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# DIPLOMARBEIT

Titel der Diplomarbeit

Influence of wastewater composition and operational mode on the community structure of nitrifying bacteria in wastewater treatment plants

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# A. Introduction

## A.1. Wastewater treatment plants and ecological theory

Cleaning wastewater is without doubt of great importance to avoid detrimental influences on the environment. Especially the development of urban agglomerations and chemical industry as well as intensified agriculture have rendered cleaning processes indispensable, as otherwise high amounts of nitrogen and phosphorous would reach the environment. Since these two elements constitute limiting growth factors for living organisms in being less available than carbon and hydrogen (Campbell, 2000), eutrophication of waters have been a frequently observed consequence of "disposal" of untreated wastewater. Apart from eutrophication, toxic effects of elevated concentrations of ammonia and nitrite on organisms have been reported (Daims *et al.*, 2006). Once wastewater treatment processes had been established, the key role of microorganisms in purifying already mechanically pre-cleaned water was recognized. Thus, numerous studies have been performed during the past decades with the aim to describe and analyse the microorganisms involved in wastewater treatment processes (e.g. Juretschko *et al.*, 2002; Snaidr *et al.*, 1997, etc.). Among these, nitrification is probably the most intensively studied process (Daims *et al.*, 2001b; Matsumoto *et al.*, 2007,;Wagner *et al.*, 1995 and others). Nitrification has obtained special attention as it is a rather unstable part of the treatment process (Wagner and Loy, 2002). Furthermore, wastewater treatment systems are favoured model systems to test ecological questions (Daims *et al.*, 2006b) since firstly chemical measurements describing influent as well as effluent, and measurements of physical parameters within the treatment plant are available. Secondly, microorganisms are replicating very fast compared to higher eukaryotic organisms, thus facilitating *in situ* observations of population changes over generations of bacteria in wastewater treatment plants (wwtps).
In the further sections a basic overview of the fundamentals of nitrogen cycling, nitrification with emphasis on ecology and phylogeny of nitrifying prokaryotes, characteristics of wwtps and at last an ecological theory principally associated with the presented investigations, will be given.

## A.2. The global nitrogen cycle

Nitrogen is, besides carbon (C), oxygen ($O_2$), hydrogen ($H_2$) and phosphorous (P), one of the key elements of life. It is an integral part of amino acids and ribonucleic acids and thus

indispensable for all living organisms. Most organisms are not able to use gaseous dinitrogen ($N_2$) and thus rely on the uptake of nitrogen compounds, mostly ammonia ($NH_3$) or ammonium ($NH_4^+$) and nitrate ($NO_3^-$). For this reason nitrogen (N), though very abundant in the atmosphere (almost 80 volume %; Campbell, 2000), constitutes a limiting growth factor for organisms. $N_2$ is inert and makes uptake and conversion very difficult. Still there exist two ways of nitrogen input into ecosystems – either by "atmospheric deposition" or fixation. As for "atmospheric deposition", after $N_2$ has been photochemically converted to $NH_3$ and $NO_x$ through intense radiation, $NH_4^+$ or $NO_3^-$ get introduced in a dissolved form with rain or in solid form through deposition of particulate matter (Heinrich and Hergt, 1990). The more important way is nitrogen fixation through the reduction of $N_2$ to $NH_3$, a reaction performed exclusively by prokaryotes, living either freely (especially in marine habitats) or in symbiosis with plants (esp. in terrestrial habitats). As the yearly input of atmospheric nitrogen to the nitrogen cycle is only around 7% of the nitrogen already present in this cycle (Heinrich and Hergt, 1990), the decomposition and conversion of organic nitrogen compounds such as amino acids (ammonification) is essential. Conversion of fully decomposed nitrogen compounds of various oxidation states is also important with regard to the retention time of nitrogen within a system. Since $NH_3$ is volatile, it can get lost from soils by vaporisation, but this contributes only a small part to the release of nitrogen compounds to the atmosphere. The main part is released in form of gaseous nitrogen compounds. Nitrogen can also get lost as $NO_3^-$, which is not volatile, but easily washed out from soils.

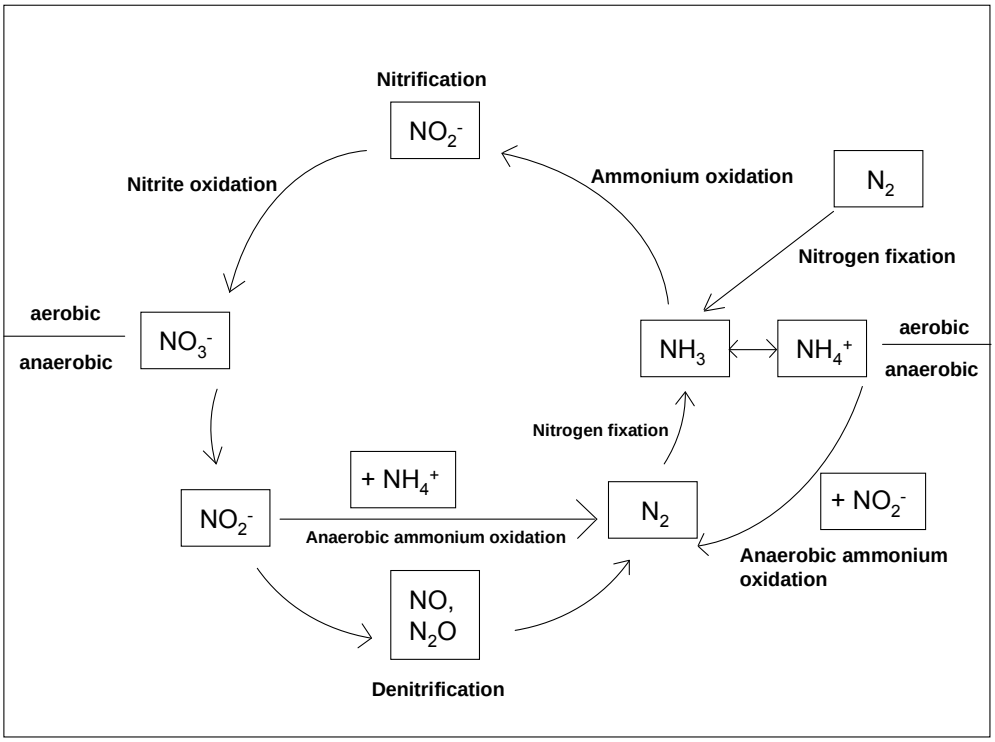



**Figure 1:** Scheme of the biogeochemical nitrogen cycle (on the basis of: Madigan and Martinko, 2006 (Redox cycle for nitrogen))

$NH_3$ is dependent on the environmental pH in chemical balance with $NH_4^+$, which can be oxidized to nitrite ($NO_2^-$) and $NO_3^-$ under aerobic conditions, a process called nitrification (see Figure 1). The first part of the reaction, the oxidation of $NH_3$ to $NO_2^-$, is assigned to autotrophic bacteria (AOB = ammonia oxidizing bacteria) (Madigan and Martinko, 2006) and archaea (AOA = ammonia oxidizing archaea) (Könneke *et al.*, 2005; Schleper *et al.*, 2005). The second part, the oxidation of the toxic intermediate $NO_2^-$ to $NO_3^-$, is attributed to the autotrophic nitrite oxidizing bacteria (NOB). Ammonia oxidation is not restricted to autotrophic prokaryotes as it has been observed for some heterotrophic bacteria, fungi and algae. Still, the production of energy by this process is solely attributed to autotrophic prokaryotes. Under anaerobic* conditions, $NH_4^+$ and $NO_2^-$ can get converted to $N_2$ (Fig. 1) in a process referred to as "anaerobic ammonium oxidation" (anammox; Strous *et al.*, 2006). Another biochemical reaction that takes place under anaerobic* conditions, is denitrification (Fig. 1). Here, $NO_3^-$ gets reduced to $NO_2^-$ and further to the gases nitric oxide (NO), nitrous oxide ($N_2O$) and $N_2$ (Fig. 1). These gases make up the main part of release of nitrogen compounds into the atmosphere, which is also discussed in context of global warming since NO and $N_2O$ are potential greenhouse gases.

### A.2.1. Ammonia-oxidizing bacteria

Ammonia-oxidizing bacteria are chemolithoautotrophic bacteria gaining energy from the oxidation of inorganic $NH_3$ to $NO_2^-$ (the first part of aerobic nitrification; Fig.1) while using $CO_2$ as carbon source. The conversion of $NH_3$ is performed in two steps: first, $NH_3$ is oxidized to hydroxylamine, a reaction catalyzed by the integral membrane-bound enzyme ammonia monooxygenase (AMO). This enzyme consists of three subunits (AmoA, B and C), from which the large subunit (AmoA) is thought to carry the active binding site (Bock and Wagner, 2006; referring to Hyman and Arp, 1992). It has been shown to be inhibited in the presence of e.g. allylthioureae (Bock and Wagner, 2006; referring to Lees, 1952), which is chelating the essential co-factor copper (Bock and Wagner, 2006; referring to Hooper and Terry, 1973 and Loveless and Painter, 1986). Furthermore, *amoA* can be used as phylogenetic marker molecule (Rotthauwe *et al.*, 1997). The growth rate of AOB has been shown to be

* „anaoerobic": without free oxygen; „anoxic": „Absence of oxygen. Usually used in reference to a microbial habitat" (Madigan and Martinko, 2006) – in these habitats lacking oxygen, other electron acceptors as $NO_2^-$ and $NO_3^-$ can be found (chemically bound $O_2$); sometimes "anaerobic" is also defined as total absence of free and chemically bound $O_2$ (online lexicon of the university of Bremen, www.wasser-wissen.de);
here the term "anaerobic" will always be referred to reactions, for which no free $O_2$ is needed, and to conditions; "anoxic" will be referred to habitats without free $O_2$ but other electron acceptors (with chemically bound $O_2$) - thus anaerobic reactions can take place in anoxic habitats; (accordingly, "aerobic" will be used when referring to reactions or conditions, and "oxic" when explicitely addressing habitats);

quite slow (7 h minimum doubling time; Wagner and Bock, 2006; referring to Bock *et al.*, 1990) as not much energy can be gained from the oxidation of $NH_3$. This is due to the high reduction potential of the electron donor $NH_3$, rendering the first part of the reaction even endergonic. In the second part, the oxidation of hydroxylamine to $NO_2^-$ by the enzyme hydroxylamine oxidoreductase, energy can finally be gained.

Interestingly, it has been shown that some AOB are either capable of growing in anoxic habitats by oxidizing $NH_3$ while reducing $NO_2^-$ to $N_2$ or $N_2O$, or to possess at least *nirK*-like genes. *NirK* encodes, like *nirS*, a class of dissimilatory nitrite reductases, which catalyze the reduction of $NO_2^-$ to NO in denitrifying bacteria. This ability is referred to as "nitrifier denitrification". Reduction of $NO_2^-$ as a way of detoxification by aerobic AOB has also been suggested as a possible reason for this unexpected metabolism. Still, the conversion rate of $NH_3$ is much higher under aerobic conditions (Jason *et al.*, 2007; Schmidt and Bock, 1997; Schmidt *et al.*, 2001). Even more surprising, NO seems to be essential for the $NH_3$-oxidation, at least for *N. eutropha*, and $N_2O$ is believed to enhance the metabolic activity of this species (Bock and Wagner, 2006; referring to Zart and Bock, 1998).

According to phylogenetic analysis of the 16S rRNA genes as well as of *amoA*, all known ammonia-oxidizing bacteria belong to two monophyletic lineages within the *Proteobacteria*, namely *β*- and *γ-Proteobacteria*. The latter one only includes the genus *Nitrosococcus*, whereas the lineage *β-Proteobacteria* contains the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*. The combination of the latter three into the genus "*Nitrosospira*" (see Fig.2) is a matter of discussion (Purkhold *et al.*, 2000 and 2003). The genus *Nitrosomonas* can be divided in six clusters (Fig.2) as revealed by treeing analysis: the *Nitrosomonas europaea/Nitrosococcus mobilis*-Cluster (Cluster 7), *Nitrsomonas communis*-Cluster (8), *Nitrosomonas crytolerans*-Cluster, *Nitrosomonas* sp. Nm143-Cluster (Cluster 9; so far without described species), *Nitrosomonas oligotropha*-Cluster (6a) and *Nitrosomonas marina*-Cluster (6b) (Fig 2). Members of Cluster 7 seem to be very tolerant towards high salt concentrations and some even require elevated concentrations for growth. Furthermore, they have been found to exhibit high affinity constants for $NH_3$ (50 - 100 µM; Bock and Wagner, 2006) and appear preferentially in nutrient-rich habitats. This fits well with the lack of the enzyme urease, catalyzing cleavage of urea, which has been reported as beneficial feature for AOB occurring in nutrient-poor habitats (low concentration of $NH_3$). Members of Cluster 8 are partly urease negative, partly positive – they are believed to occur mainly in slightly eutrophic soils and frehwaters (Bock and Wagner, 2006) whereas most members

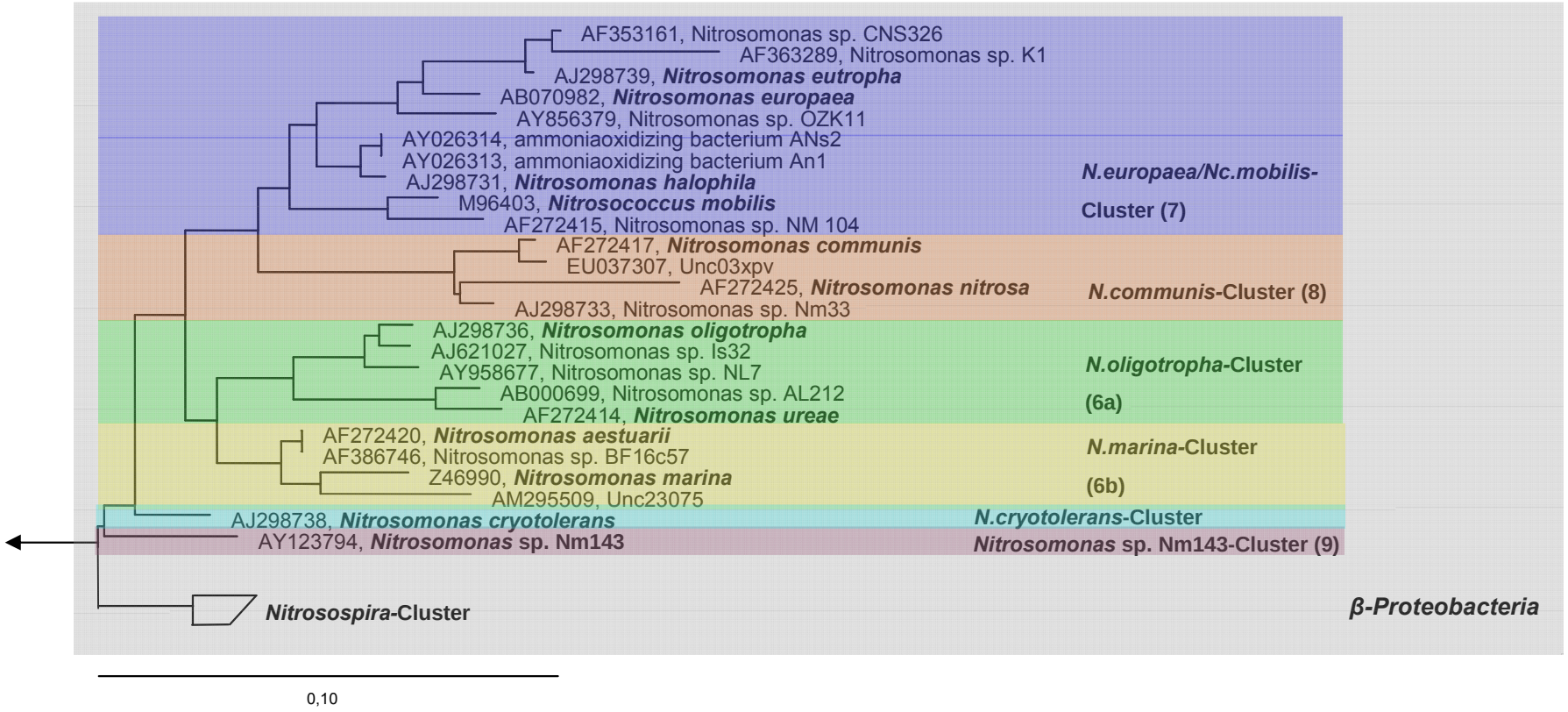



**Figure 2:** Neighbour-Joining tree showing the phylogeny of ammonia-oxidizing bacteria; the scale bar is representing 10% of the estimated sequence divergence

*Nitrosospira*-group: AJ28746/M96405/AY123790/L35505/EF175101/AY123807 (accession numbers)

of the Cluster 6 are urease positive. *N. oligotropha*-like bacteria are mostly found in oligotrophic water and a high affinity for $NH_3$ has been detected. *N. marina*-like bacteria occur mainly in marine environments, which is going well with the reported salt-dependency (Bock and Wagner, 2006). So far sequences of all described AOB species could be found in wwtps, except for *N. halophila* (isolates have been obtained from the North Sea and Mongolian soda lakes) (Koops *et al.*, 2003). Representatives of the Nm143-Cluster have been isolated from marine estuaries. However, the nitrosomonad *N. europaea-Nc. mobilis*- and *N. oligotropha*-Cluster are believed to play a main role in wastewater treatment, while *Nitrosospira*-like AOB are considered to be only of minor importance (Purkhold, 2003; Wagner and Loy, 2002). *N. communis*-like bacteria have been isolated from activated sludge samples (Purkhold, 2003), but their preferred habitats still seem to be rather pH-neutral soils (Koops *et al.*, 2003). *N. cryotolerans*-like bacteria, normally detected in sea water, have until now just once been found in a so-called A2O-wwtp, operated with an "anaerobic/anoxic/ aerobic" treatment process[*], and receiving influent high in chloride concentrations (Limpiaykorn *et al.*, 2005).

### A.2.2. Ammonia-oxidizing archaea

Existence of ammonia-oxidizing archaea has not been known until *amoA*-like sequences could be attributed to organisms affiliated to the archaeal group of *Crenarchaeota*, one of the two main archaeal phyla (cren- and euryarchaeotes) (Schleper *et al.*, 2005). Since then, archaeal oxidation of $NH_3$ could be confirmed for the first cultivated crenarchaeote "*Candidatus* Nitrosopumilis maritimus" (Könneke *et al.*, 2005). In this pure culture a long minimum generation time of 21 h was observed (Könneke *et al.*, 2005). Previously, also autotrophic C fixation could be related to some crenarchaeotes (Treusch *et al.*, 2005; referring to Wuchter *et al.*, 2003). These findings (autrotrophic C-fixation and $NH_3$ oxidation) could recently also be demonstrated for a nitrifying, moderately thermophilic (optimal growth: 45 - 80°C) crenarchaeote enriched from a hot spring. This AOA was provisionally referred to as "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler *et al.*, 2008).

Interestingly, also *nirK*-related sequences could be associated with crenarchaeotes, indicating the ability to reduce $NO_2^-$ under anaerobic conditions as previously suggested for AOB (Treusch *et al.*, 2005). Since the discovery of "nitrifying" crenarchaeotes, ubiquitous occurrence of these organisms in water and soil has been revealed (Francis *et al.*, 2005).

[*] „anaerobic/anoxic/aerobic…processes": as described by the authors; not according to the differentiation of these terms used in the present study

Crenarchaeotes appear to be highly abundant in marine ecosystems. For instance they comprise up to 40% of the bacterioplancton thriving in deep ocean water (Könneke *et al.*, 2005) and have also been proposed as most abundant ammonia-oxidizing prokaryotes in soil ecosystems (Leininger *et al.*, 2006), indicating that their role in the global biogeochemical nitrogen cycle could be of major importance. Still, high abundance and transcriptional activity do not prove their dominant role in nitrification – therefore nitrification activities and metabolic rates have to be investigated (Leininger *et al.*, 2006). First estimations of the nitrification rates of AOA have been made by Wuchter *et al.* (2006) for AOA in the North Sea: at 22 - 25°C a conversion of around 2 - 4 fmol $NH_3$/cell has been observed per day, which would fit to low estimations of *in situ* nitrification rates for AOB (approx. 5 fmol $NH_4^+$/cell/d, Wagner *et al.*, 1995). However, for different pure cultures of *Nitrosomonas* spp. and *Nitrosolobus* spp. far higher nitrification rates have been suggested (up to approx. 550 fmol $NH_4^+$/cell/d, Wagner *et al.*, 1995; referring to Belser, 1979 and Völsch *et al.*, 1990). In contrast to the proposed importance of AOA in terrestrial and aquatic nitrogen cycling, the contribution of AOA to nitrogen removal in wwtps is still unclear, though studies confirmed at least the presence of ammonia-oxidizing *Crenarchaeota* (Park *et al*., 2006). Archaeal *amoA* sequences retrieved from wwtps so far show relatedness as well to sequences obtained from soil and sediment, classified as a crenarchaeal "soil-Cluster", as to sequences obtained from marine habitats, classified as "marine-Cluster" (Park *et al.*, 2006).

### A.2.3. Nitrite-oxidizing bacteria

The chemolithoautotrophic nitrite-oxidizing bacteria (NOB) carry out the second part of aerobic nitrification (Fig.1), which is thermodynamically even more unfavourable than ammonia oxidation because of the high redox potential of nitrite (Costa, 2006). This results in slow growth rates of these organisms. As on the one hand AOB provide the substrate for NOB and on the other hand NOB remove the toxic product of ammonia oxidation, the two functional groups live in a mutualistic symbiosis and usually appear in close proximity to each other (Stein and Arp, 1998).

Some NOB, belonging to the genera *Nitrospira* (Daims *et al.*, 2001b) and *Nitrobacter* (Bock and Wagner, 2006; referring to Clark and Schmidt, 1967), which will subsequently both be described, have been found to be capable of growing mixotrophically. The enzyme catalyzing the oxidation of $NO_2^-$ to $NO_3^-$ is the nitrite oxidoreductase (Nxr). Unlike the *amoA*, *nxr* has so far not commonly been used for phylogenetic analysis though Poly *et al.* (2007) suggested

*nxrA* to be a good phylogenetic marker. However, Poly *et al.* (2007) have only performed tests with the genera *Nitrobacter* and *Nitrococcus*. As in AOB and AOA, *nirK*-like genes have also been found in *Nitrobacter winogradskyi*, an α-proteobacterial NOB. *N. winogradskyi* is capable of reducing $NO_2^-$ or $NO_3^-$ to $N_2O$ under anaerobic conditions.

Nitrite-oxidizing bacteria can be attributed to 5 distinct groups: *α-Proteobacteria* (genus *Nitrobacter*), *β-Proteobacteria* (genus *Nitrotoga*, "*Candidatus* Nitrotoga arctica"), *γ-Proteobacteria* (*Nitrococcus*), *δ-Protebacteria* (*Nitrospina*) and the phylum *Nitrospirae* (Alawi *et al.*, 2007; Bock and Wagner, 2006; Lebedeva *et al.*, 2005; Teske *et al.*, 1994). While *Nitrobacter* spp. have already been isolated from various environments like soil, fresh water and wastewater (Daims *et al.*, 2001b), "*Candidatus* Nitrotoga arctica" has just recently been isolated from arctic soil but since then also been detected in wastewater (Alawi *et al.*, 2007). Both, the genera *Nitrococcus* and *Nitrospina* comprise one described marine species, *Nitrococcus oceani* and *Nitrospina gracilis*, respectively (Bock and Wagner, 2006). The genus *Nitrospira* can be divided in four clusters (Fig.36, p.107). Cluster I encompasses mainly sequences originating from wastewater, amongst others the sequence of "*Candidatus* Nitrospira defluvii". Cluster II harbours one of the two so far validly described species for this genus, *Nitrospira moscoviensis* (Daims *et al.*, 2001b). The second described species, *Nitrospira marina*, is related to the marine Cluster IV. Cluster III encompasses sequences originating from the Nullarbor caves (Daims *et al.*, 2001b). Moderately thermophilic *Nitrospira*-like bacteria have been found in hot springs (Lebedeva *et al.*, 2005), reflecting the diversity of habitats in which *Nitrospira*-like bacteria can be found. Recently, a species forming a new lineage within this genus has been proposed and named "*Candidatus* Nitrospira bockiana" – it seems to be adapted to rather high temperatures (approx. 42°C) as well (Lebedeva *et al.*, 2008).

For a long time *Nitrobacter*-like bacteria were considered to be dominant in wwtps (Wagner and Loy, 2002). The application of molecular methods revealed that *Nitrobacter* spp. are usually of minor relevance and that *Nitrospira* spp. are the dominant nitrite-oxidizers in these systems (Daims *et al.*, 2001; Juretschko *et al.*, 1998; Schramm *et al.,* 1999). It has been proposed that occurrence of *Nitrospira* spp. and *Nitrobacter* spp. can be explained by the ecological concept of K- and r-strategists: r-strategists are characterized by a fast reproduction rate under beneficial conditions, under which they out-compete slower growing organisms – an advantage in ecosystems subjected to rapidly changing conditions. Contrary, K-strategists need longer time for reproduction but are better adapted to nutrient limited conditions, rendering survival under detrimental conditions possible. For *Nitrospira*-like bacteria a high

affinity for $NO_2^-$ and $O_2$ has been proposed (Schramm *et al.*, 1999), advantagous under limiting conditions, whereas for most *Nitrobacter* spp. a lower affinity is assumed (Schramm *et al.*, 1999; referring to Hunik *et al.*, 1993 and Prosser, 1989), which might indicate better growth at rather high nutrient concentrations. This concept has also proven to be applicable to the ecophysiology of representatives of *Nitrospira* sublineage I and II (Maixner *et al.*, 2006). Although all *Nitrospira* spp. are believed to be K-strategists (Schramm *et al.,* 1999), members of *Nitrospira*-Cluster I were suggested to be more similar to r-strategists than members of the *Nitrospira*-Cluster II. This assumption is based on the proposed differing preferences in $NO_2^-$ concentrations (Maixner *et al.*, 2006).

### A.2.4. Anaerobic ammonia-oxidizing bacteria

Anammox-bacteria convert $NH_4^+$ and $NO_2^-$ to $N_2$ under strictly anaerobic conditions. In the last step of this reaction hydrazine ($N_2H_4$), one of the intermediates, gets oxidized to $N_2$ by the hydroxylamine-oxidoreductase-like protein (HAO) (Fig.1). Bacteria capable of this reaction have so far all been attributed to the phylum *Planctomycetes* (Strous *et al.*, 2006). Details of this interesting reaction have become clear after the genome of an uncultured anammox-bacterium could be reconstructed (*Kuenenia stuttgartiensis*; Strous *et al.*, 2006). Slow growth rates of these bacteria (doubling time of approx. two weeks; Strous *et al.*, 2006) have been reported as well as a number of quite unusual features like the so-called anammoxosomes (Strous *et al.*, 2006). These are organelle-like differentiations within the cytoplasm (Strous *et al.*, 2006), which are surrounded by so-called ladderane lipids (Strous *et al.*, 2006), making the membranes extremely impermeable (Strous *et al.*, 2006). Furthermore, anammoxosomes contain the hydroxylamine-oxidoreductase-like protein (Strous *et al.*, 2006). Further investigations have shown that anammox-bacteria might play a major role in nitrogen cycling within oceans (Strous *et al.*, 2006; referring to Arrigo, 2005). Anammox-bacteria have also been observed in wwtps, partly in high numbers (Daims *et al.*, 2006b; referring to Schmid *et al.*, 2000 and Egli *et al.*, 2001). These observations have led to new strategies for nitrogen-removal, which are based on partial nitrification and anaerobic ammonia-oxidation (see A.3.) (Daims *et al.*, 2006b; referring to van Dongen *et al.*, 2001 and Wett, 2006).

## A.3. Biological nitrogen removal during wastewater treatment

One main nitrogen-compound in sewage is urea, which gets hydrolysed to $NH_4^+$ (Wagner and Loy, 2002). This $NH_4^+$ is converted to $NO_3^-$ by aerobic nitrification and can then be used as electron acceptor by denitrifying organisms in an anoxic stage, resulting in the gaseous nitrogen compounds (NO, $N_2O$, optimally $N_2$) (see A.2.). Since an aerobic environment is required for the first reaction, the respective tank gets aerated and agitated, providing a good distribution of $O_2$ and substrate. As nitrifiers are very slow growing organisms, the sludge retention time is normally set quite high to avoid wash-out of these bacteria. Denitrification then requires anaerobic conditions, which can be provided by several different operating strategies (see A.3.1.1.). A rather new way of clearing wastewater from nitrogen is by partial nitrification/anammox. Thereby $NH_3$ gets oxidized to $NO_2^-$, while NOB are inhibited, and both, $NH_4^+$ and $NO_2^-$, are then converted to $N_2$ by anammox-bacteria (Daims *et al.*, 2006). This process, which combines nitrification and denitrification in one reaction, just takes place under completely oxygen-free conditions, implicating energy conservation for the treatment plants, due to less aeration necessity. Wastewater containing high $NH_4^+$ concentrations and little C concentrations is beneficial for this process ("the online anammox resource", www.anammox.com). A disadvantage is the very slow growth rate of these organisms (Strous *et al.*, 2006)).

Still, the commonly used procedure of nitrogen removal is by aerobic nitrification and subsequent anaerobic dentrification. However, aerobic nitrification is the process most often subjected to problems and failure (Wagner and Loy, 2002). This is even more problematic since it takes some time until aerobic nitrification recovers, due to the already mentioned slow growth rates. The fragility of this process has been attributed to several factors – firstly to the sensitivity of AOB and NOB to certain environmental conditions as low temperatures, extreme pH (Koops *et al.*, 2003; Siripong *et al.*, 2007), low $O_2$ concentrations and chemical inhibitors (Siripong *et al.*, 2007). Apart from these possible causes, another hypothesis explains the instability of nitrification by the fact that dependence on intermediates renders processes less stable (Briones *et al.*, 2003), especially in case of toxic intermediates like $NO_2^-$ (Graham *et al.*, 2007). In addition, Graham *et al.* (2007), referring to chaos-theory models, believe that mutualistic systems in general exhibit chaotic behaviour, the higher the mutual benefit, the more unstable the process. Besides, Graham *et al.* (2007) hypothesize that, since other functional guilds like denitrifiers are more diverse than AOB and NOB, this limited diversity may result in instability (see A.3.4.). Aside from a general limitation in diversity in

these functional guilds, reports on the diversity of AOB occurring in wwtps, vary. Juretschko *et al.* (1998) and Daims *et al.* (2001) found plants dominated by one AOB-group only, whereas e.g. Purkhold *et al.* (2000), Daims *et al.* (2001) and Gieseke *et al.* (2003) described a high diversity of AOB in other investigated wwtps.

### A.3.1. Different design of wastewater treatment plants

Wwtps can be designed in different ways. Most common are conventional full-scale treatment plants, Sequencing Batch Reactors and trickling filters (water is trickling over natural (lava) or artifical stones). As trickling filter samples have not been studied here, this type will not be discussed in detail.

#### A.3.1.1. Conventional full-scale treatment plants

In conventional full-scale treatment plants the different reactions take place in seperate reactors with a permament flow of wastewater. The two reactors representing the two main steps are the aeration basin and the sedimentation tank (Fig. 3). In the first, degradation of mainly carbonic compounds takes place, in the second biomass is separated from cleaned water by sedimentation (of flocs). As nowadays removal of nitrogen is quite common, this simple construction has been enhanced a lot. Degradation of carbonic compounds and nitrification in the same tank can put a good separation in the clarifying sedimentation process at risk, as slugde retention times (SRTs) are extended due to the slow growth rates of nitrifiers and as a result more sludge will accumulate due to fast growing heterotrophic bacteria. Therefore C degradation and nitrification are normally separated within so-called “two-stage”-treatment plants (Dornhofer, 1993). For denitrification, anaerobic conditions have to be provided – this can be achieved in an extra tank before or after C degradation and nitrification. Denitrification before nitrification has the advantage that still enough carbonic substrate for denitrifiers is present – on the other hand $NO_3^-$ occurs mainly after nitrification. These problems can be circumvented by reflux of water after the nitrification process to the denitrification tank situated upstream of the nitrification tank or by adding external carbon sources (e.g. methanol) to downstream denitrification processes (Dornhofer, 1993). Furthermore, denitrification can also take place in the same tank as nitrification, either simultaneously or through alternating periods of aeration and solely agitation (anaerobic conditions). The principle of alternate nitrification and denitrification can also be expanded to

two tanks, which get then alternately filled and aerated (Fig. 7, 8). Finally nitrification and denitrification can be achieved in cascaded steps. Hereby either separate tanks provide alternatively aerobic or anaerobic conditions or one tank provides throughout the tank alternative aerobic and anaerobic conditions.

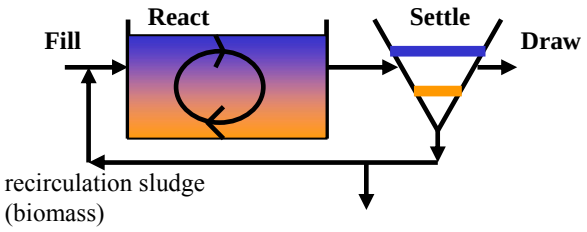



**Figure 3:** Flow-scheme of a conventional full-scale treatment plant

### A.3.1.2. Sequencing Batch Reactors

In Sequencing Batch Reactors (SBRs) the different reactions are not spatially separated, but sequenced in time. This means that all reactions take place in one reactor, but are limited to certain time periods – all time periods necessary for one process-run constitute one cycle (Schreff, 2007). Thus SBRs are loaded in batches (usually once per cycle), which is contrary to the continuous influx of wastewater in conventional systems. Normally, more than one SBRs are run in parallel (or alternating) and get loaded from a buffering tank storing the influent (Schreff, 2007). Thus, this construction is often favoured when the amount of income is strongly varying. An already more complex cycle scheme can be seen in Fig. 4.

### A.3.1.3. Fixed / Fluidized bed reactors

In fixed bed reactors the bacterial biomass is not occurring in suspended and floccular structure like in agitated activated sludge basins, but bacteria form biofilms on the surface of carrier material. The influent is conducted over the carrier material. Fluidized bed reactors also rely on biofilms attached to carrier material for the degradation of substances, but contrary to fixed bed reactors the carrier material (often grains of sand) is distributed within the reactor by agitation. SBRs can also be operated with surface-attached biofilms and are then called “Sequencing Batch Biofilm Reactors” (SBBRs) (online lexicon “wasser-wissen”, University of Bremen).

### A.3.1.4. Membrane bioreactors

Instead of the conventional way of sedimentation to separate clarified water from biomass, this can also be done by membrane filtration. Hereby the area of treatment plants can be reduced as a sedimentation tank is not needed. Another advantage is that sludge biomass in the aeration basin can be increased. This concentration is normally limited by the sedimentation process since a good separation is not secured if the sludge concentration is too high. Increasing the concentration in membrane bioreactors also allows a reduction of the volume of the bioreactor. Still, concentration of bacterial biomass is limited due to the viscosity of the biomass – high viscosity, due to enhanced production of EPS (extrapolymeric substance), deteriorates the filtration process. More recent studies have therefore questioned the potential degree of biomass increase in these reactors (Kubin, 2004; referring to Cornel *et al.*, 2001 and Coté, 2001). Effluent of these plants is extremely clean (totally free of solids) due to the small size of membrane pores (Rosenwinkel *et al.*, 2000).

### A.3.2. Different operating strategies

Operational modes of reactors can vary according to different pursued strategies; the ones considered to potentially have influence on nitrifiers will be shortly described:

- Sludge age (SA)/Sludge retention time (SRT): The SA, alternatively named SRT, describes the duration of sludge bacteria within the system before being removed as excess sludge. It is calculated in relating the bacterial biomass (dry matter) in the activated sludge basin to the daily removed amount of excess sludge. SA is a very important factor for nitrification due to the slow growth rates of nitrifiers (e.g.: stable nitritation could be obtained for a SA of 10 - 20 days according to Egli *et al.*, 2003 ).
- Regulation of pH: pH-regulation is very important, especially when considering nitrification, as nitrifying bacteria are sensitive to low pH; though this is not generally true, inhibition of nitrification has often been observed for a pH < 6 (Gieseke *et al.*, 2006; referring to Painter, 1986). The formation of hydronium ions ($H_3O^+$) during nitrification leads to an acidification of the environment (Kowalchuk *et al.*, 2001) and necessitates the pH-regulation in wwtps. One strategy used to increase alkalinity of wastewater is for instance the addition of lime water.
- Aeration: To ensure nitrification, the concentration of $O_2$ should not fall below 2 mg/l (Rosenwinkel *et al.*, 2000).

- Removal of phosphorous (P): Superfluous P can be removed biologically, or chemically by precipitation of phosphate ($PO_4^-$). Precipitation can be achieved by using lime or metallic salts (aluminium- or ferric salts), and at different steps of the treatment process: precipitation after nitrification has no influence on the nitrifying community, but pre- and simultaneous precipitation might influence nitrifiers. Though pre-precipitation has no direct influence, reduction of accumulating sludge and thus removal of excess sludge could be beneficial for nitrification. Simultaneous precipitation can lead to an increased sludge accumulation, which could require a lower SA, which can in turn effect nitrification negatively. Dilutions of metallic salts often decrease the pH, and as previously stated some metallic salts, such as ferric sulphates ($Fe(II)SO_4$-), can inhibit nitrifiers (Matsché, 1993).

For SBRs there are two more important modes of operation:

- Cycling strategy: Conventional sequencing steps within cycles of SBRs are: loading followed by agitation and subsequent aeration, then sedimentation and a final decantation step, normally followed by an idle period (break). The actual reactive time within one cycle includes all operating steps (agitation and aeration) but sedimentation and decantation. Length of cycling is dependent on the type and the amount of wastewater, which can be increased by e.g. rainfalls. Generally, shorter cycling is assumed to yield higher activity of bacteria and better sludge sedimentation (Schreff, 2007; referring to Wilderer *et al.*, 1997). Usually one cycle lasts between 6 – 12 h (Schreff, 2007) in case of dry weather. Adjustment of the different phases within one cycle might be important (e.g.: length of aeration). An interesting cycling strategy is the "differential internal cycling" (DIC; plants are referred to as DIC-SBRs; Holm *et al.*, 2000): here, two loading events with differing C/N-ratios occur. The first load, introduced before aeration and thus degradation of organic C-compounds, nitrification and P-uptake, contains less carbon and more nitrogen (low C/N-ratio). The second load (high C/N-ratio) is introduced in an anaerobic phase, in which strong denitrification and some "resolving" of phosphorous, which has already been taken up by bacteria during the first aerated stage, occur. The two differential loading events have been suggested to be beneficial for both, N- and biological P-elimination. The main advantages are seen in better denitrification due to the high carbonic load of the $2^{nd}$ loading event and in an increased P-uptake under aerobic conditions within the first internal cycle. The increased P-uptake is explained by a highly efficient release of P during the previous anaerobic agitation period, which is suggested to

be a result of the at this time prevailing low $NO_3^-$ concentrations ($NO_3^-$ is believed to inhibit P-"resolving"). During this anaerobic period far more P is resolved than during the second anaerobic period, which is characterised by higher $NO_3^-$ concentrations due to extensive previously occurring nitrification (Holm *et al.*, 2000) (Fig. 4). In the DIC-mode, sequencing is expanded by a second anaerobic agitation step and another aeration step, in which again carbon degradation and final nitrification, as well as uptake of P, occur.

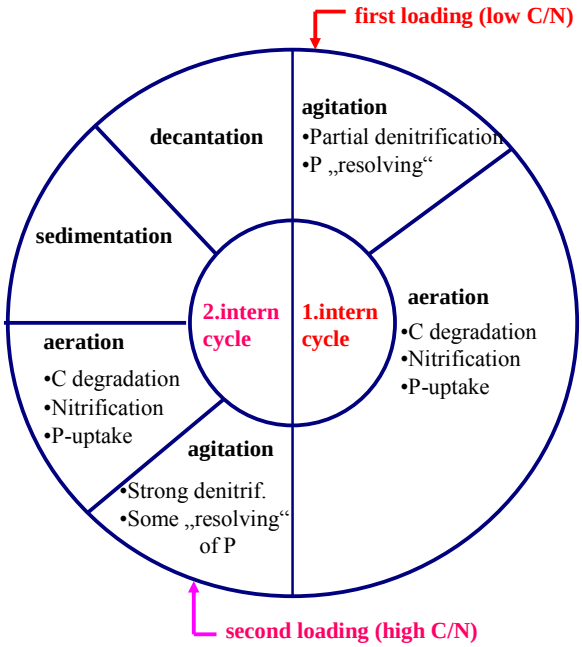



**Figure 4:** DIC-SBR-Cycle (on the basis of a scheme by Limnotec: http://www.limnotec.de/sbr.htm)

- Reactive time/loading time ($t_R/t_F$):

  This ratio describes how much of the total reactive time is dedicated to the loading process. It is important since it indicates the amount of nutrients bacteria within the system are exposed to at once – slower but steady loading might have different effects than "shock"-loading with lots of nutrients in a short time. The ratio is therefore also considered as a "controlled disturbance"-factor (Holm, personal communication). Considering that too high amounts of $NH_4^+$, "shock loading", are detrimental to nitrification (inhibition due to toxic effects on the nitrifying community), increases the relevance of loading amounts. Accumulation of $N_2O$ and $NO_2^-$ (Burgess *et al.*, 2002; referring to Anthonisen *et al.*, 1976 and Painter, 1986) have been observed as a consequence of "shock loading". Burgess *et al.* (2002) proposed that decreasing concentrations of dissolved oxygen may lead to oxidation of $NH_3$ by reduction of $NO_2^-$ ("nitrifier denitrification"), resulting in increased $N_2O$ concentrations. On the other hand depletion of oxygen could result in inhibition of NOB thus leading to accumulation of $NO_2^-$. $NO_2^-$ -accumulation could further be enforced if

saturation of $N_2O$ in the exhaust gas was reached, since produced $NO_2^-$ would not be used as electron acceptor anymore (Burgess *et al.*, 2002).

Positive correlations between the reactive and loading time-ratio and the sludge volume index have already been observed – low ratios (longer loading periods) often go along with decreased Index of Sludge Volume (ISV) (Holm, personal communication).

### A.3.3. Wastewater composition

Incoming wastewater can be characterized by various parameters:

- Biological oxygen demand (BOD5): The oxygen consumed by microorganisms during the degradation (biochemical oxidation) of biodegradable organic pollutants measured over a time period of 5 days at 20°C. After 5 d approx. 2/3 of the final BOD-values are achieved – a final BOD-value is expected after around 20 days; oxygen demand due to nitrification, which would bias the result, can be avoided by the addition of inhibitors such as allylthiourea. The BOD5 tells about the amount of carbon in the influent as most organic pollutants are carbon compounds. While the BOD5-value does not provide information about the total amount of polluting substances, it can indicate inhibitors and duration of degradation (Moser, 1993; online lexicon "wasser-wissen" of the University of Bremen).
- Chemical oxygen demand (COD): This value measures the potential "total amount of pollutants" as all oxidisable C-compounds get oxidized by wet oxidation (in a fluid phase through addition of $O_2$ at high temperature and high compression). The amount of $O_2$ needed provides information about the degree of pollution. Normally, over 95% of all oxidisable compounds are detected (Moser, 1993; online lexicon "wasser-wissen" of the University of Bremen).
- Ratio of COD/BOD5: This ratio is commonly around 2 and indicates the amount of compounds, which are difficult to degrade (Moser, 1993).
- Total Kjedahl Nitrogen (TKN): This parameter comprises the total amount of unoxidized nitrogen, which partly consists of $NH_4^+$ and organically bound N. The still bound nitrogen gets converted to inorganic nitrogen by "Kjedahl"-disintegration. The amount of still bound nitrogen depends on temperature, nature of the compounds and time period in the sewage network, in which hydrolysis already starts. In the effluent almost no TKN nitrogen should be detectable anymore (Moser, 1993).
- "Total nitrogen": comprises TKN as well as $NO_2^-$-N and $NO_3^-$-N. The latter two normally

occur in insignificant amounts in the influent. In case of good nitrification and denitrification they should also be low in the effluent (Moser, 1993).

- innorganic nitrogen: Inorganic nitrogen refers to $NH_4^+$, $NO_2^-$ and $NO_3^-$ (Moser, 1993).
- Conductivity: The conductivity reflects the amount of salts (ionic compounds) (online lexicon "wasser-wissen" of the University of Bremen) and is believed to be not highly altered within the plant (concentrations in the influent and effluent are comparable).
- Dry Matter (DM): DM refers to the remaining substance after a drying process (homogenized and filtered activated sludge gets dried at 105°C), normalized to a certain volume (g/l). It indicates the amount of biomass and can e.g. be altered by removing more excess sludge or extending the SRT (online lexicon "wasser-wissen" of the University of Bremen).
- Index of sludge volume (ISV): This parameter describes the sedimentation properties of the activated sludge, which is important for the separation of clarified water from biomass. ISV allows conclusions about the floc structure. It is calculated by relating the amount of activated sludge settled after 30 min. in a 1000 ml measuring cylinder (sludge volume) to dry matter (ml/g). ISV normally ranges from 80 to 120 ml/g – values higher than 150 ml/g indicate bulking, which puts a good separation of clarified water at risk (online lexicon "wasser-wissen" of the University of Bremen).
- Metals: Some metal salts, such as $Fe(II)SO_4$, can have an inhibitory effect on nitrifying bacteria (though concentration dependent) and should thus not occur in wastewater. Still, they are sometimes introduced by e.g. chemical phosphorous precipitation. (Matsché, 1993). Inhibitory effects have been reported for Zn, Ni, Cd and Cu (Zhiqiang *et al.*, 2003).
- Temperature (T), pH, $O_2$ concentration: These are very important parameters, which are therefore often regulated (see operational modes A.3.2.)

### A.3.4. The concept of diversity and stability

A relation between stability of ecosystems and diversity is discussed in diverse contexts – stability of large-scale ecosystems correlated to decreasing species numbers are investigated as well as the question in which way reactor processes such as nitrification in wwtps or methane-production during anaerobic sewage treatment, can be kept more stable. Even defining ecosystem stability is already challenging as various definitions of stability exist. These can be divided into two main ideas. The first idea addresses stability as a "system's dynamic stability" (McCann, 2000). According to this theory a system is stable if it is

returning to equilibrium after a perturbation and if without perturbations no variability ("variance in population densities over time") occurs. The second main idea defines stability as the "ability to defy change" (McCann, 2000). To what extent variability actually occurs after a disturbance is addressed as "resistance" of the system and believed to reflect this ability. Within this second definition, "resilience", meaning how fast the equilibrium can be reached again, is regarded as another measure of stabiliy (McCann, 2000). Stability through diversity, a quite old theory argued by well known ecologists like Robert McArthur, was challenged by Robert May's models indicating inherent dynamics in populations without perturbation events (Curtis *et al.*, 2003; referring to May, 1974) and, moreover, even increasing dynamics with higher diversity (McCann, 2000). In fact variances in population densities might even have a positive effect on the persistence of diversity on the community level (Mc Cann, 2000; Briones *et al.*, 2003 – referring to Huisman *et al.*, 2002; Huisman *et al.* 1999; Li, 2001). More variability within populations could provide these populations with a broader spectrum of alternative responses to changing conditions (McCann, 2000). Aside from this controversial debate, another theory should be introduced, which is probably of more relevance for engineering systems, and addressed as principle of "functional redundancy" or "insurance hypothesis": If more species carrying out the same function are present, or, if generally more species are present, implicating an increased probability that functional redundancy is higher, they can react differently to perturbations (McCann, 2000; referring to Naeem *et al.*, 1997 and 1998, Lawton *et al.*, 1993 and Yachi *et al.*, 1999), increasing the possibility of survival of some of these species. As previously described, aerobic nitrification in wwtps is presumably done by two main functional guilds, AOB and NOB. Applying the principle of "functional redundancy" on wastewater treatment, stability may be predictable by determining the diversity of these groups. Therefore it could be interesting to report the "total diversity" of the community, contrary to detection of the main groups by FISH (species below the detection limit will be neglected) – species of low abundance could become more important in case of disturbances. For instance a decrease of pH and substrate led to a population shift from *N. europaea* to *N. oligotropha* in an experimental reactor, though *N. oligotropha* has not been detectable by FISH before (Tarre *et al.*, 2007).

## A.4. Aims of the study

The overall aim of this study was to gain more knowledge about influences of operational mode and wastewater composition on the community structure of nitrifying bacteria. Therefore a total of 21 samples from different treatment plants mainly situated in Germany but also in Switzerland and Austria should be investigated by FISH and, partially, by clone libraries, to reveal the AOB and NOB populations present in the samples. Furthermore, NOB should be investigated in more detail, since until now mainly AOB have been intensively studied. Abundances of the genus *Nitrospira* as well as of members of *Nitrospira*-Cluster I and II, were to be determined by FISH and digital image analysis. Qualitative and quantitative data should then be analysed with regard to operational and chemical data provided by our cooperation partners at the different plants in order to detect influences of the various conditions on the community of nitrifying bacteria. Analysis of potential influences was to be done for all wwtps as well as solely for SBRs, since these represent the dominant plant type among all samples. A possible correlation of the appearance of specific representatives of AOB and NOB to some operational or chemical parameter would also be interesting regarding the often reported instability of nitrification in wwtps.

Another goal was to elucidate the role of AOA in wastewater treatment-processes. To date AOA have been detected in wwtps, but not much is known about their abundance and activity. Within this study, samples were to be screened by PCR to reveal occurrence of AOA. Cloning and phylogenetic analysis should be done for some of the samples to investigate whether certain AOA are especially adapted to wwtps. Furthermore, abundances of ammonia-oxidizing creanarchaeotes were to be determined by CARD-FISH, allowing first conclusions about their importance in nitrogen removal (Mußmann *et al.*, in preparation).

## B. Materials and Methods

All buffers and solutions were produced by using double distilled and filtered water (referred to as $H_2O_{bidest.}$), using a water purification facility (Ultra ClearTM, Barsbüttel, Germany). If not stated elsewhere, only chemicals in p.a. quality were used. pH was adjusted under the utilization of sodium hydroxid (NaOH) and hydrochloric acid (HCl).
For sterilization buffers and solutions were autoclaved for 20 min. at 121°C with a pressure of 1.013 x $10^5$ Pa (Varioclave 135S, H+P, Munich, Germany). Substances and solutions unstable at high temperatures were filtered sterile (0.22 µm pore size, Qualilab®, Merk Labor und Vertrieb GmbH, Bruchsal, Germany) and added after autoclaving (for instance antibiotics). Centrifugation steps – unless stated elsewise - were done by using a table-top centrifuge (Mikro 22R, Hettich, Tuttlingen, Germany) at room temperature.

### B.1. Investigated wastewater treatment plants

Samples were obtained from 21 wastewater treatment plants, of which five are conventional wwtps and 16 SBRs. Eight of the 16 SBRs are operated with a differential internal cycle strategy. Four conventional treatment plants and two SBRs (diverse influent) are animal rendering plants, the remaining plants receive either municipal or industrial or a mixture of wastewater. One plant receives water from sludge dewatering.

**Table 1:** Characteristics of the investigated wastewater treatment plants

| Investigated wwtps | Location | Sampling Date | Reactor Type | Wastewater-type | Population equivalents |
|---|---|---|---|---|---|
| **Plattling** | Southern Germany | 29.01.2007 | conventional wwtp | animal rendering waste | - |
| **Kraftisried** | Southern Germany | 29.01.2007 | conventional wwtp | animal rendering waste | - |
| **Oberding** | Southern Germany | 29.01.2007 | conventional wwtp | animal rendering waste | - |
| **GZM TBA Lyss** | Noth-West Switzerland | 29.01.2007 * | conventional wwtp, membrane filtration | animal rendering waste | - |
| **ARA Lyss** | Noth-West Switzerland | 29.01.2007 | conventional wwtp, fixed bed | municipal / industrial | - |
| **Ampfing** | Southern Germany | 26.03.2007 | SBR | animal rendering; milk production | 22500 |
| **Altmannstein** | Southern Germany | 24.03.2007 | SBR | municipal | 5500 |
| **Rosenheim** | Southern Germany | 30.05.2007 | SBR | industrial | 11200 |
| **Rapp-Kutzenhausen** | Southern Germany | 28.03.2007 | SBR | brewery | 20000 |
| **Langenzenn** | Southern Germany | 14.03.2007 | SBR | municipal | 16000 |
| **Waldsassen** | Southern Germany | 27.03.2007 | SBR | municipal / industrial | 13000 |

| | | | | | |
|---|---|---|---|---|---|
| **Ingolstadt** | Southern Germany | 09.05.2007 | SBR | industrial | - |
| **Seefeld** | Tyrol, Austria | 28.03.2007 | SBR | municipal | 26000 |
| **Bad Zwischenahn** [a] | Northern Germany | 23.05.2007 | DIC-SBR | municipal / industrial | 41000 |
| **Deuz** [a] | Western Germany | 21.05.2007 | DIC-SBR | municipal | 12800 |
| **Spenge** [a] | Western Germany | 22.05.2007 | DIC-SBR | municipal | 22500 |
| **Hettstedt** [a] | Southern Germany | 24.05.2007 | DIC-SBR | municipal | 30000 |
| **Weißtal** [a] | Germany | 21.05.2007 | DIC-SBR | municipal | 9500 |
| **Huntlosen** | Central Germany | 23.05.2007 | DIC-SBR | municipal | 10000 |
| **Bruchmühlen** | Germany | 22.05.2007 | DIC-SBR | municipal | 11250 |
| **Radeburg** | Northern Germany | 24.05.2007 | DIC-SBR | municipal | 15200 |

[a] online measurement; [*] repeatedly sampled

### B.1.1. Conventional full-scale wastewater treatment plants

#### B.1.1.1. Plattling

The wwtp in Plattling is an animal rendering plant. The biological stage of this wwtp is structured in a two-step cascade of denitrification- and nitrification-tanks (Figure 5). Anaerobic denitrification tanks are situated upstream of the aerobic nitrification tanks. Furthermore an anaerobic fixed bed reactor for primary treatment of wastewater exists, between the mechanical purification and biological cleaning stages. Into this anaerobic tank only around 10% of the influent is discharged. As also wastewater involved in "recycling" of blood is reaching this wwtp, high P concentrations can be found, requiring chemical P precipitation (Carozzi *et al.*, in preparation).

