cell area measured for the second image set.

The most critical step of this cell area measurement is finding an intensity threshold suitable to distinguish the probe-stained cells from background noise in the images. The current version of "Stackwizard" contains only a preliminary solution of this problem. Either the user enters suitable thresholds manually for each image set, or the quantification thresholds are determined automatically by a purely empirical method. Since this method fails to find

suitable thresholds occasionally, it will be replaced by a more reliable image analysis algorithm in a future version of the program.

Technical requirements

The current version of the program is Stackwizard 0.5.9. The program has been written in the C++ programming language. It requires the Linux operating system with kernel version 2.2.10 (or newer) and version 2.1.1 (or newer) of the Linux C library. Further requirements are X-Windows (XFree86 3.3 or newer) and the KDE 1.1 desktop environment including the Qt 1.4 libraries (or any newer KDE 1.x and Qt 1.x version, but not KDE 2.x and Qt 2.x). In addition to the basic Qt library, the Qt-OpenGL extension library (version 1.4 or any newer 1.x version) is required. Linux was chosen as the underlying operating system to ensure maximal portability to different platforms including low-cost personal computers and high-end graphics workstations. All graphical output is realized by using functions of the Qt and OpenGL libraries. OpenGL is the industry standard for computer graphics, and is available for practically all UNIX and Linux platforms. "Stackwizard" has been developed and tested with the free Mesa implementation of OpenGL (Mesa version 3.x). The Linux TIFF library (version 3.4 or newer) is required for reading and writing TIFF image files. All needed libraries are included in most standard Linux distributions.

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Summary
Zusammenfassung

Summary

The oxidation of ammonia to nitrate (nitrification) is a central step of modern wastewater treatment. Ammonia is first oxidized by ammonia-oxidizing bacteria to nitrite, which is oxidized to nitrate subsequently by nitrite-oxidizing bacteria. Detailed studies on population structure and physiology of the ammonia- and nitrite-oxidizers (nitrifiers) are urgently required due to severe instabilities of nitrification in many wastewater treatment plants. This has to be accomplished by means of cultivation-independent methods, because important nitrifiers (e. g. the nitrite-oxidizers of the genus *Nitrospira*) cannot be cultivated up to now.

In this thesis, nitrifiers living in activated sludges and biofilms were investigated *in situ* by molecular approaches. New rRNA-targeted oligonucleotide probes specific for the genus and phylum *Nitrospira* were developed for this purpose. These probes detect all known organisms that belong to this line of descent by Fluorescence *In Situ* Hybridization (FISH) in environmental samples. Furthermore, the possibilities to quantify bacterial populations (e. g. nitrifiers) *in situ* were improved. Variants of the domain-specific probe EUB338 were designed that allow to quantify the total bacterial populations in environmental samples more precisely. Absolute cell concentrations of uncultured bacteria that grow as cell aggregates (like nitrifiers) can be measured efficiently by a newly developed, semi-automatic quantification method. Nitrifiers in different wastewater treatment plants were quantified by using this method and additional, already established techniques. Based on the quantification results it was possible to estimate the average substrate turnover activity of ammonia-oxidizers in an activated sludge plant as 2.3 fmol of ammonia per hour and per cell.

Population structure and physiology of *Nitrospira*-like bacteria in wastewater treatment plants were investigated by combined applications of molecular methods. Comparative analysis of rRNA gene sequences opened new insights into the phylogeny of the genus *Nitrospira*. The complex morphology of the microcolonies formed by *Nitrospira*-like bacteria in biofilms was analyzed by FISH and Confocal Laser Scanning Microscopy (CLSM). For this purpose, a modified FISH protocol and a computer program for the 3D-visualization of CLSM image data were developed. The modified FISH protocol reduces changes of spatial structures during the investigation of environmental samples. The uptake of organic carbon sources by *Nitrospira*-like bacteria was monitored without cultivation by combining FISH with microautoradiography. These experiments demonstrated that *Nitrospira*-like bacteria in wastewater treatment plants fix CO_2 and take up pyruvate under aerobic conditions.

Zusammenfassung

Die Oxidation von Ammonium zu Nitrat (Nitrifikation) ist ein zentraler Schritt der modernen Abwasserreinigung. Das Ammonium wird zuerst von bestimmten Bakterien, den Ammoniumoxidierern, zu Nitrit und anschließend von Nitritoxidierern weiter zu Nitrat oxidiert. Wegen der instabilen Nitrifikation in vielen Kläranlagen sind detaillierte Studien zur Populationsstruktur und Physiologie der Ammonium- und Nitritoxidierer (Nitrifikanten) unbedingt erforderlich. Dies muß mit kultivierungsunabhängigen Methoden erfolgen, da wichtige Nitrifikanten (z.B. die Nitritoxidierer der Gattung *Nitrospira*) bisher nicht kultiviert werden können.