#### B.1.1.2. Kraftisried

The wwtp in Kraftisried is, like in Plattling, an animal rendering plant. It consists basically of a first stage with preliminary mechanical purification, followed by a physical-chemical stage for reduction of $NH_4^+$, an activated sludge tank and a sedimentation tank (Fig.6). The physical-chemical stage is a construction for nitrogen-stripping, which is used if necessary (mainly in winter). By increasing the pH $NH_4^+$ is converted to $NH_3$, which can be disgased. When adding sulphuric acid to the wastewater and lowering the pH, an ammonium sulphate solution can be collected and used later in agriculture or industry. In the activated sludge tank simultaneous nitrification and denitrification is ensured by aerating gradually or changing sites of aerated zones. Normally, around 50% of the tank is aerated. It is also possible to

operate an anaerobic fixed bed reactor for primary treatment upstream of the activated sludge tank (Carozzi *et al.*, in preparation).

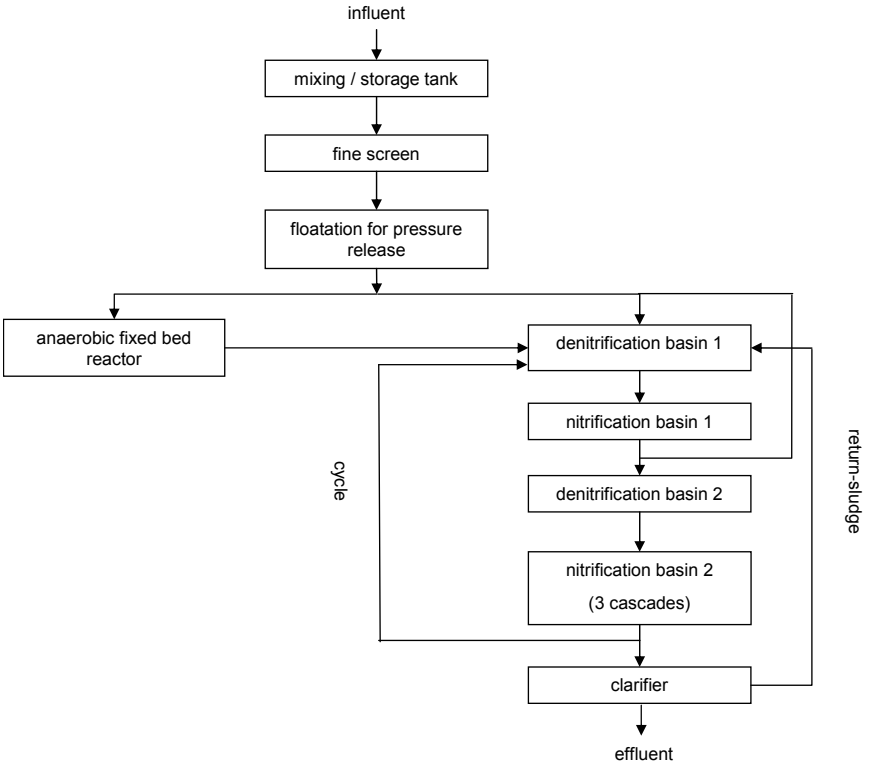



**Figure 5**: Flow scheme of the wwtp in Plattling (Temper, personal information)

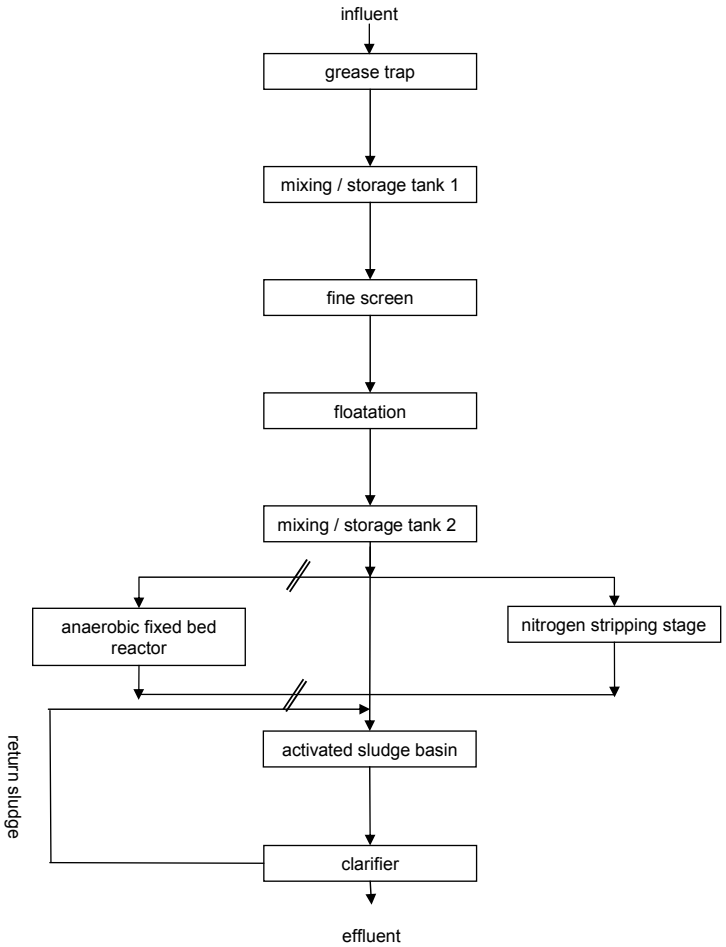



**Figure 6:** Flow scheme of the wwtp in Kraftisried (Temper, personal information)

### B.1.1.3 Oberding

Samples from Oberding have been collected from an animal rendering plant providing nitrogen-removal by intermittent denitrification (Fig.7). The biological stage consists of two tanks, which get alternately aerated and filled during agitated denitrification periods. Each period lasts for one hour. Downstream of those two tanks there is an aerobic fixed-bed reactor for final nitrification. The incoming wastewater consists, besides typical animal rendering waste, of water from densification processes from grease separation products (Carozzi *et al.*, in preparation).

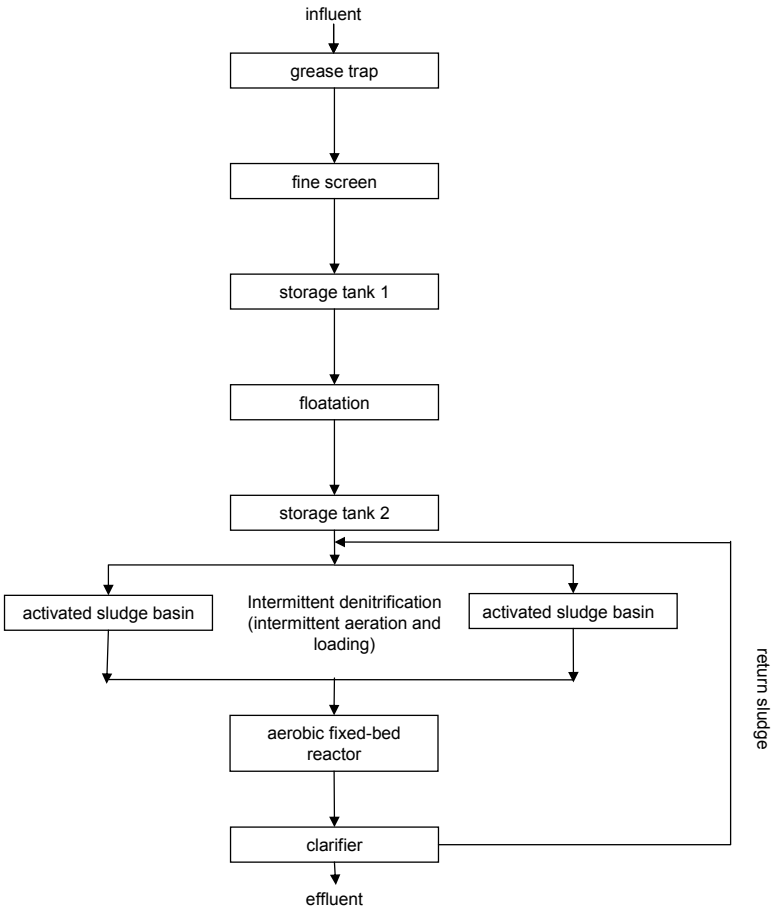



**Figure 7:** Flow scheme of wwt in Oberding (based on „Behandlung von Abwasser aus der Tierkörperbeseitigung"; Temper and Carozzi)

### B.1.1.4. GZM TBA Lyss

The animal rendering plant GZM TBA Lyss is special due to the final step of water separation, which is done by membrane filtration. As in Oberding, denitrification is achieved by intermittent denitrification in two alternately aerated (one hour periods) and filled tanks. A final aerobic tank exists for complete nitrification previous to membrane filtration (Carozzi *et al.*, in preparation).

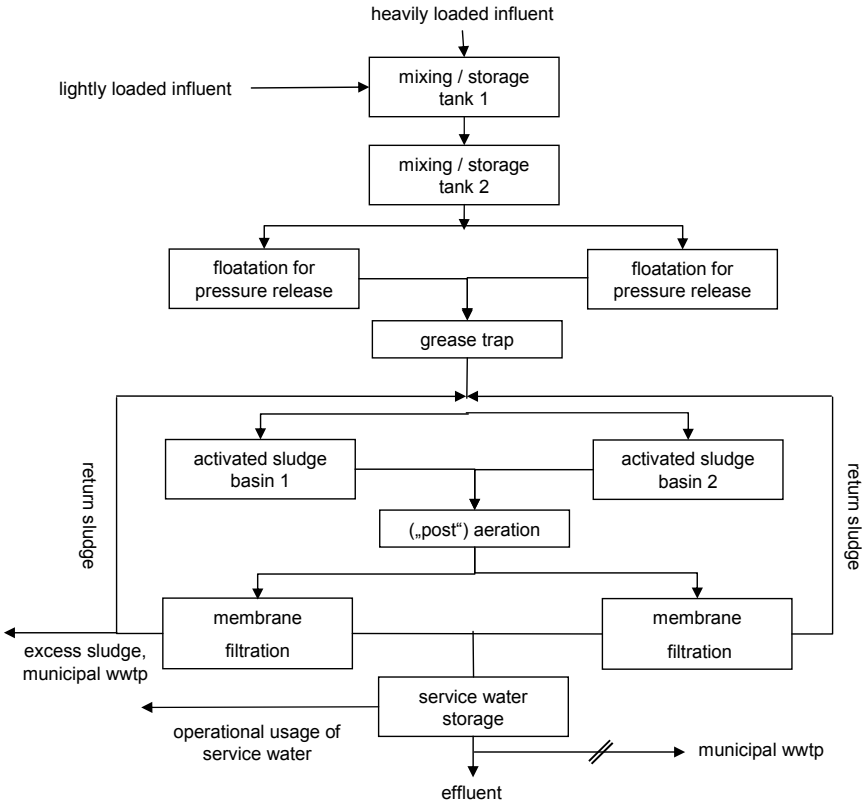



**Figure 8:** Flow scheme of the GZM TBA Lyss (Temper, personal information)

### B.1.1.5. ARA Lyss

The wwtp ARA Lyss, is the only analysed full-scale wwtp for treatment of municipal influent. Some wastewater originates from oil and grease producing industry. Only if disturbances in the GZM Lyss occur, the animal rendering wastewater gets discharged into the ARA Lyss. Another characteristic of this plant is the fixed-bed construction. The denitrification process is situated upstream of aerobic nitrification. Sludge for the inoculation of the GZM Lyss is taken from this wwtp (homepage of ARA REGION LYSS; http://www.ara-lyss.ch/; Temper, personal communication).

### B.1.2. Sequencing batch reactors

8 of the 16 SBRs sampled are operated without differential internal cycling-strategy. All except one are located in Germany, the other one (Seefeld) is located in Tyrol (Austria). The SBRs are loacted in Ampfing, Altmannstein, Rosenheim, Kutzenhausen, Langenzenn, Wald-sassen, Ingolstadt and Seefeld (Tab. 1). The SBR in Ampfing is amongst other effluents receiving water from a butchery. Rosenheim, Waldsassen and Ingolstadt receive industrial wastewater – the influent of Waldsassen is of various origins as well: partly municipal, partly

from a brewery, from companies producing soft drinks as well as from a bakery and butchery. Nevertheless input of the butchery does not appear to be high. The wwtp in Waldsassen is furthermore subjected to seasonal variations in loading due to tourism. Ingolstadt is unique concerning the influent, as it is treating water from sludge dewatering. The SBR in Kutzenhausen is connected to the brewery "Rapp" (Schreff, personal communication).

### B.1.3. DIC-Sequencing Batch Reactors

The remaining 8 samples come from SBRs (Tab. 1), which are treated by application of a differential internal cycling-strategy. Five of these reactors are controlled by online-measurements, which provide a quite extensive documentation of chemical parameters.

## B.2. Sampling

Samples have been collected by our cooperation partners at the wwtps. For all plants the samples were taken during an aerobic mixed operation phase to ensure – as far as possible – activity of nitrifying bacteria. For the ARA Lyss biofilm-samples were collected. For each plant 3 sample types were taken: For DNA-extraction 50 ml of sludge was filled in Greiner tubes without additional chemicals (see. B.10.1. Storage of sludge biomass for DNA-extraction). For FISH analysis (see B.9. Fluorescence *in situ* hybridization), 25 ml sample were mixed with either 25 ml 96%-ethanol or 25 ml 4% -paraformaldehyde (PFA). Samples have then been shipped overnight and if possible cooled to our laboratory where the fixation procedure was finalized (see B.9.1. Cell fixation). For the GZM TBA Lyss further samples have been taken: two additional samples, both added to 4%-PFA in a ratio of 1:1, and another sludge volume filled in a Greiner tube containing an equal volume of RNAlater (see B.10.2. Storage of sludge biomass for RNA-extraction).

## B.3. Software

**Table 2:** Utilized software

| **Program** | **URL** | **Reference** |
|---|---|---|
| ARB software package | http://www.arb-home.de/ | Ludwig *et al.*, 2004 |
| Basic Local Alignment Search Tool (BLAST) | http://www.ncbi.nlm.nih.gov/BLAST/ | Altschul *et al.* 1990 |
| Bellerophon | http://foo.maths.uq.edu.au/~huber/bellerophon.pl. | Huber et al, 2004 |
| Chromas | http://www.technelysium.com.au/chromas.html | Technelysium Pty Ltd |
| Daime | http://www.microbial-ecology.net/daime/ | Daims *et al.*, 2006 |

| | | |
|---|---|---|
| probeBase | http://www.microbial-ecology.net/probebase/ | Loy *et al.*, 2003 |
| probeCheck | www.microbial-ecology.net/probecheck | Loy *et al.*, unpublished |
| Ribosomal Database Project (RDP) II | http://rdp.cme.msu.edu/ | Cole *et al.*, 2003 |
| Sigma plot 8.0 | - | Systat Software Inc., Richmond, CA, USA |
| SPSS | - | Association for Institutional Research |

## B.4. Technical Equipment

**Table 3:** utilized technical equimpment

| **Equipment** | **Company** |
|---|---|
| Beadbeater Fast Prep FP 120 | Savant Instruments Inc. Holbrook, NY |
| Centrifuges:<br>Mikro 20<br><br>Rotina 35 R<br><br>Galaxy Mini Centrifuge | <br>Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany<br>Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany<br>VWR international, West Chester, PA, USA |
| Confocal Laser Scanning Microscope LSM 510 Meta | Zeiss, Jena, Germany |
| DNA Sequencer Applied Biosystems 3130 | Applied Biosystems Lincoln, USA |
| DNA Sequencer Li-cor Long Readir 4200 | MWG-Biotech, Ebersberg, Germany |
| Drier Haraeus T20 | Kendro Laboratory Products, Hanau, Germay |
| Gelcarriage:<br>HoeferTM HE 33 - gel running tray (7x10cm)<br>Sub-Cell GT UV-Transparent Gel Tray (15x15cm) | <br>Amersham Biosciences (SF) Corp., USA<br>Biorad, Munich, Germany |
| Gel Dokumentationsystem MediaSystem FlexiLine 4040 | Biostep, Jahnsdorf, Germany |
| Gelelectrophoresis:<br>HoeferTM HE 33 Mini Horizontal submarine unit<br>Sub-Cell GT | <br>Amersham Biosciences (SF) Corp., USA<br>Biorad, Munich, Germany |
| Heatblock VWR Digital Heatblock | VWR international, West Chester, PA, USA |
| Hybridisation oven UE-500 | Memmert GmbH, Schwabach, Germany |
| Laminar flow hood Safe 2010 Modell 1.2 | Holten, Jouan Nordic, Allerød, Dänemark |
| Magnetic stirrer:<br>RCT basic<br>Variomag® Maxi | <br>Biorad, Munich, Germany<br>Eppendorf, Hamburg, Germany |
| Microwave oven MD6460 | Microstar |
| Millipore Milli-Q-Biocel | Millipore Corporation; 290 Concord Road; Billerica, MA 01821 |
| PCR thermocyclers:<br>Icycler<br>Mastercycler gradient | <br>Biorad, Munich, Germany<br>Eppendorf, Hamburg, Germany |
| Pipettes Pipetman® P2 – P1000 | Gilson international, Vienna, Austria |
| pH-Meter WTW inoLab Level 1 | Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany |
| Photometers:<br>NanoDrop® ND-1000 | <br>NanoDrop Technologies, Wilmington, USA |
| Platform Shaker Innova 2300 | New Brunswick Co., Inc., Madison NJ, USA |
| Power device for gelelectrophoresis PowerPac Basic | Biorad, Munich, Germany |

| | |
|---|---|
| Transilluminator UST-30M-8E (312 nm) | Biostep GmbH, Jahnsdorf, Germany |
| Ultraviolet Sterilizing PCR Workstation | Peqlab Biotechnology GmbH, Germany |
| Vortex Genie 2 | Scientific Industries, New York, USA |
| Waterbaths:<br>DC10<br>GFL Typ 1004 | <br>Thermo Haake, Karlsruhe, Germany<br>Gesellschaft für Labortechnik GmbH, Burgwedel, Germany |
| Watervapour high pressure autoclaves:<br>Varioclav 135S H+P<br>Varioclav 25T H+P | <br>H+P, Munich, Germany<br>H+P Munich, Germany |

## B.5. Expendable items

**Table 4:** consumables

| **Consumables** | **Company** |
|---|---|
| Cover slips 24×50 mm | Paul Marienfeld, Bad Mergentheim, Germany |
| Eppendorf Reaktionsgefäße (ERT), various sizes | Eppendorf AG, Hamburg, Germany |
| Erlenmeyer-Kolben DURAN®, various sizes | Schott Glas, Mainz, Germany |
| Glas capillares (50 µl in 5.1 cm) | Idaho Technology Inc., Salt Lake City, UT, USA |
| Microseal „A“ | Film MJ Research, Waltham, MA, USA |
| U96 MicroWell™ Plates, 0.5 ml | NuncTM Serving life science, Roskilde, Denmark |
| Mikrotiterplates MicrosealTM 96, V-Boden | MJ Research, Waltham, MA, USA |
| Petri dishes 94/16 | Greiner Bio-One GmbH, Frickenhausen, Deutschland |
| Pipet tips, various sizes | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Plasticcuvettes (Halb-Mikro Greiner) | Bio-One GmbH, Frickenhausen, Germany |
| Reaction vessels with screw caps (2ml) | SorensonTMBioScience, Inc., Salt Lake City, UT, USA |
| Sampling vessels (50 ml Greiner) | Bio-One GmbH, Frickenhausen, Germany |
| Sterile filters; 0.22 µm pore size | Qualilab®, Merk<br>Labor und Vertrieb GmbH, Bruchsal, Germany |
| Test tubes | Assistent, Karl Hecht KG, Sondheim, Germany |

## B.6. Chemicals

**Table 5:** utilized chemicals

| **Chemical** | **Company** |
|---|---|
| 4’-6’-di-amidino-2-phenylindole (DAPI) | Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria |
| Acetic acid | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Acetone | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Acrylamide/bisacrylamide (40%, 37.5:1) | Biorad, Munich, Germany |
| Agar | Fluka Chemie AG, Buchs, Switzerland |
| Agarose:<br>Agarose, elecrophoresis Grade<br>NuSieve® 3:1 Agarose (low melting) | <br>Invitrogen Corp., Carlsbad, CA, USA<br>Bio Science, Rockland, Inc., Rockland, ME, USA |
| Ampicillin | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Bromphenol Blue | Sigma-Alderich Chemie GmbH, Steinhausen, Germany |
| Chloroform | Carl Roth GmbH & Co., Karlsruhe, Germany |
| chloroform/isoamyl alcohol (24:1) | Carl Roth GmbH & Co., Karlsruhe, Germany |

| | |
|---|---|
| Cititfluor AF1 | Agar Scientific Limited, Essex, England |
| Di-ethyl-pyrocarbonate (DEPC) | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| di-Sodiumhydrogenphosphate ($NaH_2PO_4$) | J.T. Baker, Deventer, Holland |
| Ethanol absolute | Merck KgaA, Darmstadt, Germany |
| Ethanol, denatured | Carl Roth GmbH & Co., Karlsruhe, Deutschland |
| Ethidium Bromide (EtBr) | FLUKA Chemie AG, Buchs, Switzerland |
| Ethylene-di-amine-tetra-acetic acid (EDTA), di-sodium salt | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Formamide (FA) | FLUKA Chemie AG, Buchs, Switzerland |
| Glycerol | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Hydrochloric acid (HCl) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Hydrogen Peroxid ($H_2O_2$), 30% | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Isopropanol (2-propanol) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Kanamycin | Sigma-Alderich Chemie GmbH, Steinhausen, Germany |
| Magnesium chloride | Carl Roth GmbH & Co., Karlsruhe, Germany |
| phenol/chloroform/isoamyl alcohol (25:24:1) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| 37% Formaldehyde | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Potassium Chloride (KCl) | Carl Roth GmbH & Co., Karlsruhe, Deutschland |
| RNA later | Solm-Ambion, Applied Biosystems, Darmstadt, Germany |
| Sodium dodecyl sulfate (SDS) | FLUKA Chemie AG, Buchs, Switzerland |
| Sodium hydroxide (NaOH) | J. T. Baker, Deventer, Holland |
| Sodium-di-hydrogenphosphate ($NaH_2PO_4$) | J.T. Baker, Deventer, Holland |
| SYBR® Green I | Cambrex Bio Science, Rockland, Inc., Rockland, ME,USA |
| Tris | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Trizol | Invitrogen Corp., Carlsbad, CA, USA |
| X-Gal | Carl Roth GmbH & Co., Karlsruhe, Germany |

## B.7. Kits

**Table 6:** used kits

| **Kit** | **Company** |
|---|---|
| Power Soil™ DNA Kit | MOBio Lab. Inc., Salana Beach, CA, USA |
| QIAquick® PCR Purification Kit | QIAgen, Hilden, Germany |
| QIAquick® Gel Extraction Kit | QIAgen, Hilden, Germany |
| RevertAid First Strand cDNA Synthesis Kit | Fermentas, St.Leon-Rot, D |
| Topo TA Cloning® Kit | Invitrogen Corporation, Carlsbad, CA, USA |

## B.8. Buffers, media and solutions

### B.8.1. General buffers

a) PBS (phosphate buffered saline) stock solution ($Na_xPO_4$)

- Solution 1: $NaH_2PO_4$ 200 mM 35.6 g/l
- Solution 2: $Na_2HPO_4$ 200 mM 27.6 g/l

pH of $NaH_2PO_4$ solution was adjusted to 7.2 - 7.4 with solution 2.

b) 1 x PBS

| | | |
|---|---|---|
| NaCl | 130 mM | 7.6 g/l |
| PBS stock solution | 10 mM | 50 ml/l |
| $H_2O_{bidest.}$ | | ad 1000 ml |
| pH 7.2 – 7.4 | | |

c) 3 x PBS

| | | |
|---|---|---|
| NaCl | 390 mM | 22.8 g/l |
| PBS stock solution | 30 mM | 150 ml/l |
| $H_2O_{bidest.}$ | | ad 1000 ml |
| pH 7.2 – 7.4 | | |

**B.8.2. Buffers, solutions and standards used for gel electrophoresis**

a) TBE buffer

*i) 10 x TBE*

| | | |
|---|---|---|
| Tris | 890 mM | 162.0 g/l |
| Boric acid | 890 mM | 27.5 g/l |
| EDTA | 20 mM | 9.3 g/l |
| $H_2O_{bidest.}$ | | ad 1000 ml |
| pH 8.3 – 8.7 | | |

*ii) 1 x TBE*

| | |
|---|---|
| 10 x TBE | 100 ml/l |
| $H_2O_{bidest.}$ | ad 1000 ml |

b) TAE buffer

*i) 50 x TAE*

| | |
|---|---|
| Tris | 2 M |
| Sodium acetate | 500 mM |
| EDTA | 50 mM |

pH was adjusted to 8.0 under with glacial acetic acid.

*ii) 1 x TAE*

| | |
|---|---|
| 50 x TAE | 20 ml/l |
| $H_2O_{bidest.}$ | ad 1000 ml |

c) TAE buffer, modified (Millipore)

*i) 50 x TAE, modified* (Millipore)

| | |
|---|---|
| Tris | 2 M |
| EDTA | 5 mM |

pH was adjusted to 8.0 under with glacial acetic acid.

*ii) 1 x TAE, modified*

| | |
|---|---|
| 50 x TAE, modified | 20 ml/l |
| $H_2O_{bidest.}$ | ad 1000 ml |

d) Loading buffer

| | |
|---|---|
| Ficoll | 25% (w/v) |
| Bromphenol blue | 0.5% (w/v) |
| Xylencyanol | 0.5% (w/v) |
| EDTA | 50 mM |

e) Ethidium bromide (EtBr) solution

*i) Ethidium bromide stock solution*

| | |
|---|---|
| Ethidium bromide | 10 mg/ml in $H_2O_{bidest.}$ |

*ii) Ethidium bromide staining solution (1:10000 dilution)*

| | |
|---|---|
| EtBr-stock solution | 100 µl |
| $H_2O_{bidest.}$ | 1000 ml |

f) SYBR® Green I solution

*i) SYBR® Green I stock solution*

| | |
|---|---|
| SYBR® Green I | 1:10000 concentrate in Di-methylsulfoxid (DMSO) |

*ii) SYBR® Green I staining solution*

| | |
|---|---|
| SYBR® Green I stock solution | 10 µl |
| TAE-buffer | 1000 ml |

g) DNA ladder

GeneRulerTM 1 kb (Fermentas, St. Leon-Rot, Germany)

GeneRulerTM 100 bp (Fermentas, St. Leon-Rot, Germany)

### B.8.3. Media for culturing of *Escherichia coli*

a) Luria Bertani medium (LB medium)

| | |
|---|---|
| Tryptone | 10.0 g/l |
| Yeast extract | 5.0 g/l |
| NaCl | 5.0 g/l |
| $H_2O_{bidest.}$ | ad 1000 ml |
| pH 7.0 - 7.5 | |

For solid media 15 g/l agar were added before autoclaving.
Liquid and solid media was stored at 4°C.

b) SOC medium

| | |
|---|---|
| Tryptone | 2 % w/v |
| Yeast extract | 0.5 % w/v |
| NaCl | 10 mM |
| KCl | 2.5 mM |
| $MgCl_2$ | 10 mM |
| $MgSO_4$ | 10 mM |
| Glucose | 20 mM |

SOC medium was stored at -20°C.

### B.8.4. Antibiotics

a) Ampicillin stock solution (Amp)

| | |
|---|---|
| Ampicillin | 100 mg/ml in 50%-$EtOH_{abs.}$ |

Amp was added to medium to a final concentration of 100 µg/µl.

b) Kanamycin stock solution (Kan)

| | |
|---|---|
| Kanamycin | 100 mg/ml in $H_2O_{bidest.}$ |

Kan was added to medium to a final concentration of 100 µg/µl.

Stock solutions of antibiotics were sterile filtered. For solid media, stock solutions of antibiotics were added after autoclaving when the media had cooled to a temperature between 50 and 60°C. Antibiotics were added to liquid media directly before usage.

### B.8.5. Selection solution

Selection solution was needed during TOPO-TA-cloning for blue/white-screening of transformed cells on the plate.

X-Gal (5-brom-4-chlor-3-indolyl-β-D-galactopyranoside) stock solution

| | |
|---|---|
| X-Gal | 40 mg/ml in DMF (Di-methylformamide) |

The stock solution was sterile filtered. X-Gal solution was stored by -20°C in the dark.

For the blue/white-screening 40 µl of X-Gal solution were spread on LB agar plates containing 100 µg/ml Kan or Amp. This was done before plating the cells.

### B.8.6. Solutions used for plasmid isolation

a) P1 buffer

| | |
|---|---|
| Tris-HCl, pH 8.0 | 50 mM |
| EDTA | 10 mM |
| RNAse A | 100 µg/ml |

b) NaOH/SDS solution

| | |
|---|---|
| $H_2O_{bidest.}$ | 8 ml |
| NaOH (2 M) | 1 ml |
| 10% SDS | 1 ml |

c) Potassium acetate/acetate solution

| | |
|---|---|
| KCl (5 M) | 6 ml |
| $H_2O_{bidest.}$ | 2.85 ml |
| Acetic acid (glacial) | 1.15 ml |

### B.9.Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization is a technique allowing the detection and identification of cells without previous cultivation but in their natural habitat. The application of fluorescently labelled oligonucleotides targeting specific parts of the 16S rRNA can be used to identify microbes from the species- to the domain-level. Before the hybridization, cells get fixed and immobilized onto a slide. Then they get hybridized with the oligonucleotide probes and

subsequently washed, both under stringent conditions. Cells can be detected by epifluorescence microscopy (Amann, 1995b).

### B.9.1. Fixation of cells

During cell fixation bacteria in the samples get killed and stabilized. This step provides long term maintenance of environmental samples (conserving the ribosome content and structure) preserving a consistent picture of the sample over time ("snap-shot").

#### B.9.1.1. Fixation with Paraformaldehyde (PFA)

For the fixation of gram-negative cells paraformaldehyde is used. This solution is cross-linking terminal amino groups of cell proteins in the cell walls. Otherwise cell walls would lyse during storage.

a) production of a 4% PFA-solution

Solutions: 37% formaldehyde
1 x PBS (pH 7.2 – 7.4)

A 37% formaldehyde stock solution was diluted with 1 x PBS (pH 7.2 – 7.4) by mixing 2.7 ml of the stock solution with 25 ml 1 x PBS. The 4% PFA solution is then mixed 1:1 with a sample to yield a final concentration of 2% PFA.

a) fixation procedure

25 ml sludge was added to the 25 ml 4% PFA solution by our cooperation partners (see B.2.). Then the samples were sent to out laboratory where the final fixation was carried out as follows: After a centrifugation step (5000 rpm, 10 min., 4°C) the supernatant was discarded and the pellet resuspended in 1 x PBS and centrifuged again (5000 rpm, 10 min., 4°C). The supernatant was removed and the pellet resuspended in about 25 ml of a 1:1 1 x PBS- and $EtOH_{abs.}$-solution. The final volume should be comparable to the initial sample volume (25 ml). The fixed samples were stored at -20°C.

### B.9.1.2. Fixation with EtOH

All samples were also fixed with EtOH, as PFA-fixation is not appropriate for archaeal cells and gram-positive bacteria. This is due to their different cell wall composition. Cross-linking the cell walls could render them impermeable for probes.

Solutions: $EtOH_{abs.}$

25 ml sludge was added to 25 ml EtOH by our cooperation partners. After their arrival these samples were stored at -20°C.

### B.9.2. *In situ* hybridization

### B.9.2.1. 16S rRNA-targeting oligonucleotide probes

Appropriate probes have been identified with the online database probeBase (Loy *et al.*, 2003).

**Table 7:** Utilized oligonucleotide probes

| Name | Sequence (5`-3` ) | Binding position (*E.Coli*) | % FA | Specificity | Reference |
|---|---|---|---|---|---|
| EUB338[a] | GCT GCC TCC CGT AGG AGT | 338-355 | 0-50 | Most *Bacteria* | (Amann *et al.*, 1990) |
| EUB338 II[a] | GCA GCC ACC CGT AGG TGT | 338-355 | 0-50 | *Planctomycetales* | (Daims *et al.*, 1999) |
| EUB338 III[a] | GCT GCC ACC CGT AGG TGT | 338-355 | 0-50 | *Verrucomicrobiales* | (Daims *et al.*, 1999) |
| NONEUB | ACT CCT ACG GGA GGC AGC | X | - | control probe complementary to EUB338 | (Wallner *et al.*, 1993) |
| Nonsense | AGA GAG AGA GAG AGA GAG | X | - | control probe | (unpublished) |
| Alf1b | CGT TCG YTC TGA GCC AG | 19-35 | 20 | *α*- and some *δ*-*Proteobacteria, Spirochaetes* | (Manz *et al.*, 1992) |
| Alf968 | GGT AAG GTT CTG CGC GTT | 968-985 | 20 | *α-Proteobacteria*, except of *Rickettsiales* | (Neef *et al.*, 1997) |
| Bet42a[b] | GCC TTC CCA CTT CGT TT | 1027-1043 | 35 | *β-Proteobacteria* | (Manz *et al.*, 1992) |
| Gam42a[b] | GCC TTC CCA CAT CGT TT | 1027-1043 | 35 | *γ-Protepbacteria* | (Manz *et al.*, 1992) |
| Pla46 | GAC TTG CAT GCC TAA TCC | 46-63 | 30 | *Planctomycetales* | (Neef *et al.*, 1998) |
| Nso190 | CGA TCC CCT GCT TTT CTC C | 189-207 | 55 | some β-proteobacterial AOB | (Mobarry *et al.*, 1996) |
| Nso1225 | CGC CAT TGT ATT ACG TGT GA | 1224-1243 | 35 | all β-proteobacterial AOB except *Nc.mobilis* | (Mobarry *et al.*, 1996) |

| | | | | | |
|---|---|---|---|---|---|
| NEU | CCC CTC TGC TGC ACT CTA | 653-670 | 40 | Most halophilic AOBs | (Wagner *et al.*, 1995) |
| Cte | TTC CAT CCC CCT CTG CCG | 659-676 | 40 | Competitor NEU (*Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., *Aquaspirillum* spp.) | (Schleifer *et al.*, 1992) |
| Ncmob (NmV) | TCC TCA GAG ACT ACG CGG | 174-191 | 35 | *Nc. mobilis* – Cluster | (Juretschko *et al.*, 1998) |
| Cluster6a 192 | CTT TCG ATC CCC TAC TTT CC | 192-212 | 35 | *N. oligotropha*- Cluster (Cluster 6a) | (Adamczyk *et al.*, 2003) |
| c6a192 | CTT TCG ATC CCC TGC TTCC | 192-212 | 35 | Competitor Cl. 6a (*N.eutropha*) | (Adamczyk *et al.*, 2003) |
| Nmar 830 | GCC TAG TAA GGC CCA ACA | 830-847 | 35 | *N. marina* – Cluster (Cluster 6b) | (Juretschko, unpublished) |
| Nolimar 712 | GCC TTC GCC ATC GAT GTT CT | 712-721 | 40 | *N. oligotropha*- & *N. marina* – Cl. (6) | (Juretschko, unpublished) |
| cNolimar 712 | CGC CTT CGG CAC CGG TGT TCC | 712-721 | 40 | Competitor Nolimar 712 | (unpublished) |
| Ncom 1025 | CTC GAT TCC CTT TCG GGC A | 1025-1044 | 35 | *N. communis*-Cluster (Cluster 8) | (Juretschko, unpublished) |
| Nsv443 | CCG TGA CCG TTT CGT TCC G | 444-462 | 30 | *Nitrosospira* spp. | (Mobarry *et al.*, 1996) |
| Amx 368 | CCT TTC GGG CAT TGC GAA | | 15 | all ANAMMOX bacteria | (Schmid, unpublished) |
| NIT3 | CCT GTG CTC CAT GCT CCG | 1035-1052 | 40 | *Nitrobacter* spp. | (Wagner *et al.*, 1996) |
| cNIT3 | CCT GTG CTC CAG GCT CCG | 1035-1052 | 40 | Competitor NIT3 (*Bradyrhizobium japonicum*, *Rhodopseudomonas palustris, Afipia clevelandis, Afipia felis*) | (Wagner *et al.*, 1996) |
| Ntspn 693 | TTC CCA ATA TCA ACG CAT TT | 694-713 | 10[c] | *Nitrospina gracilis* | (Juretschko, 2000) |
| Ntcoc 84 | TCG CCA GCC ACC TTT GCG | 85-101 | 10[c] | *Nitrococcus mobilis* | (Juretschko, 2000) |
| NTG 840 | CTAAGGAAGTCTCCTCCC | 837-854 | 10-20[c] | "*Candidatus* Nitrotoga arctica" | (Alawi *et al*, 2007) |
| Ntspa 712 | CGC CTT CGC CAC CGG CCT TCC | 712-732 | 50[cc] | Phylum *Nitrospirae* | (Daims *et al.*, 2001b) |
| cNtspa 712 | CGC CTT CGC CAC CGG TGT TCC | 712-732 | 50[cc] | Competitor *Ntspa* 712 (e.g.: *D. desulfuricans*) [d] | (Daims *et al.*, 2001b) |
| Ntspa 662 | GGA ATT CCG CGC TCC TCT | 662-679 | 35 | Genus *Nitrospira* | (Daims *et al.*, 2001b) |
| cNtspa 662 | GGA ATT CCG CTC TCC TCT | 662-679 | 35 | Competitor *Ntspa* 662 (e.g.: *B. stearothermophilus*)[dd] | (Daims *et al.*, 2001b) |
| NtspaI-1431 | TTG GCT TGG GCG ACT TCA | 1431-1448 | 35 | Cluster I *Nitrospira* | (Maixner *et al.*, 2006) |

| | | | | | |
|---|---|---|---|---|---|
| NtspaII-1151 | TTC TCC TGG GCA GTC TCT CC | 1151-1171 | 35-40 | Cluster II *Nitrospira* | (Maixner *et al.,* 2006) |
| Ntspa IV 620 | CTC GAC CTT TCC CGG TTA | 620-637 | 35 | *Ntspa. marina*-lineage (lineage IV) | (unpublished) |

[a] these 3 probes were mixed in one working solution referred to as EUBmix, for detection of all bacteria

[b] BET42a and GAM42a have been used as competitors for each other

[c] no evaluated formamid concentration was available

[cc] though the actually published FA concentration is 50, hybridizations were carried out with a FA concentration of 35 (based on previous positive experience)

[d] competitor for organisms with the same sequence as *D. desulfuricans* between *E. coli*-positions 712-732 (Damis 2001b)

[dd] competitor for organisms with the same seqeuence as *B. stearothermophilus* between *E. coli*-positions 662-679 (Daims 2001b)

To get an overview of the abundance of ammonium-oxidizing and nitrite-oxidizing bacteria in the samples, probes for the in wwtps most important AOB and NOB were mixed to a β-AOB mix and a NOB-mix (Tab. 8). Then all samples were investigated with a standard set of probes to screen for each of the most important groups of nitrifying bacteria. For AOB probes targeting representatives of the *N. oligotroha*-Cluster, the marine *Nitrosomonas*-Cluster, halophilic and –tolerant *Nitrosomonas* spp., representatives of the *Nitrosococcus mobilis*-lineage within the *Nitrosomonas europaea/Nc.mobilis*-Cluster, AOB belonging to the *N. communis*-Cluster and bacteria related to *Nitrosospira* spp., were used. Furthermore, to detect marine *Nitrosomonas*-like organisms, the probes Nolimar712 and Cluster6a-192, labelled with different dyes, were applied simultaneously to the samples (double hybridization; see B.9.2.4.). For NOB standard detection was done for the phylum and genus *Nitrospira(e)* *Nitrospira* Cluster I-like bacteria, *Nitrospira* Cluster II-like bacteria and *Nitrobacter* spp.

**Table 8:** Utilized probe-mixes

| Name | Probes mixed | % FA | Specificity |
|---|---|---|---|
| β -AOB mix | Nso1225 + Cl6a +cCl6a + NEU + Cte+ Ncom1025 + Ncmob + Nmar + Nsv443 | 35 | all known β-proteobacterial ammonia-oxidizing bacteria |
| NOB mix | NIT3+cNIT3+Ntspa662+cNtspa662 | 35 | main occurring nitrite-oxidzing bacteria in wwtps |

High Performance Liquid Chromatography (HPLC)-purified and lyophilized oligonucleotides were ordered from Thermo Electron GmbH (Ulm, Germany). Stock solutions were produced by dissolving the lyophilized oligonucleotides in $H_2O_{bidest.}$ according to the manufacturers` instructions. From these stock solutions working solutions were prepared. Final concentrations should be 50 ng/µl for Fluos-labelled probes, and 30 ng/µl for Cy3-labelled and Cy5-labelled probes. Working solutions as well as stock solutions were stored at -20°C in

the dark. The final concentration of one probe on the well should be 5 ng/µl for Fluos- and 3 ng/µl for Cy3- and Cy5-labelled probes, which is achieved by adding 1µl of the working solution of the probe to 10 µl of hybridization buffer (see B.9.2.4.). To avoid dilution of the hybridization buffer when a probe mixture with the same fluorescent labels is applied to the same well, one stock solution containing each probe, at a final concentration of 50 or 30 ng/µl was prepared. Otherwise the concentration of the probes would change. This principle was adopted when making the EUBmix as well as the β-AOB- and NOB-mix. For probes, which need a competitor, working solutions containing both, probe and competitor, have been made. Characteristics of the utilized fluorescent dyes can be seen in Table 9.

**Table 9:** Characteristic of the utilized fluorescent dyes

| Fluorescence dye | Absorption maximum (nm) | Emission maximum (nm) | Molar extinction coefficient ε (l/mol x cm) |
|---|---|---|---|
| Fluos | 494 | 518 | $7.5 \times 10^4$ |
| Cy3 | 554 | 570 | $1.3 \times 10^5$ |
| Cy5 | 650 | 667 | $\geq 2 \times 10^5$ |

### B.9.2.2. Immobilisation

The wells of the glass slides were roughed up by scratching the surface. This should increase adhesion of the sludge samples. Samples were pipetted on the wells and dried in the hybridization oven at 46°C for approximately 10 min. The sample volume differed between 4 and 10 µl depending on the density of the sample and the kind of usage, standard was 7 µl.

### B.9.2.3. Dehydration

Dehydration of the samples with ethanol increases permeability of the cells by disintegrating the cytoplasmic membranes.

Procedure:

After immobilization an increasing ethanol series was performed by dipping the slides into 50%, 80% and 96% EtOH for 3 min. each, followed by air-drying.

### B.9.2.4. Hybridization

As all hybridizations are carried out at a temperature of 46°C, stringency is adjusted by different concentrations of formamide (FA) in the hybridization buffer, which is weakening

hydrogen-bonds between nucleic acids. In the washing buffer (WB) different stringency is achieved by adjusting NaCl concentrations. NaCl-ions are stabilizing the binding of rRNA and DNA-probes by compensating repulsion, which is caused by the negatively charged backbones of the nucleic acids.

EDTA, which is added to the WBs at FA-concentration above 20%, is binding bivalent cations, which would otherwise alter the adjusted stringency in the WB (NaCl). For a FA concentration below 20% this is not necessary as the stringency is so low that additional cations will just increase an already very high stability.

a) Solutions

5 M NaCl

1 M Tris/HCl, pH 8.0

0.5 M EDTA, pH 8.0

10 % (w/v) SDS

Formamide (FA)

b) Procedure

Depending on the stringency required for the hybridization, 1 ml of the according hybridization buffer (HB) and 50 ml of the according washing buffer (WB) were made. The amounts of the different chemicals can be seen in Table 10 and 11.

**Table 10:** Composition of HB according to its FA-concentration

| FA (%) | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 50 | 55 | 70 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NaCl (µl) | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 |
| Tris (µl) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| SDS (µl) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **MQ (µl)** | **800** | **750** | **700** | **650** | **600** | **550** | **500** | **450** | **400** | **300** | **250** | **100** |
| **FA (µl)** | **0** | **50** | **100** | **150** | **200** | **250** | **300** | **350** | **400** | **500** | **550** | **700** |

**Table 11:** Composition of WB according to the FA-concentrations of the corresponding HB-buffer

| FA (%) | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 50 | 55 | 70 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tris (µl) | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| SDS (µl) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| MQ (ml) | to 50 ml | to 50 ml | to 50 ml | to 50 ml | to 50ml | to 50 ml | to 50 ml | to 50 ml | to 50 ml | to 50 ml | to 50 ml | to 50 ml |
| EDTA (µl) | 0 | 0 | 0 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 |
| **NaCl (µl)** | **9000** | **6300** | **4500** | **5400** | **2150** | **1490** | **1020** | **700** | **460** | **180** | **100** | **0** |

10 µl of hybridization buffer were pipetted onto the well and then 1µl of each probe was added. The slide was then inserted in the "hybridization chamber", a 50 ml Greiner tube, in which a tissue paper soaked with HB was placed. The closed Greiner tube was then transferred to the hybridization oven (46°C) and incubated for 1 – 3 h, standard was 2 h. After incubation the slides were washed for 10 to 20 minutes (standard: 15 min.) by transferring them into pre-warmed washing buffer (water bath, 48°C). For the final washing step the slides were shortly dipped into ice cold $H_2O_{bidest.}$ and immediately dried with compressed air. The slide was then either analysed right away or stored at -20°C in the dark.
When hybridizing with Amx368, specific at a 15%-FA, EDTA was added to the WB, though normally EDTA is only added above a FA-concentration of 20%.
Should two or more probes with different formamide concentrations be applied on the same well, serial hybridizations were carried out. First the sample is hybridized with the probe requiring the higher FA-concentration. After washing with WB and ice-cold MQ and subsequent drying, a second hybridization was started with the probe requiring the lower FA-concentration.

### B.9.3. DAPI-staining

In some cases samples were stained with 4'-6'-di-amidino-2-phenylindole (DAPI) to make all cells visible for microscopic analyses. DAPI is known to bind to double stranded DNA. A DAPI signal can be advantageous for distinguishing between real fluorescent signals, autofluorescence (AF) and inactive cells.

a) Solutions:

A 1:10000 dilution ($H_2O_{bidest.}$) of the DAPI stock solution was made and stored at 4°C in the dark.

b) Procedure:

10 µl of the DAPI working solution were pipetted onto the samples and incubated at RT in the dark for 5 min. DAPI was removed by taking off the fluid with a pipette. The sample was washed gently with 25 µl of $H_2O_{bidest.}$ to remove unbound DAPI and slides were dried at RT in the dark.

### B.9.4. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopes have the advantage – compared to conventional epifluorescense microscopes – that only light from objects in focus reach the detector and not disturbing fluorescence of other focus planes. This selective mechanism is provided by a pinhole in the beampath, which light emitted from other planes cannot pass. This makes it much easier, especially for samples with high AF (soil, sludge, etc.) and 3D-structure (biofilms, flocs, etc.), to differentiate between true signals and background noise. Furthermore objects can be scanned with lasers and reconstructed by software. Scanning throughout a sample also allows analyses of the 3-dimensional structure of specimens.

#### B.9.4.1. Detection of labelled cells

Prior to microscopic analysis, the samples on the wells were covered with Citifluor AF1 and a coverslip. This step decreases bleaching during microscopy. Analysis was done using a CLSM (LSM 510 Meta, Carl Zeiss, Jena, Germany) microscope, which was equipped with an argon-laser (430 - 514 nm) for excitation of Fluos fluophore and two helium-Neon lasers (543 and 633 nm) for excitation of Cy3 and Cy5, respectively. DAPI-staining was detected by excitation with a UV-lamp at approx. 360 nm. Plan-Neoflar objectives with 40x, 63x and 100x magnification were used combined with a 10x ocular. Furthermore the respective software was used for analysis.

#### B.9.4.2. Quantification of labelled cells

Quantification was done by measuring the area made up by the fluorescent signals of the probes targeting the population which should be quantified, and the area made up by fluorescent signals of probes binding to a hierarchically higher group. The results give the area-percentage of the specific population to the hierarchically higher group. This was done for all members of the phylum/genus *Nitrospira(e)* relative to all bacteria as well as for either members of Cluster I *Nitrospira* spp. or Cluster II *Nitrospira* spp. relative to the phylum/genus *Nitrospira(e)*. For each quantification a series of 2D images (at least 20, standard: 30) was taken to calculate the biovolume fraction of the population of interest in the sample. Pictures were taken randomly in different focus planes for quantification of members of the phylum/genus *Nitrospira(e)* relative to all bacteria. For quantification of members of

Cluster I or Cluster II relative to all *Nitrospira(e)*-like bacteria, sites with *Nitrospira*-populations were searched actively regardless of their affiliation to Cluster I or II, and recorded. Picture analysis and calculation of biovolume fractions were done with the program daime ("digital image analysis in microbial ecology"). This is a novel computer software that integrates digital image analysis and 3-D visualization functions (Daims *et al.*, 2006). The first step is referred to as "image segmentation" – hereby the program differentiates between single objects and background signals (object recognition). By the object editor the user has the possibility to eliminate artefacts individually or to modify the differentiation process done by the program. An easy way to identify artefacts is to eliminate all objects appearing with the specific probe but not with the probe having a broader coverage – therefore a congruency threshold can be set. In all quantifications the threshold was 25%, meaning that all objects appearing with the specific probe, which show less than 25% overlap with the probe targeting a hierarchically higher group, will be eliminated. Since due to different dye-intensities objects appearing with both probes sometimes do no fully overlap, the threshold was not set higher. Daime also determines the congruency of the two signals (congruency in %) and the heterogeneity of the pictures taken (Variation coefficient).

### B.9.5. Storage of slides

For later microscopic analysis, Citifluor was washed off with water, the slide was dried with compressed air and stored at -20°C in the dark.

## B.10. DNA and RNA-based analyses

### B.10.1. Storage of sludge biomass for DNA extraction

50 ml sludge samples, which had been taken by our cooperation partners and sent to our laboratory, were centrifuged (10000 rpm, 10 min., 4°C) and the supernatant was discarded. The pellet was stored at -20°C.

### B.10.2. Storage of sludge biomass for RNA extraction

25 ml sludge was filled to 25 ml RNAlater and stored at -20°C.

### B.10.3. Isolation of genomic DNA from sludge samples

For all activated sludge samples, genomic DNA was extracted by using the Power soil™ DNA Kit (MoBio Laboratories, Inc., CA, USA). Extractions were done following manufacturers instructions, only with a minor modification in the final step. Unlike in the instructions DNA was eluted in 50 µl $H_2O_{bidest}$. For each DNA isolation, a negative control containing water was treated in the same way as the actual samples to check for contaminations.

### B.10.4. Isolation of plasmid DNA from recombinant *E. coli* cells

Isolation of plasmids is based on the lysis-effect that alkaline conditions have on organic material (cells). Proteins get removed by organic precipitation and finally, plasmid DNA is precipitated by addition of 2-propanol.

a) Solutions

P1 buffer
NaOH/SDS solution
Potassium acetate/acetate solution
2-propanol
70% EtOH

b) Procedure

As described later on (in B.12.1.1. Culturing and cell harvesting), 2 x 2 ml of an overnight grown culture were centrifuged (13000 rpm, 1 min., RT) in a sterile 2 ml Eppendorf reaction tube (ERT). After discarding the supernatant, 100 µl of P1 buffer were added, the pellet resuspended and the sample incubated for 5 min. at RT. To lyse the cells 200 µl of NaOH/SDS solution were added and the ERT was inverted several times followed by 5 min. incubation at 4°C, during which the ERT was again inverted. 150 µl of potassium acetate/acetate solution were added for protein precipitation. The sample was vortexed and incubated on ice for 5 min. After centrifugation (13000 rpm, 1 min., RT) the supernatant, approximately 450 µl, was transferred to a new ERT, to which one volume of 2-propanol was added for DNA-precipitation and mixed thoroughly. The ERT was incubated at RT for at least 10 min. The sample was then centrifuged (13000 rpm, 1 min., RT), the supernatant discarded

and the DNA pellet washed in 500 µl of ice cold 70% EtOH. EtOH was removed after another centrifugation step (13000 rpm, 1 min., RT) and the DNA-pellet was air dried at RT. Finally, the dried pellet was resuspended in 50 µl $H_2O_{bidest.}$

### B.10.5. Isolation of RNA

All vessels in which caps were stored were either baked for at least 6 h at 180°C or treated with 3% $H_2O_2$ for 10 min. and then rinsed with $MQ_{DEPC}$. Only RNase-free caps and tips were used and all chemicals containing water were produced under utilization of DEPC-water.

#### B.10.5.1 Combined RNA-DNA-extraction (Lueders *et al.*, 2004)

a) Solutions:

$H_2O_{DEPC.}$

0,1% w/v DEPC 1 l $H_2O_{bidest.}$

The solution was incubated overnight at RT and then autoclaved.

120 mM $NaPO_4$-buffer:

$Na_2HPO_4$ 112.87 mM

$NaH_2PO_4$ 7.12 mM

The buffer was filter sterilized and autoclaved.

pH 8

TNS-solution:

500 mM Tris-HCl, pH 8

100 mM NaCl

10% SDS (w/v)

The buffer was filter sterilized and autoclaved; pH was adjusted with Tris-HCl.

PEG precipitation solution:

30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl

RNase-free water

Autoclaved

EB-buffer:

10 mM Tris-HCl, pH 8.5

RNase-free water

The buffer was filter sterilized and autoclaved.

b) Procedure:

All intermediate steps – if not stated elsewise – were done on ice to keep the activity of enzymes (like RNases) low.

An initial volume for liquid samples was not specified by the protocol. Accordingly, all quantities within the following steps were adjusted to the initially taken volume of 250 µl. Firstly, 500 µl $NaPO_4$-buffer were added and the mixture was incubated for 15 min. During this step RNAlater should be removed. After incubation the sample was centrifuged at maximum speed (15000 rpm, 4 min.). The supernatant was transferred into a bead beater cap, to which 750 µl of 120 mM $NaPO_4$-buffer and 250 µl TNS were added. The cap was then halfway filled – a minimum headspace of 500 µl was required for the bead beating step. Bead beating was done for 45 sec. at 6.5 m/sec. by using the FastPrep FB120 bead beating system. Immediately after, the sample was centrifuged at 4°C (15000 rpm, 4 min.). 900 µl of the supernatant were transferred to a 2 ml vial on ice. Extraction of nucleic acids was done by first adding 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1; pH 5). Using pH 5 instead of pH 8 phenol/ chloroform/isoamyl alcohol is increasing the RNA yield while decreasing the DNA yield.

After centrifuging for 4 min. (15000 rpm, 4°C) 800 µl of the supernatant were transferred to a 2 ml "Phase lock Gel Heavy"-tube, to which 1 volume chloroform/isoamyl alcohol (24:1) was added. After a centrifugation step (4 min., 15000 rpm, 4°C), 650 µl of the supernatant, which was separated from the other phase by the provided fluid in the "Phase lock Gel Heavy"-tube, were transferred to a fresh 2 ml cap and mixed with 2 volumes of PEG. Precipitation was taking place while centrifuging the mixture for 30 min. at 4°C (15000 rpm). The liquid was removed with a pipette and 500 µl of cold 70%-EtOH was added as a washing step. After spinning down (short centrifugation, 15000 rpm, 4°C) the EtOH was removed. To get rid of all EtOH the spinning step was repeated. Remaining 70%-EtOH was removed with a pipette and the pellet was resuspended in 50 µl DEPC.

### B.10.5.2 RNA-Isolation with TRIzol/Glycogen (modified)

a) Solutions:

1 x PBS
chloroform
isopropyl alcohol
chloroform/isoamyl alcohol (24:1)
phenol/chloroform/isoamylalcohol (25:24:1)
5 M ammonium acetate
glycogen stock-solution (5 mg/ml) (Ambion)
75%-$EtOH_{DEPC}$
$H_2O_{DEPC}$
RNase-inhibitor (Fermentas)

b) Procedure:

250 µl sample were pipetted into a bead-beating cap, containing one large bead as lysing matrix. 250 µl of 1 x PBS were added. The following steps (TRIzol-treatment) are supposed to remove RNAlater. After centrifugation at maximum speed (15000 rpm, 5 min., 4°C), the supernatant was removed and 250 µl of TRIzol were added immediately. The mixture was homogenized for 10 sec. at 4.5 m/s speed by using the FastPrep FB120 bead beating system. Then the bead-beating cap was centrifuged (max. 11200 rpm, 5 min., RT) and the supernatant was transferred to a new tube, in which the homogenized sample was incubated for 8 min. (5-10 min.) at RT. For nucleic extraction 50 µl chloroform (0.2 ml per 1 ml supernatant) were added and the cap was shaken vigorously by hand for 15 sec. Then the sample was incubated at RT for 5 min. A centrifugation step (9850 rpm, 15 min., 4°C) was followed by transferring the aqueous phase to a fresh tube. For RNA precipitation 125 µl isopropyl alcohol (0.5 ml isopropyl alcohol per 1 ml of supernatant; here 250 µl) and 37.5 µl 5 M ammonium acetate (0.12 ml 5 M ammonium acetate per ml of supernatant that remained after initial homogenization; to get 0.5 M) were added. Further 2.5 µl glycogen, which acts as carrier for RNA, were added (originally 5 µl of the stock solution) and the sample was incubated overnight at -20°C. In this step the amount of glycogen was not completely adjusted to the initial homogenization amount, but half (instead of approximately a quarter) of the amount according to the original protocol was taken. The sample was then centrifuged (14000 rpm, 15

min., 4°C) and after removing the supernatant, the RNA pellet was washed with 1 volume (approximately 415 µl) of 75% $EtOH_{DEPC}$. The mixture was carefully vortexed and centrifuged (7000 g, 5 min., 4°C) and the supernatant was removed. After another short centrifugation step (7000 g, 4°C), the remaining EtOH was removed and the RNA pellet was resuspended in 50 µl RNase-free $H_2O_{DEPC}$. Finally 0.5 µl RNase-inhibitor were added.

### B.10.5.3 DNase digestion of isolated RNA

For using the isolated RNA as template for reverse transcription it is important to remove all DNA from the sample.

a) Solutions:

SIGMA Deoxyribonucease I amplification grade
10 x reaction buffer (SIGMA)
RNase inhibitor (SIGMA)
$H_2O_{DEPC}$
Stop solution (Ambion)
$NaAc_{DEPC}$ (pH 5.2)
$EtOH_{abs.}$
70% $EtOH_{DEPC}$

b) Procedure:

The approach for DNase digestion, which requires 1 U DNase per 2 µg RNA, was adjusted for an amount of 6 µl RNA (measured concentration of RNA was approx. 0.5 µg/µl; $H_2O_{DEPC}$ 2.5 µl).

| | |
|---|---|
| RNA | x µl |
| 10 x reaction buffer | 1 µl |
| DNase | 1 µl |
| RNase inhibitor | 0.5 µl |
| $H_2O_{DEPC}$ | y µl (ad 10 µl) |

The mixture was incubated for 1 h at RT and the reaction was stopped by adding 1 µl stop solution.

For a subsequent reverse transcription with the RNA as template, another RNA precipitation and salt removal had to be done. The RNA-sample was filled up to 200 µl with $H_2O_{DEPC}$. Next 1/10 volume of $NaAc_{DEPC}$ (pH 5.2), 1/100 volume of glycogen and 3 volumes of $EtOH_{abs.}$ were added and the mixture was incubated for 90 min. at -80°C to allow precipitation. Salt was removed by centrifuging for 15 min. (14000 rpm, 4°C), removing the supernatant and washing with 1 volume 70% $EtOH_{DEPC}$. After centrifuging (14000 rpm, 10 min., 4°C) and subsequent removal of the supernatant, the sample was centrifuged for a short time (14000 rpm, 4°C) and the remaining supernatant of 70% $EtOH_{DEPC}$ was removed. The pellet was then resuspended in 50 µl RNase -free $MQ_{DEPC}$.

To prove total removal of DNA, Polymerase Chain Reaction was performed (see B.11.).

### B.10.6. Storage of nucleic acids

Genomic- and plasmid DNA as well as coextracted DNA and RNA were stored at -20°C. Isolated RNA was stored at -80°C prior to DNase-digestion and final precipitation. After the digestion, the final precipitation and salt removal (washing step), RNA was also stored at -20°C.

### B.10.7. Analysis of nucleic acids

#### B.10.7.1. Photometric analysis of nucleic acids

The amount of DNA and RNA as well as the purity can be determined by using a spectralphotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, USA). Thereby 1.5 µl of the nucleic acid solutions were pipetted onto the end of the fiber optic cable of the NanoDrop. The concentration of the nucleic acids was calculated (with the provided software) by measuring the extinction at a wavelength of 260 nm with regard to the different optical densities of double stranded (ds) and single stranded (ss) DNA and RNA. Information about the purity of the nucleic acids can be gained by ratios between measured extinction rates at 230 nm, 260 nm and 280 nm.

- A guideline for purity of DNA is given by Marmur (1961) and Sambrook (1989):
  E260/E280 ≥ 1.8 … no protein contaminations
  E260/E230 ≥ 2.2 ... no phenol contaminations

- A guideline for purity of RNA is:
  A280/A260 > 1.6 (optimal > 2)

### B.10.7.2. Analysis of nucleic acids by gel electrophoresis

Qualitative analyses of nucleic acids, which includes controlling whether the isolation was successful or not as well as checking the length of the DNA amplificates (see B.11.), can be done by horizontal gel electrophoresis. Thereby an electric field is built up, in which the negative loaded DNA is migrating through a gel towards the cathode. Velocity of migration is dependent on the size of the DNA.

a) Solutions
1 - 2.5% (w/v) agarose in 1 x TBE buffer
Loading buffer
DNA-ladder (KbL, 100 bp)
EtBr staining solution

b) Procedure
Depending on the kind of analysis, gels with a concentration of 1% (controlling isolated DNA/RNA, PCR-products) to 2% (RFLP) were made by adding an appropriate amount of agarose (Invitrogen Corporation, Carlsbad, CA, USA Cambrex) to1 x TBE buffer. The higher the percentage of the gel, the slower but better is the separation. Dissolving of agarose was achieved by heating the mixture in the microwave oven till the liquid was clear. When cooled down, the liquid could be poured into a gel-tray with combs for the loading pockets (Typ H3, 11 × 14 cm, Gibco-BRL, Eggenstein, Germany). After around 30 min. the gel was polymerized and could be placed in the gel electrophoresis chamber (Sub-Cell GT Biorad) filled with 1 x TBE buffer. Finally the combs were removed.
For isolated DNA or RNA an amount of 3 - 5 µl was mixed with 3 µl loading dye while for PCR-products 10 µl were mixed with 5 µl loading dye – the total amount of both mixtures was then pipetted into the pockets. Loading dye is on the one side preventing the nucleic acids from diffusing to the surface and on the other side indicating the current position of the nucleic acids in the gel. Next to the samples a size marker (Kbl, 100 bp) was pipetted to allow later size estimation of the size of the nucleic acids analysed. A voltage between 80 (RFLP) and 130 V (isolated DNA/RNA, PCR-products) was applied and the gel was run for around

45 - 60 min. (isol.DNA/RNA, PCR-prod.) and 90 min. (RFLP). After separation, the gel was stained with EtBr for 30 to 60 min. Nucleic acids could then be visualized under UV-light using the Transilluminator (UST-30M-8E (312 nm)). Documentation was done by pictures taken with a digicam and the respective software Argus XI.

### B.11. Polymerase Chain Reaction (PCR) for amplification of nucleic acid-fragments

By Polymerase Chain Reaction defined regions of the DNA can be amplified. The regions of interest are determined by the binding of complementary primer pairs (forward and reverse primer) to which the enzyme Taq Polymerase can attach. This enzyme is then elongating the primer it is attached to and produces a copy of the DNA strand. The reaction involves three main steps at different temperatures: denaturation of the double-stranded DNA, primer annealing and elongation of the primers. The number of copies is increasing exponentially with each cycle as in every cycle all so far produced copies can be used as templates. One feature of the Taq Polymerase is that it attaches dATPs to the end of the fragements, independent of the template. As those overhangs are needed in case cloning by TOPO-TA is performed later on, a final elongation step should be performed after cycling. This step allows the enzyme to fully adenylate all fragments.

#### B.11.1. Solutions and general approach

All primers used in this study were obtained from Thermo Electron GmbH (Ulm, Germany). Polymerase chain reactions were carried out in either the Icycler (Biorad, Munich, Germany) or the Mastercycler gradient (Eppendorf, Hamburg, Germany) PCR cycler.

a) Solutions:

$MgCl_2$ (25 mM) (Fermentas Inc. Hanover, MD, USA)
Nucleotide Mix (2.5 mM each dNTP)
10 x Ex Taq polymerae buffer
Taq DNA Polymerase, recombinant (5 U/µl) (Fermentas Inc. Hanover, MD, USA)
Forward Primer (50 pmol/µl)
Reverse Primer (50 pmol/µl)
Bovine Serum Albumine (BSA; 10 mg/ml) (New England BioLabs Inc., Beverly, MA, USA)
$Aqua_{bidest.}$

b) Procedure:

For one standard reaction the solutions were mixed to a final volume of 50 µl as follows:

| | |
|---|---|
| $MgCl_2$ | 4 µl |
| Buffer | 5 µl |
| dNTP – Mix | 5 µl |
| Forward primer | 0.5 µl |
| Reverse primer | 0.5 µl |
| BSA | 0.5 µl |
| Taq DNA Polymerase | 0.25 µl |
| $Aqua_{bidest.}$ | 33.25 µl |
| Template | 1 µl |

If more than one reaction was performed, the amounts of all the ingredients (except the template) were multiplied with the number of reactions. Those amounts were mixed together to a "master mix", which was then distributed to the single ERTs (0,2 ml) by pipetting 49 µl in each. Finally the appropriate template was added. For each PCR reaction two controls were done: The positive control proves that the PCR has worked. Hereby a plasmid containing the gene which should be amplified during the PCR is used. The negative control contains all reaction solutions except a template and should therefore not yield a PCR-product, unless the master mix was contaminated. In case some reactions required amounts of ingredients differing from the standard amounts, ingredients were adjusted to a final volume of 50 µl each by changing the amount of $Aqua_{bidest}$.

Concentration of $MgCl_2$ stabilizes the binding of the negatively charged template DNA and the negatively charged primer and is thus, besides the annealing temperature, influencing the stringency of the primers binding. Specific amplification can also be influenced by the concentration of the primers in the reaction. BSA is used to decrease negative effects on the amplification reaction caused by humic acids and other PCR inhibitors (Kreader, 1996).

### B.11.2. Amplification of 16S rRNA-gene fragments

**Table 12:** Primers used for the amplification of 16S rRNA gene fragments

| Primer name [a] | Sequence (5`-3`) [b] | Binding position[c] | Specificity | Annealing Temp. (°C) | Reference |
|---|---|---|---|---|---|
| 616V | AGA GTT TGA TYM TGG CTC | 7-24 | Most *Bacteria* | 58 [d] | (Juretschko *et al.*, 1998) |
| 630R | CAK AAA GGA GGT GAT CC | 1528 - 1542 | Most *Bacteria* | 58 [d] | (Juretschko *et al.*,1998) |

| | | | | | |
|---|---|---|---|---|---|
| 1492 R | GGY TAC CTT GTT ACG ACT T | 1492 - 1510 | Most *Bacteria* and *Archaea* | 52 [d] | (Lane, 1991) |
| Ntspa1158R | CCC GTT MTC CTG GGC AGT | 1158 - 1175 | Most *Nitrospira* | 58 [d] | (Maixner 2006) |
| Bact781F | A AAC AGG ATT AGA TAC CC | 781 - 797 | Most *Bacteria* | 52 | Unknown |
| Arch21F | TTC CGG TTG ATC CYG CCG GA | 7-26 | Most *Archaea* | 56 | (DeLong, 1992) |

[a] F/V = forward primer ; R = reverse primer; [b] Abbreviations according to IUPAC : K= G/T, M= A/C, Y= C/T

[c] according to *E.coli* 16S rRNA positions (Brosius, 1981); [d] modified

**Table 13:** Conditions for the amplification of 16S rRNA-genes

| PCR-step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Primary denaturation | 94-95 | 3-5 min. | 1 |
| **Denaturation** | **94-95** | **40 sec.** | **35** |
| **Annealing** | **52-58** [b] | **40 sec.** | |
| **Elongation** [a] | **72** | **90 sec.** | |
| Final elongation | 72 | 10 min. | 1 |

[a] for an estimation of the elongation time, formation of ~1000 nucleotides/min. was assumed; approximate length of all 16S rDNA-fragments ~1500 bp; though amplification with 781f and 1492R yields a shorter fragment the elongation time was not changed

[b] annealing temperature used for the different primer pairs:

| | |
|---|---|
| 616V/630R | 58°C |
| 616V/Ntspn1158R | 58°C |
| Arch21F/1492 | 56°C |
| 781F/1492R | 52°C |

## B.11.3 Amplification of crenarchaeotal *amoA*-gene fragments

**Table 14:** Primers used for the amplification of crenarchaeotal *amoA*-gene fragments

| Primer name [a] | Sequence (5`-3`) [b] | Length of amplificate | Specificity | Annealing Temp. (°C) | Ref. |
|---|---|---|---|---|---|
| CrenamoAF | AAT GGT CTG GCT WAG ACG C | 639 bp | many AOA | 56 | (Könneke *et al.*, 2005) |
| CrenamoAR | GAC CAR GCG GCC ATC CA | | many AOA | 56 | (Könneke *et al.*, 2005) |
| Francis CrenamoAF | STA ATG GTC TGG CTT AGA CG | 635 bp | many AOA | 56 [c] | (Francis *et.al.*, 2005) |
| Francis CrenamoAR | GCG GCC ATC CAT CTG TAT GT | | many AOA | 56 [c] | (Francis *et.al.*, 2005) |

[a] F/V = forward primer ; R = reverse primer;

[b] abbreviations according to IUPAC: R = A/G, S = C/G, W = A/T; [c] modified

**Table 15:** Conditions for the amplification of crenarchaeotal *amoA*-genes

| PCR-step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Primary denaturation | 94 | 5 min. | 1 |
| **Denaturation** | **94** | **40 sec.** | **35** |
| **Annealing** | **56** | **40 sec.** | |
| **Elongation** [a] | **72** | **20 sec.** | |
| Final elongation | 72 | 5 min. | 1 |

[a] for an estimation of the elongation time, formation of ~1000 nucleotides/min. was assumed; approximate length of all crenarchaeotal *amoA*- fragments ~650 bp; to decrease unspecific amplification the elongation time was kept very short

### B.11.4. Nested PCR

Nested PCR consists of two PCR-reactions – the amplificons of the first PCR are used as template in the second PCR. This method can be used to increase specificity of the PCR-product in so far as unspecific amplificons produced during the first PCR (unspecific primer binding) will most probably not be amplified by the second primer pair. These should have the same specificity but bind within the region flanked by the first primer pair.
Here, Nested-PCR was used to screen for contaminations in the samples. To exclude contaminations with plasmids, Nested-PCRs were performed using primers fitting to primer binding sites on TOPO-TA and TOPO-XL vectors (see Table 16). In case of a positive result, the second PCR was done by using primers specific for bacterial groups investigated in this study, mainly *Nitrospira* (16S rDNA) and *Crenarchaeota* (crenarchaeotal *amoA*).

**Table 16:** Primers used for the first amplification of Nested PCRs

| Primer name [a] | Sequence (5`-3`) | Specificity | Annealing Temp.(°C) |
|---|---|---|---|
| M13F | GTA AAA CGA CGG CCA G | Vector pCR®II-TOPO/pCR®2.1 | 60 [b] |
| M13R | CAG GAA ACA GCT ATG AC | Vector pCR®II-TOPO/pCR®2.1 | 60 [b] |
| TOPO F | AGC TTG GTA CCG AGC T | Vector pCR®II-TOPO/pCR®2.1 | 48 [b/c] |
| TOPO R | TCT AGA TGC ATG CTC GA | Vector pCR®II-TOPO/pCR®2.1 | 48 [b/c] |

[a] F/V = forward primer ; R = reverse primer

[b] annealing temperature not stringent for PCR with environm. samples; unspecific amplification

[c] annealing temperature appropriate for sequencing (isolated plasmids) but not tested for "standard-PCR"

The standard reaction mixture had a final volume of 50µl and was handled as previously described. Just for PCRs with M13-specific primers the $MgCl_2$-concentration was reduced to 2 mM to increase stringency.

**Table 17:** Conditions for the amplification of 16S rRNA-genes; M13-primers (60°C); TOPO-primers (48°C)

| PCR-step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Primary denaturation | 94 | 3 | 1 |
| **Denaturation** | **94** | **40 sec.** | **35** |
| **Annealing** | **48-60** | **40 sec.** | |
| **Elongation** | **72** | **90 sec.** | |
| Final elongation | 72 | 10 min. | 1 |

The second PCRs were performed as previously described (B.11.) apart from the usage of 1µl of the product of the preceding PCR as template. To be sure that non-appearance of PCR-products indicated the purity of the sample and was not caused by inhibition of PCR due to template-inhibition (too much template is – immediately after denaturation – forming comp-

lexes again and thereby restraining primer binding), another positive control was included in the approach. The positive control of the first PCR-control was also used as template for the second PCR. Positive PCR-products resulting from the second PCR could also be caused by genomic DNA still present in the PCR-products of the first PCR and thus do not guarantee presence of the insert of interest on the plasmid. Genomic DNA, present during the first PCR-essay, is diluted 1:50 when using 1 µl of this first PCR-essay in the second PCR-essay (50 µl). To control whether positive PCR-products of the second PCR are due to present genomic DNA or if the the concentration of genomic DNA is already too diluted to be amplified, a PCR was performed with the genomic DNA diluted 1:50 as template. To confirm that in principle genomic DNA – if present – still could be amplified, isolated DNA was also amplified with the primer pair targeting the gene of interest (which had been amplified for cloning or other analysis).
For contamination-screening, M13 primers were also combined with CrenamoA-primers in one reaction (M13f and Francis-CrenamoA R; M13r and Francis-CrenamoA F) – the annealing temperature was 56°C and the elongation time 20 sec. (annealing temperature of Francis-CrenamoA R; elongation time for CrenamoA-specific PCRs; see Table 14 and 15).

### B.11.5. Gradient PCR

Gradient-PCRs were performed to determine optimal stringent conditions for amplification reactions in environmental samples.

#### B.11.5.1. Temperature gradient PCR

One possibility to increase stringency is by increasing the annealing temperature for primers. To find out the most stringent annealing temperature, reactions were done with different annealing temperatures. Negative controls were run at the lowest temperature. All other steps did not differ from standard PCR approaches.

#### B.11.5.2. $MgCl_2$ gradient PCR

Another possibility to increase stringency is by decreasing stability of the template-primer-complex by reduction of $MgCl_2$ concentration. PCRs were therefore performed with different $MgCl_2$ concentrations. Since an own master mix was needed for each $MgCl_2$ concentration, negative controls were run with every concentration.

### B.11.6. Reverse transcription for amplification of RNA-fragments

Reverse transcription (RT) was performed by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, D). Reactions were done according to manufacturers` instructions for "Synthesis of First Strand cDNA Suitable for PCR Amplification". As template total RNA (Tillmann extraction, DNase digestion) with a concentration of approx. 30 ng/µl was used. In total 6 reactions were performed as two different primers were used, two different amounts of template per primer and a positive and a negative control. One primer, Francis-CrenamoA R, was used because the goal was to target mRNA of this gene (gene-expression). Reverse transcriptase with 630R (16S rDNA, most bacteria) was applied as control of RNA integrity. The sample should contain 16S rRNA, but strongly shredded RNA could prevent successful RT (see Tab.12 and 14 for a more detailed description of primers). Reaction mixtures for the first step of cDNA-synthesis were as follows for the different approaches:

| | | |
|---|---|---|
| i) FrancisCrenamoAR and 630R: | | |
| total RNA | 2 µl | 4 µl |
| sequence-specific primer | 0.1 µl | 0.1µl |
| DEPC-treated water (provided) | 9.9 µl | 7.9 µl |
| | | |
| ii) Positive control: | | |
| control RNA (provided) | | 2 µl |
| control primer (provided) | | 2 µl |
| DEPC-treated water (provided) | | 8 µl |
| | | |
| iii) Negative Control: | | |
| control primer (provided) | | 0.1 µl |
| DEPC-treated water (provided) | | 9.9 µl |

The one hour incubation step was done at 44°C instead of 42°C to prevent negative influence of secondary structure. cDNA was stored at -20°C.

Synthesis of cDNA was controlled by gel electrophoresis – 6 µl of the cDNA were therefore mixed with 2 µl loading buffer and pipetted on a 1% agarose gel, which was subsequently stained with EtBr (see B.10.7.2.). The produced cDNA was also amplified with different primer pairs using a standard PCR approach. (see B.11.)

## B.12. Cloning of PCR products

### B.12.1. Cultivation and maintenance of bacterial cultures

#### B.12.1.1. Culturing and cell harvesting

a) Solutions

Culturing medium (LB-medium for *E. coli*)

Antibiotic stock solutions

c) Procedure:

Culturing of recombinant *E. coli* cells was done on solid media in plates and in liquid media at 37°C. 100 µg/ml Kan or Amp were previously added to the media to avoid growth of *E. coli* cells without plasmid. Inoculation of the liquid cell cultures was done under sterile conditions in the laminar flow by picking a single colony from the plates with a toothpick, which was then transferred to a test tube containing 5 ml medium. The inoculated liquid media were incubated at 37°C on an orbital shaker at 200 rpm overnight. For isolation of plasmids, cells were harvested by two centrifugation steps (2 ml culture each) in sterile ERTs (see B.10.4.).

#### B.12.1.2. Maintenance and storage

For short conservation single clones were transferred to LB-plates, referred to as "master plates", incubated overnight at 37°C or at RT (room temperature) and finally stored at 4°C.

For long term preservation, 700 µl of an ON-culture were transferred into a 1.5 ml screw cap and mixed with 300 µl 50% glycerol, or less of both, the cultures and 50% glycerol (according to a ratio of 2,3 to 1) were transferred into a well of a 96-well plate (Nunc™ Serving life science, Roskilde, Denmark). Microtiterplates were sealed with well caps (Nunc™ Serving life science, Roskilde, Denmark). The cells were then incubated at 4°C for 1 h and finally frozen at -80°C.

### B.12.2. Purification of PCR-products

#### B.12.2.1. Gel-purification

a) Solutions:

2% (w/v) Nusieve 3:1 agarose (low-melting-point) in 1 x TAE buffer

Loading buffer

DNA ladder (KbL)

SYBR® Green I staining solution

TE buffer

Tris 10 mM

EDTA 5 mM

pH was adjusted to 8.0 with HCl.

b) Procedure:

PCR-products mixed with loading dye were separated on a 2%-low-melting gel (made with 1 x TAE-buffer) with a current of 100 mA for about 90 min. in a gel electrophoresis chamber filled with 1 x TAE buffer. Low-melting gel was used for a better elution efficiency of DNA-fragments from the gel by heating. The gel was stained in SYBR Green I, which is less harmful to DNA (cross-linking) than EtBR-staining, for about one hour. Gel bands of the right height were excised from low-melting-point agarose gels with 50 µl glass capillaries and transferred to sterile ERTs. 80 µl TE-buffer was added and the samples – after spinning them down – put on a heating block (VWR international, West Chester, PA, USA) and melted at 80°C for 10 min.

PCR-products purified in this way were used for cloning (B.12.3.).

#### B.12.2.2. Purification by QIAquick® PCR Purification Kit

Purification under utilization of the QIAquick® PCR Purification Kit (QIAgen, Hilden, Germany) was done following the manufacturers` instructions. 20 - 30 µl of PCR-product were diluted in 125 µl PBI-buffer in the first step. In the final step the samples were eluted in 30 µl $H_2O_{bidest}$.

PCR-products used for sequencing were purified this way (B.14.).

### B.12.2.3. Purification by QIAquick® Gel Extraction Kit

a) Solutions:

1 x $TAE_{mod.}$

b) Procedure:

For previous gel-electrophoresis a gel was made by mixing appropriate amounts of agarose and 1 x $TAE_{mod.}$, staining was done with SYBR Green I (see 12.2.1.). Bands which had been cut out of the gel using a scalpel were further purified according to the manufacturers` instructions.

PCR-products purified in this way were used for another PCR (B.11.).

### B.12.3. Cloning of PCR products with the TOPO TA Cloning® kit

16S rDNA and crenarchaeotal *amoA*-clone libraries were constructed by cloning the respective PCR products into the vector pCR®II-TOPO (Table 18).

**Table 18:** Characteristics of the cloning vector

| Vector | Size | Properties | Reference |
|---|---|---|---|
| pCR®II-TOPO | 3973 bp | *ampR*, *kanR*, *lacZα*, T7 promotor | Invitrogen Corp., Carlsbad, CA, USA |

Both, Ampicillin and Kanamycin resistance is encoded on the TOPO TA vector. This makes it possible to select for cells which have taken up a plasmid during transformation by adding antibiotics to the media used for cell culturing. Furthermore the α-fragment of the enzyme β-galactosidase (lacZα) is encoded on the vector. This makes a blue/white-screening of transformed cells possible (see B.12.7. Identification of recombinant clones).

### B.12.4. Description of the used *E. coli* strain

Just one *E. coli* strain was used for cloning (see Table 19).

**Table 19:** Characteristics of the *E. coli* strain used for cloning

| Strain | Type of transformation | Genotype | Optimal T (°C) | Medium |
|---|---|---|---|---|
| *E.coli* TOP 10 | Chemical | F'[lacIq Tn10(tetR)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 λ- | 37 | LB |

### B.12.5. Ligation of gene amplificons into vectors

Poly-A-tails added by the Taq-polymerase to the amplificons during PCR (B.11.), so called "sticky ends", can be connected to single Poly-T overhangs of the vector by the enzyme topo-isomerase, which is already attached to the TOPO-TA vector. For cloning only PCR-products purified by gel-purification (B.12.2.1.) were used. After cutting and dissolving the gel pieces in TE-buffer by incubating for 10 min. at 80°C, 8 µl of the fluid was pipetted into a 0.2 ml ERT and 1µl of Salt Solution and 1µl of Vector (pCR®II) were added. The mix was centrifuged briefly and incubated for 15 min at RT before transformation.

### B.12.6. Transformation in recombinant cells

a) Solutions:

SOC-medium

b) Procedure:

All of the ligation reaction mixture (10 µl) was pipetted to chemical competent cells, which were then gently stirred before incubation on ice for 30 min. After 30 min. the cells got "heat-shocked" for exactly 30 sec. at 42°C in a water bath (Gesellschaft für Labortechnik GmbH, Burgwedel, Germany). The cells were put on ice again and 250 µl of SOC-medium were added to the competent cells followed by an incubation step on an orbital shaker (200 rpm) at 37°C for 1 h. This incubation step allows the shocked cells to recover and express antibiotic resistance. For each cloning reaction 100 and 150 µl of the incubated cells were then spread under sterile conditions (laminar flow) on two LB-plates containing either 100 µg/ml Kan or Amp. Previously, 40 µl X-Gal had been spread on each plate. Plates were incubated at 37°C overnight.

### B.12.7. Identification of recombinant clones

Due to the antibiotics within the LB-medium, only cells which have taken up a vector during transformation can grow (encoded Kan and Amp resistance; see Table 18). To distinguish between cells, which took up a vector with insert and those that took up a vector without insert, X-Gal is used. Insert positive clones can be identified by blue/white-screening. The screening is based on the fact that one fragment of the enzyme β-galactosidase, capable of cleaving X-Gal, is encoded on the vector (α-fragment), whereas the second fragment is encoded on the genome. Only expression of both fragments, leading to a complementation of

the two fragments to the whole enzyme β-galactosidase, allows cleavage of X-Gal whereby blue colouring is produced. As the insertion site on the vector lies within the coding region of the α-fragment, expression of this part of the enzyme after successful ligation of DNA into the vector is impossible. As a consequence, cells which took up a vector with insert cannot cleave X-Gal (white colonies).
White colonies were picked under sterile conditions (see B.12.1.1.) and transferred to a master plate. Picked clones were, after having inoculated the master plate, simultaneously used for a M13-screening PCR.

### B.12.8. Controlling insert size by M13-screening PCR

Cells which had been positive in so far that they took up a vector with insert, were now controlled for having the right insert by testing the size of the insert. As the multiple cloning site of the vector is flanked with primer binding sites for the M13-primer pair, a PCR with this primer pair will yield amplicons for all inserts even without knowledge of the insert sequence. PCR is performed with whole cells (without isolation of the plasmid), therefore the primary denaturation step is elongated to ensure cell lysis. Furthermore, no BSA was added and the final volume of each reaction mixture was reduced to 25 µl.
For one standard reaction the solutions were mixed to a final volume of 25 µl as follows:

| | |
|---|---|
| $MgCl_2$ | 2 µl |
| Buffer | 2.5 µl |
| dNTP – Mix | 2.5 µl |
| Forward primer | 0.125 µl |
| Reverse primer | 0.125 µl |
| Taq DNA Polymerase | 0.125 µl |
| $Aqua_{bidest.}$ | 17.625 µl |

Procedure:

The master mix was pipetted in cavities of a 96-well microtiterplate (25 µl/well) (MJ Research, Waltham, MA, USA), which was used for screening PCRs. With a sterile toothpick a colony was picked and resuspended in the respective well by stirring gently with the toothpick. The microtiterplate was sealed with a thermostable foil (MJ Research,Waltham, MA, USA) and the PCR was started under the conditions listed in Table 20. Details of the M13-primer pair can be seen in Table 16.

**Table 20:** Conditions for the amplification of cloned inserts with M13-primers (insert screening)

| PCR-step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Primary denaturation | 94 | 5-10 min. | 1 |
| **Denaturation** | **94** | **40 sec.** | **35** |
| **Annealing** | **60** | **40 sec.** | |
| **Elongation** [a] | **72** | **40 – 90 sec.** | |
| Final elongation | 72 | 10 min. | 1 |

[a] the elongation time was either 40 sec. or 90 sec. according to the size of the insert (~1,000 nt/min)

The PCR-product was analysed by separation on a 1% agarose gel, staining with EtBr and subsequent detection under UV-light. Clones with the right insert size were used for further analysis.

## B.13. Restriction fragment length polymorphism (RFLP)

RFLP is a method providing a rough overview of the sequence diversity within a clone library as sequences get cut by restriction endonucleases at specific recognition sites. Distances between those recognition sites are varying depending on the respective sequence. This is reflected in different lengths of DNA fragments for each sequence and can be seen as varying band patterns after separation by gel electrophoresis.

a) Solutions:

Restriction enzymes (MspI/RsaI/AluI)

Restriction buffers (Tango)

$H_2O_{bidest.}$

b) Procedure:

RFLP was done with the M13-PCR-products for both, 16S rRNA-gene libraries and crenarchaeotal amoA-gene libraries. Characteristics of the used enzymes can be seen in Table 21.

**Table 21:** Characteristics of enzymes used for RFLP

| Enzyme | Restriction site [a] | Buffer | Incubation temp. (°C) | Company | Application - Gene library [b] |
|---|---|---|---|---|---|
| MspI | C↓CGG | Tango | 37 | Fermentas Life Sciences Inc., Hanover, MD, USA | 16S rDNA |
| RsaI | GT↓AC | Tango | 37 | Fermentas Life Sciences Inc., Hanover, MD, USA | crenarchaeotal *amoA* |
| AluI | AG↓CT | Tango | 37 | Fermentas Life Sciences Inc., Hanover, MD, USA | crenarchaeotal *amoA* |

[a] arrow indicates site of restriction

[b] Application-Gene library : shows which enzymes were used for the analysis of which gene-specific diversity

Standard reaction mix (10 µl)

| | |
|---|---|
| Restriction enzyme (10 U/µl) | 1 µl |
| Buffer | 1 µl |
| $H_2O_{bidest.}$ | 3 µl |
| PCR-product | 5 µl |

The amount of ingredients was muliplied with the number of reactions and a master mix containing everything except the PCR-product was produced. 5µl of the master mix were then pipetted in the wells of a 96-well microtiterplate (MJ Research, Waltham, MA, USA) and the appropriate PCR-products were added. The reaction mix was incubated for 3 h at 37°C. After incubation the reaction was stopped by adding 5µl loading dye per cavity. The whole mixture could then directly be separated by gel electrophoresis (see B.10.7.2.). For each pattern the plasmid was isolated and subsequently sequenced. For patterns occurring more often, at least three plasmids were isolated and sequenced, as sequences producing identical restriction patterns are not necessarily the same.

### B.14. Sequencing

The applied DNA-sequencing technique is based on both, Sanger's di-deoxy mediated chain termination method (Sanger *et al.*, 1977) and Polymerase Chain Reaction (Saiki *et al.*, 1988). In Sanger`s sequencing approach basically elongation of one primer is performed by using dNTPs as well as di-deoxy-NTPs (ddNTPs), which lack the 3`-OH-group. Integration of ddNTPs happens by chance and leads to a termination of the elongation process because the polymerase cannot add further nucleotides without a free 3`-OH-group. Labelling of the four ddNTPs (ddATP, ddCTP, ddGTP and ddTTP) and using a thermostable DNA-polymerase allows DNA-sequencing in only one reaction. The differently labelled ddNTPs at the end of the DNA-fragments (varying in length) can be automatically detected after having been separated by length in capillaries. Sequencing was done with the DNA Sequencer Applied Biosystems 3130 either with primer TopoSeq-F or TopoSeq-R (see Tab.16). Sequences were exported in FASTA-format, which could then be imported into the software program ARB (Ludwig *et al.*, 2004),

## B.15. Sequence analyses

### B.15.1. Quick analysis of sequences using the search engine BLAST

Obtained sequences were compared to online databases to get a first impression of the taxonomic affiliation. This was done using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (NCBI, USA). The searching mechanism of this tool is based on specific algorithms with which similar sequences are found and ranked according to their relevance.

### B.15.2. Analysis of sequences using ARB

Phylogenetic analysis was done using the ARB software package (Ludwig 2004). ARB combines differrent programs and tools for construction and management of databases, comparative sequence analysis and calculation of phylogenetic trees. Trees reflecting phylogenetic relations of proteins can be calculated based on DNA as well as on basis of the inferred amino acid sequences. At the beginning of the analysis sequences were imported and aligned according to their homologous regions. Based on the alignment estimated evolutionary distances can be calculated in different ways and visualized as "trees". Differences between the sequences are then reflected in the distances between branches. Furthermore ARB can be used to design primers and probes.

#### B.15.2.1. Alignment of sequences

Imported sequences were firstly automatically aligned using the "fast aligner" tool of the ARB-editor. Automatic alignment was manually curated by taking into account the secondary structure, base matches within this secondary structure and visual comparison of the position of bases between similar sequences (of the same taxonomic group). Vector sequences were deleted. The sequence was proofread by comparing it to the chromatogram using the program ChromasPro (Technelysium Pty Ltd). Due to the length of the 16S rRNA-gene (~ 1500 bp), sequencing resulted in incomplete sequences (~ 800 – 1000 bp). Sequencing the same insert once with the forward and once with the reverse primer provides two parts of the same insert with a central overlap. These parts can be combined to a full sequence in the editor.

### B.15.2.2. Alignment and analysis of protein sequences

DNA-sequences of crenarchaeotal *amoA*-like genes were translated into amino acid sequences using the tool "Sequence-Perform Translation". The right reading frame was chosen by checking the amino acid-sequence for stop-codons, which are encoded by the base triplets "UAA", "UAG" and "UGA". As the crenarchaeotal *amoA*-gene is a functional gene and should be translated at some time, stop codons within the gene would indicate a pseudogene. Alignment of amino acid sequences was curated manually.

### B.15.3. Detection of chimeric sequences

To detect possible chimeric sequences generated during PCR, the base pair positions 1 - 513, 514 - 1026 and 1027 - 1539 (corresponding to *E. coli* positions; Brosius *et al.*, 1981) were independently subjected to phylogenetic analysis by using the tool "Parsimony interactive-Quick add marked", which adds sequences to an existing tree providing a rough phylogenetic affiliation. Adding just parts of the full sequence was done by using filters, with which defined regions of base pairs can be excluded from phylogenetic analysis. Sequences, whose fragments showed inconsistent phylogenetic affiliation, were interpreted as chimeras. Other programs used for detection of chimeric sequences were "chimera-check" (RDP) and Bellerophon, but the highest trust was set in the results by ARB.

## B.16. Phylogeny

Phylogenetic trees were calculated with the obtained sequences and selected reference sequences from the database, both for the 16S rRNA-gene and for the crenarchaeotal *amoA*-gene. To exclude highly variable regions so called "conservation filters" were used. For the 16S rDNA based trees "50% conservation" filters were used, meaning that only alignment positions conserved in at least 50% of sequences were considered (filter: nitrospirae_rr5_dec04). For trees based on crenarchaeotal *amoA*-genes, "terminal" filters were used, excluding additional bases at the beginning and at the end of the compared sequences. Furthermore, a filter excluding every third base position was used – this is because some amino acids can be encoded by different base triplets with varying third base position. Excluding the so called "wobble"-position will probably give more reliable results. To get a good phylogenetic resolution only reference sequences longer than 1400 bp were used for 16S rRNA phylogeny. Trees were calculated for reference sequences only and for a combination

of reference sequences and the ones obtained during this study – from these trees one consensus tree was constructed. For crenarchaeotal *amoA*-analysis 584 bp (when applying the terminal filters) were used (except 5 sequences, which were shorter than 606 bp; the shortest sequence was 365 bp) and a consensus trees was constructed. Partial sequences were added afterwards to calculated trees using the Parsimony interactive tool of ARB. For 16S rRNA-based tree calculations an outgroup (containing homologous but unrelated sequences) was incorporated. According to the position of the outgroup the trees were rooted manually. Trees based on crenarchaeotal *amoA* were calculated without an outgroup. The different treeing methods (Ludwig *et al.*, 1998) used are described and discussed in the following chapters.

### B.16.1. Neighbour joining (NJ)

Neighbour joining trees are, contrary to the other treeing methods, based on analysis of the data by distance matrices and construction of the trees by clustering based on sequence similarity. Dissimilarities between sequences are believed to reflect the number of evolutionary changes between those sequences. Therefore dissimilarities between all sequence pairs are summed up and used to build a distance matrix. The original sequences do not have any further importance in the algorithm, which is the major disadvantage of this method. Only visible base differences can be used ignoring the fact that the difference could be due to multiple substitution events, which might have even occurred without resulting in any now observable difference in the sequences. This can lead to an underestimation of difference. Furthermore, differing probabilities of transition and transversion events within substitutions and varying frequencies of the four bases (varying GC-content of different sequences or even between different genes) will lead to misinterpretations. Therefore various correction models can be used. One of the simplest models, which has been applied during this study, is the Jukes-Cantor correction model, including the probability of substitutions but assuming that all four bases are equally frequent and that there is no difference between transition and transversion events. No evolutionary models are involved in this treeing process. Still, NJ is a very fast construction method and thus often used.

### B.16.2. Maximum parsimony (MP)

The more time consuming maximum parsimony method is like maximum likelihood based on data analysis by discrete methods, which are considering each nucleotide site directly

(variable sites have less importance than conserved sites). Optimality methods are used to construct the trees. Thereby various trees are evaluated with regard to a certain model of evolution. As not all possible trees can be considered, a subset of all trees is searched for the tree explaining best the phylogenetic relations of the sequences and the optimal tree is "simply" expected to be part of the examined subset. The evaluation follows a "heuristic" search, starting at any tree and then rearranging it by altering the branches in order to improve its agreement with the theory of evolution. Maximum parsimony searches the tree with the fewest necessary base substitution events. Different substitution possibilities are included. A disadvantage of this method is that no branch lengths are calculated.

### B.16.3. Maximum likelihood (ML)

Here, the tree with the highest likelihood of representing the input data, with regard to the underlying evolution model, is searched. Anyway it does not give any clue about the actual probability that the underlying model is right. Distances between sequences are compared by looking at probabilities of sequences having certain bases at certain sites. This algorithm is calculating branch lengths reflecting evolutionary distances. Any evolution model can be applied, therefore different substitution events and unequal frequencies of bases can be incorporated.

### B.16.4. Consensus trees

To increase the reliability of phylogenetic relations of sequences, consensus trees were constructed. In these trees only branching patterns observed in all different treeing methods applied (NJ, MP, ML), were retained – branches differing between the trees were reassigned in multifurcations. Branch lengths of ML-trees were maintained. For 16S rRNA-trees, the tree topology of a consensus tree of all trees calculated with reference sequences, was considered to be most reliable as only sequences longer than 1400 bp were included. The consensus tree of all trees calculated including the shorter sequences obtained during this study, was adapted to the reference-consensus tree topology. Branch lengths of the ML tree including shorter sequences were maintained when constructing the overall consensus tree.

## B.17. Statistics

### B.17.1. Data editing

For subsequent statistical analysis but also to enable a better comparison of the conditions dominating in the different treatment plants, data was prepared as follows:
For all parameters, which were measured regularly, average and standard deviation were calculated. These average values were used for all further analysis. Parameters, which were measured only once, were ignored unless it was either T (temperature), pH, SA (sludge age), SVI (sludge volume index), DM (dry matter) and $O_2$ concentration. All values of this kind are marked in the respective Tables (Tab.26&27, p.76&79). Some parameters were combined to "overall parameters" or unified: Conductivity measured either in the influent, the effluent or within the treatment plants, depending on the different wwtps, were pooled. This means that regardless of where the measurement took place, the values are all given in the column "Conductivity" (see D.1.). For a comparison of the nitrogen concentrations the bacteria are exposed to, a parameter named "$N_{in}$" (mg/l) was established. For all conventional full-scale wwtps this parameter was created by summing up the parameters "$NH_4$", (or, if provided, "TKN"), $NO_3^-$ and $NO_2^-$ , all measured in the effluent (see D.1.). In case "total N" was provided for the effluent, these values were considered as equivalent to "$N_{in}$". "$N_{in}$" for conventional full-scale plants was additionally entitled as "inside conc." ("inside concentrations") (see D.1.). For all SBRs either "TKN" or "total N" in the influent were considered being equivalent to "$N_{in}$" and resembling "beginning concentrations" (see D.1.). For SBRs operated in the DIC-SBR-mode, "$N_{in}$" was modified in regard to C/N concentrations of the first loading event (see D.1.). Here, initial N concentrations were reduced by approx. 30%. "$N_{in}$" for DIC-SBRs were also regarded as "beginning concentrations" (see D.1.). COD- and BOD5-values measured in the effluent were considered as the concentrations resembling "inside concentrations" ("inside conc.") within the sludge basins of conventional full-scale wwtps. For SBRs incoming COD- and BOD5-values were taken into account, for DIC-SBRs initial values were reduced by approx. 50%. COD and BOD5 for SBRs and DIC-SBRs were considered as "beginning concentrations" (see D.1.).

### B.17.2. Tests

Spearman`s Correlation coefficient was calculated by using SPSS to find correlations between biological and physical data, among physical parameters or within biological data. For the calculations average values of the respective parameters in each wwtp were used.
Unfortunately the incompleteness of the dataset did not allow to apply any statistical test aimed at searching for a combination of factors explaining appearance, abundance or absence of the investigated nitrifying bacteria.

# C. Results

## C.1. Diversity in wastewater treatment samples as revealed by FISH

To avoid misinterpretations, samples were examined for false positive signals caused by unspecific binding of probes to non-targets or autofluorescence (AF). The sample from Kraftisried showed high AF by laser excitation at 543 nm. This could be due to the addition of lime water for pH-regulation (Daims; personal communication). A sample of another plant (Weißtal), in which lime is added as well, did not exhibit this problem. While in the wwtp in Kraftisried lime was continuously added in the time before sampling, this is not known for the wwtp in Weißtal. Possibly no lime was added shortly before the sample was taken. As far as possible positive controls in form of pure cultures or already characterized samples were used.

### C.1.1. AOB

All samples were first of all investigated with a standard set of probes to allow detection of the in wwtps most important groups (see B.9.2.1.). All bacteria targeted with Nolimar712 but not with Cluster6a-192 were expected to be related to members of the *N. marina*-Cluster as Nolimar712 is detecting the whole so far known Cluster 6, comprising *N. marina*-like (Cluster 6b) and *N. oligotropha*-like (Cluster 6a) AOB. According to the multiple probe concept positive signals were confirmed by applying either the probe Nso190, targeting most AOB, or the probe Bet42a, detecting all *β-Proteobacteria*, and EUBmix (targeting all bacteria).

As visible in Table 22, only one sample (Rapp-Kutzenhausen) did not give any signal for AOB and *β-Proteobacteria*. However, bacteria could be detected by using the EUBmix. In all other samples *N. oligotropha*-like bacteria were seen. The abundance of members of Cluster 6a was estimated and categorized in “no-”, “few-”, “some-” and “many - Cluster 6a”. According to these categories only few bacteria related to Cluster 6a were found in samples from Huntlosen, Rosenheim, TBA GZM Lyss and Oberding, some in the plants in Kraftisried, ARA Lyss, Ingolstadt, Ampfing, Altmannstein, Langenzenn, Seefeld, Deuz and Weißtal and many in Plattling, Waldsassen, Radeburg, Bad Zwischenahn, Bruchmühlen, Hettstedt and Spenge. Halophilic and –tolerant AOB were detected in the plants in Plattling, Rosenheim, Ingolstadt, Radeburg and Hettstedt. Additionally, in the sample from Ingolstadt bacteria

related to the *Nc. mobilis*- and in Rosenheim AOB related to the *N. communis*-Cluster were detected..

**Table 22:** Results of screening for various AOB by FISH

| Treatment plant | type of reactor | type of waste water | halo.AOB[a] | amount Cl.6a[b] | N.com.-Cl.[a] | env.Cl.6a[a] | *Nc.mobilis** |
|---|---|---|---|---|---|---|---|
| Kraftisried | conventional wwtp | animal rendering | - | ++ | - | - | - |
| Plattling | conventional wwtp | animal rendering | + | +++ | - | - | - |
| Oberding | conventional wwtp | animal rendering | - | + | - | - | - |
| TBA GZM Lyss | conventional wwtp, membrane filtration | animal rendering | - | + | - | + | - |
| ARA Lyss | conventional wwtp, fixed bed | Municipal | - | ++ | - | - | - |
| Rosenheim | SBR | Municipal | + | + | + | - | - |
| Rapp-Kutzenhausen | SBR | Brewery | - | - | - | - | - |
| Ingolstadt | SBR | Sludge drainiage | + | ++ | - | + | + |
| Waldsassen | SBR | Industrial | - | +++ | - | - | - |
| Ampfing | SBR | animal rendering, milk production | - | ++ | - | - | - |
| Altmannstein | SBR | Municipal | - | ++ | - | - | - |
| Langenzenn | SBR | Municipal | - | ++ | - | - | - |
| Seefeld | SBR | Municipal | - | ++ | - | - | - |
| Radeburg | DIC-SBR | Municipal | + | +++ | - | - | - |
| Bad Zwischenahn | DIC-SBR | municipal, industrial influence | - | +++ | - | - | - |
| Deuz | DIC-SBR | Municipal | - | ++ | - | - | - |
| Bruchmühlen | DIC-SBR | Municipal | - | +++ | - | - | - |
| Weißtal SBR | DIC-SBR | Municipal | - | ++ | - | - | - |
| Huntlosen | DIC-SBR | Municipal | - | + | - | - | - |
| Hettstedt | DIC-SBR | municipal, external faecal sludge | + | +++ | - | - | - |
| Spenge | DIC-SBR | Municipal | - | +++ | - | - | - |

[a] - = not detected/ + = detected

[b] estimated amount of Cluster 6a-like organisms, in the categories: -(not detected), +(few), ++(some), +++(many)

In two samples, Ingolstadt and Oberding, signals were obtained when applying the probe Nolimar712, which did not overlap with signals of the probe Cluster6a-192. These samples, as well as all other samples, did not show any signal with the probe targeting *N. marina*-like AOB. Organisms, exhibiting this hybridization pattern have been considered as representatives of an "environmental Cluster 6a", referring to a previous study (Anneser, 2004; see D.2.2.), though this assumption was not confirmed in the present study. Whereas members of Cluster 6a occured in all samples harbouring AOB, other AOB occurred only in few plants. The wwtps in Ingolstadt and Rosenheim seemed to harbour a quite diverse community of AOB.