In dieser Arbeit wurden Nitrifikanten in Belebtschlämmen und Biofilmen mit molekularen Ansätzen zur *in situ* Analyse von Bakterienpopulationen untersucht. Dazu wurden für die Gattung und das Phylum *Nitrospira* spezifische, rRNS-gerichtete Oligonukleotidsonden entwickelt. Diese Sonden erlauben erstmals den *in situ* Nachweis aller Organismen dieser Entwicklungslinie mittels Fluoreszenz *in situ* Hybridisierung (FISH) in Umweltproben. Weiterhin wurden die Möglichkeiten zur *in situ* Quantifizierung von Bakterienpopulationen, z. B. Nitrifikanten, verbessert. Neu entwickelte Oligonukleotidsonden ergänzen die bakterienspezifische Sonde EUB338 und ermöglichen eine umfassendere Quantifizierung der Gesamtbakterienpopulation in Umweltproben. Eine halbautomatische Quantifizierungsmethode wurde entwickelt, mit der Zellkonzentrationen nicht-kultivierter Bakterien, die in Aggregaten wachsen (z. B. Nitrifikanten), effizient meßbar sind. Mit dieser Methode und anderen, bereits etablierten Techniken wurden Nitrifikanten in verschiedenen Kläranlagen quantifiziert. Aufgrund dieser Ergebnisse wurde für Ammoniumoxidierer in einer Belebtschlammanlage eine geschätzte, durchschnittliche Umsatzrate von 2,3 fmol Ammonium pro Stunde und pro Zelle ermittelt.

Die Populationsstruktur und Physiologie *Nitrospira*-ähnlicher Bakterien in Kläranlagen wurden durch den kombinierten Einsatz molekularer Methoden untersucht. Eine vergleichende Analyse von rRNS-Gensequenzen ergab neue Einblicke in die Phylogenie der Gattung *Nitrospira*. Mittels FISH und konfokaler Laser-Scanning-Mikroskopie (CLSM) wurde erstmals die komplexe Morphologie von *Nitrospira*-Mikrokolonien in Biofilmen analysiert. Zu diesem Zweck wurden ein modifiziertes FISH-Protokoll sowie ein Computerprogramm zur 3D-Visualisierung von CLSM-Daten entwickelt. Das modifizierte FISH-Protokoll verringert Veränderungen der räumlichen Struktur in den untersuchten Umweltproben. Die Aufnahme organischer Kohlenstoffquellen durch *Nitrospira*-ähnliche Bakterien wurde kultivierungsunabhängig durch Kombination von FISH und Mikroautoradiographie beobachtet. Es zeigte sich, daß diese Bakterien unter aeroben Bedingungen CO_2 fixieren und zusätzlich Pyruvat aufnehmen.

List of Publications

Contributions to the manuscripts presented in this dissertation are listed in brackets.

1. **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. System. Appl. Microbiol. **22**: 434-444.
[*concept by M.W., experimental work by H.D., digital image analysis by A.B., writing by H.D. with help of the co-authors*]

2. **Daims, H., Nielsen, P. H., Nielsen, J. L., Juretschko, S. and Wagner, M.** (2000). Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and *in situ* physiology. Wat. Sci. Tech. **41**: 85-90.

3. **Daims, H., Purkhold, U., Bjerrum, L., Arnold, E., Wilderer, P. A. and Wagner, M.** (2001). Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. Wat. Sci. Tech. **43**(3): 9-18.
[*outline of the project by M.W., experimental work by H.D., U.P. and L.B., technical advice by E.A., writing by H.D. with editorial help of the co-authors*]

4. **H. Daims, N. B. Ramsing, K.-H. Schleifer, 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**(12): 5810-5818.
[*concept by H.D. and M.W., experimental work by H.D., writing by H.D. with help of the co-authors*]

5. **H. Daims, J. L. Nielsen, P. H. Nielsen, K.-H. Schleifer, and M. Wagner** (2001). *In Situ* Characterization of *Nitrospira*-like Nitrite-oxidizing Bacteria Active in Wastewater Treatment Plants. Appl. Environ. Microbiol. **67**(11): 5273-5284.
[*outline of the project by M.W., concept by H.D. and M.W., experimental work by H.D. and J.L.N., visualization software development by H.D., writing by H.D. with help of the co-authors*]

6. **A. Loy, H. Daims, and M. Wagner** (in press). Activated Sludge – Molecular Techniques for Determining Community Composition. Accepted for publication as book chapter in The Encyclopedia of Environmental Microbiology (John Wiley & Sons, Inc., New York).
[*writing by M.W., A.L. and H.D., contribution of experimental data by A.L. and H.D.*]

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