### C.1.2. NOB

All samples were firstly investigated with a standard set of probes to screen for the in wwtps most important NOB (see B.9.2.1.). *Nitrospira*-like NOB could be detected in all samples but Rapp-Kutzenhausen, Langenzenn and Bad Zwischenahn. Wherever members of *Nitrospira* were detected, *Nitrospira* Cluster I like bacteria (which will from now on simply be addressed as "Cluster I") could be found apart from one sample taken from Ampfing, whereas *Nitrospira* Cluster II-like bacteria (referred to as "Cluster II") could not be found in samples from ARA Lyss, Waldsassen, Seefeld, Radeburg, Bruchmühlen and Spenge. "Cluster II" was less represented in the samples and only found in 12 of 18 plants, in which *Nitrospira*-like organisms were generally present (Tab. 23).
To look for bacteria affiliated to the genus *Nitrospira* apart from "Cluster I" and "Cluster II", a mix of the probes specific for these two clusters was hybridized simultaneously in combination with the genus-specific probe. Total overlap of the probes indicated absence of further *Nitrospira*-like bacteria whereas signals with the genus-specific probe, but without the "Cluster I and II-mix" suggested the presence of "other *Nitrospira*"-like populations. Those samples, taken from Oberding, Rosenheim, Ingolstadt, Ampfing and Hettstedt, were subsequently screened for *Nitrospira*-Cluster IV-like organisms (marine representatives), but none could be detected, supporting the assumption of "other *Nitrospira*"-like populations, which will from now on be addressed as "other *Nitrospira*". In all but one (Ampfing) of the samples harbouring "other *Nitrospira*", "Cluster I" and "Cluster II" could be found as well. Therefore these four samples (from Oberding, Hettstedt, Rosenheim and Ingolstadt) comprised a higher diversity of *Nitrospira*-like organisms than the other plants (Tab. 23). The latter two plants also showed a relatively high diversity of AOB. *Nitrobacter*-like NOB could not be detected in any of the investigated samples (see Tab. 23).

### C.1.3. Further investigations

Especially samples, in which no NOB could be detected by FISH during the standard hybridizations, were screened for the presence of anammox-bacteria (Amx368). In all samples examined in this respect, namely the samples from Rapp-Kutzenhausen, Langenzenn, Bad Zwischenahn, as well as in Weißtal, Spenge, Deuz and Hettstedt, no signals were obtained with the anammox-specific probe. The first three plants, without *Nitrospira*- and *Nitrobacter*-like NOB, were further screened for *Nitrococcus mobilis* (Ntococ 84), *Nitrospina gracilis* (Ntspn 693) and "*Candidatus* Nitrotoga arctica" (NTG 840). No signals were obtained for the

first two, but signals were obtained for "*Candidatus* Nitrotoga arctica" in the sample taken from Bad Zwischenahn. These signals overlapped with signals of the probe specific for the hierarchically higher *β–Proteobactera* (Bet42a), supporting the result. However, it can only be seen as indication of an occurrence of these NOB since the appropriate FA concentration had not been determined yet and therefore false positive signals could not be ruled out. This was the reason for excluding the result from further data analysis.

**Table 23**: Results of screening for various *Nitrospira*-lineages by FISH

| Treatment plant | type of reactor | type of waste water | Cl.I *Ntspa*[a] | Cl.II *Ntspa*[a] | „other" *Ntpsa*.[a] |
|---|---|---|---|---|---|
| Kraftisried | conventional wwtp | animal rendering | + | + | - |
| Plattling | conventional wwtp | animal rendering | + | + | - |
| Oberding | conventional wwtp | animal rendering | + | + | + |
| TBA GZM Lyss | conventional wwtp, membrane filtration | animal rendering | + | + | - |
| ARA Lyss | conventional wwtp, fixed bed | municipal | + | - | - |
| Rosenheim | SBR | municipal | + | + | + |
| Rapp-Kutzenhausen | SBR | Brewery | - | - | - |
| Ingolstadt | SBR | sludge drainiage | + | + | + |
| Waldsassen | SBR | municip.+industrial | + | - | - |
| Ampfing | SBR | animal rendering, milk producition | - | + | + |
| Altmannstein | SBR | municipal | + | + | - |
| Langenzenn | SBR | municipal | - | - | - |
| Seefeld | SBR | municipal | + | - | - |
| Radeburg | DIC-SBR | municipal | + | - | - |
| Bad Zwischenahn | DIC-SBR | municipal, industrial influence | - | - | - |
| Deuz | DIC-SBR | municipal | + | + | - |
| Bruchmühlen | DIC-SBR | municipal | + | - | - |
| Weißtal SBR | DIC-SBR | municipal | + | + | - |
| Huntlosen | DIC-SBR | municipal | + | + | - |
| Hettstedt | DIC-SBR | municipal, external faecal sludge | + | + | + |
| Spenge | DIC-SBR | municipal | + | - | - |

[a] - = not detected/ + = detected; Cl. I *Ntspa* = "Cluster I" detected; Cl. II *Ntspa* = "Cluster II" detected
"other *Ntspa*." = "other *Nitrospira*" detected

## C.2. Abundance of *Nitrospira*-like NOB in the samples revealed by quantitative FISH

In all samples, showing presence of *Nitrospira*-like bacteria, the percentage of all *Nitrospira*-like organisms relative to all bacteria was determined (referred to as abundance of the "genus *Nitrospira"*), applying FISH and digital image analysis (Tab. 24). Further, the percentage of

each, “Cluster I” and “Cluster II”, relative to the total abundance of *Nitrospira*-like organisms, was determined (Tab. 24).

**Table 24:** Abundances of the genus *Nitrospira*, “Cluster I” and “Cluster II” as well as the sum of “Cluster I+II”, for each sample

| Treatment plant | type of reactor | type of waste water | *Ntspa*(%) | Std. Error | Cl.I *Ntspa*(%) | Std. Error | Cl.II *Ntspa*(%) | Std. Error | Cluster I + Cluster II (%) |
|---|---|---|---|---|---|---|---|---|---|
| Kraftisried | conv.wwtp | animal rendering | 4.0 | 1.2 | 14.9 | 2.8 | 85.2 | 3.5 | 100.1 |
| Plattling | conv.wwtp | animal rendering | 3.3 | 0.3 | 37.8 | 3.5 | 64.3 | 5.8 | 102.1 |
| Oberding | conv.wwtp | animal rendering | 3.4 | 0.2 | 22.6 | 3.3 | 77.6 | 4.1 | 100.2 |
| TBA GZM Lyss | conv.wwtp membrane filtration | animal rendering | 4.8 | 0.9 | 80.6 | 6.6 | 12.4 | 4.0 | 93.0 |
| ARA Lyss | conv.wwtp fixed bed | Municipal | 1.2 | 1.3 | 100[a] | - | 0 | - | 100 |
| Rosenheim | SBR | Municipal | 8.4 | 2.2 | 4.6 | 2.8 | 62.3 | 10.2 | 66.9 |
| Rapp-Kutzenhausen | SBR | Brewery | 0 | - | 0 | - | 0 | - | 0 |
| Ingolstadt | SBR | sludge drainiage | 16.5 | 1.9 | 81.9 | 6.2 | 3.3 | 1.8 | 85.2 |
| Waldsassen | SBR | municip.+Indus. | 3.4 | 0.9 | 100[a] | - | 0 | - | 100 |
| Ampfing | SBR | animal rendering, milk production | 3.1 | 1.1 | 0 | - | 92.6 | 2.7 | 92.6 |
| Altmannstein | SBR | Municipal | 0.1 | 0.1 | 88.5 | 10.5 | 13.6 | 9.2 | 102.1 |
| Langenzenn | SBR | Municipal | 0 | - | 0 | - | 0 | - | 0 |
| Seefeld | SBR | Municipal | 1.6 | 0.2 | 100[a] | - | 0 | - | 100 |
| Radeburg | DIC-SBR | Municipal | 2.8 | 0.8 | 100[a] | - | 0 | - | 100 |
| Bad Zwischenahn | DIC-SBR | municipal, ind. influence | 0 | - | 0 | - | 0 | - | 0 |
| Deuz | DIC-SBR | Municipal | 14.1 | 5.0 | 61.7 | 5.5 | 31.9 | 5.9 | 93.6 |
| Bruchmühlen | DIC-SBR | Municipal | 4.4 | 1.1 | 100[a] | - | 0 | - | 100 |
| Weißtal SBR | DIC-SBR | Municipal | 3.3 | 0.7 | 83.9 | 2.6 | 9.6 | 2.5 | 93.5 |
| Huntlosen | DIC-SBR | Municipal | 1.3 | 0.6 | 53.2 | 7.1 | 36.9 | 8.0 | 90.1 |
| Hettstedt | DIC-SBR | municipal, external faecal sludge | 2.3 | 0.4 | 80.4 | 2.2 | 3.3 | 2.5 | 83.7 |
| Spenge | DIC-SBR | Municipal | 0.2 | 0.1 | 100[a] | - | 0 | - | 100 |

conv.wwtp = conventional wastewater treatment plant; *Ntspa* = genus *Nitrospira*

Std. error = standard error of quantification as given by the program daime (digital image analysis)

[a] not quantified but assumed based on qualitative FISH-results (In case only “Cluster I” or “Cluster II” and no “other *Ntpsa*” have been detected, the abundance of the found Cluster makes up 100%); Variation coefficient and congruency for each series of digital image analysis can be seen in Tab.37 (appendix)

The abundance of *Nitrospira*-like bacteria relative to all bacteria varied from 0.1% in the sample taken from Altmannstein (apart from samples with no *Nitrospira*) to a maximum of 16.5% (SBR Ingolstadt). In most of the plants, *Nitrospira* accounted for less than 5%. Only in the SBR in Ingolstadt (16.5%), Deuz (14.1%) and Rosenheim (8.4%) *Nitrospira*-like bacteria constituted more than 5%. Less than 2% were found in samples from the conventional plant ARA Lyss and the SBRs in Altmannstein, Seefeld, Huntlosen and Spenge. The standard error

of the genus-specific quantification was quite high for the samples from ARA Lyss, Ampfing and Deuz, Huntlosen and Spenge (standard error > a third of the actual value). Congruency of the signals ranged from 79% to 99% (see Tab.36, appendix). In 11 of the 18 plants, in which *Nitrospira*-like organisms were detected, "Cluster I and II" co-occurred. In six of these plants "Cluster I" dominated (more than 60%) over "Cluster II" and in four plants "Cluster II" dominated over "Cluster I". Three of the latter are animal rendering conventional treatment plants. In one of the samples (Huntlosen), "Cluster I" accounted for more than 50%, but the standard error of both, the quantifications of "Cluster I" and "Cluster II", was high (7.1% and 8%). Standard errors were also high for the quantifications of "Cluster I and II" in the sample from Altmannstein (10.5% and 9.2%).

If no "other *Nitrospira*" than "Cluster I" and "Cluster II" could be found in the sample, quantification of "Cluster I" and "Cluster II" was believed to sum up to approx. 100% - a deviation up to 10% was tolerated (see D.2.). In the samples from Rosenheim, Ingolstadt and Hettstedt "Cluster I and II" accounted together for 66.9%, 85.2% and 83.7%, respectively. This supports the assumption of "other *Nitrospira*" in those plants. In the two remaining plants expected to harbour "other *Nitrospira*", members of *Nitrospira*-Cluster I and II nevertheless accounted for around 100% (Oberding) and 93% (Ampfing). Since for the sample from the wwtp in Oberding only few very small cell clusters were identified as potential "other *Nitrospira*"-population, the quantification result is not contradictory to the assumption based on qualitative FISH. Less than 80% congruency of the images was calculated for the picture series taken for the quantification of "Cluster I or II" against all *Nitrospira*-like bacteria for the samples taken from Plattling, Oberding, Ingolstadt, Ampfing, Rosenheim, Deuz, Spenge and Weißtal. More detailed information is given in Tab.36 (see appendix).

Table 25 shows the abundance of "Cluster I" and "Cluster II" related to all bacteria (adressed as Cl.I *Ntspa*B and Cl.II *Ntspa*B). These values were calculated according to the formula shown in Equation 1.

Cl. I or II *Ntspa*B(%) of all bacteria = *Ntspa*(%) / 100 * Cl. I or II *Ntspa*(%)

**Equation 1:** Cl. I or II *Ntspa*B(%) = abundance of "Cluster I" or "Cluster II" relativ to all bacteria
*Ntspa*(%) = percentage of all *Nitrospira*-like bacteria within each sample
Cl. I or II *Ntspa*(%) = percentage of *Nitrospira* -Cluster I-like or Cluster II-like bacteria relative to all *Nitrospira*-like organisms

**Table 25:** Abundances of "Cluster I and II" relative to all bacteria (Cl.I and II*Ntspa*B) and relative to all *Nitrospira* (Cl. I and II *Ntspa*)

| Treatment plant | type of reactor | type of waste water | Cl.I *Ntspa*B (%) | Cl.II *Ntspa*B (%) | *Ntspa* (%) | Cl.I *Ntspa*(%) | Cl.II *Ntspa*(%) | ratio Cl.I to Cl.II |
|---|---|---|---|---|---|---|---|---|
| Kraftisried | conv. wwtp | animal rendering | 0.6 | 3.4 | 4.0 | 14.9 | 85.2 | 1:5.7 |
| Plattling | conv. wwtp | animal rendering | 1.2 | 2.1 | 3.3 | 37.8 | 64.3 | 1:1.7 |
| Oberding | conv. wwtp | animal rendering | 0.8 | 2.6 | 3.4 | 22.6 | 77.6 | 1:3.4 |
| TBA GZM Lyss | conv. wwtp, membrane filtration | animal rendering | 3.9 | 0.6 | 4.8 | 80.6 | 12.4 | 1:0.15 |
| ARA Lyss | conv. wwtp, fixed bed | Municipal | 1.2 | 0 | 1.2 | 100* | 0 | - |
| Rosenheim | SBR | Municipal | 0.4 | 5.2 | 8.4 | 4.6 | 62.3 | 1:13.5 |
| Rapp-Kutzenhausen | SBR | Brewery | 0 | 0 | 0 | 0 | 0 | - |
| Ingolstadt | SBR | sludge drainiage | 13.5 | 0.5 | 16.5 | 81.9 | 3.3 | 1:0.04 |
| Waldsassen | SBR | municip.+ industrial | 3.4 | 0 | 3.4 | 100* | 0 | - |
| Ampfing | SBR | animal rendering, milk production | 0 | 2.9 | 3.1 | 0 | 92.6 | - |
| Altmannstein | SBR | Municipal | 0.1 | 0 | 0.1 | 88.5 | 13.6 | 1:0.15 |
| Langenzenn | SBR | Municipal | 0 | 0 | 0 | 0 | 0 | - |
| Seefeld | SBR | Municipal | 1.6 | 0 | 1.6 | 100* | 0 | - |
| Radeburg | DIC-SBR | Municipal | 2.8 | 0 | 2.8 | 100* | 0 | - |
| Bad Zwischenahn | DIC-SBR | municipal, industrial influence | 0 | 0 | 0 | 0 | 0 | - |
| Deuz | DIC-SBR | Municipal | 8.7 | 4.5 | 14.1 | 61.7 | 31.9 | 1:0.52 |
| Bruchmühlen | DIC-SBR | Municipal | 4.4 | 0 | 4.4 | 100* | 0 | - |
| Weißtal SBR | DIC-SBR | Municipal | 2.8 | 0.3 | 3.3 | 83.9 | 9.6 | 1:0.1 |
| Huntlosen | DIC-SBR | Municipal | 0.7 | 0.5 | 1.3 | 53.2 | 36.9 | 1:0.7 |
| Hettstedt | DIC-SBR | municipal, external faecal sludge | 1.8 | 0.1 | 2.3 | 80.4 | 3.3 | 1:0.04 |
| Spenge | DIC-SBR | Municipal | 0.2 | 0 | 0.2 | 100* | 0 | - |

conv. wwtp = conventional wastewater treatment plant

abundance of the genus *Nitrospira* to all bacteria (*Ntspa*); ratio Cl. I to Cl.II (ratio of Cl.I *Ntspa* to Cl.II *Ntspa*)

* not quantified but assumed based on qualitative FISH-results (If only "Cluster I or II" were detected and no "other *Ntpsa*" occurred, the abundance of the found Cluster makes up 100%.)

Variation coefficient and congruency for each series of digital image analysis can be seen in Tab.37 (appendix)

## C.3. Nitrifiers-diversity and abundance revealed by FISH and characteristics of plants

### C.3.1. Characteristics of the different treatment plants

Conductivity in the different treatment plants varied between approximately 400 µS/cm and 5000 µS/cm – the highest values (3000 - 5000 µS/cm) occured in the four animal rendering plants, for which conductivity measurements were available. These plants are also conventional full-scale treatment plants. The conductivity for the only conventional plant which is not an animal rendering plant, was lower (1000 µS/cm). For the SBRs treating partly wastewater from butcheries (Ampfing, Waldsassen), no conductivity measurements were

available. The SBR in Rapp-Kutzenhausen also showed a high conductivity (1900 µS/cm) compared to other values of SBRs. At least for all the SBRs operated in a DIC-mode, conductivity levels seemed to vary strongly. Conductivity also seems to vary a lot between night and day (Holm, personal communication).

Overall, COD-values varied between 30 and approx. 1800 mg/l. In conventional plants the COD (named "inside-conc. COD") varied between 30 (Plattling) and approx. 46 mg/l (Kraftisried) whereas for SBRs the COD (named "beginning-conc. COD") ranged from approx. 300 (Deuz) to 1800 mg/l (Rapp-Kutzenhausen). The high discrepancies between COD-values of conventional wwtps and SBRs reflect that these values do represent different situations in the reactors (this is a rather constant concentration in conventional wwtps and the initial concentration of a concentration gradient in SBRs). Thus COD-values did not seem to be comparable between conventional wwtps and SBRs (see D.1.1.-D.1.3.). The same is true for BOD5-values: for conventional plants "inside-conc. BOD5"-values ranged from 5 (Plattling, Oberding, TBA GZM Lyss) to 12 mg/l (Kraftisried), for SBRs "beginning-conc. BOD5"-values varied from around 100 (Seefeld) to 1250 mg/l (Rapp-Kutzenhausen). Unlike for conductivity, values of the sample taken from the ARA Lyss (municipal but conventional plant) were in the range of the other conventional full-scale treatment plants and values of the samples taken from Ampfing and Waldsassen were in the range of the other SBRs, thus indicating that these discrepancies were not observable between animal rendering and non-animal rendering plants, but between conventional plants and SBRs. "Inside-conc. N" of conventional plants varied from 5.5 (Plattling, Oberding) to 45.6 mg/l (Kraftisried) and "beginning-conc. N" for SBRs varied between approx. 20 (Waldsassen) and 160 mg/l (Radeburg). For this last parameter there existed no high discrepancy between the two main types. Looking at ratios describing the nutrient-availability in the respective wastewater of the various plants, for both, the COD/BOD5-ratio and also the BOD5/N-ratio, a high discrepancy could be seen between the two design types of plants again, due to the previously described differences of COD and BOD5-values for conventional- and sequencing batch reactors. The "inside-ratio COD/BOD5" for conventional plants ranged from approx. 4 (Kraftisried) to 7.5 (ARA Lyss) and for SBRs the "beginning-ratio COD/BOD5" was in between approx. 1.4 (Ampfing) and 2.2 (Altmannstein).

Unlike the previously discussed parameters, temperature, pH and dry matter have been compared between conventional plants and SBRs. Temperatures in the plants ranged from 7 (Kraftisried) to approx. 38°C (Rosenheim). The pH, which is often regulated in wwtps, varied from 6.4 (Waldsassen) to 7.8 (ARA Lyss). The lowest amount of dry matter was 2.2 g/l

(Altmannstein), the highest 9.5 g/l (Ingolstadt). The Index of Sludge Volume (ISV) was only available for SBRs and ranged from 45.8 ml/g (Ingolstadt) to 200 ml/g (Rosenheim). The ratio of loading time ($t_F$) to reaction time ($t_R$) is describing different operational strategies within SBRs – here data were available for all but one DIC-SBR and varied between < 0.2 (Radeburg) and 0.4 (Deuz). All data can be seen in Tab.27 (p.79).

For most plants minimum and maximum $O_2$ concentrations were given, which did not seem appropriate for further analysis (Tab. 26). SA (SRT) was quite similar between the plants (but given in large intervals), ranging from around 13 (Waldsassen) to a potential maximum of 50 days (d). In all conventioal reactors the SRT ranged from 20 to 50 d. Overall, in most plants the sludge stayed around 20 d within the system. Most of the SRT listed in Tab. 26 were not determined during the investigated period of time, but are "general averaged values" resembling averages determined during a longer period of time like annual averages and "long-term experience" (see B.17.1., p.66).

**Table 26:** Oxygen-concentrations and sludge ages of the various plants

| Treatment Plant | type of reactor | Inside average O2 (mg/L) | st.dev. O2 (mg/L) | SRT (d) |
|---|---|---|---|---|
| Kraftisried | 3 | 0.0-10 | | >20-50[a] |
| Plattling | 3 | 0-1.5 | | >20-50[a] |
| Oberding | 3 | 0-2 | | >20-50[a] |
| TBA GZM Lyss | 5 | 0-3 | | >20-50[a] |
| ARA Lyss | 4 | 8 | | >20-50[a] |
| Rosenheim | 1 | 1.8 | 0.5 | 30 |
| Rapp-Kutzenhausen | 1 | 1.5-2.5 | | |
| Ingolstadt | 1 | 1.8[a] | 1.72 | |
| Waldsassen | 1 | 2-3.7 | 1 | 12.9 |
| Ampfing | 1 | 2.7 | 0.2 | 21.8 |
| Altmannstein | 1 | 0.1-2.9 | | |
| Langenzenn | 1 | 1-2 | | 15 |
| Seefeld | 1 | 0-3 | 1.6 | |
| Radeburg | 2 | | | 25 |
| Bad Zwischenahn | 2 | 1.3 | 0.1 | ~20[a] |
| Deuz | 2 | 0.6 | 0.17 | ~20[a] |
| Bruchmühlen | 2 | 0.32 | 0.11 | ~20[a] |
| Weißtal SBR | 2 | | | ~20[a] |
| Huntlosen | 2 | 0.1-1.28 | | ~20[a] |
| Hettstedt | 2 | | | ~20[a] |
| Spenge | 2 | 0.4 | 0.07 | ~20[a] |

empty caskets = not detected; st.dev.=standard deviation; [a] "general averaged values"

type of reactor: 1 = SBR / 2 = DIC-SBR / 3 = conventional wwtp / 4 = conventional wwtp, fixed bed/ 5 = conventional wwtp, membrane filtration

### C.3.2. Correlation between diversity and abundance of different nitrifiers

#### C.3.2.1. Determination of diversity

One aspect investigated is the diversity of nitrifiers within the samples. For AOB, diversity was determined based on the occurrence of members of the main clusters (as the number of different AOB-clusters present):

- *N. oligotropha*-Cluster 6a (as indicated by positive signals of the probe Cluster6a-192)
- *N. communis*-Cluster (as indicated by positive signals of the probe Ncom1025)
- *N.europaea/Nc.mobilis*-Cluster (as indicated by pos. signals of probes NEU or Ncmob)
- *N. marina*-Cluster 6b (as indicated by positive signals of the probe Nmar830)
- *Nitrosospira*-Cluster (as indicated by positive signals of the probe Nsv443)

The number of different AOB-clusters of all samples ranged from 0 to 3 as no representatives of the *N. marina*-Cluster and the *Nitrosospira*-Cluster could be detected in any sample.

NOB diversity was solely based on the occurrence of *Nitrospira*-clusters as members of the genera *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrotoga* (not confirmed), were not detected (and since occurrence of the latter three genera was not investigated for all samples):

- Cluster I (as indicated by positive signals of the probe Ntspa1431)
- Cluster II (as indicated by positive signals of the probe Ntspa1151)
- "other *Nitrospira*" (as indicated by genus-specific signals (Ntspa662) without overlap with Ntspa1431+ Ntspa1151+Ntspa620)

The number of NOB-clusters of all samples ranged from 0 to 3. Furthermore, diversity was determined as the sum of AOB- and NOB-clusters (possible number of clusters: 0 - 6).

#### C.3.2.2. Correlation with the diversity of AOB- and NOB-clusters

In Tab.28, samples and the respective collected biological data are ordered according to the number of different AOB-clusters. In the sample Rapp-Kutzenhausen, in which no AOB could be found, no NOB were found either. There seemed to be hardly any interrelations between the different occurrence and abundance of clusters: Co-occurence of members of the *Nitrospira*-clusters I and II ("Cl. I+II") did for instance not seem to be related to the diversity of AOB-clusters. Nor did samples with higher AOB-diversity show a greater abundance (%) of *Nitrospira*-like bacteria. A trend linking the abundance of *N. oligotropha*-like bacteria to

the number of different AOB-clusters could not be observed either. Occurrence of halophilic and –tolerant AOB did not seem to coincide with a greater abundance of either "Cluster I or Cluster II" of all *Nitrospira*-like organisms. Yet in 3 of the 5 samples harbouring "other *Nitrospira*", halophilic and -tolerant AOB were found as well (see C.3.3. and D.5.5. for a further discussion). This trend was also visible considering only SBRs since 4 of the 5 plants harbouring "other *Nitrospira*" were SBRs and in 3 of those 4, both "other *Nitrospira*" and halophilic and –tolerant AOB were present. The abundances of "Cluster I or Cluster II" relative to all *Nitrospira*-like bacteria were not correlated with the occurrence of halophilic and –tolerant AOB in SBRs either. Tab.29 shows samples and biological data arranged in accordance to the number of different NOB-clusters. An ascending diversity of AOB-clusters with the increasing number of NOB-clusters is indicated considering all plants or only SBRs.

### C.3.2.3. Correlation with the abundance of *Nitrospira*-like bacteria

In Tab.30 samples are ordered according to the abundance of the genus *Nitrospira* in the samples. There seemed to be no interrelation between appearance and abundance of certain clusters with the abundance of *Nitrospira*-like bacteria. In the four samples considering all wwtps or in the three samples taking only SBRs into account, which showed the highest amounts of the genus *Nitrospira* (4,8% TBA GZM Lyss; 8,4% SBR Rosenheim; 14,1% SBR Deuz and 16,5% SBR Ingolstadt), both, *Nitrospira*-Cluster I-like organisms and *Nitrospira*-Cluster II –like organisms were present, as well as in the sample taken from the SBR in Altmannstein, which exhibited the lowest abundance of *Nitrospira*-like organisms (Table 30).

**Table 27:** Physical parameters of the different treatment plants

| Treatment Plant | type of reactor | average Cond. (µS/cm) | beginning conc.[a] average COD (mg/l) | inside conc. average COD (mg/l) | beginning conc.[a] average BOD5 (mg/l) | Inside-conc. Average BOD5 (mg/l) | Beginning ratio [a] COD/ BOD5 | inside ratio COD/ BOD5 | beginning conc.[a] Average „Nin“ (mg/l) | inside-conc. average „Nin“ (mg/l) | beginning ratio.[a] BOD5/ „Nin“ | inside-ratio BOD5/ „Nin“ | Inside Average T (°C) | inside average pH | average DM (g/l) | average ISV (ml/g) | tF/tR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kraftisried[b] | 3 | 5000 | | 46.3 | | 12 | | 3.9 | | 45.6 | | 0.26 | 7 | 7.3 | 5 | | |
| Plattling[b] | 3 | 4000 | | 30 | | 5 | | 6 | | 5.5 | | 0.91 | 30 | 7 | 6.5 | | |
| Oberding[b] | 3 | 4000 | | 35 | | 5 | | 7 | | 5.5 | | 0.91 | 26 | 6.8 | 3.5 | | |
| TBA GZM Lyss [b] | 5 | 3000 | | 35 | | 5 | | 7 | | 15.5 | | 0.32 | 30 | 7 | 7 | | |
| ARA Lyss | 4 | 1000 | | 45 | | 6 | | 7.5 | | 20.1 | | 0.3 | 12 | 7.8 | | | |
| Rosenheim | 1 | | | | | | | | | | | | 38.2 | 7.7 | 8.2[c] | 200 | |
| Rapp-Kutzenhausen | 1 | 1900 | 1797 | | 1250 | | 1.44 | | 55 | | 22.7 | | | 7.4 | 5.6 | 68.3 | |
| Ingolstadt | 1 | | | | | | | | | | | | 27.4 | 7.2 | 9.5[c] | 45.8[c] | |
| Waldsassen([b]) | 1 | | 410 | | 235 | | 1.74 | | 18.5 | | 12.7 | | 9 | 6.4 | 3.4 | 132.3 | |
| Ampfing([b]) | 1 | | 875.4 | | 648.2 | | 1.35 | | 88.7 | | 7.3 | | | | 5.3 | 102.4 | |
| Altmannstein | 1 | | 443.7 | | 206.7 | | 2.15 | | | | | | | | 2.22 | | |
| Langenzenn | 1 | | 259.8 | | 131.25 | | 1.98 | | | | | | 9.2 | 7.8 | 3.84 | 78.1 | |
| Seefeld | 1 | | 203.8 | | 103.5 | | 1.97 | | | | | | | | 3.18 | 141.5 | |
| Radeburg | 2 | 1437.7 | 534.85 (1069.7) | | 275 (550) | | 1.94 (1.9) | | 110.81 (158.3) | | 2.48 (3.5) | | | | 3.6 | 78 | <0.20 |
| Bad Zwischenahn | 2 | | | | | | | | | | | | | | 3.19 | 52.75 | 0.3 |
| Deuz | 2 | 547.1 | 153.5 (307) | | | | | | 24.37 (34.8) | | | | 13 | 6.8 | 3.35 | 82.2 | 0.4 |
| Bruchmühlen | 2 | 475.4 | 286.7 (573.4) | | 151.25 (302.5) | | 1.89 (1.9) | | 39.76 (56.8) | | 3.8 (5.3) | | 15 | 6.6 | 3.23 | 93.41 | 0.35 |
| Weißtal SBR | 2 | 397.8 | | | | | | | | | | | | 6.5 | 4.8 | 66.75 | 0.2 |
| Huntlosen | 2 | | 311 (622) | | 190 (380) | | 1.64 (1.6) | | | | | | 16.7 | | 5.95 | | |
| Hettstedt | 2 | 1282.3 | 520.5 (1041) | | 302.5 (605) | | 1.72 (1.7) | | 78.75 (112.5) | | 3.84 (5.4) | | 15.25 | 7.3 | 6.8 | 89.6 | 0.35 |
| Spenge | 2 | 961.9 | 192.9 (385.8) | | 101.9 (203.8) | | 1.89 (1.9) | | 24.67 (35.25) | | 4.1 (5.8) | | 14.02 | 6.8 | 3.12 | 109.7 | 0.35 |

[a] COD, BOD5 and “Nin” refer to the first charging: 30% less “Nin”, 50% less C – values given in brackets for these parameters are measurements of the “original” influent before reaching the buffering tank

[b] animal rendering plants; ([b]) partly treating animal rendering waste; 1=SBR/ 2=DIC-SBR/ 3=conventional wwtp/ 4=conventional wwtp,fixed bed/ 5=conventional wwtp, ,membr.filtration; [c] ”general averaged values”

**Table 28:** Collected biological data; ranked according to the number of different AOB-clusters within the samples (_); different grey-shading according to the number of AOB-clusters 0-3

| Treatment plant | N AOB- Cl. | N NOB- Cl. | N AOB+ NOB | Cl.I *Ntspa*[a] | Cl.II *Ntspa*[a] | „other" *Ntpsa** | *Ntspa* (%) | Cl.I *Ntspa* (%) | Cl.II *Ntspa* (%) | Cl.I *Ntspa*B (%) | Cl.II *Ntspa*B (%) | halo. AOB[a] | Cluster 6a[a] | amount cl.6a[b] | *N.com.*- Cl.[a] | „env. Cl.6a"[a] | *Nc.mobilis**- Cl.[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rapp-Kutzenhausen | 0 | 0 | 0 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | - | - | - | - | - |
| **Kraftisried** | **1** | **2** | **3** | **+** | **+** | **-** | **4.0** | **14.9** | **85.2** | **0.6** | **3.4** | **-** | **+** | **++** | **-** | **-** | **-** |
| **Oberding** | **1** | **3** | **4** | **+** | **+** | **+** | **3.4** | **22.6** | **77.6** | **0.8** | **2.6** | **-** | **+** | **+** | **-** | **-** | **-** |
| **TBA GZM Lyss** | **1** | **2** | **3** | **+** | **+** | **-** | **4.8** | **80.6** | **12.4** | **3.9** | **0.6** | **-** | **+** | **+** | **-** | **+** | **-** |
| **ARA Lyss** | **1** | **2** | **3** | **+** | **-** | **-** | **1.2** | **100.0** | **0.0** | **1.2** | **0.0** | **-** | **+** | **++** | **-** | **-** | **-** |
| Waldsassen | 1 | 1 | 2 | + | - | - | 3.4 | 100.0 | 0.0 | 3.4 | 0.0 | - | + | +++ | - | - | - |
| Ampfing | 1 | 2 | 3 | - | + | + | 3.1 | 0.0 | 92.6 | 0.0 | 2.9 | - | + | ++ | - | - | - |
| Altmannstein | 1 | 2 | 3 | + | + | - | 0.1 | 88.5 | 13.6 | 0.1 | 0.0 | - | + | ++ | - | - | - |
| Langenzenn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | ++ | - | - | - |
| Seefeld | 1 | 1 | 2 | + | - | - | 1.6 | 100.0 | 0.0 | 1.6 | 0.0 | - | + | ++ | - | - | - |
| Bad Zwischenahn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | +++ | - | - | - |
| Deuz | 1 | 2 | 3 | + | + | - | 14.1 | 61.7 | 31.9 | 8.7 | 4.5 | - | + | ++ | - | - | - |
| Bruchmühlen | 1 | 1 | 2 | + | - | - | 4.4 | 100.0 | 0.0 | 4.4 | 0.0 | - | + | +++ | - | - | - |
| Weißtal SBR | 1 | 2 | 3 | + | + | - | 3.3 | 83.9 | 9.6 | 2.8 | 0.3 | - | + | ++ | - | - | - |
| Huntlosen | 1 | 2 | 3 | + | + | - | 1.3 | 53.2 | 36.9 | 0.7 | 0.5 | - | + | + | - | - | - |
| Spenge | 1 | 2 | 3 | + | - | - | 0.2 | 100.0 | 0.0 | 0.2 | 0.0 | - | + | +++ | - | - | - |
| **Plattling** | **2** | **2** | **4** | **+** | **+** | **-** | **3.3** | **37.8** | **64.3** | **1.2** | **2.1** | **+** | **+** | **+++** | **-** | **-** | **-** |
| Ingolstadt | 2 | 3 | 5 | + | + | + | 16.5 | 81.9 | 3.3 | 13.5 | 0.5 | + | + | ++ | - | + | + |
| Radeburg | 2 | 1 | 3 | + | - | - | 2.8 | 100.0 | 0.0 | 2.8 | 0.0 | + | + | +++ | - | - | - |
| Hettstedt | 2 | 3 | 5 | + | + | + | 2.3 | 80.4 | 3.3 | 1.8 | 0.1 | + | + | +++ | - | - | - |
| Rosenheim | 3 | 3 | 6 | + | + | + | 8.4 | 4.6 | 62.3 | 0.4 | 5.2 | + | + | + | + | - | - |

conventional treatment plants and the corresponding data are marked by appearing in **bold** letters

"N" = number / "cl." = cluster

[a] appearance of the respective cluster (+ = yes; - = no) / [b] amount Cl.6a = estimated abundance of Cluster6a-like bacteria in the categories -=no, +=few, ++=some and +++=many

"Cl. I *Ntspa*" = occurrence of "Cluster I" / "Cl II *Ntspa*" = occurrence of "Cluster II" / "other *Ntspa*" = occurrence of "other *Nitrospira*"

cl.I *Ntspa*B(%) and cl.II *Ntspa*B(%) = percentage of *Nitrospira*- "Cluster I and II" relative to all bacteria

halo.AOB = halophilic and –tolerant AOB (indicating *N.europaea/Nc.mobilis*-Cluster)

"*N.com.*-Cl" = *N. communis*-like AOB / "env.Cl.6a" = "environmental Cluster6a" / *Nc.mobilis*-Cl = *Nitrosococcus mobilis*-lineage

**Table 29:** Collected biological data; ranked according to the number of different NOB-clusters within the samples (_); different grey-shading according to the number of NOB-clusters 0-3

| Treatment plant | N AOB-Cl. | N NOB-Cl. | N AOB+ NOB | Cl.I *Ntspa*[a] | Cl.II *Ntspa*[a] | „other" *Ntpsa*[a] | *Ntspa* (%) | Cl.I *Ntspa* (%) | Cl.II *Ntspa* (%) | Cl.I *Ntspa*B (%) | Cl.II *Ntspa*B (%) | halo. AOB[a] | Cluster 6a[a] | amount Cl.6a[b] | *N.com.*-Cl.[a] | „env. Cl.6a"[a] | *Nc.mobilis*-Cl.[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rapp-Kutzenhausen | 0 | 0 | 0 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | - | - | - | - | - |
| Langenzenn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | ++ | - | - | - |
| Bad Zwischenahn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | +++ | - | - | - |
| Waldsassen | 1 | 1 | 2 | + | - | - | 3.4 | 100.0 | 0.0 | 3.4 | 0.0 | - | + | +++ | - | - | - |
| Seefeld | 1 | 1 | 2 | + | - | - | 1.6 | 100.0 | 0.0 | 1.6 | 0.0 | - | + | ++ | - | - | - |
| Bruchmühlen | 1 | 1 | 2 | + | - | - | 4.4 | 100.0 | 0.0 | 4.4 | 0.0 | - | + | +++ | - | - | - |
| Radeburg | 2 | 1 | 3 | + | - | - | 2.8 | 100.0 | 0.0 | 2.8 | 0.0 | + | + | +++ | - | - | - |
| **Kraftisried** | **1** | **2** | **3** | **+** | **+** | **-** | **4.0** | **14.9** | **85.2** | **0.6** | **3.4** | **-** | **+** | **++** | **-** | **-** | **-** |
| **TBA GZM Lyss** | **1** | **2** | **3** | **+** | **+** | **-** | **4.8** | **80.6** | **12.4** | **3.9** | **0.6** | **-** | **+** | **+** | **-** | **+** | **-** |
| **ARA Lyss** | **1** | **2** | **3** | **+** | **-** | **-** | **1.2** | **100.0** | **0.0** | **1.2** | **0.0** | **-** | **+** | **++** | **-** | **-** | **-** |
| Ampfing | 1 | 2 | 3 | - | + | + | 3.1 | 0.0 | 92.6 | 0.0 | 2.9 | - | + | ++ | - | - | - |
| Altmannstein | 1 | 2 | 3 | + | + | - | 0.1 | 88.5 | 13.6 | 0.1 | 0.0 | - | + | ++ | - | - | - |
| Deuz | 1 | 2 | 3 | + | + | - | 14.1 | 61.7 | 31.9 | 8.7 | 4.5 | - | + | ++ | - | - | - |
| Weißtal SBR | 1 | 2 | 3 | + | + | - | 3.3 | 83.9 | 9.6 | 2.8 | 0.3 | - | + | ++ | - | - | - |
| Huntlosen | 1 | 2 | 3 | + | + | - | 1.3 | 53.2 | 36.9 | 0.7 | 0.5 | - | + | + | - | - | - |
| Spenge | 1 | 2 | 3 | + | - | - | 0.2 | 100.0 | 0.0 | 0.2 | 0.0 | - | + | +++ | - | - | - |
| **Plattling** | **2** | **2** | **4** | **+** | **+** | **-** | **3.3** | **37.8** | **64.3** | **1.2** | **2.1** | **+** | **+** | **+++** | **-** | **-** | **-** |
| **Oberding** | **1** | **3** | **4** | **+** | **+** | **+** | **3.4** | **22.6** | **77.6** | **0.8** | **2.6** | **-** | **+** | **+** | **-** | **-** | **-** |
| Ingolstadt | 2 | 3 | 5 | + | + | + | 16.5 | 81.9 | 3.3 | 13.5 | 0.5 | + | + | ++ | - | + | + |
| Hettstedt | 2 | 3 | 5 | + | + | + | 2.3 | 80.4 | 3.3 | 1.8 | 0.1 | + | + | +++ | - | - | - |
| Rosenheim | 3 | 3 | 6 | + | + | + | 8.4 | 4.6 | 62.3 | 0.4 | 5.2 | + | + | + | + | - | - |

conventional treatment plants and the corresponding data are marked by appearing in **bold** letters

"N" = number / "cl." = cluster

[a] appearance of the respective cluster (+ = yes; - = no) / [b] amount Cl.6a = estimated abundance of Cluster6a-like bacteria in the categories -=no, +=few, ++=some and +++=many

"Cl. I *Ntspa*" = occurrence of "Cluster I" / "Cl II *Ntspa*" = occurrence of "Cluster II" / "other *Ntspa*" = occurrence of "other *Nitrospira*"

cl.I *Ntspa*B(%) and cl.II *Ntspa*B(%) = percentage of *Nitrospira*- "Cluster I and II" relative to all bacteria

halo.AOB = halophilic and –tolerant AOB (indicating *N.europaea/Nc.mobilis*-Cluster)

"*N.com.*-Cl" = *N. communis*-like AOB / "env.Cl.6a" = "environmental Cluster6a" / *Nc.mobilis*-Cl =*.Nitrosococcus mobilis*-lineage

**Table 30:** Collected biological data; ranked according to the abundance of *Nitrospira* within the samples (_)

| Treatment plant | N AOB- Cl. | N NOB- Cl. | N AOB+ NOB | Cl.I Ntspa[a] | Cl.II Ntspa[a] | „other“ Ntpsa[a] | Ntspa (%) | Cl.I Ntspa (%) | Cl.II Ntspa (%) | Cl.I NtspaB (%) | Cl.II NtspaB (%) | halo. AOB[a] | Cluster 6a[a] | amount Cl.6a[b] | N.com.-Cl.[a] | „env. Cl.6a“ [a] | Nc.mobilis[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rapp-Kutzenhausen | 0 | 0 | 0 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | - | - | - | - | - |
| Langenzenn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | ++ | - | - | - |
| Bad Zwischenahn | 1 | 0 | 1 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - | + | +++ | - | - | - |
| Altmannstein | 1 | 2 | 3 | + | + | - | 0.1 | 88.5 | 13.6 | 0.1 | 0.0 | - | + | ++ | - | - | - |
| Spenge | 1 | 2 | 3 | + | - | - | 0.2 | 100.0 | 0.0 | 0.2 | 0.0 | - | + | +++ | - | - | - |
| **ARA Lyss** | **1** | **2** | **3** | **+** | **-** | **-** | **1.2** | **100.0** | **0.0** | **1.2** | **0.0** | **-** | **+** | **++** | **-** | **-** | **-** |
| Huntlosen | 1 | 2 | 3 | + | + | - | 1.3 | 53.2 | 36.9 | 0.7 | 0.5 | - | + | + | - | - | - |
| Seefeld | 1 | 1 | 2 | + | - | - | 1.6 | 100.0 | 0.0 | 1.6 | 0.0 | - | + | ++ | - | - | - |
| Hettstedt | 2 | 3 | 5 | + | + | + | 2.3 | 80.4 | 3.3 | 1.8 | 0.1 | + | + | +++ | - | - | - |
| Radeburg | 2 | 1 | 3 | + | - | - | 2.8 | 100.0 | 0.0 | 2.8 | 0.0 | + | + | +++ | - | - | - |
| Ampfing | 1 | 2 | 3 | - | + | + | 3.1 | 0.0 | 92.6 | 0.0 | 2.9 | - | + | ++ | - | - | - |
| Weißtal SBR | 1 | 2 | 3 | + | + | - | 3.3 | 83.9 | 9.6 | 2.8 | 0.3 | - | + | ++ | - | - | - |
| **Plattling** | **2** | **2** | **4** | **+** | **+** | **-** | **3.3** | **37.8** | **64.3** | **1.2** | **2.1** | **+** | **+** | **+++** | **-** | **-** | **-** |
| Waldsassen | 1 | 1 | 2 | + | - | - | 3.4 | 100.0 | 0.0 | 3.4 | 0.0 | - | + | +++ | - | - | - |
| **Oberding** | **1** | **3** | **4** | **+** | **+** | **+** | **3.4** | **22.6** | **77.6** | **0.8** | **2.6** | **-** | **+** | **+** | **-** | **-** | **-** |
| **Kraftisried** | **1** | **2** | **3** | **+** | **+** | **-** | **4.0** | **14.9** | **85.2** | **0.6** | **3.4** | **-** | **+** | **++** | **-** | **-** | **-** |
| Bruchmühlen | 1 | 1 | 2 | + | - | - | 4.4 | 100.0 | 0.0 | 4.4 | 0.0 | - | + | +++ | - | - | - |
| **TBA GZM Lyss** | **1** | **2** | **3** | **+** | **+** | **-** | **4.8** | **80.6** | **12.4** | **3.9** | **0.6** | **-** | **+** | **+** | **-** | **+** | **-** |
| Rosenheim | 3 | 3 | 6 | + | + | + | 8.4 | 4.6 | 62.3 | 0.4 | 5.2 | + | + | + | + | - | - |
| Deuz | 1 | 2 | 3 | + | + | - | 14.1 | 61.7 | 31.9 | 8.7 | 4.5 | - | + | ++ | - | - | - |
| Ingolstadt | 2 | 3 | 5 | + | + | + | 16.5 | 81.9 | 3.3 | 13.5 | 0.5 | + | + | ++ | - | + | + |

conventional treatment plants and the corresponding data are marked by appearing in **bold** letters

“N” = number / “cl.” = cluster

[a] appearance of the respective cluster (+ = yes; - = no) / [b] amount Cl.6a = estimated abundance of Cluster6a-like bacteria in the categories -=no, +=few, ++=some and +++=many

“Cl. I *Ntspa*” = occurrence of “Cluster I” / “Cl II *Ntspa*” = occurrence of “Cluster II” / “other *Ntspa*” = occurrence of ”other *Nitrospira*”

cl.I *Ntspa*B(%) and cl.II *Ntspa*B(%) = percentage of *Nitrospira*- “Cluster I and II” to all bacteria

halo.AOB = halophilic and –tolerant AOB (indicating *N.europaea/Nc.mobilis*-Cluster)

“*N.com.*-Cl” = *N. communis*-like AOB / “env.Cl.6a” = ”environmental Cluster6a” / *Nc.mobilis*-Cl = *.Nitrosococcus mobilis*-lineage

Dominance of "Cluster I" or "Cluster II" did not seem to be linked to the overall percentage of all *Nitrospira*-like NOB within the samples (sample size = 18) (Fig. 9) Fig. 10 shows the ("actual") abundance of *Nitrospira*-Cluster I- and II-like organisms relative to all bacteria plotted against the percentage of the genus *Nitrospira*. "Actual" abundances of "Cluster I and II" were naturally to some extent correlated to the percentage of the genus *Nitrospira*.

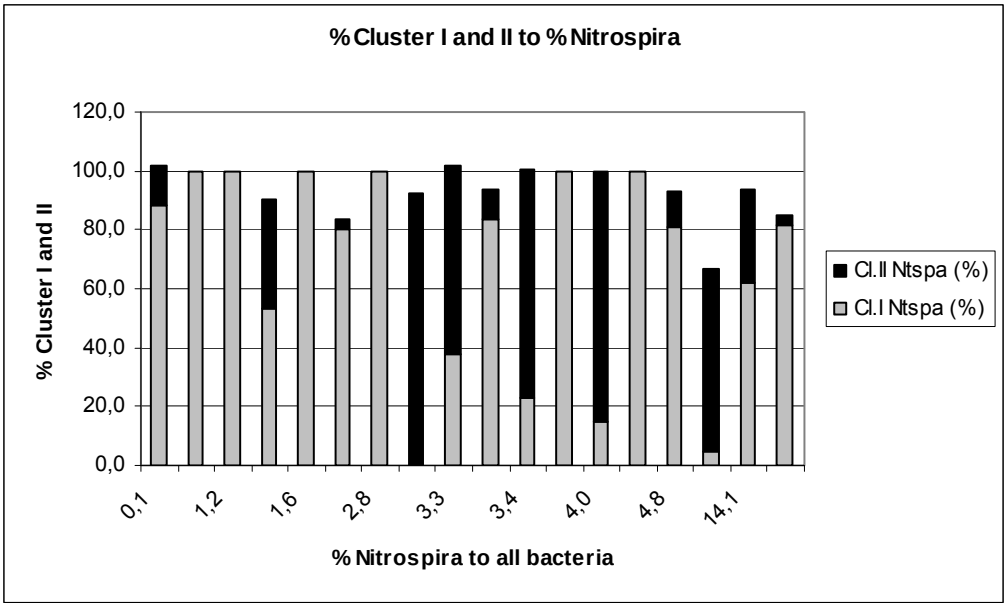



**Figure 9:** Abundances of "Cluster I and II" relative to all *Nitrospira*-like bacteria plotted against the abundance of the genus *Nitrospira* (N=18)

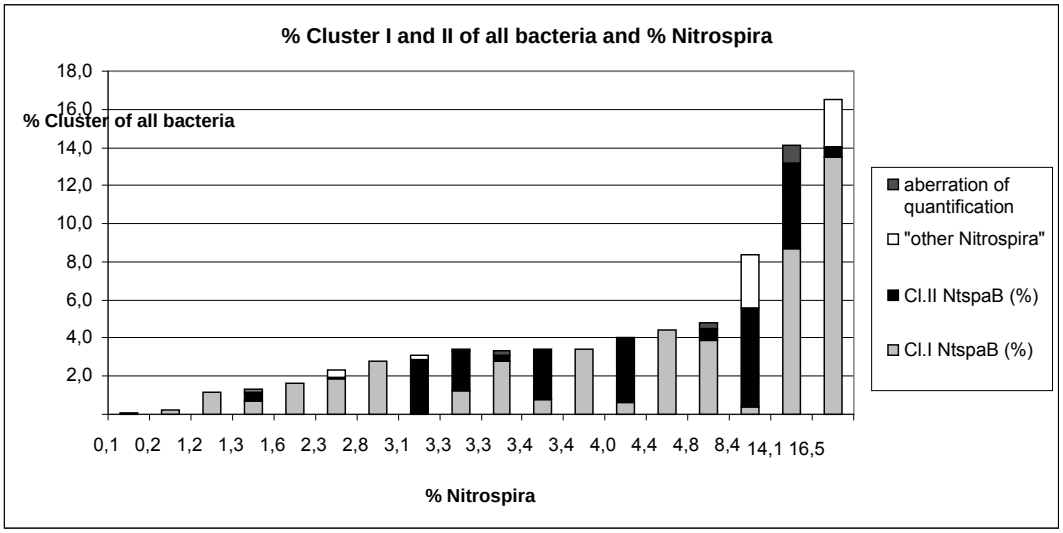



**Figure 10:** Abundances of "Cluster I and II" relative to all bacteria plotted against the abundance of the genus *Nitrospira* (N=18)
"other *Nitrospira*": percentage of "other *Nitrospira*" in the samples (5 samples)
"aberration of quantification": Without the occurrence of "other *Nitrospira*", Cluster I and II should sum up to 100% of the genus *Nitrospira*. An aberration of maximally -/+10%, which could be due to differing intensities of the fluorescent signals, was tolerated (see D.2.).

### C.3.2.4. Co-occurrence of different AOB- and NOB-clusters

Comparing the varying appearance of different AOB- and NOB-clusters, co-occurrence of clusters was analysed in more detail. Considering only AOB, in 15 out of 20 samples only members of Cluster 6a could be found; in 4 samples (Plattling, Ingolstadt, Radeburg, Hettstedt) both, Cluster 6a-like AOB and halophilic and –tolerant AOB, were found (Tab. 31). *N. communis*-like bacteria were only found in one sample, also harbouring Cluster 6a-like and halophilic and –tolerant AOB (Rosenheim).

**Table 31:** Co-occurrence of different AOB-clusters

| | Cluster6a | halo.AOB | N.com | Cluster6a + halo.AOB |
|---|---|---|---|---|
| Cluster 6a | 15 | 4 | 0 | |
| halo.AOB | 4 | 0 | 0 | |
| N.com | 0 | 0 | 0 | 1 |
| Cluster6a + halo.AOB | | | 1 | |

numbers of samples harbouring the specific co-occurrences are given; sample size=20

different AOB-clusters: Cluster6a / *Nc.mobilis/N.europaea*-Cluster ("halo.AOB") / *N.communis*-Cluster ("N.com.")

Regarding the co-occurrence of different *Nitrospira*-Clusters (N = 18), "Cluster I" alone appeared in six samples (from ARA Lyss, Waldsassen, Seefeld, Radeburg, Bruchmühlen, Spenge) whereas "Cluster II" could not be found without any other *Nitrospira*-cluster (Table 32). In 7 samples (Kraftisried, TBA GZM Lyss, Altmannstein, Deuz, Weißtal, Huntlosen, Plattling), members of both, "Cluster I and II", could be found. "Other *Nitrospira*" (counted as an extra NOB-cluster) occurred in one sample together with "Cluster II" (Ampfing) and in four samples (Oberding, Rosenheim, Ingolstadt, Hettstedt) together with "Cluster I and II".

**Table 32:** Co-occurrence of different *Nitrospira*-like bacteria

| | Cluster I | Cluster II | other *Nitrospira* | Cluster I + Cluster II |
|---|---|---|---|---|
| Cluster I | 6 | 7 | 0 | |
| Cluster II | 7 | 0 | 1 | |
| other Nitrospira | 0 | 1 | 0 | 4 |
| Cluster I + Cluster II | | | 4 | |

numbers of samples harbouring the specific co-occurrences are given; sample size=18

different *Nitrospira*-clusters: *Nitrospira*-Cluster I / *Nitrospira*-Cluster II / "other *Nitrospira"*-Cluster

Analysis of the observed combinations of AOB and NOB-clusters in all samples showed that Cluster 6a-like AOB appeared in every combination and members of *Nitrospira*-clusters could be found in most combinations (Table 33). The most frequent combinations were occurrence of members of Cluster 6a and Cluster I (5 samples) and of Cluster 6a, Cluster I and Cluster II (6 samples). "Other *Nitrospira*" occurred in 4 of the 9 observed combinations.

In two samples members of all detected clusters except the *N. communis*-Cluster were found, and in one sample members of all clusters occurred (Tab. 33; Fig. 11).

Samples harbouring the same richness of AOB did not show a high similarity in the abundance of Cluster 6a-like bacteria. Occurrence of both, *Nitrospira*-Cluster I- and II-like bacteria, either with or without appearance of "other *Nitrospira*", did not coincide with a similar ratio of Cluster I to Cluster II in the samples. For instance, of the 4 samples, in which members of "Cluster I", "Cluster II" and "other *Nitrospira*" occured, those from Oberding and Rosenheim showed more "Cluster II" than "Cluster I", but for the remaining two it was vice versa (Ingolstadt, Hettstedt). There was no resemblance in the percentage of "Cluster I or II" relative to all bacteria between these samples. The same co-occurrence of AOB and NOB in the samples did not come along with similar abundances of Cluster 6a-like bacteria, different ratios of Cluster I to II or different abundances of members of Cluster I and II relative to all bacteria.

**Table 33:** Co-occurrences of different AOB- and NOB-clusters; N=20

| A | AD | ABD | AEF | ADE | ADEF | ABDE | ABDEF | ABCDEF |
|---|---|---|---|---|---|---|---|---|
| 2 | 5 | 1 | 1 | 6 | 1 | 1 | 2 | 1 |

A=Cluster 6a / B=halophilic and –tolerant AOB (*Nc.mobilis/N.europaea*-Cluster) / C=*N.communis*-Cluster /
D=Cluster I-*Nitrospira* / E=Cluster II-*Nitrospira* / F="other *Ntspa.*"
Samples harbouring the different combinations, respectively:
A: Langenzenn, Bad Zwischenahn / AD: ARA Lyss, Waldsassen, Seefeld, Bruchmühlen, Spenge /
ABD: Radeburg / AEF: Ampfing / ADE: Kraftisried, TBA GZM Lyss, Altmannstein, Deuz, Weißtal, Huntlosen /
ADEF: Oberding / ABDE: Plattling / ABDEF: Ingolstadt, Hettstedt; ABCDEF: Rosenheim;
Colours are according to connecting lines between co-occuring AOB&NOB in Fig.11

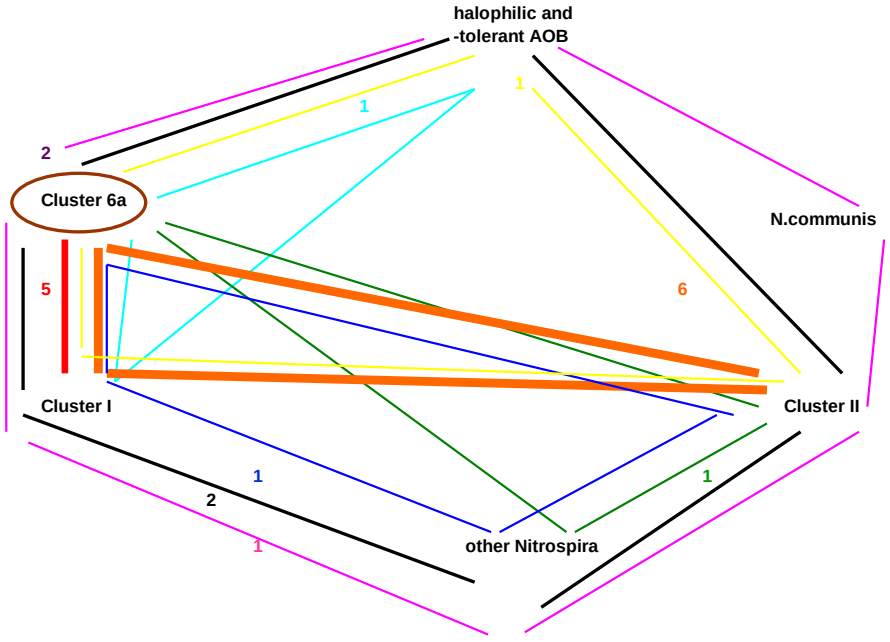



**Figure 11**: Co-occurrences of different AOB- and NOB-clusters; N=20
AOB and NOB co-occurring in different combinations dependent on the sample are connected with differently coloured lines– numbers and thickness of lines indicate the amount of samples, in which this combination has been found; colours are according to Table 36

### C.3.3. Correlation of wastewater composition and operational mode

For characteristic parameters, for which enough data were available, possible correlations with AOB and/or NOB-diversity, abundance of *Nitrospira*-like bacteria (in particular *Nitrospira*-Cluster I-like and II-like bacteria) and abundance of Cluster 6a-like AOB, have been investigated.

#### C.3.3.1. Temperature

Temperature-measurements were available for 14 of the 21 investigated plants. By comparing the numbers of different AOB- or NOB-clusters with the average temperature (T) in each sample, a trend of higher diversity with increasing T was visible (Fig. 12, 13) This was also the case when comparing T and the number of AOB- plus NOB-clusters (Fig. 14). Though at low T higher diversity could be seen as well (Fig. 12), additional clusters seemed to occur more often at a higher T. The slightly visible correlation (low sample size for some categories, defined as the number of clusters) was tested by calculating Spearman`s Rho, which was significant for T and number of AOB-clusters (<0.05; r=0.631; N=14), T and number of NOB-clusters (<0,05; r=0,598; N=14) and T and number of AOB- plus NOB-clusters (<0.01; r=0.696; N=14). This means that approximately 40%, 36% and 48% ($r^2$) of the varying numbers of different clusters (thus cluster-diversity) could be explained by differing temperatures. Correlations between T and cluster-diversities were also significant considering solely SBRs. Variations in the numbers of different clusters could be even better explained with the differing T of the corresponding samples by considering only SBRs (AOB-clusters and T: <0.05; r=0.757; $r^2$=57%; N=9 / NOB-clusters and T: <0.05; r=0.797; $r^2$=64%; N=9 / AOB+NOB-clusters; <0.01; r=0.821; $r^2$=67%; N=9).

To see how similar (referring to T) samples are in regard to co-occurrences of certain AOB and/or NOB-clusters, in addition to comparisons of the diversity just in terms of numbers of different clusters, correlations between categories of certain cluster co-occurrences (e.g.: category ABD…co-occurrence of *N. oligotropha*-like AOB, *Nc. mobilis/N. europaea*-like AOB and *Nitrospira*-Cluster I-like NOB) and T should be investigated. However, this did not make much sense, because too few data were available for the high number of categories (see Tab.33; 9 categories; N=20 (AOB) and 14 (NOB)). No pattern could be seen by plotting the different abundance of members of Cluster6a (not shown) and the different occurrence of *Nitrospira*-like organisms against the temperatures of the respective plants (Fig. 15).

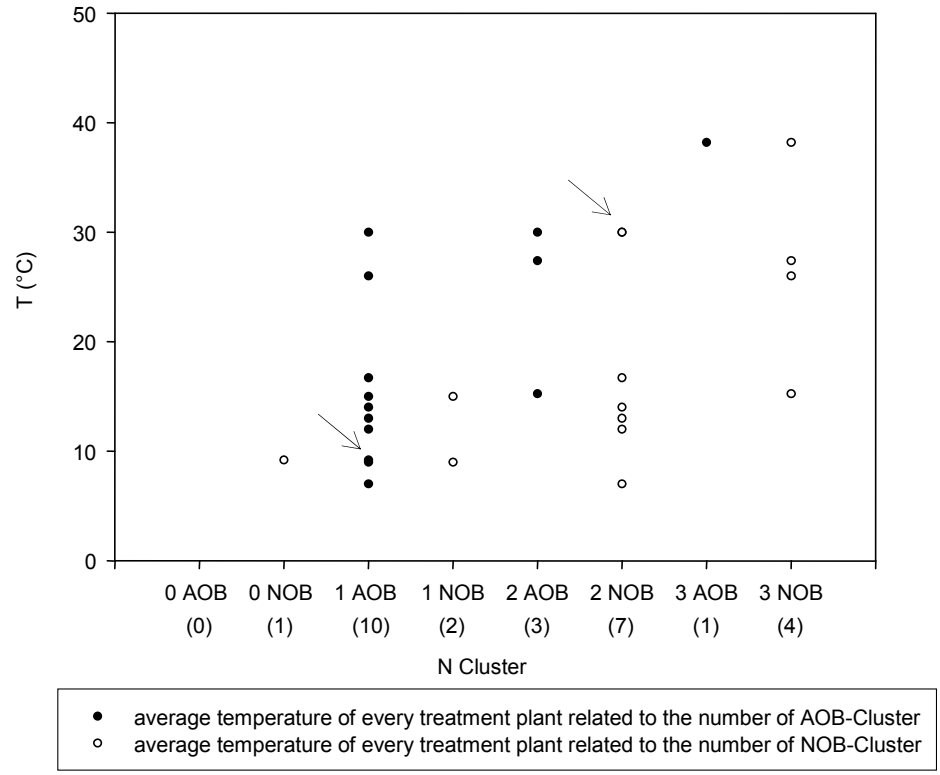



**Figure 12**: Correlation cluster-diversity (number of clusters) and temperature for AOB and NOB; total sample size = 14
Points are representing the different wwtps falling into the respective categories (once for AOB-clusters and once for NOB-clusters).
Sample sizes N for each category of number of clusters (0-3) are given in brackets underneath the respective categories.
⟶ 2 wwtp represented by one point (exhibiting approx. the same T)

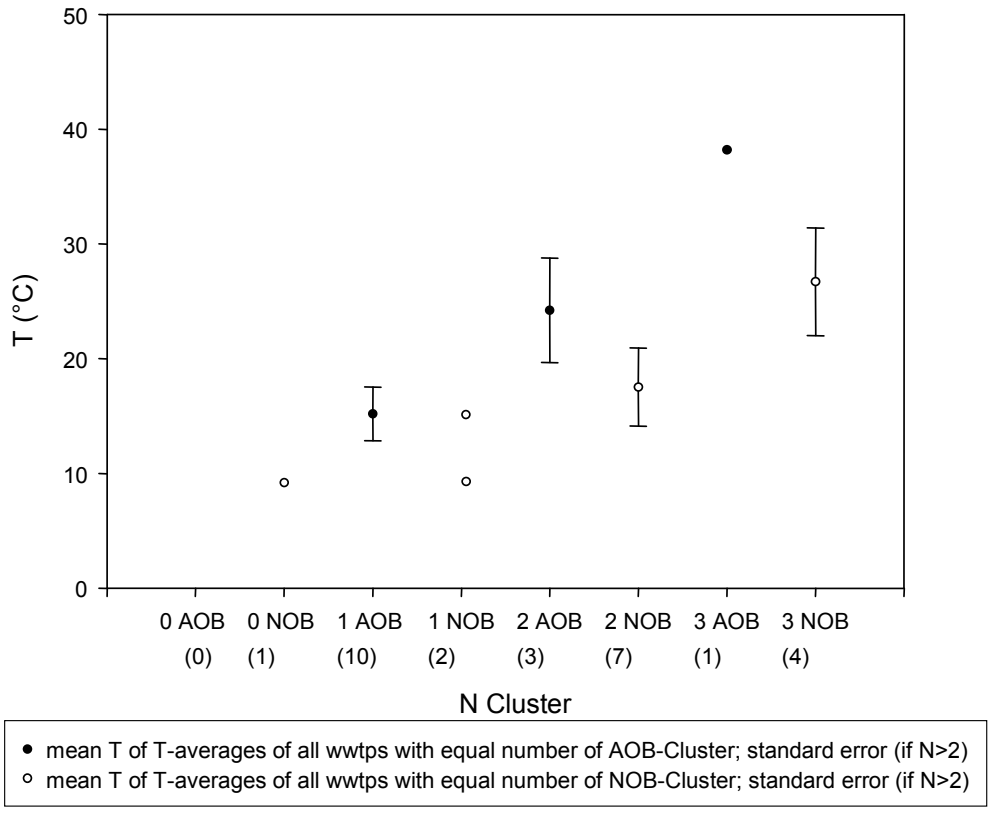



**Figure 13:** Correlation cluster-diversity (number of clusters) and temperature for AOB and NOB; total sample size = 14
mean T of all mean temperatures of the wwtps falling into a certain category of number of clusters (0-3) + standard errors
Sample sizes N for each category of number of clusters (0-3) are given in brackets underneath the respective categories.

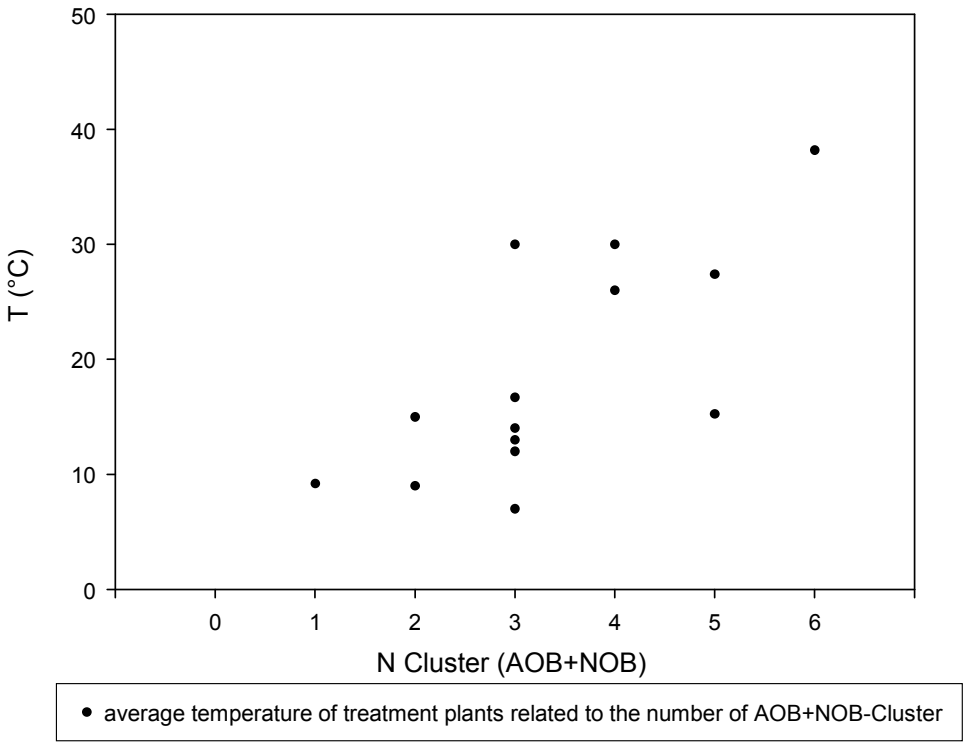



**Figure 14:** Correlation cluster-diversity (number of AOB+NOB-clusters) and temperature; total sample size = 14 average temperatures of the different wwtps falling into a certain category of number of clusters (0-6)

No *Nitrospira*-like bacteria ("No *Ntspa*.") were found in a sample with rather low temperature (Langenzenn; 9.2°C), but anyway *Nitrospira*-like NOB could be found in the sample with the lowest temperature (Kraftisried; 7°C). Organisms affiliated either to *Nitrospira* Cluster I or II (regardless which one; "Cl.IorII") seemed to occur at somehow lower temperatures, at which samples containing members of both, *Nitrospira*-Cluster I and II ("Cl.I+II"), could nevertheless be found as well (Fig.15). Representatives of *Nitrospira* Cluster I as well as of *Nitrospira* Cluster II (no matter if they were appearing together or not; "Cl.I" and "Cl.II"), were found at low and at high temperatures. Only "other *Nitrospira*" did not occur at low T. This contributed to a good part to the correlation between NOB richness and higher T.

*N. europaea/Nc. mobilis*-like organisms, the only ones apart from Cluster 6a-like organisms which have been found in more than 2 samples, were not found at low temperatures either. Still, the temperatures of the samples in which these AOB appeared, were varying a lot (Hettstedt T=15.3°C; Rosenheim T=38.2°C). Co-occurrence of "other *Nitrospira*" and halophilic and –tolerant AOB, which were both present at rather elevated temperatures (though within a quite broad range), was observed in three samples.

Abundances of *Nitrospira*-like bacteria (Fig.17) as well as abundances of "Cluster I and II" (relative to all bacteria) (not shown) did not seem to be influenced by the T in any way.

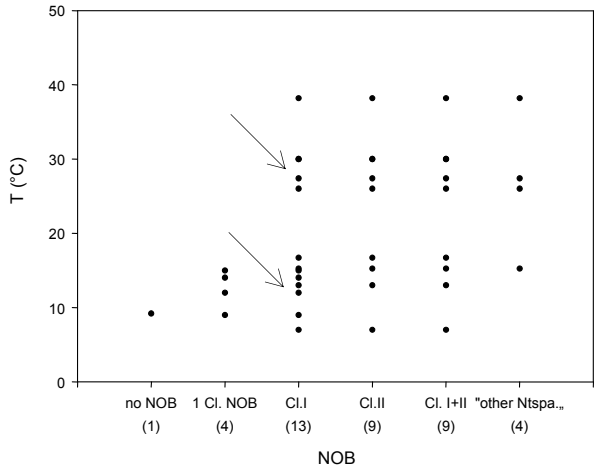



**Figure 15:** Differences in occurrence of NOB related to T (total sample size=14); N of each category are given in brackets underneath the respective categories.
Points resemble the mean temperatures of the respective plants and fall into the different categories according to the occurrence of *Nitrospira*-like NOB in these wwtps.
"no NOB" = no NOB detected
"1 Cl. NOB" = either "Cluster I" or "Cluster II" detected
"Cl.I" = "Cluster I" was detected – solely or with other *Nitrospira*-cluster
"Cl.II" = "Cluster II" was detected – solely or with other *Nitrospira*-cluster
"other *Ntpsa*." = "other *Nitrospira*" detected
⟶ 2 wwtps with the same T

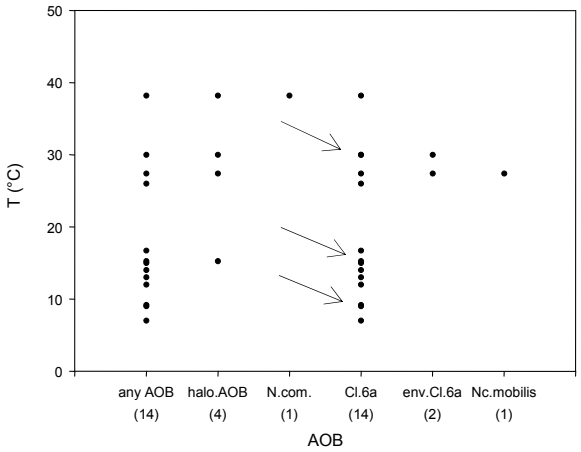



**Figure 16:** Differences in occurrence of AOB related to T (total sample size=14); N of each category are given in brackets underneath the respective categories.
Points resemble the mean temperatures of the respective plants and fall into the different categories according to the occurrence of *Nitrospira*-like NOB in these wwtps.
"any AOB" = T-range within which all different AOB have been found; "halo.AOB" = occurrence of halophilic and -tolerant AOB; "N.com" = *N.communis*-like bacteria detected
"Cl.6a" = Cluster 6a-like bacteria detected
"env.Cl.6a" = "environ. Cluster 6a"-like bacteria detected
"Nc.mobilis" = *Nitrosococcus mobilis*-like bacteria detected
⟶ 2 wwtps with the same or similarly high T

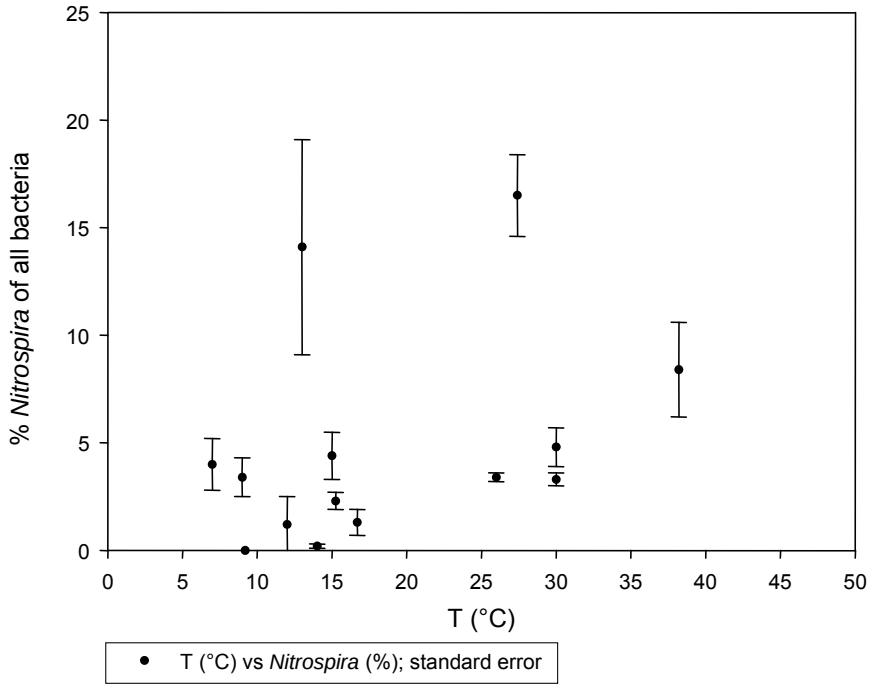



**Figure 17:** Abundance of *Nitrospira*-like bacteria plotted against the mean temperature in the respective wwtps; sample size=14

### C.3.3.2. pH and Conductivity

No pattern could be seen for an influence of pH and average conductivity on diversity, the occurrence of AOB (Fig.18) or *Nitrospira*-like NOB and the abundance of the genus *Nitrospira.*

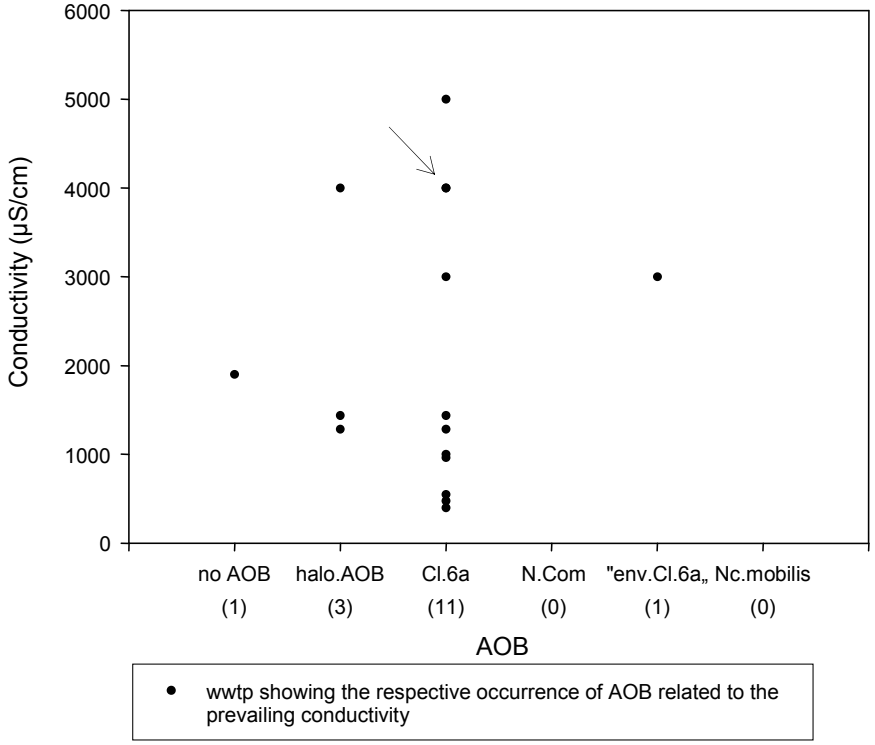



**Figure 18:** Differences in occurrence of AOB related to the prevailing conductivity
total sample size = 12; N of each category is given are brackets underneath of the respective categories.
"no AOB" = no AOB detected; "halo.AOB" = occurrence of halophilic &-tolerant AOB; "N.com" = *N.communis*-like bacteria detected
"Cl.6a" = Cluster 6a-like bacteria detected; "env.Cl.6a" = "environmental Cluster 6a"-like bacteria detected
"Nc.mobilis" = *Nitrosococcus mobilis*-like bacteria detected; $\rightarrow$ 2 wwtps represented by one point (same mean conductivity)

The same kind of analysis, performed with SBRs only, indicated that halophilic and –tolerant AOB occurred at elevated conductivities (Fig.19). This was also indicated for "other *Nitrospira*", but only based on one SBR harbouring this group, for which conductivity values were available. However, this separate analysis should be questioned (see D.5.5.).
Increasing conductivity in samples (N=11) came along with a higher abundance of "Cluster II" and lower abundance of "Cluster I" (Fig. 20). This was, though less distinct, also reflected in the abundances of "Cluster I" as well as "Cluster II" relative to all bacteria (Fig. 21). A rapid decline in abundance of "Cl. I" and a rapid increase of "Cl. II" can be seen at around 3500 µS/cm (Fig. 20). Referring the abundances of "Cluster I and II" to all bacteria weakend this trend. "Cluster II" was once also more abundant at a low conductivity (Deuz; 4.5%; 547 µS/cm) and abundance of "Cluster I" was partly also very low at lower conductivities. Spearman`s Rho showed a significant decline of the abundance of "Cluster I" relative to all

*Nitrospira*-like NOB with rising conductivity (<0.05; r=0.645; N=12). Neither the increase of "Cluster II" nor trends for "Cluster I and II" relative to all bacteria were significant.

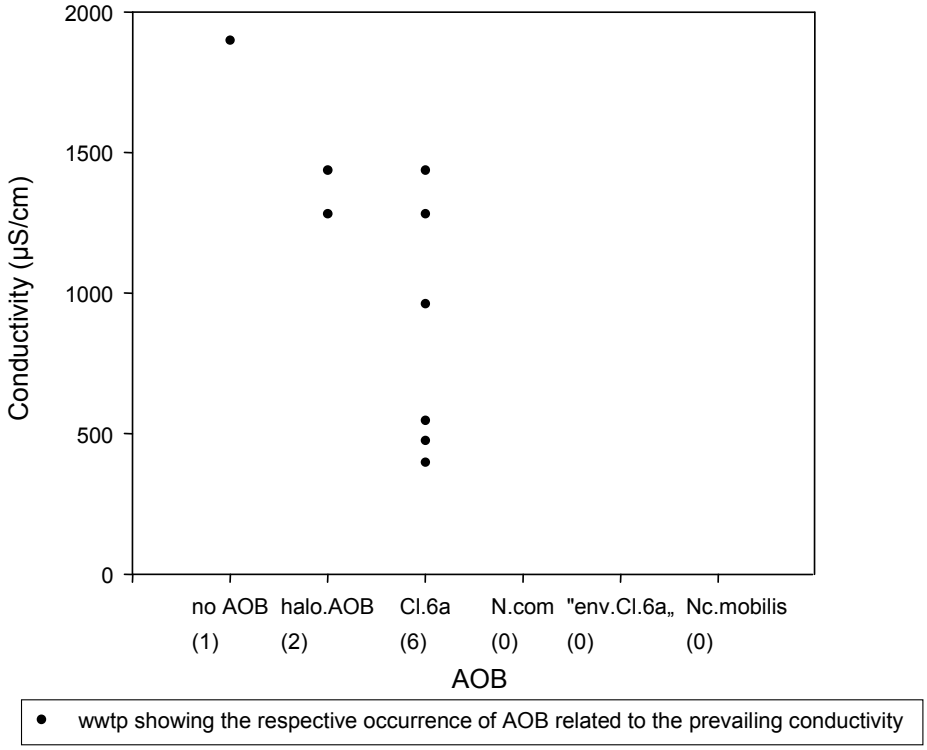



**Figure 19:** Differences in occurrence of AOB related to the prevailing conductivity for SBRs
total sample size = 7; N of each category are given in brackets underneath the respective categories.
for each wwtp the mean conductivity is given; "no AOB" = no AOB detected; "halo.AOB" = occurrence of halophilic &-tolerant AOB
"N.com" = *N. communis*-like bacteria detected; "Cl.6a" =Cluster 6a-like bacteria detected
"env.Cl.6a" = "environmental Cluster 6a"-like bacteria detected; "Nc.mobilis" = *Nitrosococcus mobilis*-like bacteria detected

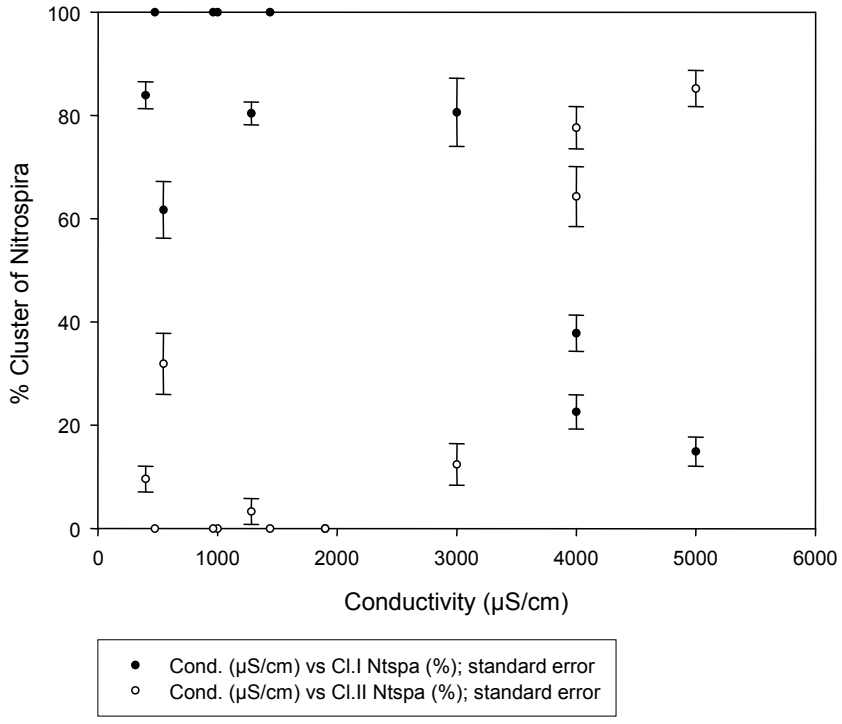



**Figure 20:** Relative abundances of "Cluster I" and "Cluster II" relative to all *Nitrospira*-like bacteria plotted against the mean conductivtiy of the respective wwtps (N=12)

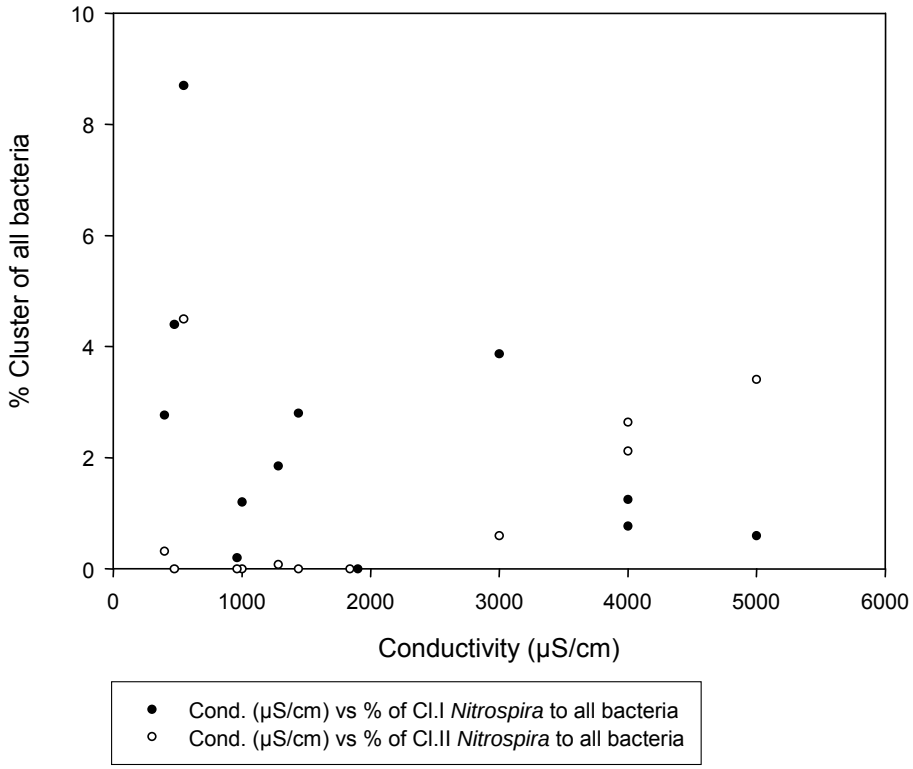



**Figure 21:** Relative abundances of *Nitrospira* "Cluster I" and "Cluster II" relative to all bacteria plotted against the mean conductivtiy of the respective wwtps (N=12)

### C.3.3.3. Dry Matter

For 20 of the 21 wwtps, dry matter-values were available (including Rapp-Kutzenhausen harbouring no AOB and NOB). Increasing diversity (number of different AOB and/or NOB-clusters within one sample) could be observed in samples containing on average a higher concentration of DM (Fig.22 and 23).

The correlation of DM with the cluster-diversity was, according to Spearman`s Rho correlation coefficient, significant. More precisely, the correlation between DM and the number of different AOB accounted for around 22% of the variation in AOB-diversity between the samples (<0.05; r=0.469; N=20). Variation in the number of NOB-clusters could also be explained to approx. 22% by varying amounts of dry matter (<0.05; r=0.47; N=20). For AOB+NOB-clusters the correlation coefficient r was 0.532 (<0.05; N=20) and thus could be interpreted as explaining 28% of divergence. The same kind of analysis, performed with SBRs only, showed that correlations between numbers of different AOB- and/or NOB-clusters and DM were not significant, but the same trends still visible.

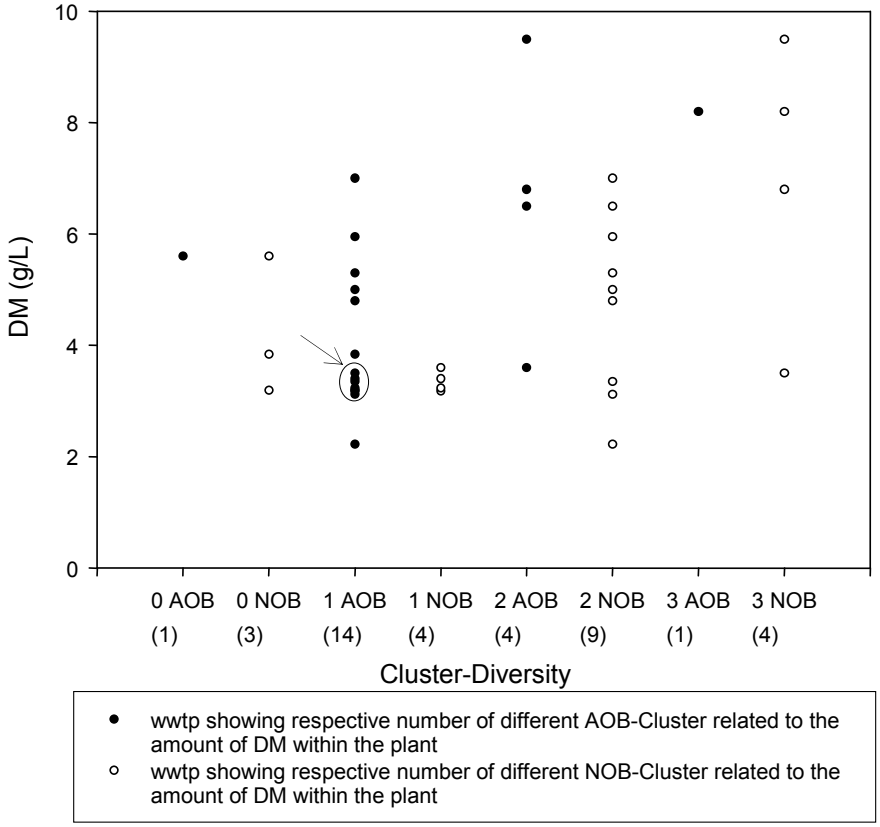



**Figure 22:** AOB- and NOB-cluster-diversities (determined as number of different AOB- or NOB-clusters) related to the amount of DM
Points represent wwtps falling into the corresponding category (0-3 for each, AOB and NOB) referred to the mean amount of dry-matter in the sample:, total sample size = 20; N of each category are given in brackets underneath the respective categories.
Categories: number of clusters (0 AOB-Clusters, etc.); → ◯ …contains 7 points representing 7 wwtps very similar in DM-conc:

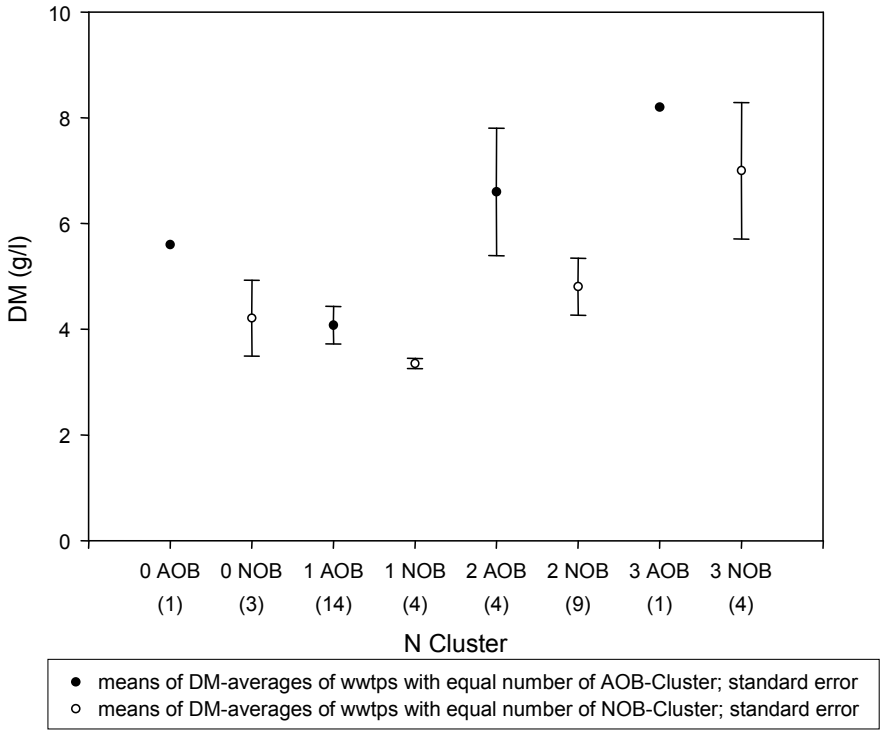



**Figure 23:** AOB- and NOB-cluster-diversities related to DM
mean concentration of all mean DM-concentrations of the wwtps falling into the corresponding categories (0-3 for each, AOB and NOB), shown with standard errors; total sample size = 20; N of each category are given in brackets underneath the respective categories.
Categories: number of clusters (0 AOB = 0 AOB-clusters detected)

The amount of dry matter has also been found to correlate significantly with the temperature (<0.05; r=0.66; N=13) (see Figure 24). Considering only SBRs, this correlation remained significant (<0.05; r=0.667; N=9).

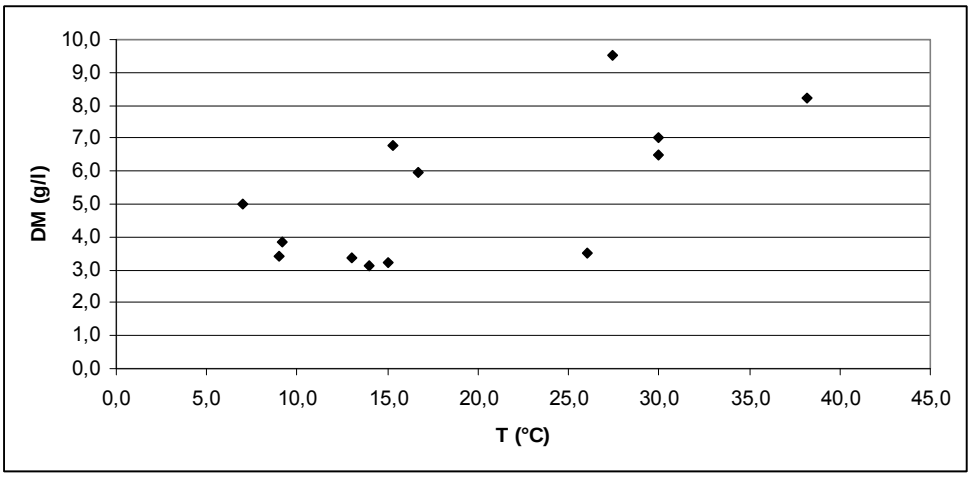



**Figure 24**: Mean amounts of DM plotted against the mean temperatures in the respective wwtps (N=13); all plants considered

DM did not trend to increase with larger nitrogen input or the BOD5/"Nin"-ratio. DM and BOD5 for SBRs (for DIC-SBRs the BOD5-values of the "original" influent have been considered; see Tab. 27, p.79) correlated significantly (Fig.25; Spearman Correlation; <0.05; N=11; r=0.691), whereas DM and BOD5 for all plants did not correlate significantly.

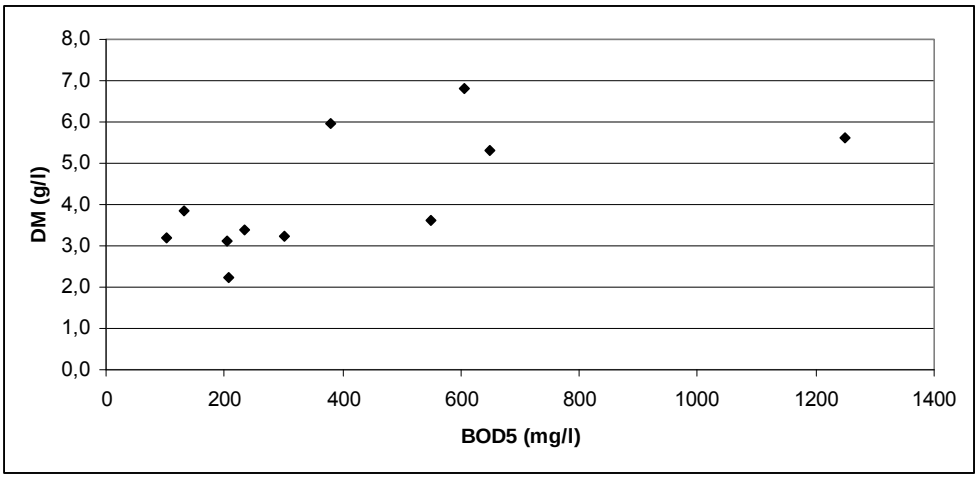



**Figure 25:** Mean amount of DM plotted against the mean BOD5-concentrations for SBRs (N=11); only "original" influent-conc. considered

No interrelations could be found between the abundances of all *Nitrospira*-like NOB in general and the abundances of representatives of each *Nitrospira*-cluster when related to the amount of DM. Furthermore, the appearance of "Cluster I or II" or the co-occurrence of both "Cluster I and II" did not coincide with the concentration of DM. Occurrence of halophilic and –tolerant AOB has not been found to be related to DM either.

### C.3.3.4. BOD5-concentrations

As the concentration of biodegradable C is better represented by the parameter BOD5, all further analysis referring to C concentrations was done with BOD5- instead of COD-values (see D.5.5.1.). Due to bad comparability of this parameter between conventional wwtps and SBRs (see D.1.1. - D.1.4., p.113-117), analysis was done for SBRs only. The sample size for an analysis of only conventioal plants seemed to be too low (N=5), besides they were not very diverse in their average BOD5-concentration.

Abundances of the genus *Nitrospira* as well as of *Nitrospira* "Cluster I and II" did not show any correlation with the mean BOD5-values. The same was visible for the diversity of NOB- and AOB-clusters, although conclusions can hardly be drawn, as in samples, for which this parameter was available, only Cluster 6a-like AOB or Cluster 6a-like and halophilic and –tolerant AOB occurred. It could be seen that the sample harbouring no AOB and NOB exhibited the highest BOD5-values (Rapp-Kutzenhausen; 1250 mg/l). Evaluating the appearance and absence of different AOB-clusters, showed that *N. oligotropha*-like AOB appeared quite ubiquitous over the whole range of varying BOD5-values while halophilic and –tolerant AOB were observable at higher BOD5-concentrations (see Fig. 26).

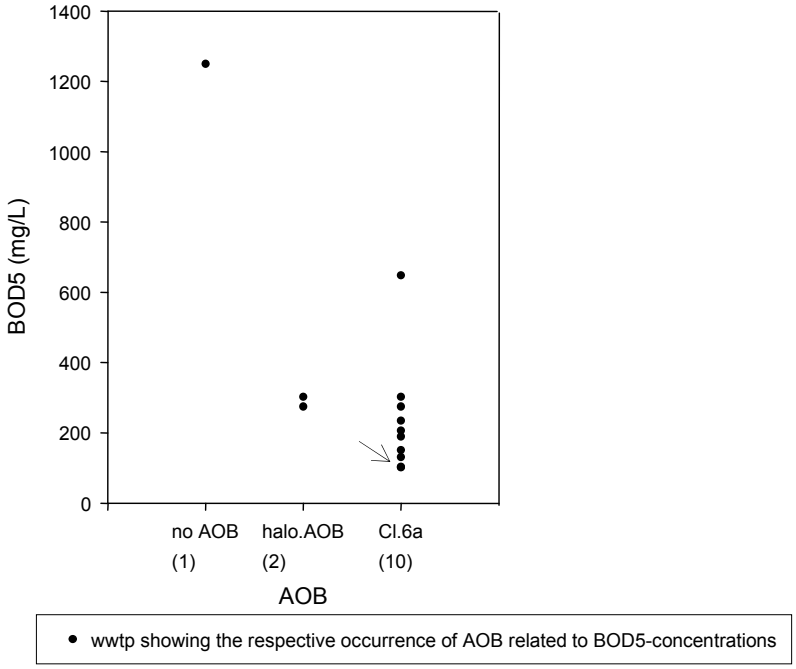



**Figure26:** Difference in occurrence of AOB related to BOD5; the mean BOD5-values of each wwtp are given
total sample size = 11; N of each category are given in brackets underneath the respective categories.
no AOB = no AOB have been found; halo.AOB = occurrence of halophilic &-tolerant AOB
Cl.6a = Cluster 6a detected
→ 2 wwtps represented by one point (as showing highly similar BOD5-conc.)

This trend could interestingly also be observed for the appearance of "other *Nitrospira*" as those seemed to occur only at elevated BOD5-concentrations (Fig.27). Appearance of

"Cluster I and II" seemed to be independent of BOD5-values. They occured at various BOD5-concentrations, though "Cluster I" occured not at the highest BOD5-concentrations, contrary to "Cluster II". However, the latter observation was just based on one sample exhibiting an especially high BOD5-value. Though "other *Nitrospira*" and halophilic and –tolerant AOB occurred together in some of the SBRs, unfortunantely data concerning this parameter were not available for all of those plants, apart from one (Hettstedt; BOD5 = 302.5 mg/l). Therefore the second sample in Fig. 26 harbouring halophilic and –tolerant AOB and the second sample harbouring "other *Nitrospira*" in Fig. 27, visible at elevated BOD5-concentrations, do not resemble the same plant. No correlation was visible for the abundance of Cluster 6a-like bacteria.

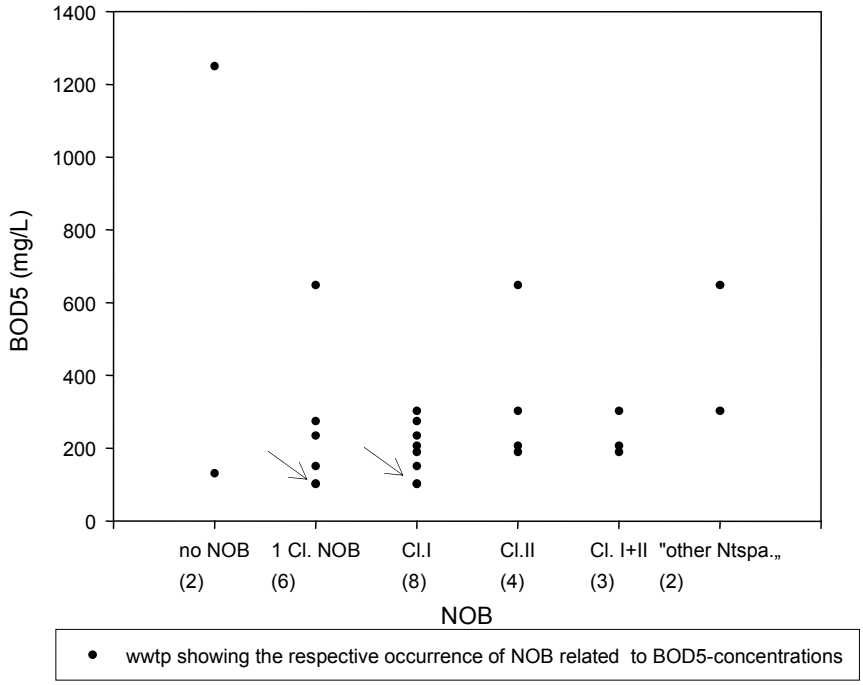



**Figure27:** Differences in occurrence of NOB related to BOD5; the mean BOD5-value of each wwtp is given
total sample number = 11; N of each category are given in bracksets underneath the respective categories.
"no NOB." = no NOB could be found; "1 Cl. NOB" = either Cluster I or II detected
"Cl.I" = "Cluster I" detected – both, solely "Cluster I" as well as together with "Cluster II" counted
"Cl.II" = "Cluster II" detected – both, solely "Cluster II" as well as together with "Cluster I" counted
"Cl. I+II" = both, "Cluster I and II" detected; "other Ntspa." = "other *Nitrospira*" detected
→ 2 wwtps represented by one point (having highly similar BOD5-concentrations)

### C.3.3.5. COD/BOD5-ratio

The ratio of COD to BOD5 is interesting because it indicates the amount of carbon-containing substances difficult to degrade, and was done separately for the two main types of wwtps. AOB- and NOB-diversity of both types of wwtps did not show any correlation with the respective COD/BOD5-values (based on average COD and BOD5-values for each plant).

Occurrence of different NOB- and AOB-clusters as well as abundance of members of Cluster 6a seemed to be independent of the ratio. No correlation between abundance of the genus *Nitrospira* or "Cluster I" and "Cluster II" with the ratio was visible.

### C.3.3.6. "$N_{in}$"-concentrations

Analysis was done according to the same considerations as for BOD5-values (only for SBRs). The average "$N_{in}$"-value of the SBR in Rapp-Kutzenhausen was, compared to the corresponding high average BOD5-value, not higher than the mean values of other treatment plants (as reflected in the extremely high BOD5/"$N_{in}$"-ratio of Rapp-Kutzenhausen). Again no correlations could be found for any data concerning abundance of the genus *Nitrospira* and "Cluster I" and "Cluster II", nor for differences in the abundance of Cluster 6a-like AOB. Both, halophilic and –tolerant AOB and "other *Nitrospira*" occurred at increased nitrogen concentrations (see Fig.28 and 29). Again, trends were not based on the same wwtps (apart from Hettstedt which was harbouring both groups), as for the other plants, showing co-occurrence of halophilic and –tolerant AOB and "other *Nitrospira*", no data concerning the prevailing nitrogen concentrations could be obtained. The diversity of AOB- and NOB-clusters could not be investigated since only values for samples harbouring maximally two different clusters, have been available.

### C.3.3.7 BOD5/"$N_{in}$"-ratio

These ratios are based on the average BOD5- and "$N_{in}$"-concentrations of each plant. In SBRs no correlation could be seen between BOD5/"$N_{in}$"-ratios and abundances of *Nitrospira*-like bacteria, *Nitrospira*-"Cluster I" and -"Cluster II". Noticeable was the high ratio of the SBR in Rapp-Kutzenhausen (22.7) compared to the ratios of the other SBRs. This sample was the only one that contained no detectable NOB or AOB. The other two samples without detectable NOB originated from the wwtps in Langenzenn and Bad Zwischenahn (though for the latter one evidence for the occurrence of "*Candidatus* Nitrotoga arctica" existed). However, BOD5/"$N_{in}$"-ratios for these two plants were not included in the analysis, since, firstly for both only measurements of $NH_3$ were available. Secondly, for Bad Zwischenahn $NH_3$ concentrations were measured only once during the time of investigation and thus only a single value was available. Furthermore, no BOD5-measurements existed for the investigated time-period for the SBR in Bad Zwischenahn.

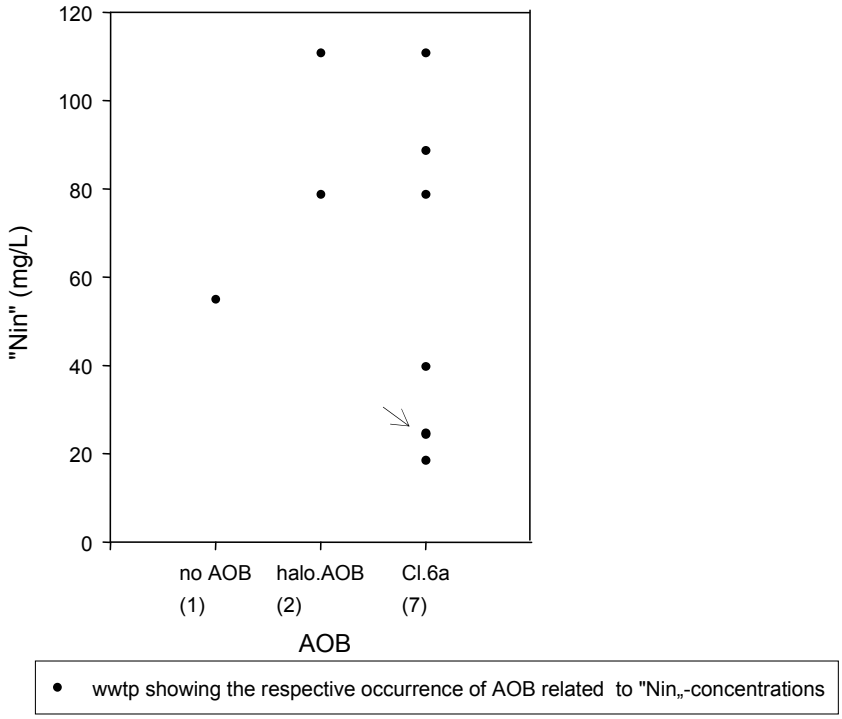



**Figure 28:** Difference in occurrence of AOB related to "$N_{in}$"; for each wwtp the respective mean "Nin"-concentration is given
total sample size = 8; N for each category are given in brackets underneath the respective categories.
no AOB = no AOB have been found; halo.AOB = occurrence of halophilic &-tolerant AOB
Cl.6a = Cluster 6a detected
→ 2 wwtps represented by one point (same "Nin"-concentrations)

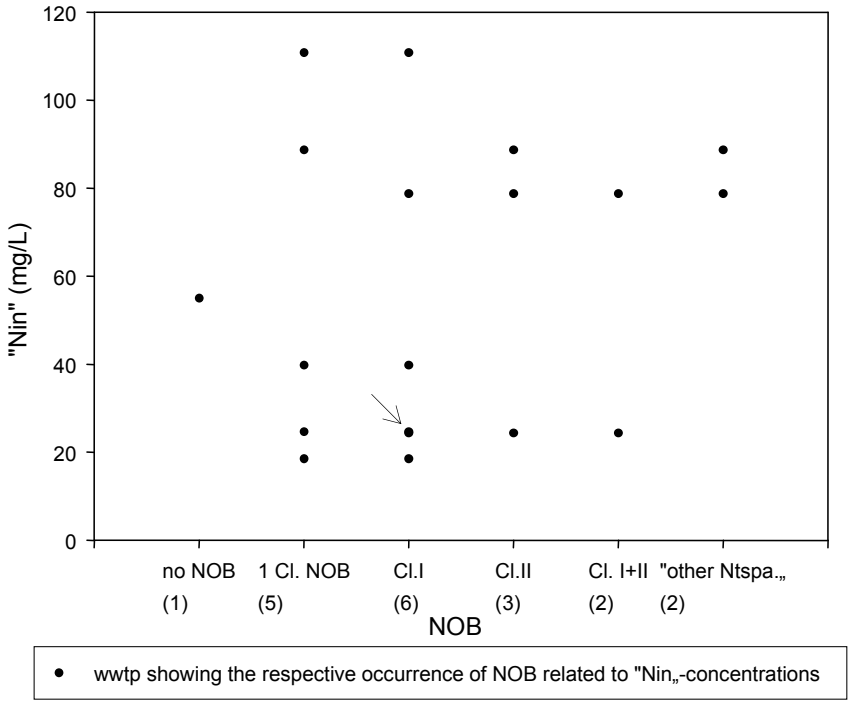



**Figure29:** Difference in occurrence of NOB related to "$N_{in}$"; for each plant the respective mean "Nin"-concentration is given;
Total sample size = 8; N for each category are given in brackets underneath the respective categories.
"no NOB" = no NOB could be found; "1 Cl. NOB" = either "Cluster I" or "Cluster II" detected
"Cl.I" = "Cluster I" detected – both, solely "Cluster I" as well as together with "Cluster II" counted
"Cl.II" = "Cluster II" detected – both, solely "Cluster II" as well as together with "Cluster I" counted
"Cl. I+II" = both, "Cluster I and II" detected; "other Ntspa." = "other *Nitrospira*" detected
→ 2 wwtps represented by one point (having highly similar "Nin"-concentrations)

Halophilic and –tolerant AOB occurred at low ratios while members of Cluster 6a appeared throughout the range of BOD5/"$N_{in}$"-values (Fig. 30).

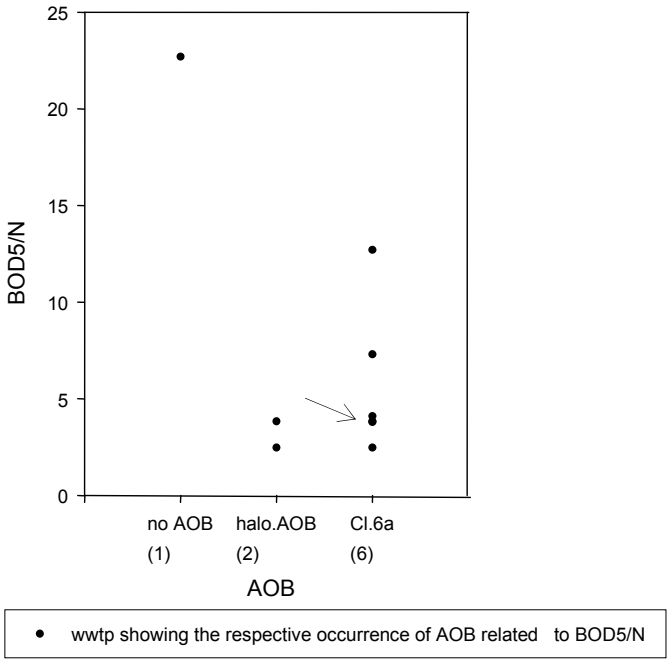



**Figure 30:** Difference in occurrence of AOB related to BOD5/N; ratios are based on the mean BOD5- and N-concentrations of the respective plant; total sample size = 7; N of each category are given in brackets underneath the respective categories.
no AOB = no AOB have been found; halo.AOB = occurrence of halophilic &-tolerant AOB
Cl.6a = Cluster 6a detected
→ 2 wwtps represented by one point (same BOD5/"Nin"-ratio)

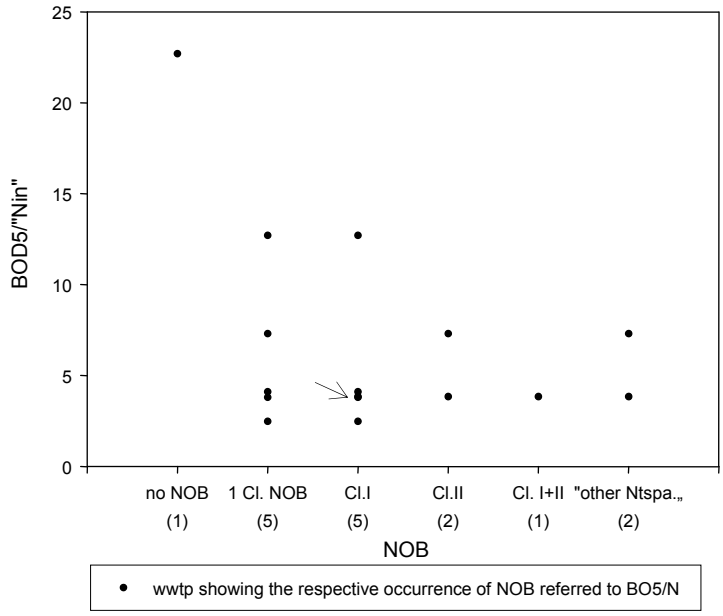



**Figure31:** Difference in occurrence of NOB related to BOD5/"$N_{in}$"; ratios are based on the mean BOD5- and N-concentrations of the respective plant; total sample number = 7; N of each category are given in brackets underneath the respective category.
"no NOB.": no NOB could be found; "1 Cl. NOB": either "Cluster I" or "Cluster II" detected
"Cl.I" = "Cluster I" detected – both, solely "Cluster I" as well as together with "Cluster II" counted
"Cl.II" = "Cluster II" detected – both, solely "Cluster II" as well as together with "Cluster I" counted
"Cl. I+II" = both, "Cluster I and II" detected; "other Ntspa." = "other *Nitrospira*" detected
→ 2 wwtps represented by one point (having the same BOD5/"Nin"-ratio)

"Other *Nitrospira*" occurred indeed in wwtps with low BOD5/"Nin"-ratios as well, but no difference to the occurrence of *Nitrospira*-Cluster II-like bacteria could be seen. Furthermore, *Nitrospira*-Cluster I-like bacteria also occurred in wwtps with low BOD5/"Nin"-ratios apart from one wwtp with a ratio of 12.7 (Waldsassen) (Fig.31).

### C.3.3.8. Index of sludge volume (ISV)

The average ISV, for which values were available for most SBRs but no conventioal treatment plants, did not exhibit any influence on AOB- and NOB-diversity nor on the occurrence of specific AOB and NOB. No effect of the ISV on abundances of the genus *Nitrospira* and "Cluster I and II" was visible.

### C.3.3.9 Ratio of "loading time" to "reaction time" ($t_F/t_R$-ratio)

Data for this operational parameter were available for 7 of the 8 sampled DIC-SBRs. The diversity in these plants (numbers of different AOB- and/or NOB-clusters), as well as the occurrence of certain AOB-clusters and NOB-clusters, were not related to this operational parameter, nor was the abundance of the genus *Nitrospira*. Abundances of "Cluster I" and "Cluster II" did not seem to be influenced by the $t_F/t_R$-ratio – though the sample with the highest ratio (Deuz; 0.4) exhibited a lower abundance of "Cluster I" relative to all *Nitrospira*-like bacteria than the other samples. This resulted in the impression that the abundance of "Cluster I" was declining with an increasing $t_F/t_R$-ratio (Fig.32). However, the abundances of both, "Cluster I and II" relative to all bacteria, were higher in the sample taken from Deuz than in the other samples (Figure 33).

### C.3.3.10. Further characterization of specific samples

The most diverse samples in terms of numbers of different AOB- and/or NOB-clusters were from the SBRs in Rosenheim and Ingolstadt. These two plants had the highest concentrations of DM and a rather high T in common (Rosenheim: 8.2 g/l and 38.2°C; Ingolstadt: 9.5 g/l; 27.2°C). The SBR Ingolstadt was the only plant, in which *Nc. mobilis*-like AOB (as detected with the probe Ncmob) and the SBR Rosenheim the only one, in which *N. communis*-like AOB were found – however, not much data (conductivity, BOD5, "Nin") were available for these plants and thus conclusions can hardly be drawn.

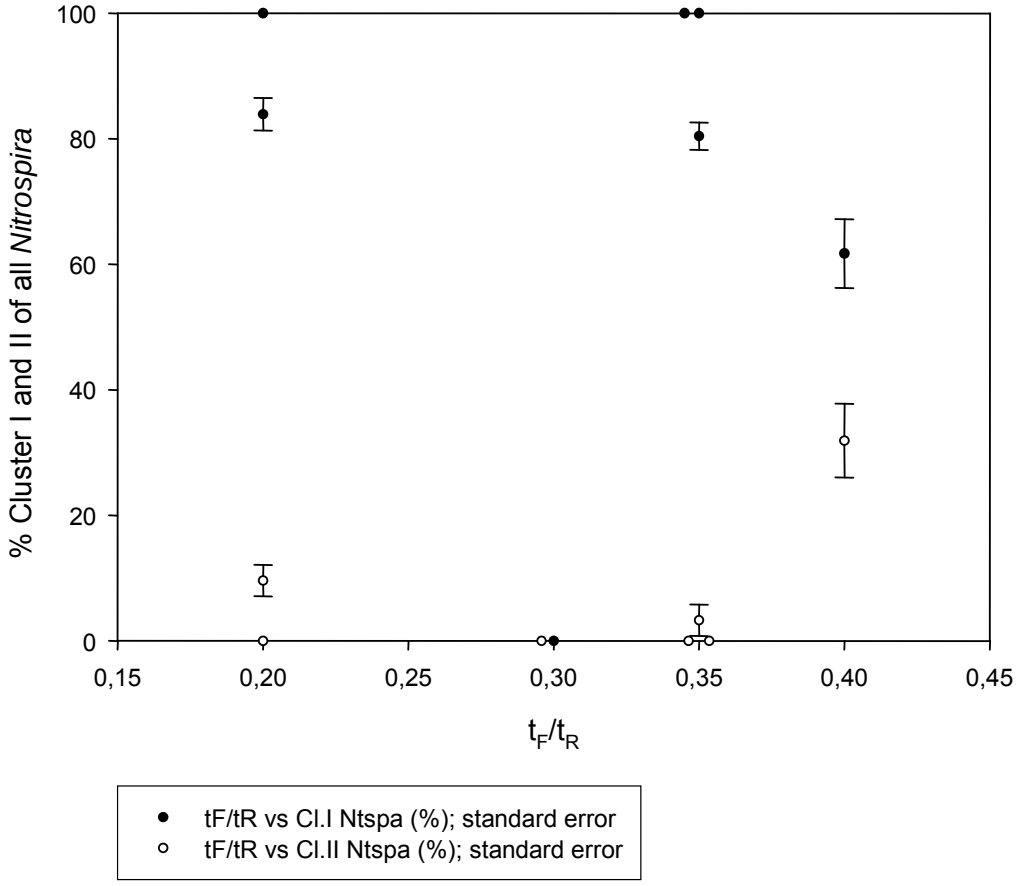



**Figure 32:** % *Nitrospira*-Cluster I and II relative to all *Nitrospira*-like bacteria plotting against the ratio $t_F/t_R$; sample size N=7

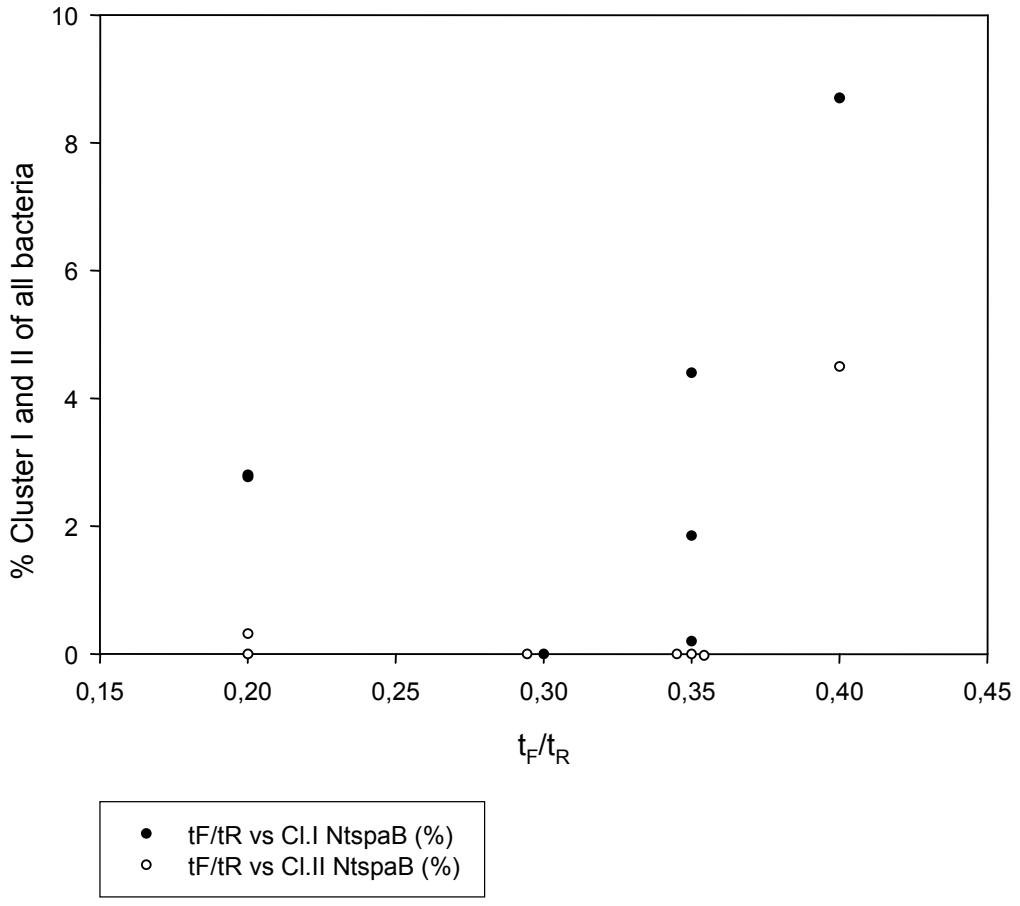



**Figure 33:** % *Nitrospira*-Cluster I and II relative to all bacteria plotted against the ratio $t_F/t_R$ sample size N=7

Samples taken from Rosenheim, Ingolstadt, Plattling, TBA GZM Lyss, Hettstedt and Oberding contained more than 4 different AOB- plus NOB-clusters. All except the sample from Oberding showed a relatively high concentration of DM (> 6 g/l) and all apart from the sample from Hettstedt exhibited T > 27°C (Hettstedt T = 15.3°C). Samples harbouring bacteria affiliated to just one *Nitrospira*-cluster originated from the wwtps in Waldsassen, Seefeld, Radeburg and Bruchmühlen. The pH in the SBRs in Waldsassen and Bruchmühlen was rather low (6.4; 6.6) within the narrow range of pH values of all plants. Still, a low pH was also found in the SBR in Weißtal (6.5); for the SBRs in Seefeld and Radeburg no values were available. Bacteria belonging to the suggested "environmental Cluster 6a" occurred in samples from Ingolstadt and TBA GZM Lyss, for which no common characteristics could be identified, apart from the rather high concentration of DM and the rather high T, which could also be found in other plants.

Co-occurrence of "other *Nitrospira*" and halophilic and –tolerant AOB could be detected in the samples from Rosenheim, Ingolstadt and Hettstedt. Unfortunately not much data was available for those plants – the first two showed higher T and all showed elevated DM concentrations. Higher DM concentrations could nevertheless also be seen in other plants not harbouring those groups. When considering SBRs only, especially halophilic and –tolerant AOB seemed to occur at a rather high conductivity. Still, firstly these assumptions were based on a low sample size and secondly separate analysis of the two main wwtp-types, when considering parameters apart from those describing C- and N-concentrations, is questionable (see D.5.5.2.). Both groups seemed to appear at elevated BOD5- and "$N_{in}$"-concentrations and halophilic and –tolerant AOB furthermore at low BOD5/"$N_{in}$"-ratios (see C.3.3.4., C.3.3.6. and C.3.3.7.).

## C.4. Diversity analysis by PCR

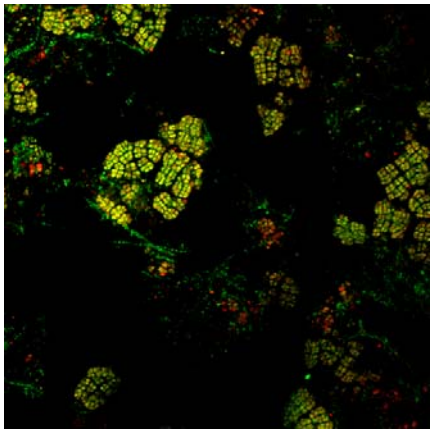
DNA of all samples was extracted by using the PowerSoil-Kit and could be amplified by PCR. Concentrations of DNA ranged between approx. 20 and 120 ng/µl. Contamination was excluded by negative controls for the extraction and for the PCR, but also by screening all isolated DNA for contaminations by plasmids by performing PCR with M13-primers and, if necessary, by further doing a Nested-PCR-approach (see B.11.4.).

### C.4.1. 16S rDNA analysis by PCR

#### C.4.1.1 16S rDNA analysis by PCR for Rapp-Kutzenhausen

For Rapp-Kutzenhausen the 16S rRNA-gene was amplified using the primer pair 616V and 630R targeting most bacteria, to get an idea which bacteria could dominate the sample as it seemed to contain almost only one cell type (Fig. 34), but no AOB and NOB. This cell type occured mainly in coccal tetrads or clusters.

**Figure 34:** Dominant cell-type in Rapp-Kutzenhausen;
(FLUOS: EUBmix; Cy3: *α-Proteobacteria*-specific probe)



30 clones were screened for an insert of the right size. 24 clones with an insert of the right size were further characterized by RFLP and 9 different patterns obtained. The plasmid of 14 clones was isolated and the insert sequenced. Analysis in ARB revealed affiliation of the sequences to the phyla *Proteobacteria* (*α-Proteobacteria:* 8 sequences; *γ-Proteobacteria*: 1), *Acidobacteria* (1), *Planctomycetes* (1), *Firmicutes* (1) and the suggested phylum OP11 (2) (see Fig.35). 7 of the 8 sequences affiliated to the *α-Proteobacteria* showed strong affiliation to *Defluviicoccus* (*vanus*), which belongs to the *Rhodospirillaceae* (*α-Proteobacteria*). Applying FISH with probes specific for *α-Proteobacteria* (Alf1b) and for all bacteria (EUBmix) revealed that almost all bacteria were targeted by both probes (Fig. 34).

#### C.4.1.2. Analysis of *Nitrospira*-like 16S rDNA

For the two samples apart from Rapp-Kutzenhausen, which did not give NOB-specific FISH-signal, taken from Langenzenn and Bad Zwischenahn (no assured FISH-signal), as well as for three of the five samples containing "other *Nitrospira*", Ingolstadt, Oberding and Hettstedt, and furthermore of the samples from Huntlosen, Bruchmühlen and Radeburg, 16S rDNA of

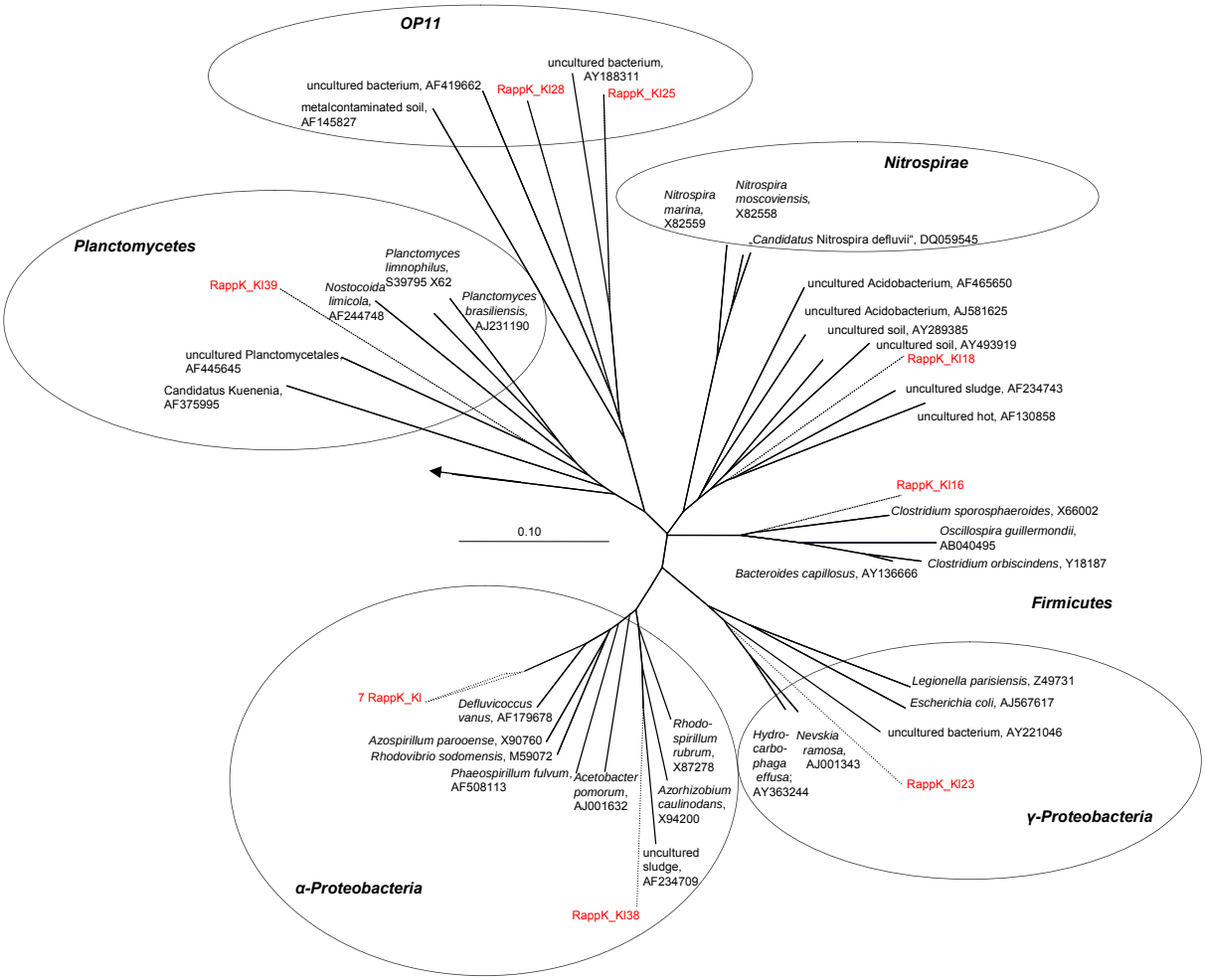



**Figure 35:** NJ-tree, showing the phyla to which the obtained sequences from the sample Rapp-Kutzenhausen were affiliated; additionally the phylum *Nitrospirae* is shown; scale bar indicates 10% sequences divergence; ····· clones obtained during this study (marked in red)

*Nitrospira*-like bacteria was amplified with the general forward primer 616V and the specific reverse primer 1158R. Details of the cloning procedure like the number of clones screened for the right insert size can be seen in Tab. 34. RFLP was then done with the respective clones and the results of this step in terms of the number of different obtained patterns, can be seen in Tab. 34 as well. Furthermore the number of sequenced clones, sequences showing affiliation to *Nitrospira*-specific sequences and obtained full sequences are listed in Tab. 34.

None of the sequences originating from the sample from Langenzenn was affiliated to the phylum *Nitrospirae*. The nearest BLAST hits showed sequences from *β-Proteobacteria* (*Rhodoferax*), *Acidobacteria* and the suggested phyla TM7 and SR1. 19 sequences obtained from the sample from Bad Zwischenahn were analysed and one of those could be affiliated to the genus *Nitrospira*, contradicting the previously obtained FISH result (no *Nitrospira*-like organisms within the sample). The other sequences were assigned to *Acidobacteria*, *Chloroflexi*, *γ-Proteobacteria* and *Verrucomicrobia*.

Full sequences were obtained for some sequences of the different wwtps (Tab.34), showing differing affiliation within the genus *Nitrospira* (Fig.36).

**Table 34:** Cloning and sequence data of the wwtp-samples (*Nitrospira*-specific 16S rRNA approach); N = number

| Treatment plant | N of clones Screened | clones with right insert (N) | different patterns (RFLP) (N) | sequenced clones (N) | *Nitrospira*-specific sequences (N) | N of full sequences |
|---|---|---|---|---|---|---|
| Bruchmühlen | 30 | 29 | 3 | 10 | 6 | 4 |
| Radeburg | 30 | 21 | 2 | 9 | 9 | 7 |
| Hettstedt | 30 | 27 | 8 | 17 | 10 | 3 |
| Huntlosen | 30 | 17 | 3 | 6 | 5 | 2 |
| Oberding | 60 | 41 | - * | 31 | 30 | 9 |
| Ingolstadt | 60 | 54 | - * | 20 | 19 | 4 |
| Bad Zwischenahn | 30 | 23 | 11 | 19 | 1 | 1 |
| Langenzenn | 30 | 27 | 12 | 20 | - | - |

* RFLP was not applied to these samples but clones with the right insert size were randomly chosen and the plasmid sequenced.

All full sequences were tested by ARB, rdp-chimera check and bellerophon for being chimerae. Seven chimeric sequences, from the plants in Ingolstadt, Oberding and Hettstedt, could be detected and were subsequently deleted. All remaining full sequences fell into Cluster I or II – no sequence related to *N. marina* could be found, nor did any sequence support the assumed "other *Nitrospira*" in the samples from Ingolstadt, Oberding and Hettstedt. *Nitrospira* Cluster I-like sequences were represented in all samples, for which sequences could be obtained: Bruchmühlen (4 sequences), Radeburg (7), Hettstedt (2), Huntlosen (1), Bad Zwischenahn (1), Oberding (7) and Ingolstadt (4). Almost all sequences fell in close proximity to "*Candidatus* Nitrospira defluvii" and most showed >99% similarity to each other and to "*Candidatus* Nitrospira defluvii". Sequences of all different plants clustered together, meaning that no sub-groups containing sequences of only one sample or reactor type could be identified. However, within and around the cluster comprising "*Candidatus* Nitrospira defluvii" in the consensus tree, there was not much phylogenetic resolution anymore due to differing branching patterns obtained by the various applied treeing methods. Sequences of the samples taken from Ingolstadt and Oberding seemed to be most diverse within Cluster I and fell also partly at the root of the cluster. Furthermore, the SBR in Ingolstadt was the only wwtp not represented by sequences strongly affiliated to "*Candidatus* Nitrospira defluvii".

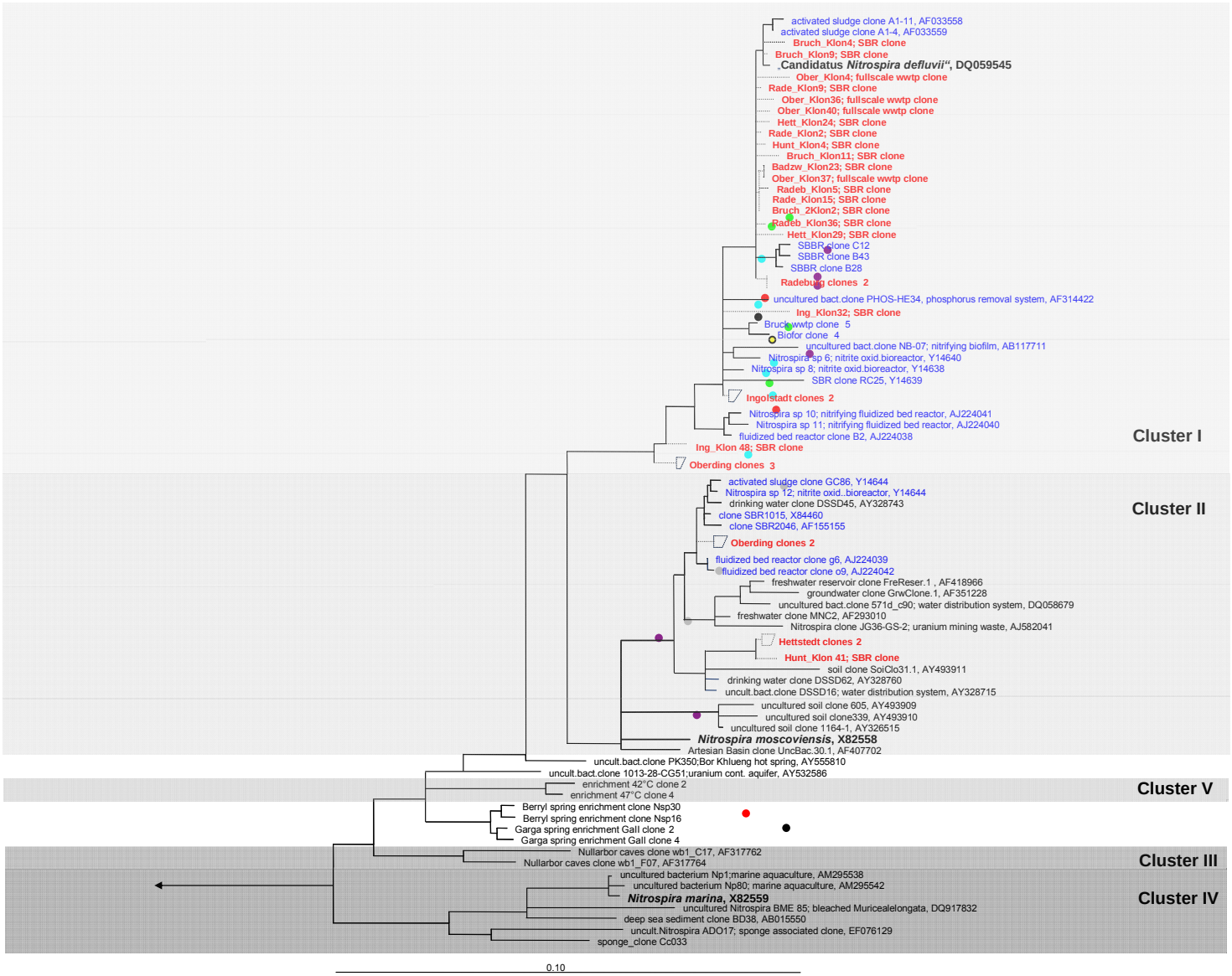
Only sequences obtained from the samples from Oberding (2), Hettstedt (2) and Huntlosen (1) were related to Cluster II. Sequences from the wwtp in Oberding fell very close to the other sequences from wwtps in Cluster II, while sequences of the other two plants fell close to clones obtained from soil and drinking water distribution systems. Cluster II-like sequences

clustered closer together and to other sequences from wwtps than to *Nitrospira moscoviensis*. None of the sequences from the SBR in Ingolstadt could be assigned to Cluster II, though FISH-results revealed appearance of Cluster II-*Nitrospira*-like bacteria. Apart from the samples Ingolstadt and Bad Zwischenahn, results of the 16S rRNA analysis were consistent with the previously obtained FISH results.

**Figure 36:** Consensus-tree for Nitrospira-sequences; scale bar indicates 10% sequences divergence

● Bruchmühlen; ● Oberding; ● Radeburg; ● Huntlosen; ● Hettstedt; ○ Bad Zwischenahn; ● Ingolstadt

····· clones obtained during this study (marked in red); reference clones originating from wwtps are marked in blue


activated sludge clone A1-11, AF033558
activated sludge clone A1-4, AF033559
Bruch_Klon4; SBR clone
Bruch_Klon9; SBR clone
„Candidatus Nitrospira defluvii“, DQ059545
Ober_Klon4; fullscale wwtp clone
Rade_Klon9; SBR clone
Ober_Klon36; fullscale wwtp clone
Ober_Klon40; fullscale wwtp clone
Hett_Klon24; SBR clone
Rade_Klon2; SBR clone
Hunt_Klon4; SBR clone
Bruch_Klon11; SBR clone
Badzw_Klon23; SBR clone
Ober_Klon37; fullscale wwtp clone
Radeb_Klon5; SBR clone
Rade_Klon15; SBR clone
Bruch_2Klon2; SBR clone
Radeb_Klon36; SBR clone
Hett_Klon29; SBR clone
SBBR clone C12
SBBR clone B43
SBBR clone B28
Radeburg clones 2
uncultured bact.clone PHOS-HE34, phosphorus removal system, AF314422
Ing_Klon32; SBR clone
Bruch wwtp clone 5
Biofor clone 4
uncultured bact.clone NB-07; nitrifying biofilm, AB117711
Nitrospira sp 6; nitrite oxid.bioreactor, Y14640
Nitrospira sp 8; nitrite oxid.bioreactor, Y14638
SBR clone RC25, Y14639
Ingolstadt clones 2
Nitrospira sp 10; nitrifying fluidized bed reactor, AJ224041
Nitrospira sp 11; nitrifying fluidized bed reactor, AJ224040
fluidized bed reactor clone B2, AJ224038
Ing_Klon 48; SBR clone
Oberding clones 3
Cluster I
activated sludge clone GC86, Y14644
Nitrospira sp 12; nitrite oxid..bioreactor, Y14644
drinking water clone DSSD45, AY328743
clone SBR1015, X84460
clone SBR2046, AF155155
Oberding clones 2
fluidized bed reactor clone g6, AJ224039
fluidized bed reactor clone o9, AJ224042
freshwater reservoir clone FreReser.1 , AF418966
groundwater clone GrwClone.1, AF351228
uncultured bact.clone 571d_c90; water distribution system, DQ058679
freshwater clone MNC2, AF293010
Nitrospira clone JG36-GS-2; uranium mining waste, AJ582041
Hettstedt clones 2
Hunt_Klon 41; SBR clone
soil clone SoiClo31.1, AY493911
drinking water clone DSSD62, AY328760
uncult.bact.clone DSSD16; water distribution system, AY328715
uncultured soil clone 605, AY493909
uncultured soil clone339, AY493910
uncultured soil clone 1164-1, AY326515
Nitrospira moscoviensis, X82558
Artesian Basin clone UncBac.30.1, AF407702
Cluster II
uncult.bact.clone PK350;Bor Khlueng hot spring, AY555810
uncult.bact.clone 1013-28-CG51;uranium cont. aquifer, AY532586
enrichment 42°C clone 2
enrichment 47°C clone 4
Cluster V
Berryl spring enrichment clone Nsp30
Berryl spring enrichment clone Nsp16
Garga spring enrichment GalI clone 2
Garga spring enrichment GalI clone 4
Nullarbor caves clone wb1_C17, AF317762
Nullarbor caves clone wb1_F07, AF317764
Cluster III
uncultured bacterium Np1;marine aquaculture, AM295538
uncultured bacterium Np80; marine aquaculture, AM295542
Nitrospira marina, X82559
uncultured Nitrospira BME 85; bleached Muricealelongata, DQ917832
deep sea sediment clone BD38, AB015550
uncult.Nitrospira ADO17; sponge associated clone, EF076129
sponge_clone Cc033
Cluster IV
0.10

### C.4.2. Crenarchaeotal *amoA* analysis by PCR

All wwtps were screened for ammonia-oxidizing crenarchaeotes in order to find out more about their potential role in nitrogen removal from wastewater.

#### C.4.2.1. Amplification of crenarchaeotal *amoA*-like sequences

Amplification of crenarchaeotal *amoA*-like sequences was tried with different primer pairs (B.11.3., Tab.14). Firstly the CrenamoA F/R-primer pair (Könneke) was used. As decreasing the concentration of the primers used (50 to 25 pmol/µl), dilution of the template (1:100) and performance of a gradient-PCR for determination of the optimal $MgCl_2$ concentration (1 to 2 mM in the reaction tubes), did not result in single specific bands, further analysis was done with the primer pair Francis CrenamoA F&R. Amplification with this primer pair did not show specific products either, therefore a temperature-gradient PCR was performed, ranging from 53°C to 62°C. Although the result of this gradient PCR was not clear, an annealing temperature of 56°C was chosen. Applying this temperature did not prevent unspecific amplification. Despite this fact, products were cloned, but firstly purified by transferring bands of the right size from agarose gels. Cloning was performed for the samples from TBA GZM Lyss (TBA), Plattling, Ampfing (AM) and Altmannstein (AL). RFLP was applied with two different enzymes, nevertheless plasmids of all clones with the right insert size were isolated and sequenced, due to the low number of clones and the minor differences between the inserts as revealed by RFLP (20;10;25;25; number of clones screened and sequenced for each sample). After controlling by BLAST-search whether all the sequences were indeed crenarchaeotal *amoA*-like sequences, all verified sequences were imported into ARB: 19 sequences for the sample from TBA GZM Lyss, 9 for the SBR in Ampfing, 9 for the SBR in Altmannstein and none for the wwtp in Plattling (no crenarchaeotal *amoA*-like sequences). Sequences were compared on the level of nucleotides and of amino acids. Derived amino acid sequences showed no internal stop codons apart from one sequence: Clone 1 from AL exhibited 4 stop codons at the amino acid positions 96, 140, 178 and 201.

#### C.4.2.2. Phylogenetic analysis of crenarchaeotal *amoA*-like sequences

Most sequences from the three investigated samples (AM, AL, TBA), fell into Cluster I.1b., the “soil-sediment”-Cluster (Fig.37). 3 sequences fell into Cluster I.1a., the rather “marine”-Cluster. Clones AM_9 and AM_16 fell quite close to other activated sludge sequences (Park

*et al.*, 2006), which had been assigned to the marine cluster. The third sequence, AL_1, could not be clearly assigned to a position within Cluster I.1.a., since its phylogenetic affiliation was varying a lot in the different treeing methods. The quite abundant multifurcations in the consensus tree did not give much information about how the different wwtps were clustering together – anyway, many sequences within Cluster I.1b. showed >99%-similarity to each other (see Fig. 37) and only some clusters appeared to be rather distinct (the cluster containing mainly TBA- and the one comprising mainly AM-sequences).

### C.4.2.3. Activity analysis by Reverse Transcription PCR

Coextraction of DNA and RNA (Lueders *et al.*, 2004) from the sample from the TBA GZM Lyss worked well as could be seen by gel electrophoresis. Still, the concentration of extracted RNA was not high (approx. 53 ng/µl). RNA extraction by TRIzol/Glycogen also worked well according to the measured concentration of the isolated RNA (~ 56 and 163 ng/µl), but controlling by gel electrophoresis showed just very light bands. After digestion with DNase, for both extraction approaches no bands were visible anymore. As measurements of RNA-concentrations after digestion showed only some RNA (approx. 30 ng/µl) in the sample extracted according to the first protocol (Lueders *et al.*, 2004), further analysis was done with this approach. PCR with primers specific for 16S rDNA and primers specific for crenarchaeotal *amoA*-like genes showed no product for the sample after digestion. The same PCRs were done before digestion, but showed just a product for the amplification of 16S rDNA. Dilution of the template (1:10; 1:100) did not result in amplification of crenarchaeotal *amoA*-genes either. cDNA was produced with a reverse primer for crenarchaeotal *amoA*- (Francis CrenamoA-R) and 16S rRNA-genes (630R).

Though the cDNA could not be amplified with the primer pairs 616V/630R and 616V/1492R, utilization of 781f and 1492r, flanking a shorter piece of the 16S rDNA, did show a PCR-product. No product was obtained for amplification of crenarchaeotal *amoA*-specific cDNA. Amplification of crenarchaeotal *amoA*-genes of the samples, from which sequences affiliated to crenarchaeotal *amoA* had already been amplified, did not work either.

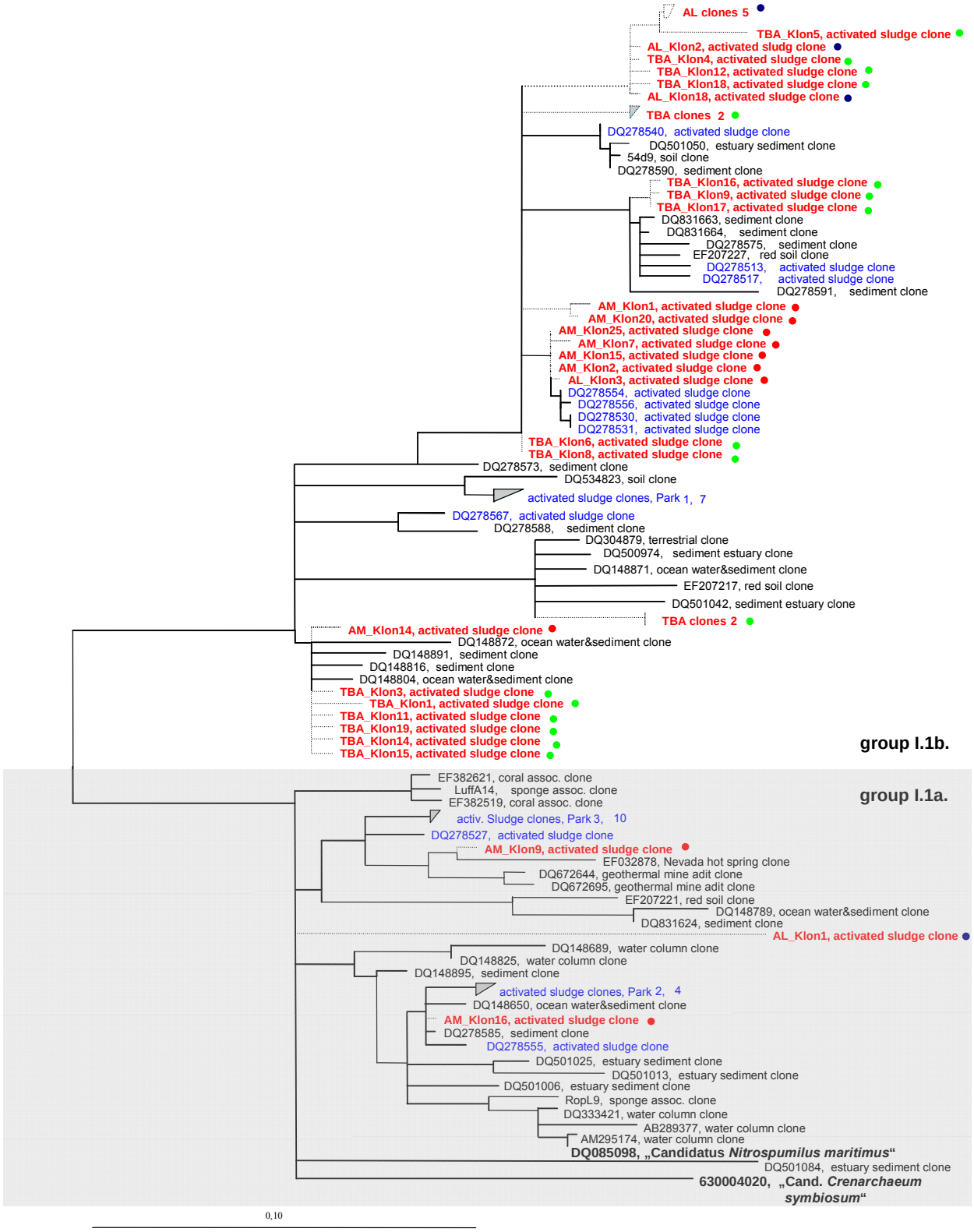



**Figure 37:** Consensus-tree of crenarchaeotal *amoA*-like sequences (nucleotides; without considering every third nucleotide (wobble position))
activated sludge Park 1 = DQ278541, DQ278551, DQ278533, DQ278546, DQ278548, DQ278498 and DQ278511; activated sludge Park 2 = DQ278558, DQ278560, DQ278561, DQ278564; activated sludge Park 3 = DQ278525, DQ278526, DQ278528, DQ278529, DQ278519, DQ278520, DQ278514, DQ278515, DQ278516, DQ278524
in red: during this study obtained sequences ..... ; in blue: activated sludge sequences from other studies (Park *et al.*, 2006)
scale bar: representing 10% of proposed sequence divergence; ● AL; ● TBA; ● AM
(no outgroup)

### C.4.2.4. Screening for contaminations

It is important to keep in mind that amplification assays of the crenarchaeotal *amoA*-genes of the samples (TBA GZM Lyss, Ampfing, Altmannstein), from which sequences had already been obtained, first yielded positive products when repeating the PCR approach (with varying annealing T), but did after the fourth time not yield any bands anymore. Amplification of the 16S rDNA from those samples was still possible.

#### C.4.2.4.1. Screening with Nested PCRs

Screening for plasmid-contamination was done with both, M13- and TOPO-primers. For both assays no distinct bands were visible, but either lots of bands or something best described as a large "smearing band", covering a size-range of about 200 to ~1000 base pairs (Fig. 38). As these bands were also visible at the height at which crenarchaeotal *amoA*-inserts (amplified from a plasmid and therefore also containing flanking vector sequence) would be detectable (~880 bp), nested PCRs were performed with the M13- and TOPO-PCR-products as templates. Nested PCRs with either the PCR-product of the M13-PCR or the product of the TOPO-PCR, showed no bands when "reamplified" with Francis CrenamoA-F&R primers, apart from the positive controls. Besides the normal PCR-positive control, a PCR-product of the positive control of a previously performed PCR of crenarchaeotal *amoA*, was reamplified. A positive reamplification of this control allowed excluding template inhibition as a reason for the absence of bands (B.11.4.). The same nested PCRs were carried out with the Könneke CrenamoA F&R primers. Both nested PCRs, but more the PCR with TOPO-PCR-products as template, showed some bands, though rather at a lower height than the one at which crenarchaeotal *amoA* would appear (Fig. 39). Still, as described previously, direct amplification of crenarchaeotal *amoA*-genes within the samples, did not show any products for both primer pairs (Francis, Könneke) although 16S rDNA could still be amplified.
Nested PCRs suggested that the samples are not contaminated as did the phylogenetic affiliation of the sequences. Phylogenetic affiliation was analysed together with all other crenarchaeotal *amoA*-like sequences obtained previously in this laboratory (results not shown; see Disc. D.7.3.). Sequences obtained during this study did not fall in close proximity to those other sequences.

**Figure 38:** Screening for plasmid-contaminations with TOPO-primers (left) and M13-primers (right)
For 3 samples each; "-" = negative control

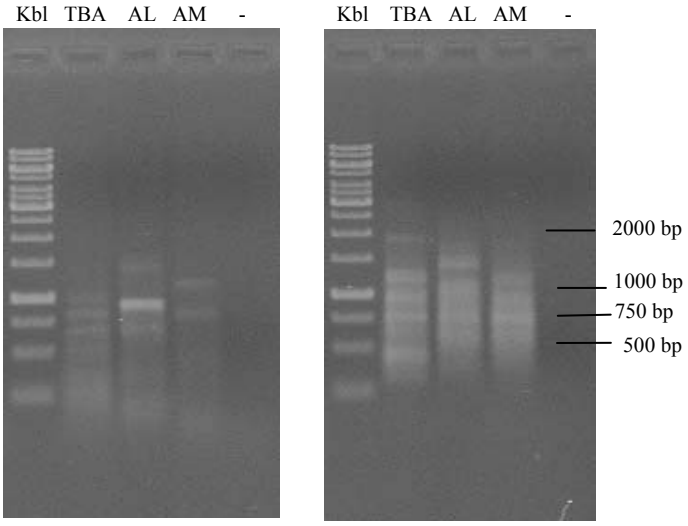
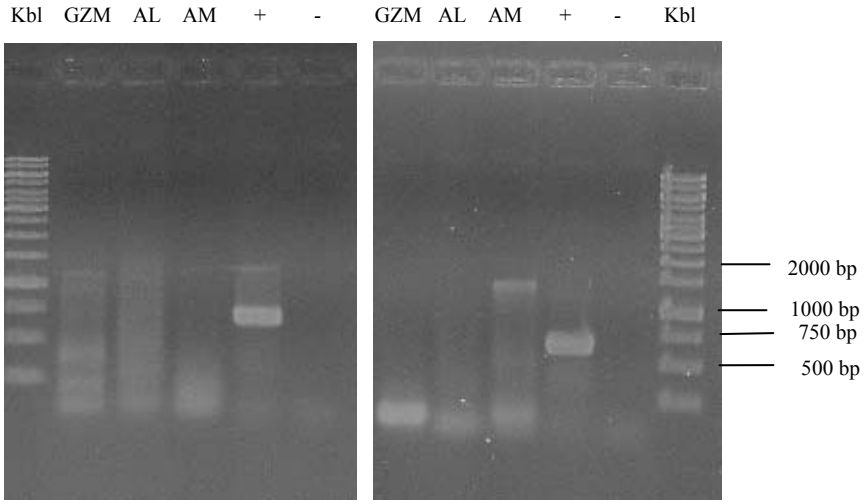



**Figure 39**: Screening for plasmid-contaminations by Nested PCR; reamplification with Könneke-CrenamoA-primers
Left: reamplification of PCR-products (TOPO-primers),; right: reampl. of PCR-products (M13-primers)
For 3 samples each; "+" = positive control; "-" = negative control




### C.4.2.4.2. Screening by primer combination

Screening by PCR with a combination of M13- and Francis crenarchaeotal *amoA*-specific primers was applied. Using one M13- and one Francis-primer should secure that a product is due to a plasmid containing a crenarchaeotal *amoA*-like sequence as insert. Because the putative insert could be integrated in two directions (into the vector), two approaches were carried out: one combining M13F and Francis-CrenamoA-R, and secondly M13F and Francis-CrenamoA-F. Both combinations showed no bands, but the first combination showed a band with the positive control.

# D. Discussion

## D.1. Data editing

Data were obtained for 3 to 31 days before the sampling date and on average data for the last 20 days were analysed. This was done, because the last 2-3 weeks (comparable to the average sludge age) before sampling should reflect the conditions influencing the monitored nitrifier populations. The big differences in intervals of measurements, ranging from daily to once in two weeks (see appendix, Tab.35) were mostly disregarded to provide more completeness of the dataset. However, single measurements were excluded, but this restriction was eased for factors like T (temperature), pH, SA (sludge age), SVI (sludge volume index), DM (dry matter) and $O_2$ concentration. T and pH were used after consultation with the respective contact person who could provide information about the constancy of these parameters. SA, SVI and DM were considered as being not that fluctuating and thus included. $O_2$ concentration was regarded as rather stable since this parameter is normally regulated – the same is true for the pH. For some of the latter parameters, values determined after the sampling date were available. For the SA general averaged values, not determined shortly before or after sampling (e.g.: annual values), were accepted. All values of this kind are marked in the respective tables (see Tab.26, p.76 and Tab.27, p.79; Tab. 35, appendix). Firstly, comparability of data from sampling points in January, March and May was questioned in regard to temperature. However, the high temperature in three of the 5 conventional plants, which have been sampled in January (26 - 30°C; Oberding, Plattling and TBA GZM Lyss), confirm, at least for these plants, independence of the outside temperature. Further editing, aimed at providing more comparable and representative data, is described in the next sections.

### D.1.1. Carbon concentrations in conventional wastewater treatment plants

Concentration of organic carbon in wastewater is reflected best in the amount of COD or BOD5, the latter one more referring to easily biodegradable carbonic compounds. However, concentration of COD and BOD5 of the influent do not reflect the actual concentrations within the activated sludge basin. When entering the basin, concentrations get immediately diluted to much lower values. As the basin is continuously mixed, concentrations within the basin are everywhere the same, reflecting a mixture of already decomposed and not yet decomposed compounds (Daims, personal communication) Consequently, effluent concen-

trations for COD and BOD5 have been considered for all further analysis (referred to as "inside" COD or BOD5 concentrations). Interestingly, following this approach, the investigated animal rendering plants look less like eutrophic habitats as previously assumed.

### D.1.2. Nitrogen concentrations in conventional wastewater treatment plants

The immediate dilution of influent concentrations applies also to nitrogen-input in conventional full-scale treatment plants. But as the available ammonia ($NH_3$) is oxidized to nitrite ($NO_2^-$) and further to nitrate ($NO_3^-$), $NH_3$ concentrations are hardly detectable in the effluent. Instead of, firstly the concentration of $NO_3^-$ in the effluent, as mainly representing the fully oxidized $NH_3$, as well as the concentration of $NO_2^-$ in the effluent, representing not fully oxidized $NH_3$, and last remaining $NH_3$ in the effluent, have been summed up to the parameter "N-inside" ("$N_{in}$"). In case TKN has been measured in the effluent, this value has been considered as equal to measured $NH_3$ concentrations as most organic bound nitrogen should already be available (Moser, 1993). Measurements of "total nitrogen" in the effluent were regarded as equivalent to "N-inside". Still, nitrogen concentrations in the effluent will of course not reach initial diluted $NH_3$ concentrations, as nitrogen is removed from the system by denitrification. When aerobic nitrification and denitrification occur in the same basin (simultaneous nitrification and denitrification), nitrifying bacteria will just be exposed to nitrogen concentrations already partly decreased by denitrification. This is also true if denitrification occurs upstream of the nitrification process.

### D.1.3. C and N concentrations in SBRs

Concerning SBRs the situation is quite different from conventional treatment plants. Here, firstly the dilution of the influent entering the SBR is not that high, though the incoming load is mixed with the remaining sludge inside the SBR after decantation. Secondly, there is no continuous effluent but only at the end of the cycle after processing of wastewater. Therefore conditions inside are not well represented in using values measured in the effluent and concentrations would be probably best described in form of a gradient. Because of this for COD and BOD5, incoming concentrations have been considered (referred to as "beginning"-concentrations), which differ strongly from COD and BOD5 values of conventioal treatment plants and can hence rather not or only with caution be compared between SBRs and conventional full-scale treatment plants. These values represent rather the highest

concentrations bacteria are suddenly exposed to. The same is true for nitrogen input, especially as dentrification is generally conducted in a phase following nitrification – although at least for DIC-SBRs there is finally another aerated period prior to sedimentation, in which "final" nitrification of remaining $NH_3$ occurs. Focussing on the "beginning" nitrogen concentrations seemed to be the most reliable variant of comparing nitrogen input at least among SBRs. Both, TKN and "total N" were used as "beginning N concentrations" for the further analysis as either one or the other has been measured in the various plants. "Total N" can be regarded as equivalent to TKN values since "total N" is indeed including $NO_3^-$ and $NO_2^-$ concentrations as well but these can normally be neglected in the influent (Moser, 1993).

One issue rendering comparability of concentrations between the different plants uncertain, is the unknown influence that storage of wastewater in a buffering tank has on its composition. All examined SBRs are filled from a buffering tank and concentrations of the influent are partly measured before reaching this buffering tank. Operation of buffering tanks can vary quite a lot – sometimes sludge is returned to these tanks (from other stages of the cleaning process), sometimes they are mixed (more particulate matter will reach the SBRs) or even aerated and partly sludge is removed (Schreff, personal communication). It is likely that reactions already take place as has been observed for sewage networks (Moser, 1993) and is indicated by frequently happening acidification (Schreff, personal communication). Still, residence time of wastewater in the buffering tank is generally only around 2 – 4 h and the amount of biomass low (Schreff, personal communication), suggesting negligible changes. However, as not more was known about this potential influence, concentrations were used without further considerations.

Though actually the same troubles as for COD and BOD5 values seem to exist when trying to compare N input between conventional treatment plants and SBRs, N concentrations are much more similar.

### D.1.4. C and N concentrations in DIC-SBRs

Principally the same system as for all other SBRs was applied here, meaning that influent-concentrations have been considered. The DIC-SBR operational mode includes two loading events – the first time wastewater with a low C/N-ratio gets introduced, and the second time with a high C/N-ratio to enhance denitrification (see A.3.2). Nitrifiers could benefit from low carbon concentrations because heterotrophic bacteria, when provided with high carbon concentrations, out-compete the slow growing nitrifiers (Dornhofer, 1993; Zhang *et al.*, 1995).

Furthermore, inhibition of nitrifying bacteria at high concentrations of organic carbon has been reported (Yang and Zhang, 1995). Contrary, for denitrification an organic carbon source is needed. This source often has to be externally added to treatment plants to provide functioning denitrification (Ginige *et al.*, 2004). Nitrifiers are assumed to be mainly influenced by the concentrations of COD or BOD5 and N input (reflected either as TKN or "total N") of the firstly loaded wastewater, since nitrification should be highest after the first loading event. Thus, concentrations representing this low C/N-ratio have been analysed. Measurements of COD, BOD5 and TKN or "total N" have been done before the influent reaches the buffering tank, from which wastewater with different C/N-ratio is then conducted to the SBRs. Therefore these measured "original" concentrations do not reflect the composition of the first or the second loading event. In the buffering tank particulate carbon and nitrogen sink to the bottom while some C- and N-compounds dissolve. Only about 20-30% of the incoming nitrogen compounds do not dissolve while for carbon-containing compounds the rate is around 50%. For the first loading event wastewater is taken from the upper part of the tank, which contains now ~50% less carbon and ~30% less nitrogen (Holm, personal communication). Though this is not very accurate, all measured COD and BOD5 values have been decreased by 50% and N values by 30%. Alternatively, original values of the influent (C/N-ratio not modified) could have been used, arguing that latest after the second loading event (wastewater is taken from the lower-middle of the tank) approximately the amounts of the original influent should have reached the SBR. Nitrifiers could also be influenced by the second load as some have been shown to reduce $NO_2^-$ to $N_2$ or $N_2O$ in anoxic envrionments instead of being inactive (Jason *et al.*, 2007; Manser, 2005; Schmidt and Bock, 1997; Schmidt *et al*., 2001). However, this activity is low and thus influences during this period can be neglected. Nevertheless, nitrifiers are certainly influenced by the second loading event, as this step is followed by another period of firstly only agitation but then also aeration, during which nitrification of remaining $NH_3$ occurs. Still, the second load has perhaps less importance since the second aeration period is shorter and the C/N-ratio is not that beneficial for nitrification. Furthermore, even if in total the "original"-concentrations do reach the SBR after the second loading event, bacteria are never exposed to these concentrations at once but succsessively to two differing concentrations representing the two loading events. Besides, concentrations of the secondly loaded wastewater do not really reach nitrifiers during aeration within the second internal cycle as they are likely to be fundamentally altered after the intense denitrification-period. All in all, though usage of concentrations resembling the low C/N-concentrations of the first loading process is not fully

reflecting the concentrations nitrifying bacteria are exposed to in DIC-SBRs, it still seems to be the better choice as little is known about the concentrations reaching those bacteria during their active period after the second loading event.

### D.2. FISH as main technique

FISH was the method of preference to get a well-founded overview of the diversity of AOB and NOB in the various samples in reasonable time. This technique can identify only bacteria, for which probes are already available. However, as nitrifying bacteria have already been investigated quite intensively (Daims *et al.*, 2001 and 2001b; Matsumoto *et al.*, 2007; Wagner *et al.*, 1995 and others), the most frequent and important nitrifiers in wwtps seem to be already known (Koops *et al.*, 2003). Furthermore, applying the hierarchical probe concept can reveal previously unidentified bacteria when probes targeting a phylogenetically broader group do not fully overlap with probes targeting the specific clusters within this group.

Still, FISH has also some drawbacks such as detection limits. These will be briefly explained in the following section: The concentration of rRNA within cells could be very low, resulting in a weak fluorescent signal, which does not allow detection unless the signal is electronically or enzymatically amplified (Hoshino *et al.*, 2008). A certain threshold of targeted ribosomes within a cell is required and has been determined as 1400 16S rRNA copies per cell in activated sludge (Hoshino *et al.*, 2008). For a long time a high ribosome content has been believed to be positively correlated to the growth rate of bacteria and thus to indicate activity (Amann *et al.*, 1995; referring to DeLong *et al.* 1989 and Wallner *et al.*, 1993). Consequently, bacteria with a very low ribosome-content could be considered to play only a minor role in wastewater treatment. However, a positive correlation between growth rate and ribosome content has turned out to be wrong for the slow-growing nitrifying bacteria (Wagner *et al.*, 1995). For most other prokaryotes it is unknown whether a correlation exists or not.

Furthermore, a very low number of target cells renders the detection of positively targeted cells by eye under the microscope difficult and less probable. This amount has been suggested to be around $10^3$-$10^4$ cells/ml (Amann, 1995). Although for PCR a required amount of target molecules (depending on the PCR-approach) has been reported as well, PCR is a more sensitive approach than FISH as even amplification of target genes of single cells is possible. This can explain inconsistent results of FISH- and PCR-analysis, which was the case for the sample taken from Bad Zwischenahn. While FISH did not reveal occurrence of NOB in this sample, a sequence, most similar to the 16S rDNA of *Nitrospira*-Cluster I, could be obtained.

However, though diversity could be investigated in more detail by applying the 16S rRNA approach, this approach is very time-consuming. Besides, 16S rRNA analysis requires amplification of the target genes by PCR at the beginning of the approach. As it has been shown that the number of group-specific (e.g. species-specific) PCR-products does not resemble the initial abundance of microorganisms in the investigated sample (PCR-bias), quantitative conclusions cannot be drawn (Witzingerode, 1997). Thus, while 16S rRNA analysis would not allow to name the main groups in N removal, abundances can be determined by applying quantitative FISH.

When determining the abundance of *Nitrospira*-like organisms within the samples, the areas made up by signals of the targeted bacteria can be over- or underestimated due to varying intensities of fluorescent signals. Varying signal intensities can for instance be a consequence of differing accessibility of the probe binding sites (Fuchs, 1998; Behrens *et al.*, 2003) or quenching* (Wagner *et al.*, 2003; referring to Marras *et al.*, 2002 and Torimura *et al.*, 2001). However, detection of signal intensities of two probes can be adjusted during picture recording with the CLSM. Still, adjustment is difficult and thus, in case the abundances of members of *Nitrospira*-Cluster I and II related to all *Nitrospira*-like bacteria should sum up to 100%, an aberration of 10% was tolerated.

### D.3. Determination of diversity

Diversity has been approached in terms of recording the occurrence of different AOB-, NOB- or the number of AOB- and NOB-clusters together. Thereby occurrence of members of the *Nc. mobilis*-lineage was not recorded separately for AOB, as this lineage is included in the *N. europaea/Nc. mobilis*-Cluster, which was already recorded in case of positive NEU-signals. Though in this way information gets lost, otherwise diversity would perhaps not be truly reflected. As the probe NEU is targeting three of the four species within the *N. europaea/Nc. mobilis*-Cluster, a positive signal can represent a single species as well as all three species, whereas a positive signal of the probe Ncmob (targeting members of the *Nc. mobilis*-lineage within the *N. europaea/Nc. mobilis*-Cluster) reveals solely occurrence of *Nc. mobilis*-like AOB. "Environmental Cluster 6a" was not recorded separately either, because it clusters within Cluster 6a. For NOB, Cluster I, II and "other *Nitrospira*" were each counted as own NOB-cluster.

* The intensity of a fluorescent dye can be influenced by "photo-induced electron transfer between the fluorescent dyes used for probe labelling and nucleotides of the probe or target molecule". (Wagner *et al.*, 2003; referring to Marras *et al.*, 2002 and Torimura *et al.*, 2001)

Diversity of AOB and NOB seems to be important in context of stability of ecosystems based on functional redundancy (A.3.4.) Analysis has been conducted on this high phylogenetic level (cluster) as screening for the so far known different species would have been more time-consuming and as clusters were considered to reflect to some degree ecological niches of these bacterial groups. For instance are members of Cluster 6, comprising the *N. oligotropha*-Cluster as well as the *N. marina*-Cluster, assumed to occur in less eutrophic habitats while *N. europaea*, *N. eutropha* and *Nc. mobilis* are believed to occur in more nutrient-rich environments (D.5.5.1., D.5.5.2.). Diversity, in the context of functional redundancy, is only interesting as long as it describes how many different groups occur which would probably react differentially to changing conditions, as a consequence of varying adaptations. Determining the numbers of different clusters in the samples is therefore questionable as in fact fewer clusters do not necessarily imply less diversity in terms of species and possible responses to changes. Even on the species level these considerations remain the same as reflected in the current debate on microbial species concepts. Recently it has been illustrated that "a named bacterial species is typically an assemblage of closely related but ecologically distinct populations" (Cohan and Perry, 2007). Examples consulted are among others different adaptations to radiation in *Bacillus simplex* and *B. licheniformis* (Cohan and Perry, 2007; referring to Sikorski and Nevo, 2005) and different utilization of nutrient resources by *Prochlorococcus marinus* (Cohan and Perry, 2007; referring to Coleman *et al.*, 2006) (appropriaty of the latter example has been questioned by Achtman and Wagner (2008)). Based on these observations, a species concept relying on "ecotypes" was suggested (Cohan and Perry, 2007), being defined as "a group of bacteria that are ecologically similar to one another, so similar that genetic diversity within the ecotype is limited by a cohesive force, either periodic selection or genetic drift, or both" (Cohan and Perry, 2007). This concept goes along with the definition of species as "metapopulation* lineages": "A lineage can be thought of as a metapopulation that extends through time, "occupies an adaptive zone minimally different from any other lineage in its range" and "evolves separately from all other lineages outside its range"." (Achtman and Wagner, 2008; referring to de Queiroz, 2005). As mentioned before, different ecophysiologies have also been proposed for *N. oligotropha* (Lydmark *et al.*, 2007) as varying responses to nutrient availability of different populations of this species could be observed. Following this approach, it seems like even within *N. oligotropha* different

* metapopulation: „are "sets of connected subpopulations, mayimally inclusive populations" whose limits are set by evolutionary cohesive forces" (Achtman and Wagner, 2008; referring to de Queiroz, 2005)

reactions can occur, thus questioning again how appropriate it is to determine diversity in context of functional redundancy by measuring numbers of clusters (or also species). Still, lacking another solution, this approach is reasonable as it gives at least some information on diversity.

Another point of criticism might be using FISH solely for a determination of diversity. FISH is appropriate to determine which bacteria are mainly involved in processes and was a good choice as those groups should be linked to physical parameters of the diverse plants (see D.2.). However, in regard to stability, also minor populations should be considered, since they may play a more important role after eventually occurring perturbation events.

## D.4. Diversity of AOB and NOB

### D.4.1. Occurrence of AOB

For AOB, probes targeting the genus *Nitrosospira* and all described clusters within the genus *Nitrosomonas* (except for the *N. cryotolerans*-Cluster and the *Nitrosomonas* sp. Nm143-Cluster) have been used as all these have previously been found in wwtp-systems (Gieseke *et al.*, 2000; Purkhold *et al.*, 2000). Members of the *N. cryotolerans*-Cluster have been found in only one wwtp (Limpiyakron *et al.*, 2005), and *Nitrosomonas* sp. Nm143-like organisms have not yet been described to occur in wastewater (Foesel *et al.*, 2008; Purkhold *et al.*, 2000; Purkhold, 2003). In the present study, within the genus *Nitrosomonas*, appearance of all of the investigated different clusters could be detected apart from the *N. marina*-Cluster (*N. oligotropha*-Cluster, *N. communis*-Cluster, *N. europaea/Nc. mobilis*-Cluster). Members of the *Nc. mobilis*-lineage within the *N. europaea/Nc. mobilis*-Cluster could be found as well, but no bacteria related to the genus *Nitrosospira* were detected. The absence of *Nitrosospira*-like bacteria in the present study supports the already suggested minor role of these AOB in wastewater treatment. *Nitrosospira*-like bacteria have been credited with little importance based on their rare occurrence (Purkhold, 2003; Wagner and Loy, 2002) and their low abundance (Mota *et al.*, 2005). In the latter study low abundances of these AOB have been related to high nitrogen-loads within the observed plant. This goes well with findings presenting these bacteria as dominating AOB in a nitrifying fluidized bed reactor operated with low $NH_4^+$ concentrations, simulating freshwater-like conditions (Schramm *et al.*, 1998), which indicates that their occurrence might be related to low $NH_4^+$ concentrations. Interestingly, almost exclusively sequences related to *Nitrosospira* spp. have been retrieved

from some constructed wetlands for wastewater treatment (Purkhold, 2003; referring to Haleem *et al.*, 2000). This is in accordance with the assumption that *Nitrosospira*-like bacteria are mainly occurring in soils and occasionally in freshwater habitats (Koops *et al.*, 2003).

Members of Cluster 6a (*N. oligotropha*-Cluster) were found in all samples in which AOB were detected. Estimated abundances varied, but generally Cluster 6a-like AOB seemed to be quite abundant – still, abundances should be handled critically as the subjective assignment to the defined abundance classes might have varied at different time points. Bacteria detected with the probe Nolimar712, targeting members of Cluster 6a and Cluster 6b (*N. marina*-like bacteria), but neither with the probe Cluster6a-192 nor Nmar830 were referred to as "environmental Cluster 6a"-like bacteria. Those bacteria could either not be detected by the probe Nolimar712 despite being representatives of one of the included clusters (6a/6b) not targeted by the respective specific probe, or constitute an additional Cluster within Cluster 6 as previously reported. Similar hybridization patterns in another study on nitrifiers in wwtps (Anneser, 2004), in which cloning and subsequent sequence analysis of *amoA* has been done, revealed formation of a distinct cluster within the *N. oligotropha*-Cluster. This cluster could not be confirmed by analysis of the 16S rRNA-gene of AOB. Still, *amoA*- and FISH-results have been interpreted to reveal a separate "environmental Cluster 6". This classification was adopted in the present study (here: "environmental Cluster 6a"), though this was not verified by further analysis here. Since only the average influent and effluent nitrogen concentrations of the previously investigated wwtp in Bruck an der Leitha could be found (48.1 mg/l and ~8.3 mg/l, respectively; Anneser, 2004), as well as the estimated cleaning performance for carbonic compounds (approx. 90 mg/l; Anneser, 2004), comparison with the here investigated wwtps is limited. As little data were also available for one of the two investigated plants harbouring this putative "environmental Cluster 6a", no conclusion about the factors supporting its occurrence can be drawn.

Halophilic and –tolerant AOB, which were for a long time been believed to be mainly responsible for the oxidation of $NH_3$ in wwtps, were only detected in 5 of the 20 samples harbouring AOB. This result confirms the occurrence of *N. europaea/Nc. mobilis*-like bacteria in some systems, but also supports the assumption that their dominance in wastewater treatment is limited (Wagner *et al.*, 2002). In contrast, the ubiquitous occurrence of members of the *N. oligotropha*-Cluster in the investigated samples is in accordance with their already suggested importance in wwtps (Lydmark *et al.*, 2007; Anneser, 2004; Siripong *et al.*, 2007). Occurrence of these two *Nitrosomonas*-clusters will be discussed in more detail and in context with the investigated parameters in the next chapters (see D.5.5.1, D.5.5.2.).

Members of the *N. communis*-Cluster could just be detected in the sample taken from the Rosenheim wwtp, meaning that it just occurs in this single plant or that a detection by FISH was not possible in the other samples (see D.2.). Interestingly in one study (Limpiyakorn *et al.*, 2005) fluctuating concentrations of $O_2$ up to time periods without $O_2$, were suggested as favourable for the occurrence of *N. communis*-like AOB. In this way the authors explained appearance in the examined systems with A2O (anaerobic/anoxic/aerobic)- and AO (anaerobic/aerobic)-treatment processes and absence of this lineage in conventional activated sludge-systems. Although *N. communis*-like bacteria can be found in wwtps and several isolates from this habitat exist, these AOB are not believed to occur typically in wwtps (Koops *et al.*, 2003; Purkold *et al.*, 2003). This assumption is also supported by the results of the present study.

Although in the plants investigated here most frequently only members of Cluster 6a were detected and co-occurrence of different AOB-clusters was limited to five samples, co-occurrence (as detectable by FISH) has been proposed as a common scenario in some studies (Daims *et al.*, 2001; Gieseke *et al.*, 2005; Lydmark *et al.*, 2007; Siripong *et al.*, 2007).

### D.4.2. Occurrence and abundance of NOB

#### D.4.2.1. Occurrence of NOB

*Nitrobacter* spp., considered being r-strategists that thrive best in presence of high substrate concentrations (Schramm *et al.*, 1999), could not be detected in any of the samples by FISH. This accords well with other results ascribing little importance to *Nitrobacter* spp. in wastewater treatment, while indicating *Nitrospira*-like bacteria as most important NOB in these systems (Daims *et al.*, 2001b). However, *Nitrobacter* spp. could already be found in wwtps; possible factors influencing their appearance will be discussed in the sections D.5.5.1. and D.5.5.2.

*Nitrospira*-like NOB were found in almost all samples (see D.5.5.1, D.5.5.2.). Only in two samples, in which AOB could be found, and in one sample without the detection of AOB, no *Nitrospira*-like organisms could be detected by FISH. These samples did not give signals with a probe specific for anammox-bacteria either. In one of the two samples harbouring AOB but lacking NOB, *Nitrospira*-specific sequences (Cluster I) could be obtained by PCR (amplification was done with the *Nitrospira*-specific reverse primer 1158R), suggesting either extremely low abundance or a too low ribosome content of the cells for a detection by FISH.

In this sample FISH also indicated occurrence of "*Candidatus* Nitrotoga arctica" (based on binding of the probes NTG 840 and Bet42a), which has already been found in activated sludge before (Alawi *et al.*, 2007). However, false positive targeting due to unstringent binding of the probe NTG 840 could have occurred as the FA-concentration for the hybridization was suggested to be between 10 and 20%, but not experimentally determined. Consequently, this result was excluded from all further analysis as occurrence of these just recently suggested NOB still has to be confirmed. For Langenzenn, the 16S rRNA-approach (amplification was done with the *Nitrospira*-specific reverse primer 1158R) did not reveal occurrence of representatives of *Nitrospira*. All obtained sequences showed different phylogenetic affiliation (see C.4.1.2.), which seems to be a result of unstringent primer binding. Maybe analysis of additional clones would have revealed *Nitrospira*-specific sequences. As it seems improbable that AOB but no NOB occur (accumulation of $NO_2^-$ unless the present AOB are not active), either other known NOB (*Nitrobacter-, Nitrospina-,* "*Candidatus* Nitrotoga"- or *Nitrococcus*-like bacteria) not detectable by FISH, or yet unknown NOB could be present in this sample. However, the occurrence of NOB in too low numbers for detection by FISH would not explain the functioning nitrification in these two SBRs. In conventional full-scale wwtps exhibiting efficient nitrification, abundance of *Nitrospira* has for instance been reported to be around 12% (Juretschko *et al.*, 2002) and between 25 to 40% (Lydmark *et al.*, 2007). Far lower abundances of *Nitrospira*-like bacteria have been determined in the present study for conventional full-scale wwtps and SBRs (see chapter D.4.2.2.).

Another possibility explaining the absence of NOB is that in these wwtps simply no nitrification occurs. Nitrification is indicated by decreased N concentrations in the effluent as well as by a rather low C/N-ratio of the incoming water (see D.5.5.1.). Still, the C/N-ratio of the influent does suggest nitrification, at least for the SBR in Langenzenn (BOD5/"$N_{in}$" = 6.2; see D.5.5.1. for a further discussion). The ratio of the influent indicates high N concentrations, rendering nitrification necessary, though "$N_{in}$" concentrations might have been even underestimated as only measurements of $NH_3$ have been available. Because of this, N concentrations of the influent of the plant in Langenzenn have not been used for further analyses. On the other hand, the incoming amount of $NH_3$ was not very high (~21 mg/l) compared to other "beginning concentrations" of $NH_3$ in SBRs. Despite the low concentration of incoming $NH_3$, an average concentration of ~8 mg/l was still present in the effluent together with a $NO_3^-$ concentration of ~3 mg/l. The first value is not supporting the assumption of occurring nitrification as the lower $NH_3$ concentration could have been only due to assimilation of nitrogen into biomass as well, but formation of $NO_3^-$ is, as the

occurrence of AOB, again indicating nitrification, though possibly only to a minor degree. Still, $NO_3^-$ does not necessarily have to be a result of occurring nitrification, but could also be introduced with the influent, though this is rather unlikely; unfortunately no measurements of $NO_3^-$ were available for the influent of this plant. In the SBR in Bad Zwischenahn, influent and effluent $NH_3$ concentrations (~60 mg/l and 0.25 mg/l respectively) as well as effluent $NO_3^-$ concentrations (~6.5 mg/l) suggest nitrification although no NOB apart from putative "*Candidatus* Nitrotoga arctica"-like bacteria could be found and those appeared only in rather low abundance (estimation is based on the by FISH detected cell-clusters). Again, also for this SBR no measurements of incoming $NO_3^-$ concentrations were available. However, "$N_{in}$" concentrations for Bad Zwischenahn have been excluded from further analyses as only a single measurement for $NH_4^+$ was available and consequently reliability was questioned. Unfortunately, BOD5 measurements, which would have allowed analysis of an approximate C/N-ratio, were totally missing. Hence, to elucidate the absence of known NOB in amounts detectable by FISH in these samples, firstly occurring nitrification should be confirmed. If nitrification is confirmed but occurs only to a minor degree, a low abundance of NOB, as indicated for Bad Zwischenahn by the *Nitrospira*-like sequence which could be obtained, might however be sufficient. Another possible explanation of functioning N removal in this plant is that only incomplete nitrification accompanied by nitrite respiration occurs; AOB could convert $NH_3$ to $NO_2^-$, which is then not further oxidized to $NO_3^-$ by NOB but gets instead reduced by denitrifying bacteria to NO, $N_2O$ and $N_2$. This scenario seems to be plausible, amongst others, because denitrification has been shown to be not limited to totally anaerobic conditions, but to occur possibly at the same time as $O_2$-usage or even under totally aerobic conditions, depending on the respective denitrifying organism ( Zumft, 1992). Finally there remains the possibility that "comammox" (complete ammonium oxidation) occurred in these SBRs. Existence of these organisms, being able to oxidize $NH_3$ to $NO_3^-$, has been proposed by Costa *et al.* (2006) based on theoretical considerations.

"Other *Nitrospira*" could be previously totally unknown *Nitrospira*-like bacteria as well as bacteria similar to *Nitrospira*-like clones from the Nullarbor caves (Cluster III) or to other deep-branching sequences (not affiliated to an already described cluster) within the genus *Nitrospira*. Besides this, the putative "other *Nitrospira*" could have been false negatively detected by the cluster-specific *Nitrospira*-probes as well. A false positive detection by the probe specific for the genus *Nitrospira* is unlikely since these bacteria were also detected with the probe specific for the phylum *Nitrospirae*. The pylum *Nitrospirae* also comprises the

genera *Leptospirillum*, *Thermodesulfovibrio* and "*Candidatus* Magnetobacterium", which would be targeted by the probe specific for the phylum *Nitrospirae* but should not be detected by the probe specific for the genus *Nitrospira*. However, appearance of members of these genera in activated sludge is rather unlikely since they seem to be adapted to totally different habitats (*Leptospirillum* iron-oxidizing bacteria; *Thermodesulfovibrio*: thermophilic sulphate-reducing bacteria; "*Candidatus* Magnetobacterium": iron-dependent energy-metabolism was suggested; Madigan and Martinko, 2006 and Spring *et al.* 1993). Nevertheless, FISH with probes targeting these genera should have been used to exclude this possibility. Furthermore, FISH-MAR could provide more information about the energy-metabolism of these organisms. Occurrence of "other *Nitrospira*" was tried to be confirmed by cloning and sequencing of *Nitrospira*-specific 16S rDNA with subsequent phylogenetic analysis. For the amplification of the 16S rDNA at the beginning of this approach, the reverse primer 1158R was used. This primer targets because of a wobble position both "Cluster I and II" and the amplification of the 16S rDNA of novel *Nitrospira*-like bacteria seems probable. Still, no such sequences could be obtained – all sequenced clones fell in either Cluster I or II. Possibly too few clones have been sequenced. An alternative would have been to use the phylum specific probe Ntspa712 as backward primer to increase the probability of amplification of unidentified members of the genus *Nitrospira*. This approach has already been used with success for the amplification of unknown *Nitrospira*-like NOB from hot springs (Hauzmayer, personal communication). Possible influences of the investigated parameters on the occurrence of "other *Nitrospira*" will be discussed in section D.5.5.1.

Analysis of the phylogenetic affiliations of newly obtained sequences, as illustrated in a phylogenetic tree (p.107, Fig.36), revealed high similarity of the clones to each other. Most sequences of the different wwtps falling into Cluster I, showed >99% similarity to "*Candidatus* Nitrospira defluvii" and most also shared >99% sequence similarity as did many reference sequences. Cross-contamination as explanation for the high similarity was not supported by the results of contamination-screenings by PCR. Clustering of sequences did not seem to reflect their source (no formation of clusters based on the plant setup), though for confirming this preliminary result more clones should have been analysed.

### D.4.2.2. Abundance of *Nitrospira*-like bacteria

In this study abundance of the genus *Nitrospira* varied between a minimum of 0.1% and a maximum of 16.5%, but most samples contained less than 5%. These values were somewhat

lower than abundances of *Nitrospira* reported in other studies. Surprisingly, whereas in the present study the fraction of all *Nitrospira*-like bacteria was determined to be ~4% of all bacteria in the sample taken from Kraftisried, an abundance of approx. 12% was observed for a sample from the same plant taken in 1996 (Juretschko *et al.*, 2002). In the meantime this plant has been reconstructed and the incoming N-load reduced (see D.5.5.1.). So far it is unclear why abundances (for conventional wwtps as well as for SBRs) presented in this study are on average lower than abundances reported elsewhere (Juretschko *et al.*, 2002; Lydmark *et al.*, 2007). In these two studies, abundances of *Nitrospira*-like organisms have been determined for conventional wwtps; equivalent studies in which abundances have been determined for SBRs were not available.

If "other *Nitrospira*" were not detected in the samples, *Nitrospira*-Cluster I-like NOB ("*Candidatus* Nitrospira defluvii"-like NOB; referred to as "Cluster I") and *Nitrospira*-Cluster-II-like NOB (*Nitrospira moscoviensis*-like NOB; referred to as "Cluster II") should sum up to around 100% of all *Nitrospira*-like bacteria. An aberration up to 10% was tolerated as different intensities of fluorescently labelled probes can result in a biased measurement of the areas formed by the signals of the different probes. Although signal intensities can be adjusted during detection, minor differences might remain and affect the result (see D.2.).
Abundances of Cluster I and Cluster II have been related to the abundance of the whole genus *Nitrospira* within the corresponding samples. In this way abundances of "Cluster I and II" relative to all bacteria have been determined (see equation 1, p.73). These abundances yield more information on the with the different investigated parameters varying competitiveness of Cluster I and II compared to all bacteria.
Correlations of the abundances of all *Nitrospira*-like bacteria as well as of Cluster I and Cluster II with the investigated parameters will be discussed subsequently (see section D.5.).

## D.5. Influences of wastewater composition, operational mode and plant design

### D.5.1. Design of wastewater treatment plants

Two main wwtp designs have been investigated during this study: conventional full-scale treatment plants and SBRs. Of the conventional plants, one was operated as a fixed-bed reactor and one with membrane filtration. In a previous study (Manser, 2005) comparing populations of ammonia-oxidizing and nitrite-oxidizing bacteria between a conventional activated sludge process and a membrane aerated bioreactor (MAR), only slight differences

have been observed, which were interpreted to be more a result of differing wastewater composition and SA. Here, no influence on the community composition of AOB and NOB could be seen either. Similarly no differences were observed between the fixed-bed reactor and the other wwtps. As biofilms on fixed-beds are believed to harbour various microenvironments along nutrient- and oxygen-gradients (Gieseke *et al.*, 2000; Gieseke *et al.*, 2003), this sample could have been expected to be more diverse – but only representatives of the *N. oligotropha*-Cluster and of the *Nitrospira*-Cluster I were detected. A comparably low diversity has been observed in a nitrifying fluidized bed reactor driven with low $NH_4^+$ concentrations (Schramm *et al.*, 1998).

Since in SBRs organisms are exposed to strongly changing but periodically reoccurring conditions (Gieseke *et al.*, 2003), on the one hand appearance of only some bacterial groups resisting these variable conditions and on the other hand, due to various niches, appearance of a more diverse community composition can be hypothesized. Greater diversity in terms of different co-occurring AOB and NOB-Clusters for SBRs was nevertheless not observed. Furthermore, the variable conditions within SBRs may also be favourable for specific nitrifying bacteria, which has been proposed for *Nitrobacter* spp. (Dytcak *et al*, 2008). Dytcak *et al.* (2008) suggested alternating aerobic and anaerobic conditions to be advantageous for these organisms (see D.5.5.1. for further discussion). Although the two most diverse samples in the present study (from Ingolstadt and Rosenheim) came from SBRs, no profound difference in diversity between the two main design types could be seen.

Head *et al.* (2003) found differences in community composition of AOB between a biological aerated filter (BAF) and a trickling filter consisting of two filter beds. The trickling filter harboured in total (primary and secondary filter bed) more different AOB than the BAF though for BAF and the primary filter bed, the diversity of AOB was similar. These two reactors received the same influent. BAF and the secondary filter bed were less similar, which was interpreted as caused by the income of already modified wastewater in the second filter bed (coming from the first filter bed). Therefore, wastewater composition might be concluded more important than the design of the wwtp.

### D.5.2. Operational mode

#### D.5.2.1. Ratio of “loading time” to “reaction time” ($t_F/t_R$)

The ratio of “loading time” to “reactive time” was related to the biological data since it describes the amount of nutrients the bacteria within the SBRs are exposed to at once. This

could influence the community structure of bacteria. As the ratio has been calculated in a different way for DIC-SBRs (loading time to active time; Holm; personal communication) and for SBRs (loading time to cycling time; Schreff, 2007), only ratios for DIC-SBRs have been compared. However, no trend was visible. This could have been caused by the small range in which the ratios varied (0.2 – 0.4), or maybe this ratio makes only sense when referred to the actual concentration of carbon and nitrogen in the influent. However, the ratio $t_F/t_R$ is suggested to influence the ISV as far as lower ratios correlate with lower ISV (Holm, personal communication). This indicates at least influences on the structure of flocs. Combining this parameter with incoming N concentrations appears to be important in view of detrimental effects of extremely high $NH_3$ concentrations on nitrifiers (Burgess *et al.*, 2002). Burgess *et al.* (2002) reported accumulation of $NO_2^-$ and nitrous oxide ($N_2O$) as a consequence of $NH_3$ shock loading. A possible explanation for this observed effect is that a decrease in oxygen concentration, which is likely a result of elevated nitrification, caused nitrifying bacteria to use $NO_2^-$ instead of $O_2$ as electron acceptor for the oxidation of $NH_3$ ("nitrifier denitrification"). Thereby $N_2O$ accumulated in the exhaust gas. Furthermore, $O_2$-depletion possibly led to an inhibition of NOB resulting in the observed $NO_2^-$-accumulation. Saturation of $N_2O$ in the exhaust gas may have contributed to the accumulation of $NO_2^-$ (Burgess *et al.*, 2002). $NH_3$ shock loading seems to be less probable for conventional full-scale treatment plants (dilution of $NH_3$).

### D.5.2.2. Sludge retention time and pH

Other parameters linked to the operational mode are for instance the sludge retention time (SRT) and the pH. SRT is supposed to be one of the most important factors influencing growth of the slow-growing nitrifying bacteria in reactors since wash-out of these bacteria can easily occur at short SRTs. However, SRT was very high in almost all investigated plants. Furthermore, a comparison between the SRTs of the different wwtps was diffucult since for most plants only minimum and maximum SRTs were available (e.g. SRT > 20 - 50 d for all conventional wwtps).

The pH did not show any interrelation with presence or absence of AOB and NOB either. However, all measured pH ranged between 6.4 and 7.8; the lowest pH occurred in the SBRs in Waldsassen (6.4) and Weißtal (6.5). Optimal growth of nitrifiers has been reported at a pH around 7.6 – 7.8; nevertheless, nitrifiers have also been found in habitats with a pH ~4 or between 9.7 - 10. As a possible explanation for growth under very acidic conditions, usage of

urea (urease enzyme) has been suggested (Bock and Wagner, 2006; referring to De Boer *et al.*, 1991 and Sorokin *et al.*, 2001). Efficient aerobic nitrification in bioreactors at a pH of 4.5 has been reported to correlate with a population shift from *N. europaea*-like to *N. oligotropha*-like AOB. *Nitrospira*-like NOB were present throughout the whole experiment (Tarre *et al.*, 2007). Still, pH-sensitivity of nitrifiers is assumed to be a critical factor in wastewater treatment (Wagner *et al.*, 1995; referring to Rittmann and Whiteman, 1994). Here, in the two plants with the lowest pH, only *N. oligotropha*-like bacteria were detected, but these were also the only AOB in some other samples with a higher pH (as detected by FISH).

### D.5.3. Temperature

The diversity of AOB- and NOB-clusters correlated significantly with this parameter. The two samples with the highest temperature (T) (Rosenheim: 38.2°C and Ingolstadt: 27.4°C) also showed the highest diversity of different AOB- and NOB-clusters. Approx. 25 - 30°C have been suggested to be optimal for nitrification in wwtps (Martin *et al.*, 2005, referring to Bitton, 1999; Siripong *et al.*, 2007, referring to Watson *et al.*, 1989). Generally, aerobic nitrification seems to occur within a much broader temperature range since nitrifiers have been isolated from various extreme habitats with for instance temperatures below 0°C (-12°C) in permafrost soils (Alawi *et al.*, 2007) and up to 47°C (Bock and Wagner, 2006; referring to Ehrich *et al.*, 1995) or even 70°C (Golovacheva, 1976). In wwtps, moderate temperatures (5 - 20°C) and treatment conditions have been proposed to favour growth of NOB, for AOB 35 - 40°C have been reported as favourable T to out-compete NOB (Egli *et al.*, 2003; referring to Bae *et al.*, 2001, Hellinga *et al.*, 1999 and 1998 and Logemann *et al.*, 1998). This is interesting as ~38°C in Rosenheim firstly appeared to be quite high, but is obviously in the range of good growth temperatures for at least AOB. Optimal conditions could favour growth of various nitrifiers as, unlike under detrimental conditions, not only some nitrifying bacteria especially adapted to very low or high T, will occur. However, according to this, high diversity of either NOB, AOB or both, should have been found in all samples with a T within the proposed optimal temperatures, ranging from ~25° to ~40°C. However, at higher T, diversity can also be low. Schramm *et al.* (1998) found (by FISH) *Nitrosospira* spp. as the only occurring AOB in a nitrifying fluidized bed reactor operated at 30°C. Contrary to the present study, Siripong *et al.* (2007) proposed higher diversity at lower T, arguing that rather fast-growing *N. europaea*-like AOB will outcompete others at optimal T, while at lower temperature slower growing AOB as *Nitrosospira* spp. can exist as well. Although this hypothesis is based on inconsistent T-

RFLP results, the idea of an advantage of fast growing bacteria at optimal conditions, thereby suppressing growth of others, should be considered although it disagrees with results obtained in the present study. A potential cause of less favourable conditions at lower T was discussed by Manser (2005): Diffusion resistance for $O_2$ was found to be higher at lower temperatures, but at the same time decreased microbial activity was observed and hence constant $K_o$-values (half saturation constant for $O_2$) explained. Unfortunately for the two most diverse plants many parameters concerning wastewater composition, which would perhaps yield more insights, were not available. It should further be noted that more data (more samples) would render the correlation between diversity and T more reliable.

### D.5.4. Dry Matter

The second parameter, which was found to be significantly correlated to cluster-diversity within this study, is the concentration of dry matter, describing the amount of biomass within the reactor. Furthermore, a significant positive correlation has been found between dry matter and T. As elevated T is up to a certain limit believed to increase productivity (accelerated reactions), more biomass at increasing T seems reasonable.

In this study, statistical analysis showed a significant correlation between DM and BOD5 values of the "original" influent of SBRs. Since heterotrophic bacteria are believed to constitute the major part of the biomass within wwtps and since those are probably equally influenced by the concentrations of both loading events in DIC-SBRs, "original" BOD5-influent values have been used. A correlation between DM and nitrogen concentrations in the first loading event could not be seen. For this analysis "$N_{in}$" concentrations representing the first loading event have been considered since these concentrations are believed to have possibly influences on the abundance of nitrifying bacteria (see D.1.4.). Still, higher "$N_{in}$" concentrations seemed to go along (not significant) with higher BOD5 concentrations (both, "original" influent concentrations and concentrations according to the composition of the first loading event) in wastewater. The correlations of DM with T and BOD5 ("original" values) seem to indicate higher growth rates of heterotrophic bacteria at elevated T and BOD5 values, at least within the investigated SBRs, but do not tell much about nitrifying bacteria in the samples. Still, growth of heterotrophic bacteria could influence nitrifying bacteria, as could a higher input of carbonic compounds: These C compounds could be of differing availability and influence AOB and NOB as well, since representatives of the genus *Nitrosomonas*, *Nitrobacter* (Bock and Wagner, 2006; referring to Clark and Schmidt, 1967) and *Nitrospira*

(Daims et al., 2001b) have been shown to be able to grow mixotrophically. Influences on nitrifying bacteria could be both, beneficial and detrimental. Elevated concentrations of degradable organic carbon have for instance been reported to inhibit nitrification in a rotating biological contractor* (Yang and Zhang, 1995; referring to Klees and Silverstein, 1992). Another suggested detrimental effect of elevated C concentrations is overgrowth of nitrifying bacteria by heterotrophic bacteria resulting in limited $O_2$-supply, as observed in a biofilm with high SRT (Nogueira *et al.*, 2002). Therefore, an increasing biomass, presumably to a good part attributable to heterotrophic bacteria, may also be disadvantageous for nitrifiers, especially in conventional full-scale plants. So far, the previously discussed correlations indicate an increasing amount of biomass (dry matter) with increasing T and increasing input of carbonic compounds. The last correlation was only significant when considering solely SBRs and concentrations representing both loading events, which are assumed to influence heterotrophic bacteria equally but could also influence nitrifying bacteria.

In any case, assuming that more input of nutrients goes along with more biomass and thus indicates a higher productivity, a correlation between higher productivity and greater diversity remains questionable. Firstly, the correlation between cluster-diversity and DM was just significant considering all wwtps but not when performed for SBRs only, though a trend was still visible. However, only for SBRs the correlation between BOD5 and DM could be seen. This means that in the wwtps, for which a correlation between higher nutrient input and higher biomass (reflecting higher productivity) could be shown, the correlation between higher productivity and diversity was not significant. Thus, it is arguable to explain a higher diversity of AOB and NOB with an increased productivity, which is on the other hand attributed to elevated nutrient concentrations. Still, as discussed earlier (see D.1.1. – D.1.4.), BOD5-values are hardly comparable between SBRs and conventional treatment plants and thus could explain that a correlation between nutrient-input (BOD5) and biomass/productivity (DM) could not be seen for all wwtps.

The second aspect questioning the plausibility of a correlation between productivity and cluster-diversity, is that the amount of biomass within wwtps (measured as DM), is not implicitly reflecting productivity of bacteria within a plant (Holm, personal communication) even though DM seemed to be positively correlated to the "original" BOD5 values of the influent of SBRs. This is due to the fact that the concentration of dry matter is regulated

* "rotating biological contractor": based on planes harbouring biofilms; planes are immersed into the wastewater and rotated (online lexicon "wasser-wissen" of the University of Bremen)

within wwtps. As a good separation of cleaned water from sludge must be ensured in the sedimentation tank and as this separation can be negatively influenced by a too high concentration of DM, though the main parameter influencing separation is the sludge volume (Kroiß, 2007), the amount of DM in the activated sludge basin is regulated by the amount of removed excess sludge. Removal of excess sludge is also determining sludge age (Kroiß, 2007). The fact that productivity does not have to be reflected in the amount of biomass is maybe best illustrated regarding "light" and "high" load-systems. Depending on the nutrient concentrations that reach bacteria involved in the cleaning process, "light" and "high" loads are defined – in the first one only few nutrients are available whereas in the latter one bacteria are confronted with high concentrations of nutrients that can hardly be fully degraded (Kroiß, 2007). Thus, for instance in a "light load-system", the amount of biomass is higher than actually necessary according to the incoming nutrients (Holm, personal communication). As a consequence, these two systems do not fit the previously introduced direct correlation between nutrients-loading and biomass concentration.

Analysing different factors influencing the amount of biomass within bioreactors, another hypothesis might be interesting, according to which elevated sludge age (less withdrawal of excess sludge) and arising temperature go along with less bacterial biomass (Kroiß, 2007). This observation has been attributed to endogenous respiration and lysis of cells (Kroiß, 2007) and seems to be appropriate for increasing T but constant input of nutrients in the system. However, in the present study, a correlation between T and DM as well as, for SBRs, between BOD5 and DM could be seen, which suggests higher input of nutrients in the plants and higher T at the same time, therefore rendering endogenous respiration less likely. However, concerning this discussion, data of different sampling dates of one plant, in which one or more of these parameters are changing (T, BOD5, DM) would probably allow more conclusions than comparing data from one sampling event between totally different plants.

Assuming now that elevated dry matter would indeed be a consequence of higher productivity – though this is rather unclear considering the previous discussed points – does higher productivity have any influence on the diversity of organisms ? This question addresses a long-running discussion in ecology. On the one hand more available resources and thus a higher productivity are likely to lead to an increase in species richness. As increased substrate does not necessarily imply more different resources but might just mean a greater amount of the same resource, more individuals per species can be a more likely outcome than increased

species numbers. Still, even if the resources are not of great variety, the fact that there are more of them enhances the probability that extra species that have been out-competed under conditions of limited substrate, emerge (Townsend *et al.*, 2000). Also plausible is a contrary scenario of high population growth due to enhanced substrate, leading to extinction of species as a result of competitive exclusion (Townsend *et al.*, 2000). Guo and Berry (1998) proposed that microhabitats, initially rather poor in biomass, exhibit positive correlations of biomass growth and increase in species richness while diversity decreases with further arising of an already high biomass. As previous studies have focused mainly on terrestrial systems and macroorganisms, it is questionable whether it can be assumed for microcosms as well. Still, if so, high diversity might be related to the observed initial increase in species richness prior to this critical range. However, diversity is here only reflected for two functional guilds and not for heterotrophic bacteria comprising the higher fraction of biomass. Furthermore, referring to nitrifying bacteria, a correlation between "$N_{in}$" concentrations and the amount of biomass would be more reasonable for this discussion, but could not be observed. All in all, reliability of this correlation should be questioned, as a positive relation of productivity to the amount of dry matter is doubtful. Furthermore, as mentioned before, the correlation was not significant when considering SBRs only. Unfortunately anaylsis of cluster-diversity related to C and N concentrations does not yield more informations, as in all samples, for which values have been available, only one or two different AOB- or NOB-clusters occurred.

### D.5.5. Wastewater composition

#### D.5.5.1. C and N concentrations

The grade of pollution of the influent is best described by the parameters COD, BOD5 and TKN or "total N". $NH_4^+$ concentrations are also often used, but in doing so nitrogen loads could be underestimated by excluding still organically bound nitrogen. Ratios of COD to BOD5 and of BOD5 to N seem to be even more interesting than each of the parameters alone. BOD5 has been chosen for the second ratio, as considering certainly biodegradable substances seemed more reasonable than overestimating the available C concentrations by including persistent carbon-compounds (COD). COD values alone do not allow reliable conclusions about the availability of carbon nor about the amount of persistent carbonic pollutants. The amount of hardly degradable substances can be determined by looking at the ratio of COD to BOD5. The ratio of BOD5 to "$N_{in}$" can be considered as a kind of C/N-ratio, which is

essential to estimate how well nutrients can be assimilated by bacteria. A C:N:P-ratio between 100:14:3 (C/N=7) and 100:10:1 (C/N=10) is considered as optimal for growth – an empirical ratio for municipal wastewater is 100:20:5 (C/N=5) (Online-lexicon, University of Bremen). This illustrates that the influent contains usually too high N concentrations (and P concentrations), which cannot be removed solely by assimilation into biomass, making nitrification and P removal important. Empirical average influent values for COD, BOD5 and N for municipal wastewater are ~600 mg/l, ~300 mg/l and 55 mg/l, respectively (Moser, 1993). Most plants investigated here had somewhat lower concentrations in the influent. The ratio of BOD5/N did mostly reflect the empirical ratio of 100:20:(5); the influent of the SBR in Waldsassen seemed to have had an optimal ratio (~7) meaning that probably all nitrogen could be assimilated into biomass along with carbon. Interestingly, the BOD5/N-ratio for incoming wastewater in the SBR in Rapp Kutzenhausen was around 23, which means that much carbon is available, which can be related to the absence of AOB and NOB in this sample. The respective ratio of the sample from Langenzenn (~6), in which no NOB could be detected, was similar to the ratio in the influent of the SBR in Waldsassen and suggests removal of nitrogen solely by assimilation into biomass (see D.4.2.1.). On the other hand AOB were detected in the sample from Langenzenn. This contradicts the idea of nitrogen removal by assimiliation into biomass alone, as $NH_4^+$ can be better assimilated than $NO_2^-$ and thus AOB should be absent as well.

Occurrence of different AOB-clusters in SBRs seemed to be influenced by BOD5 and N concentrations. Halophilic and –tolerant AOB appeared at elevated N and BOD5 values and at low BOD5/N-ratios. Higher N concentrations seemed to go along with higher C concentrations (not significant), hence occurrence of these AOB at elevated BOD5-values could be based on the corresponding N concentrations as well. Furthermore, occurrence could be traced back to the prevailing conductivity within these wwtps since besides elevated BOD5 and N values, increased conductivity values could be found. However, the influence of conductivity levels should be regarded critically (see D.5.5.2.). Based on their rather high half-saturation constants for substrate ($K_s$) (highest within the genus *Nitrosomonas*) (Lydmark *et al.*, 2007, referring to Koops and Pommering-Röser, 2001), low $NH_3$-affinity and thus occurrence at rather elevated $NH_3$ concentrations has been suggested for *N. europaea*, *N. eutropha* and *N. halophila* (Koops *et al.*, 2003), going well with the above described observations. Due to the low sample size, these results should just be considered as indications.

Results for conventional plants do not seem to reflect the results for SBRs. The only sample (Plattling) containing bacteria detected with the probe NEU (*N. europaea/Nc. mobilis*-Cluster), exhibited rather low N and BOD5 concentrations (compared to the other conventional plants). In these plants other factors may have had more influence on the occurrence of AOB. However, the latter argument is just based on 5 samples.

*Nc. mobilis*-like bacteria, belonging to the *N. europaea/Nc. mobilis*-Cluster, have been found solely in the sample taken from the SBR in Ingolstadt, but have been reported quite often and sometimes as the most abundant AOB in treatment plants (Daims *et al.*, 2001; Juretschko *et al.*, 1998). In the wwtp in Ingolstadt *Nc. mobilis*-like AOB were very abundant. This plant is the running plant of the pilot wwtp in Ingolstadt, which was investigated by Daims and others in 2001. In the present study as well as in the study from 2001, *Nc. mobilis*-like and other organisms affiliated to the *N. europaea/Nc. mobilis*-Cluster (*N. europaea, N. eutropha*) could be detected. During the present study *N. oligotropha*-like bacteria could be found as well. Both, the pilot plant and the plant now in operation, treat reject water from sludge dewatering, which has been shown to be very high in nutrients for the pilot plant (COD = 300 mg/l; $NH_4^+$-N = 400 - 500 mg/l and Cond. = 5000 - 6000 µS/cm; Daims *et al.*, 2001). Compared to the other SBRs recently investigated, incoming concentration of $NH_4^+$-N and conductivity are extremely high; conductivity values are even higher than in the animal rendering plants for which those values have been available (see D.5.5.2.). Unfortunately, these values were not available for the SBR in Ingolstadt presently in use. Differences in the design of the former (SBBR) and now sampled (SBR) plant could have altered the community composition, which would mean that the appearance of *N. oligotropha*-like but not *Nc. mobilis*-like bacteria is influenced by the design of wwtps; an indication, which is, at least for *N. oligotropha*-like bacteria (ubiquitous occurrence in the investigated wwtps), not represented in the present study.

*Nc. mobilis*-like bacteria could, as already mentioned, just be found in the sample taken from Ingolstadt. Consequently, these AOB did not occur in the animal rendering plant in Kraftisried; still, in a previous study investigating the nitrifying community composition of the wwtp in Kraftisried, *Nc. mobilis*-like bacteria were found to be even the dominant ammonia-oxidizers (Juretschko *et al.*, 1998). Furthermore, hybridization patterns indicative for *N. europaea*- and *N. eutropha*-like organisms were seen in this former study, whereas in the present study only AOB belonging to the *N. oligotropha*-Cluster could be found. Maybe changes in the community composition can on the one hand be explained by the fact that the wwtp has been reconstructed in the meanwhile; on the other hand, assuming equally high

conductivity levels of the wastewater in the previous study and in the present study (see D.5.5.2.), nitrogen loads, reaching the former wwtp in Kraftisried seem to have been much higher than now (up to 5000 mg $NH_4^+$/l; nowadays ~800 before and ~400 mg/l incoming TKN after nitrogen stripping (usually done in winter); TKN is, if at all, higher than the incoming $NH_4^+$ concentration as organically still bound N is included). This could influence the appearance of *N. europaea*-related AOB. Though, as discussed in the sections D.1.1. to D.1.4., high incoming concentrations do not really reflect concentrations affecting bacteria in the activated sludge basin due to dilution processes – these might nevertheless differ from the current degree of dilution (diff. volumes, incoming rates).

Contrary to members of the *N. europaea/Nc. mobilis*-Cluster, members of the *N. oligotropha*-Cluster are considered as well adapted to harsh conditions according to their determined low $K_s$ values (Lydmark *et al.*, 2007 referring to Koops and Pommering-Röser, 2001). Nevertheless, *N. oligotroha*-like AOB have been shown to thrive in systems with high $NH_4^+$ concentrations as well. In the present study, members of the *N. oligotropha*-Cluster have been detected at minimum and maximum "$N_{in}$" concentrations of the various samples and thus can be assumed to be well adapted to different substrate-availability. Based on the occurrence along a broad range of $NH_4^+$ concentrations, which has already been observed before, even altering adaptations and different ecophysiologies between *N. oligotropha* populations (Lydmark *et al.*, 2007) have been proposed. As *N. oligotropha* is for instance also capable to survive at a lower pH (Koops *et al.*, 2003; Tarre *et al.*, 2007), *N. oligotropha*-like bacteria might be considered as K-strategists as being able to maintain at various and perhaps even detrimental conditions.

Previously, members of the whole genus *Nitrosomonas* have been suggested to resemble r-strategists compared to members of *Nitrosospira* (Schramm *et al.*, 1998). This has anyway been questioned again in the same study by referring to the low $K_s$-values observed for *N. oligotropha*. Koops et al (2003) suggested a high production of extrapolymeric substances (EPS) as reason for the ubiquitous occurrence in industrial wwpts (referring to Koops et Harms, 1985 and Suwa *et al.*, 1997). EPS would render those bacteria less sensitive to heavy-metals, which may occur in the influent (referring to Stehr *et al.*, 1995). On the other hand, occurrence of Cluster 6a-like AOB (*N. oligotropha*-like bacteria) throughout the different BOD5 and N concentrations could also lead to the conclusion that occurrence of these AOB cannot be fully explained by the investigated parameters.

While *Nitrospira*-like bacteria could be found in almost all plants, *Nitrobacter* spp. were not detected in any of the investigated wwtps. This is, as already mentioned, supporting the hypothesis that *Nitrospira*-like and not *Nitrobacter*-like organisms are the most important NOB in wwtps (Daims *et al.*, 2001b). *Nitrobacter*-like bacteria have for instance been found in "lab-scale" reactors with a high nitrogen load (~300 mg/l), though also in these reactors *Nitrobacter*-like bacteria were less abundant than *Nitrospira*-like bacteria (Mota *et al.*, 2005). Still, the degree of dilution of these high N concentrations when entering the basins of the "lab-scale" reactors, is unclear, as no information about effluent concentrations and volumes of the reactors are available. *Nitrobacter* spp. could also be detected *in situ* by FISH in the pilot plant in Ingolstadt in 2001 (Daims *et al.*, 2001b), and, by performing a PCR-essay (not conducted during the present study), in the former wwtp in Kraftisried in 1998 (Juretschko *et al.*, 1998). Negative FISH-results and positive detection by PCR in the latter wwtp suggest primarily extremely low abundance, but possibly also a low ribosome content of these NOB (detection limit, see D.2.). The differing result of the recent investigations of the running plant in Ingolstadt (SBR) from the results of the pilot plant in Ingolstadt (SBBR) could be due to changes in the design on the wwtp as well as to changes in wastewater composition, although the influent still derives from sludge dewatering processes. Disregarding conductivity levels, which will be discussed in D.5.5.2., both, the former SBBR in Ingolstadt and the former wwtp in Kraftisried, showed high influent N concentrations (though the influent concentration does not say much about the conditions prevailing in the activated sludge basin of the wwtp in Kraftisried; see D.1.1. and D.1.2.). In addition, a high $NO_2^-$ peak was observed once per cycle in the SBBR in Ingolstadt, which might have selected for *Nitrobacter* spp. Wwtps investigated in the present study, exhibiting high "beginning"- or "inside"-N concentrations, did not show occurrrence of *Nitrobacter* spp., at least not as determined by FISH. Still, for these plants no information on $NO_2^-$ concentrations within the reactors were available, which could yet be the most important factor determining the appearance of *Nitrobacter*-like NOB. Moreover, alternating aerobic and anaerobic conditions in a SBR have been proposed to select for NOB related to *Nitrobacter* (Dytczak *et al.*, 2008).. This has been referred to better $O_2$-availability as a consequence of C removal during the anaerobic reaction, reducing the competition with heterotrophic bacteria in aerobic periods, and, furthermore, to accumulated $NO_2^-$ during anaerobic periods, resulting from incomplete denitrification (Dytczak *et al.*, 2008). This leads once again to the previously presented assumption, that *Nitrobacter* spp. might be well adapted to elevated $NO_2^-$ concentrations.

Neither BOD5 nor "$N_{in}$" concentrations showed any correlation with the abundance of all *Nitrospira*-like bacteria. The same is true for the abundances of *Nitrospira*-"Cluster I" and "Cluster II". This is, at least for "$N_{in}$", somehow surprising as dominance of *Nitrospira* Cluster I or II has been hypothesized to be associated with available $NO_2^-$ concentrations. Cluster I-like bacteria were suggested to be more similar to r-strategists than Cluster II-like bacteria and to dominate at higher concentrations while members of both, Cluster I and Cluster II, seemed to occur in rather equal abundances at lower $NO_2^-$ concentrations (Maixner *et al.*, 2006). Still, maybe other parameters had more influence on the distribution of those bacteria.

Interestingly, "other *Nitrospira*" have firstly been found to occur often in the same samples as halophilic and –tolerant AOB and secondly, they also seem to occur at rather elevated BOD5 and "Nin" concentrations, comparing SBRs; occurrence at rather low BOD5/"Nin"-ratios was less distinctive than for halophilic and –tolerant AOB. Unfortunately there was not much data for the samples harbouring both groups (Rosenheim, Ingolstadt) and the before mentioned interrelations (occurrence of halophilic and –tolerant AOB and "other Nitrospira") at elevated C and N concentrations) are in fact just partly based on samples harbouring both groups. However, factors driving the appearance of this proposed group, are unclear, but might be similar to factors driving the occurrence of halophilic and –tolerant AOB.

### D.5.5.2. Conductivity

Conductivity, describing the concentration of salts in wastewater, did not correlate with the appearance of halophilic and –tolerant AOB. Only when comparing solely SBRs, occurrence seemed to be related to elevated salinity. This trend goes well with the reported characteristics of these AOB. Members of the *N. europaea/Nc. mobilis*-lineage seem to tolerate high salinity levels and some have even been shown to require elevated salinity for growth (*N. halophila*, *Nc. mobilis*) (Koops *et al.*, 2003). However, this trend should be examined critically since performing the analysis only for SBRs is questionable. Conductivity levels were, contrary to C and N concentrations (see D.5.5.1.), considered to be comparable between SBRs and conventional full-scale treatment plants. Yet in conventional wwtps other factors might have more influence on the appearance of these AOB than the conductivity. Such putative factors, characteristic for the operational mode of conventional wwtps, could be a possible explanation for the absence of the trend when all wwtps are considered, but would contradict previous suggestions, reporting wwtp design as an only minor influential factor on nitrifying

bacteria compared to the wastewater composition (Manser, 2005; Head *et al.*, 2003). In addition, conductivity levels were extremely high in most conventional treatment plants (3000 – 5000 µS/cm) and would therefore be expected to have some influence on the appearance of bacteria.

Maybe the occurrence is also dependent on a combination of high conductivites and BOD5 values. For two of the three SBRs harbouring halophilic and –tolerant AOB, data concerning these parameters were available. Both, conductivity levels and BOD5 concentrations were found to be rather high in these wwtps (conductivity = 1282.3 µS/cm and 1437.7 µS/cm; BOD5 = 302.5 mg/l and 275 mg/l for Hettstedt and Radeburg, respectively). However, in the conventional wwtp, in which these AOB were detected (Plattling), BOD5 concentrations were not especially high compared to the other conventional wwtps. Still, analyzing the influence of a combination of these parameters is difficult. On the one hand, because the comparability of BOD5 concentrations between the different types of wwtps seems to be limited. On the other hand the incompleteness of the dataset did not allow an application of statistical tests aimed at finding combinations of factors with significant influence.

As previously mentioned, *Nc. mobilis*-like bacteria, which require elevated salt concentrations for growth and have amongst others been isolated from brackish water and seawater (Koops *et al.*, 2003; Purkhold *et al*., 2000), were detected only in the sample from Ingolstadt. These bacteria were also found in the former pilot plant in Ingolstadt (Daims *et al.*, 2001b) and in the former wwpt in Kraftisried in 1998 (Juretschko *et al.*, 1998). Apart from presumably high N input (for the former wwtp in Kraftisried only influent values were available; see D.1.1., D.2.2.), the two plants seem to have extremely high conductivity values in common. At least the pilot plant in Ingolstadt exhibited very high conductivity values, indicating high ionic concentrations (5000 – 6000 µS/cm; Daims *et al.*, 2001b). Recent data for Kraftisried showed a high conductivity as well (~5000 µS/cm), as did the other animal rendering plants for which data were available. Generally, raw effluent of flaying companies not processing blood, feathers and bones, has conductivities with minimal values of 3000 µS/cm and maxima of 12000 µS/cm (Carozzi *et al.*; in preparation). This could be partly related to chlorides from processing of skins. However, not in all animal rendering plants such processes are carried out, but still high conductivities can be found (Temper; personal communication). High conductivities can also be attributed to elevated concentrations of $NH_4^+$ or organic acids. For the latter one no data were collected. Concerning $NH_4^+$ concentrations, higher conductivities did not correlate with higher nitrogen concentrations (and thus $NH_4^+$ concentrations) in the

investigated plants. However, other wwtps in the present study, also with high conductivities, did not show occurrrence of *Nc. mobilis*-like bacteria, at least not as determined by FISH.

As already described, *Nitrobacter* spp. were not found in any of the samples. However, in the former SBBR in Ingolstadt *Nitrobacter* spp. were observed. These findings do not seem to be referable to elevated N concentrations and high conductivities solely, since *Nitrobacter* spp. should then also be detectable in the wwtps sampled during this study, which exhibited these characteristics. However, as has been previously discussed in section D.5.5.1., a possible explanation for the appearance of *Nitrobacter* spp. in the former SBBR in Ingolstadt could be the high $NO_2^-$ peak, which has been observed in every cycle (Daims *et al.*, 2001b). Unfortunately no data on the varying $NO_2^-$ concentrations in the wwtps investigated during the present study, were available.

A correlation could be shown (by comparing all plants) for increasing conductivity with on the one hand lower abundance of *Nitrospira*-Cluster I-like- and on the other hand higher abundance of *Nitrospira*-Cluster II-like bacteria (both relative to all *Nitrospira*-like organisms), though only the correlation with "Cluster I" was significant. This trend was only weakly visible when considering abundances of "Cluster I and II" relative to all bacteria and should be interpreted cautiously. So far it is unclear whether members of *Nitrospira*-Cluster I and II react differently to differing salt concentrations. Attention should be paid to the fact that the three treatment plants that actually account for the observable trend are all animal rendering plants and at the same time conventional full-scale plants. Another animal rendering plant, TBA GZM Lyss, also exhibiting very high conductivity (~3000 µS/cm), was unlike the others dominated by "Cluster I"; conductivity values for the SBRs receiving partly water from animal rendering have not been available. The only conventional plant (ARA Lyss) not treating animal waste did not show extremely high salt concentrations and harboured only "Cluster I". The exclusive appearance of "Cluster I" in the wwtp ARA Lyss and the predominant appearance in the wwtp TBA GZM Lyss, suggest that the elevated abundance of Cluster II is not correlated with parameters specific for conventional wwtps (wwtp design). The trend cannot be attributed to characteristics of animal rendering plants either as results for the sample from TBA GZM Lyss (dominance of "Cluster I") would not go with such assumptions (though it could also be an exception). Unfortunately not more data points exist between 2000 and 4000 µS/cm, which could perhaps show whether the wwtp in TBA GZM Lyss constituted an exception. A rather sharp decline of the abundance of Cluster I was

visible at conductivity levels over 3000 µS/cm (TBA GZM Lyss), which could indicate some kind of threshold as well as some unknown characteristics, which the three plants, in which Cluster II was dominating over Cluster I, have in common. The observable trend is further challenged by the dominant abuncance of Cluster I in the sample taken from Ingolstadt. Although no recent conductivity levels were available for this plant, rather high levels can be assumed since it is receiving reject water from sludge dewatering just like the fromer pilot plant. In the pilot plant in Ingolstadt extremely high conductivity levels (5000 – 6000 µS/cm) have been observed. However, without actual conductivity measurements for the SBR now in use, these con-clusions are highly speculative. All in all the trend is questionable, especially since it is less distinctive for abundances of *Nitrospira*-Cluster I and II-like bacteria relative to all bacteria.

### D.5.6. Further possible, non-examined influences

As occurrence and abundance of AOB and NOB cannot easily be explained by the considered parameters, other factors might also have influence. One factor of interest could be the structure of flocs or biofilms, in which nitrifying bacteria occur. Floc- and biofilm-structures may influence occurrence and abundance of different bacteria as they are relevant for the supply with $O_2$ and nutrients. It might be shaped through aeration (pressure of aeration) as proposed by Manser (2005) (though within this study no differences in community composition were observed) or differences in agitation. One factor investigated, but just for few plants, is the loading strategy ($t_F/t_R$), already suggested to interrelate with ISV (floc structure) (Holm, personal communication).

A biotic factor not investigated at all is predation of bacteria by protozoa and metazoa as well as viral attacks. Little is known about the role predation has in these microcosms till now, though the role of protozoa for good effluent quality has been recognized (Moussa *et al.*, 2005; referring to Curds and Cockburn, 1970). So far no discrimination in predation of different bacteria has been reported (Moussa *et al.*, 2005; referring to Girffiths, 1989), apart from one negative example of protozoan impact, demonstrated by Bouchez *et al.* (2000), in which overgrowth of protozoans grazing on aerobic denitrifying bacteria resulted in failure of N-removal. Diversity of predators could be furthermore important, as more diversity has been found to have more impact on prey biomass (Duffy, 2002; referring to Sommer *et al.*, 2001, Holt and Loreau, 2002). An influence of predation might be essential for maintaining bacterial diversity, which could otherwise be reduced due to potential further growth of biomass

accompanied by competitive exclusion (D.5.4.). Complex food web structure and interactions are believed to be significant for stability (McCann, 2000; referring to Chesson and Huntley, 1997; Townsend *et al.*, 2000; referring to McArthur, 1955).

## D.6. Investigation of the predominating bacterial morphotype in Rapp-Kutzenhausen

In the sample from the SBR in Rapp-Kutzenhausen neither AOB- nor NOB-signals were observed, which maybe due to the extremely high C/N-ratio (23) of the influent in this SBR. The high ratio suggests that all nitrogen could be assimilated into biomass along with carbon and as a consequence no nitrification occurs (see D.4.2.1. and D.5.5.1. for further discussion). Therefore and because the sample appeared to contain mostly one bacterial morphotype occurring in tetrads, a 16S rDNA-approach was carried out (based on amplification of the respective gene, cloning and sequencing, followed by comparative sequence analysis). Most of the obtained partial sequences showed phylogenetic affiliation to *Defluviicoccus vanus* (*Rhodospirillaceae; α-Proteobacteria*). When applying FISH with a probe targeting *α-Proteobacteria*, the predominating bacteria were detected. Still, this result is just pointing out that dominating organisms could be related to the genus *Defluviicoccus*, since the FISH-probe applied was too general to allow detailed conclusions. In addition, this probe has been reported to be rather unspecific. Assuming *Defluviicoccus*-like bacteria as the dominant bacteria in this sample is still tempting as those are reported to occur often as distinctive coccal cells in tetrads or clusters (Maszenan *et al.*, 2005). Interestingly, these bacteria are suggested to be glycogen-accumulating organisms (GAO), which are thought to decrease efficiency of EBPR (Enhanced biological phosphorus removal systems) by competing with the polyphosphate accumulating organisms (PAO) (Burow *et al.*, 2007; Meyer et al, 2006). Total P concentrations in the effluent of the different SBRs of this plant fell almost completely below a value of 2 mg/l, which seems to be normal considering incoming $P_{total}$ concentrations of approx. 10 mg/l, since the European guideline for municipal wastewater treatment 91/271/EWG; 1991 (partly changed in 1998; Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft - Sektion VII, 2006) demands a reduction of P concentrations of approx. 80% for wwtps having population equivalents ($EW_{60}$*) > 10000 (Rapp Kutzenhausen: population $equivalents_{60}$ = 20000). Even though the investigated reactor (SBR3) is the only one with a slightly higher effluent concentration (< 2.8 mg/l), tracing back

* population $equivalent_{60}$: This parameter is describing the degree of contamination of the influent and referred to the average amount of organic pollutants, one inhabitant is contributing to the overall contamination of wastewater per day (60 – 65 g BOD5/inhabitant/d) (www.wasser-wissen.de, online-lexicon of the university of Bremen).

this elevated concentrations to the potential occurrence of GAOs should be regarded critically as in this wwtp P gets removed chemically by Fe-precipitation. Biological P removal could occur as well, but no additional information was available on this issue.

### D.7. Screening for ammonia-oxidizing *Crenarchaeota*

#### D.7.1. Analysis of crenarchaeotal *amoA*-like sequences

Sequences of crenarchaeotic *amoA*-like genes were obtained for three plants (TBA GZM Lyss, Altmannstein and Ampfing). For the cloning procedure, PCR-products were purified as PCR-amplification did not result in one specific band each, but in some at different heights. Even increasing the annealing temperature did not prevent unspecific binding of the primers. Until sequences were finally obtained, this was seen as a hint that actually no ammonia-oxidizing *Crenarchaeota* occured in the samples and the primers annealed unspecifically. Interestingly, when translating the obtained sequences in amino acids in ARB, one sequence (AL_1) showed 4 stop codons, which would mean that the gene cannot be fully translated. This can be argued in different ways: firstly, a translation from nucleotide into protein sequences by ARB is done based on the bacterial genetic code encompassing three possible stop-codons (UAG, UGA and UAA). However, deviations from the standard genetic code, referred to as "recoding", have been observed to occur in some bacteria, archaea and eukaryotes as well as viruses (Cobuzzi-Ponzano *et al.*, 2005; referring to Farabaugh, 1996 and Baranov *et al.*, 2002). "Recoding" is believed to occur either in form of "ribosome programmed -/+1 frameshifting", "ribosome hopping" or "stop codon read-through" (Cobuzzi -Ponzano *et al.*, 2005). Two unusual amino acids, not formed by chemical modification, have been reported apart from the standard 20 L-amino acids, which normally constitute proteins of organisms: selenocystein (21$^{st}$ amino acid) encoded by the stop codon "UGA" (named opal) has been found in some organisms such as methanogenic archaea, animals and some bacteria (Cobuzzi-Ponzano *et al.* 2005; Fenske *et al.* 2003); and pyrrolysine (22$^{nd}$ amino acid), encoded by the stop-codon "UAG" (amber) and as well translated by "stop codon readthrough". The latter one has been identified in bacteria and archaea as well as in eukaryotes, but within the archaea so far only in one branch of methanogenic archaea (Cobuzzi-Ponzano *et al.* 2005). The stop codons found in AL_1 were encoded by the triplets "UGA" and "UAA" (3 times). Taking this into account, the stop codons found, could be linked to on the one hand incorporation of selenocystein, which has indeed only been reported for methanogenic archaea till now and on

the other hand to unknown “stop codon readthrough”. However, this is not likely, since stop codons were just found in one sequence. Alternatively, stop codons in a functional gene can be seen as a result of the loss of the function of a gene or gene duplication and subsequent alternation of one gene under eased constraints (Zhang *et al.*, 2003). Such events can lead to the formation of pseudogenes (possibly followed by deletion from the genome), but also to development of novel functions (Zhang *et al.*, 2003; referring to Ohno, 1970, Lynch *et al.*, 2000, Walsh *et al.*, 1995, Lynch *et al.*, 2001 and Harisson *et al.*, 2002). Another, more simple explanation is the occurrence of an error during PCR, though occurrence of 4 errors seems to be rather unlikely. A sequencing error could be excluded since repeated sequencing did not alter the result.

### D.7.2. Activity analysis by mRNA extraction

Extraction of RNA from a new sample of one of the plants (TBA GZM Lyss) and subsequent detection of *amoA*-mRNA should have provided new information on the activity and participation of AOA in N removal in wwtps. Though extraction of RNA and transcription in cDNA did work, crenarchaeotal *amoA*-like cDNA could not be amplified. Extraction of DNA from the newly obtained sample from the TBA GZM Lyss, followed by PCR did not result in amplificates of crenarchaeotal *amoA*-like genes either. This could be due to a disturbance in terms of $NO_2^-$ accumulation in the wwtp, which occurred shortly before the new sample was taken. Elevated $NO_2^-$ concentrations could have had detrimental influences on AOA, though FISH revealed that AOB present in the previously taken sample could also be detected in the new sample. As could be expected, no NOB were observable after the break-down. In this context crenarchaeotal *amoA*-specific amplification of the extracted DNA of the samples, from which sequences had already been obtained, was repeated, but no amplificons were obtained, not even after lowering the annealing temperature. Both, the primers (the positive control could still be amplified), as well as DNA-quality (16S rDNA could still be amplified), could be excluded as sources of error. One remaining possibility is that the previously successful amplifications were a consequence of contaminations, for moreover by CARD-FISH no AOA were detectable in these samples. Furthermore, no bands could be detected by PCR with primers specific for crenarchaeal 16S rDNA (Mußmann *et al.*, in preparation). However, contaminations should be amplifiable again as well. More details concerning this subject can be seen in the next section.

### D.7.3. Contamination screening

Screening by nested-PCR (Könneke primers) indicated no contamination as no distinct bands at the right height were observed. Screening by using a primer-combination (crenarchaeotal *amoA*-specific and M13-primers) did not yield amplificates with the samples, but only with the positive control. However, using the CrenamoA-Francis primer pair, no bands could be detected at all anymore (even in the nested-PCR-approach), unless for the positive control. All in all PCR-screening did not indicate contamination, though the results are inconsistent (C.4.2.4.). Phylogenetic analysis of the obtained sequences in ARB performed to test whether high similarities to other crenarchaeotal *amoA*-like sequences, obtained in this laboratory, do exist, further reduced the probability of contamination since no affiliation between these sequences could be seen.

### D.7.4. Relevance of AOA in wastewater treatment plants

Importance of ammonia-oxidizing *Crenarchaeota* based on quantitative data has been suggested for marine and soil habitats. In fact it has been questioned whether the significant role attributed to AOB in N-cycling should not be assigned to AOA. In wastewater treatment though, the possible contribution of AOA to N removal is still unclear. Sequences of crenarchaeotal *amoA*-like genes could be obtained from wwtps and high SRT was introduced as a possible factor determining occurrence of AOA (Park *et al.*, 2006), but neither any information about activity nor abundance of AOA was gathered in this study. Abundance has been investigated recently by CARD-FISH. Just in few of the investigated plants AOA could be detected by CARD-FISH or PCR, a result already challenging the role of AOA in activated sludge (Mußmann *et al.*, in preparation). Less dissolved $O_2$ was proposed as a factor which could favour higher abundances of archaea instead of AOB. In one of the wwtps investigated during this study, in which most ammonia-oxidizing *Crenarchaeota* could be seen by applying CARD-FISH, no AOB could be detected by FISH (Mußmann *et al.*, in preparation). In the samples, from which crenarchaeotal *amoA*-like sequences could be obtained in the present study, no AOA could be detected by CARD-FISH, indicating either very low abundances of cells or extremely low rRNA-concentrations per cell, since for detection by CARD-FISH already ~50 rRNA-copies/cell have been reported to be sufficient (Hoshino *et al.*, 2008). Considering this and also the fact that in all those plants AOB could be detected, AOA seem to play a minor role in N removal in these plants. Although the consensus tree

only provides low phylogenetic resolution due to the varying branching patterns obtained by the different applied treeing methods, phylogenetic affiliation of the obtained sequences within the tree supports the idea of little importance of AOA in wwtps. Clusters containing sequences of all of the three investigated wwtps would indicate AOA, which are well-adapted to the prevailing conditions in activated sludge and thus represented in all plants. However, such clusters were not observed. However, coverage of the diversity of the clone-libraries was probably not reached. Overall, especially the absence of signals after CARD-FISH (Mußmann *et al*., in preparation) suggests minor importance of archaea in the wwtps. Finally, a successful analysis of mRNA, assuming crenarchaeotal *amoA*-like mRNA was indeed present, would have revealed more about their role in wastewater treatment

## D.8. Summary

Nitrification is essential for nitrogen removal in wastewater treatment and primarily attributed to the two functional guilds of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, though importance of ammonia-oxidizing archaea is a matter of current discussions. As this process is still subjected to failures now and then, it is frequently investigated, also with regard to an ecological model proposing a process as more stable if the same function within the system is carried out by different species, which can react differently to changing conditions, thus increasing the probability of maintenance of the process even under detrimental conditions (functional redundancy).

In 21 samples taken from wastewater treatment plants differing in design, operational mode and wastewater composition, the occurrence of AOB and NOB as well as abundances of *Nitrospira*-clusters have been determined by FISH. Linking this biological data to physical parameters of the investigated plants should provide information about factors influencing the occurrence or abundance of certain nitrifiers. Overall, representatives of the *N. oligotropha*-Cluster and the *Nitrospira*-Cluster I were found to occur most frequently in the wwtps, suggesting good capability of adapting to varying conditions or differently responding species or populations within these clusters. Halophilic and –tolerant AOB have been found at elevated carbon and nitrogen concentrations and at elelvated conductivity-levels considering solely SBRs, agreeing with previous assumptions of preferred nutrient-rich habitats, high salt-tolerance and partly even high salt-requirement of these AOB. Occurrence of these AOB did not correlate with elevated carbon, nitrogen and conductivity levels when the same analysis was performed for both, SBRs and conventional full-scale wwtps. However, comparability of carbon and nitrogen concentrations between SBRs and conventional wwtps is questionable. Conductivity, on the other hand, was comparable between all wwtps and a weak trend was visible proposing increasing abundances of *Nitrospira*-Cluster II-like and declining abundances of *Nitrospira*-Cluster I-like bacteria with increasing conductivity. However, more wwtps should be analyzed to confirm this observation.

Significant correlations have been found for increasing diversity (in terms of the number of different AOB- and/or NOB-clusters in the samples) and increasing temperature as well as increasing concentrations of dry matter (biomass). The correlation between diversity and concentration of dry matter should be regarded critically, since this concentration is regulated by removal of excess sludge and thus allows only limited interpretation. However, as at least for SBRs the concentrations of dry matter seemed to be related to the amount of incoming C-

compounds, relating the amount of biomass to the productivity of the system and the productivity in turn to the diversity within the system, is tempting. Apart from increasing the overall abundance of bacteria, more resources might also increase species richness in a system.

Furthermore, FISH-analysis indicated presence of yet unknown "other *Nitrospira*" in some of the samples, but this observation could not be confirmed by 16S rDNA sequence data. This proposed group has been found to co-occur with halophilic and –tolerant AOB in some samples, and to occur at elevated BOD5- and "Nin"-conc. like halophlic and –tolerant AOB, considering only SBRs.

As all in all the investigated parameters do not sufficiently explain the biological data, other factors could be of more importance.

Presence of putative ammonia-oxidizing crenarchaeotes could be shown for three samples. Still, this result does not tell much about the role of these organisms in nitrogen removal. However, negative results of specific CARD-FISH with these samples indicate extremely low abundance or growth and thus, if at all, little importance of these archaea.

## F. Literature

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## Abbreviations:

| | |
|---|---|
| α | alpha |
| β | beta |
| γ | gamma |
| δ | delta |
| ε | molar extinction coefficient |
| λ | lambda (unit for wavelength) |
| % | percentage |
| °C | degree Celsius |
| µ | micro- |
| 2D | 2-dimensional |
| 3D | 3-dimensional |
| $A_x$ | absorption at the wavelength x |
| abs. | absolute |
| AF | autofluorescence |
| *amoA* | ammonia monooxygenase A-gene |
| AMO | ammonia monooxygenase |
| Amp | ampicillin |
| ANAMMOX | anaerobic ammonium oxidation |
| AOA | ammonia oxidizing archaea |
| AOB | ammonia oxidizing bacteria |
| approx. | approximately |
| ATP | Adenosine triphosphate |
| BAF | biological aerated filter |
| bidest. | double distilled and filtered |
| BLAST | Basic local alignment search tool |
| BOD5 | Biological oxygen demand |
| bp | base pairs |
| BSA | bovine serum albumine |
| C | carbon |
| CARD-FISH | catalyzed reporter deposition-fluorescence in situ hybridization |
| Cd | cadmium |
| cDNA | complementary DNA |
| $CHCl_3$ | chloroform |
| Cl. (here) | Cluster |
| Cl.I Ntspa.B (here) | abundance of Cluster I *Nitrospira* spp. related to all batceria |
| Cl.II Ntspa.B (here) | abundance of Cluster II *Nitrospira* spp. related to all Bacteria |
| CLSM | confocal laser scanning microscope |

| | |
|---|---|
| cm | centimeter |
| $CO_2$ | carbon dioxide |
| COD | Chemical oxygen demand |
| Comp. (c) | Competitor |
| Cond. | Conductivity |
| crenarchaotal *amoA* | Crenarchaeal ammonia monooxygenase A-gene |
| Cu | copper |
| Cy3 | 5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy3.18-derivative N-hydroxysuccimidester |
| Cy5 | 5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy5.18- |
| d | days |
| DAPI | 4'-6'-di-amidino-2-phenylindole |
| dNTP (N=A/G/C/T) | deoxy-nucelotide-triphosphate (adenine/guanine/cytosine/thymine) |
| ddNTP (N=A/G/C/T) | dideoxy-nucelotide-triphosphate (adenine/guanine/cytosine/thymine) |
| denitrif. (here) | denitrification |
| DEPC | Di-ethyl-pyrocarbonate |
| DIC | differential internal cycling |
| diff. | different |
| Disc. | Discussion |
| DM | dry matter |
| DMF | Di-methylformamide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| ds | double stranded |
| $E_x$ | extinction at a wavelength x |
| *E. coli* | Escherichia coli |
| e.g. | "exemplia gratia" (for example) |
| EBPR | enhanced biological phosphorous removal |
| EDTA | ethylene-di-amine-tetra-acetic acid |
| environ./env. (here) | environmental |
| EPS | extrapolymeric substance |
| ERT | Eppendorf reaction tube |
| Esp. | especially |
| et. al. | et alteri ("and others") |
| EtBR | ethidium bromide |
| Etc. | et cetera ("and other things") |
| EtOH | ethanol |
| Ev. | eventually |

| | |
|---|---|
| F | forward (usually related to primers) |
| FA | formamide |
| $Fe(II)SO_4$ | ferric sulphate |
| fig. | figure |
| FISH | fluorescence in situ hybridization |
| FLUOS | 5,(6)-carboxyfluorescein-N-hydroxysuccimidester |
| fmol | femtomol |
| g | “Earth`s gravity” (normally connected to centrifugation) |
| g | gram |
| GAO | glycogen-accumulating organisms |
| GC-content | GC base ratio: % of DNA consisting of guanine and cytosine (in mol% GC) |
| h | hour |
| $H^+$ | hydrogen ion |
| $H_2O$ | water |
| $H_2O_2$ | hydrogen peroxide |
| halo. (here) | halophilic and -tolerant |
| HAO | hydroxylamine-oxidoreductase |
| HB | hybridization buffer |
| HCl | hydrochloric acid |
| HPLC | High Performance Liquid Chromatography |
| ind./indus. (here) | industrial |
| Intr. | Introduction |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| ISV | index of sludge volume |
| Kan | kanamycin |
| Kbl | “kilo-basepair-ladder” |
| KCl | potassium chloride |
| $K_o$ | Half-saturation constant (oxygen) of Monod kinetics |
| $K_s$ | Half-saturation constant (substrate) of Monod kinetics |
| l | liter |
| LB-medium | Luria Bertani medium |
| M | molar |
| MAR | membrane aerated bioreactor |
| mg | milligram |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4^-$ | magnesium sulphate |
| min. | minutes |
| ML | maximum likelihood |
| ml | milliliter |
| mM | millimolar |

| | |
|---|---|
| MP | maximum parsimony |
| mRNA | messenger RNA |
| municip. | Municipal |
| N | nitrogen |
| N | sample size |
| n.d. | not determined |
| n.k. | not known |
| *N*….(*oligotropha*), etc. | *Nitrosomonas*…(*oligotroha*), etc. |
| $N_2$ | dinitrogen |
| $N_2H_4$ | hydrazine |
| $N_2O$ | nitrous oxide |
| $Na_2HPO_4$ | di-sodium-hydrogenphosphate |
| NaAc | natrium acetate |
| NaCl | sodium chloride |
| $NaH_2PO_4$ | Sodium-di-hydrogenphosphate |
| NaOH | sodium hydroxide |
| *Nc.* | *Nitrosococcus* |
| NCBI | National Center for Biotechnology Information |
| ng | nanogram |
| $NH_3$ | ammonia |
| $NH_4^+$ / $NH_4^+$ -N | ammonium /ammonium-nitrogen |
| Ni | nickel |
| *NirK*/*nirS* | nitrite reductase-genes (differing in structure) |
| NJ | neighbour joining |
| nm | nanometer |
| Nm143 | *Nitrosomonas* strain Nm143 |
| NO | nitric oxide |
| $NO_2^-$ / $NO_2^-$ -N | nitrite / nitrite nitrogen |
| $NO_3^-$ / $NO_3^-$ -N | nitrate / nitrate nitrogen |
| NOB | nitrite oxidizing bacteria |
| $NO_x$ | $NO_3^-$or $NO_2^-$ (reduced nitrogen) |
| Ntspa. | Nitrospira |
| *nxr* | nitrite-oxidoreductase-gene |
| $O_2$ | oxygen |
| OD | optical density |
| ON | overnight |
| P | phosphorous |
| Pa | Pascal |
| PAO | polyphosphate-accumulating organisms |
| PBS | phosphate buffered saline |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PFA | paraformaldehyde |
| Pmol (pM) | picomolar |
| $PO_4^-$ | phosphate |
| pos. | positive |
| r | correlation coefficient |
| R | reverse (normally related to primers) |
| RDP | Ribosomal database project |
| reampl. (here) | reamplified |
| rel.abundance | relative abundance |
| RFLP | restriction fragment length polymorphism |
| RNase | ribonuclease |
| rpm | rotations per minute |
| rRNA | ribosomal ribonucleic acid |
| RT | reverse transcriptase |
| RT | room temperature |
| S | Siemens |
| SA | sludge age |
| SBBR | Sequencing Biofilm Batch Reactor |
| SBR | Sequencing Batch Reactor |
| SDS | sodium dodecyl sulphate |
| sec. | seconds |
| spp. | species (plural) |
| SRT | sludge retention time |
| ss | single stranded |
| Subst. | substitution |
| T/Temp. | temperature |
| tab. | tables |
| TAE | Tris-actetate-EDTA |
| Taq | thermostable DNA-polymerase from *Thermus aquaticus* |
| TBE | Tris-boric acid-EDTA |
| TE | Tris-EDTA |
| $t_F$ | loading time (filling time) |
| TKN | total Kjedahl nitrogen |
| $t_R$ | reactive time |
| T-RFLP | terminal-restriction length polymorphism |
| Tris | tris-(hydroxymethyl-)aminomethane |
| tRNA | transfer RNA |
| UV | ultraviolet |
| V | Volt |
| w/v | weight/volume |

| | |
|---|---|
| WB | washing buffer |
| wwtp | wastewater treatment plant |
| X-Gal | 5-brom-4-chlor-3-indolyl-β-D-galactopyranoside |
| Zn | zinc |

## Appendix:

Tables can be seen on the following pages.

**Table 35:** Appendix: overview of the data used in the present study

"time period of sampling": time during which data has been collected

"amount of measurements": how often have measurements in general been taken during "time period of sampling"

n.d. = not detected

n. k. = not known

| Treatment plant | type of reactor | type of waste water | Population equivalent | sampling date | time-period of data (d) | (amount of measurements) | constance of data acquisition | number of rainy days |
|---|---|---|---|---|---|---|---|---|
| Kraftisried | full-scale | animal rendering | | 29.01.07 | 29 | 29 | Daily-weekly (marked: less than daily) | n.d. |
| Plattling | full-scale | animal rendering | | 29.01.07 | 7 | n.k. | n.k. | n.d. |
| Oberding | full-scale | animal rendering | | 29.01.07 | 7 | n.k. | n.k. | n.d. |
| TBA GZM Lyss | full-scale, membrane filtration | animal rendering | | 29.01.07 | 7 | n.k. | n.k. | n.d. |
| ARA Lyss | full-scale, fixed bed | municipal | | 29.01.07 | 7 | n.k. | n.k. | n.d. |
| Rosenheim | SBR | municipal | 11200 | 30.05.07 | 3 | 3 | Every 3 days | n.d. |
| Rapp-Kutzenhausen | SBR | Brewery | 20000 | 28.03.07 | 22 | 4 | Weekly (marked: not weekly) | n.d. |
| Ingolstadt | SBR | sludge drainiage | | 09.05.07 | 22 | 15 | 3-4times/week | n.d. |
| Waldsassen | SBR | municip.+industrial | 13000 | 27.03.07 | 27 | 2-27 | daily (marked: not daily) | 9 |
| Ampfing | SBR | animal rendering, milk production | 22500 | 26.03.07 | 26 | 26-4 | Daily-weekly (marked: less than daily) | 13 |
| Altmannstein | SBR | municipal | 5500 | 24.03.07 | 31 | 3 | ~every two weeks | n.d. |
| Langenzenn | SBR | municipal | 16000 | 14.03.07 | 29 | 4/7 | Weekly | n.d. |
| Seefeld | SBR | municipal | 26000 | 28.03.07 | 25 | 12 | every 2nd day (marked: less often) | n.d. |
| Radeburg | DIC-SBR | municipal | 15200 | 24.05.07 | 15 | 3 | Weekly | n.d. |
| Bad Zwischenahn | DIC-SBR | municipa, industrial influence | 41000 | 23.05.07 | 23 | ~23 | daily (marked: less) | n.d. |
| Deuz | DIC-SBR | municipal | 12800 | 21.05.07 | 21 | 21/3 | daily-weekly (otherwise marked) | 12 |
| Bruchmühlen | DIC-SBR | municipal | 11250 | 22.05.07 | 28 | 28-4 | daily (marked: not daily) | 16 |
| Weisstal SBR | DIC-SBR | municipal | 9500 | 21.05.07 | 27 | 4 | ~ weekly | n.d. |
| Huntlosen | DIC-SBR | municipal | 10000 | 23.05.07 | 22 | 2 | two measurements from the first of three weeks before sampling | n.d. |
| Hettstedt | DIC-SBR | municipal, external faecal sludge | 30000 | 24.05.07 | 16 | 16 | daily (marked: less) | 10 |
| Spenge | DIC-SBR | municipal | 22500 | 22.05.07 | 22 | 13-19 / ~4 | daily-weekly (marked: less than daily) | 9 |

**Table 35:** continued; data, before further analysis, but already measured values are already given as averages and standard deviations (st. dev.)

[a] inside, outside, within

"marked in yellow" = not corresponding to "amount of measurements"; sample size = actual amount of measurements

[b] st.dev. was provided

| Treatment plant | conductivity[a] (µS/cm) | | | income COD (mg/l) | | | outcome COD (mg/l) | | | income BOD5 (mg/l) | | | outcome BOD5 (mg/l) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | average | st.dev. | sample size | average | st.dev. | sample size | average | st.dev. | sample size | average | st.dev. | sample size | average | st.dev. | sample size |
| Kraftisried | 5000 | | n.k. | 3875 | 809.8 | 4 | 46.3 | 6 | 3 | 3000 | | | 12 | | |
| Plattling | 4000 | | n.k. | 5000 | | | 30 | | | 3500 | | | 5 | | |
| Oberding | 4000 | | n.k. | 5000 | | | 35 | | | 3500 | | | 5 | | |
| TBA GZM Lyss | 3000 | | n.k. | 4500 | | | 35 | | | 3000 | | | 5 | | |
| ARA Lyss | 1000 | | n.k. | 270 | | | 45 | | | 110 | | | 6 | | |
| Rosenheim | | | | | | | | | | | | | | | |
| Rapp-Kutzenhausen | 1900 | | 1 | 1797 | 160.6 | | 56.8 | 4.79 | | 1250 | 129.1 | | | | |
| Ingolstadt | | | | | | | | | | | | | | | |
| Waldsassen | | | | 410 | 55.2 | 2 | 20.5 | 1 | 4 | 235 | 63.6 | 2 | 2.3 | 1.5 | 4 |
| Ampfing | | | | 875.4 | 80.4 | 4 | 20.8 | 2.2 | 4 | 648.2 | 230.7 | | 4.3 | 1.3 | 4 |
| Altmannstein | | | | 443.7 | 237.9 | | 19.7 | 4.9 | | 206.7 | 100.7 | | 5.3 | 2.1 | |
| Langenzenn | | | | 259.8 | 56.77 | | 19 | 1.7 | | 131.3 | 26.6 | | 2.1 | 0.3 | |
| Seefeld | | | | 203.8 | 48 | | 13.12 | 1.29 | | 103.5 | 23.95 | | 3 | 0.6 | |
| Radeburg | 1437.7 | 140.59 | | 1069.7 | 128.4 | | 49.3 | 2.9 | | 550 | 36.1 | | 1.7 | 0.6 | |
| Bad Zwischenahn | | | | | | | 59.2 | 6.2 | | | | | 3 | | |
| Deuz | 547.1 | 170.3 | | 307 | 168.6 | 3 | 14.3 | 3.59 | 4 | | | | | | |
| Bruchmühlen | 475.4 | 128.5 | | 573.4 | 267.5 | 5 | 24.5 | 4.1 | 6 | 302.5 | 119.6 | 4 | 10.2 | 8.4 | 5 |
| Weisstal SBR | 397.8 | 102.2 | | | | | 10.5 | 3.3 | | | | | 2 | 0 | |
| Huntlosen | | | | 622 | 84.9 | | 28 | 1.4 | | 380 | 56.6 | | 3.5 | 0.7 | |
| Hettstedt | 1282.3 | 143 [b] | 1 | 1041 | 185.3 | 2 | 24 | 3.6 | 8 | 605 | 49.5 | 2 | 1 | | 1 |
| Spenge | 961.9 | 346.2 | | 385.8 | 197 | 4 | 27.8 | 8.34 | 4 | 203.8 | 105.6 | 4 | 5.3 | 1.71 | 4 |

**Table 35:** continued; data, before further analysis, but measured values are already given as averages and standard deviations (st. dev.)

"marked in yellow" = not corresponding to "amount of measurements"; sample size = actual amount of measurements

| | Income | | | outcome | | | income | | | outcome | Income | | | outcome | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N "total" (mg/l) | | | N "total" (mg/l) | | | TKN (mg/l) | | | TKN (mg/l) | NH4 (mg/l) | | | NH4 (mg/l) | | |
| Treatment plant | average | st.dev. | sample size | average | st.dev. | sample size | average | st.dev. | sample size | average | average | st.dev. | sample size | average | st.dev. | sample size |
| Kraftisried | | | | | | | 400 | | | 22 | 397.5 | 51.9 | 4 | 35.3 | 29 | 4 |
| Plattling | | | | | | | 800 | | | 2 | 750 | | | 1 | | |
| Oberding | | | | | | | 500 | | | 1 | 450 | | | <1 | | |
| TBA GZM Lyss | | | | | | | 750 | | | 1 | 700 | | | <1 | | |
| ARA Lyss | | | | | | | 35 | | | 2 | 20 | | | 1 | | |
| Rosenheim | | | | | | | | | | | | | | | | |
| Rapp-Kutzenhausen | 55 | 2.83 | 2 | | | | | | | | 14 | 3.86 | | 0.8 | 1.35 | |
| Ingolstadt | | | | | | | | | | | | | | | | |
| Waldsassen | 18.5 | 3.7 | 2 | | | | 18.5 | 3.7 | 2 | | 18.5 | 3.7 | 2 | < 0.1 | | |
| Ampfing | 88.7 | 4.6 | 4 | 3.5 | 0.5 | 4 | | | | | | | | 0.06 | 0.016 | 4 |
| Altmannstein | | | | | | | | | | | 54.7 | 27.5 | | 9.18 | 6.79 | |
| Langenzenn | | | | | | | | | | | 21.25 | 5.25 | | 7.96 | 2.43 | |
| Seefeld | | | | | | | | | | | 8.32 | 1.57 | | 1.59 | 0.4 | |
| Radeburg | | | | | | | 158.3 | 22.48 | | | | | | 0 | 0 | |
| Bad Zwischenahn | | | | | | | | | | | 60 | | 1 | 0.25 | 0.11 | |
| Deuz | 34.8 | 9.8 | 3 | 3.78 | 2.62 | 4 | | | | | | | | 0.33 | 0.39 | 4 |
| Bruchmühlen | 56.8 | 27.76 | 5 | 5.25 | 2.9 | 6 | | | | | 36 | 17.48 | 5 | 0.53 | 1.1 | 6 |
| Weisstal SBR | | | | 4.6 | 1.4 | | | | | | | | | 0 | 0 | |
| Huntlosen | | | | | | | | | | | 68 | 1.4 | | 0.13 | 0.007 | |
| Hettstedt | 112.5 | 45.96 | 2 | | | | | | | | 56 | 5.7 | 2 | 12.35 | 7.8 | 12 |
| Spenge | 35.25 | 21.23 | 4 | 2.28 | 0.33 | 4 | | | | | 24 | 14.35 | 4 | ≤0.2 | | |

**Table 35:** continued; data, before further analysis, but measured values are already given as averages and standard deviations (st. dev.)

"marked in yellow" = not corresponding to "amount of measurements"; sample size = actual amount of measurements

[c] "general averaged values"

| Treatment plant | income $NO_3^-$ (mg/l) | outcome $NO_3^-$ (mg/l) | | | outcome $NO_2^-$ (mg/l) | | | inside T (°C) | | | Inside pH | | | inside $O_2$ (mg/l) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | average | st.dev. | sample size | average | st.dev. | sample size | Average | st.dev. | sample size | average | st.dev. | sample size | average | st.dev. |
| Kraftisried | 0 | 17.4 | 4.7 | 4 | 6.2 | 11.9 | 4 | 7 | | | 7.3 | | | 0.0-10 | |
| Plattling | 0 | 3 | | | <0.5 | | | 30 | | | 7 | | | 0-1.5 | |
| Oberding | 0 | 4 | | | <0.5 | | | 26 | | | 6.8 | | | 0-2 | |
| TBA GZM Lyss | 0 | 14 | | | <0.5 | | | 30 | | | 7 | | | 0-3 | |
| ARA Lyss | 2 | 18 | | | 0.1 | | | 12 | | | 7.8 | | | 8 | |
| Rosenheim | | | | | | | | 38.2 | 2.7 | | 7.7 | 0.1 | | 1.8 | 0.5 |
| Rapp-Kutzenhausen | < 0.5 | < 0.5 | | | < 0.1 | | | | | | 7.1-7.7 | | | 1.5-2.5 | |
| Ingolstadt | | | | | | | | 27.4 | 2.7 | | 7.2 | 0.3 | | 1.8 [c] | 1.72 |
| Waldsassen | | 3.45 | 1.8 | | | | | 9 | 0.9 | 12 | 6.4 | | | 2-3.7 | 1 |
| Ampfing | | 3.5 | 0.5 | 4 | 0.03 | | 1 | | | | | | | 2.7 | 0.2 |
| Altmannstein | | 0.72 | 0.17 | | 0.48 | 0.31 | | | | | | | | 0.1-2.9 | |
| Langenzenn | | 3.1 | 0.61 | | 0.42 | 0.22 | | 9.2 | 0.5 | | 7.8 | 0.08 | | 1-2 | |
| Seefeld | | 1.73 | 0.53 | | | | | | | | | | | 0-3 | 1.6 |
| Radeburg | | 3.3 | 0.2 | | 0.05 | 0.01 | | | | | | | | | |
| Bad Zwischenahn | | 6.5 | 2.7 | | | | | | | | | | | 1.3 | 0.1 |
| Deuz | | 3.46 | 2.3 | 4 | 0.09 | 0.07 | 4 | 13 | 0.4 | | 6.8 | 0.2 | | 0.6 | 0.17 |
| Bruchmühlen | | 4.53 | 2.7 | 6 | 0.09 | 0.09 | 6 | 15 | 0.4 | | 6.6 | 0.06 | | 0.32 | 0.11 |
| Weisstal SBR | | 4.4 | 0.87 | | 0.02 | 0.003 | | | | | 6.5 | 0.06 | | | |
| Huntlosen | | 2.2 | 0 | | 0.03 | 0 | | 16.7 | 0.14 | | | | | 0.1-1.28 | |
| Hettstedt | | 3 | 0.6 | 9 | 0.24 | 0.06 | 9 | 15.25 | 1.1 | 2 | 7.3 | 0.3 | 2 | | |
| Spenge | <0.3-0.8 | 1.38 | 0.28 | 4 | 0.05 | 0 | 4 | 14.02 | 0.58 | | 6.8 | 0.17 | | 0.4 | 0.07 |

**Table 35:** continued; data, before further analysis, but measured values are already given as averages and standard deviations (st. dev.)

"marked in yellow" = not corresponding to "amount of measurements"; sample size = actual amount of measurements

n.d. = not detected

[c] "general averaged values"

[d] calculated by dividing the sludge volume (SV) by the amount of dry matter (DM) (either using averages of all respective SV- and the corresponding DM-values, or calculated for each measured SV- and corresponding DM-value followed by averaging all calculated ISV-values)

| | DM (g/l) | | | ISV (ml/g) | | | SRT (d) | $t_F/t_R$ |
|---|---|---|---|---|---|---|---|---|
| Treatment plant | average | st.dev. | sample size | average | st.dev. | sample size | | |
| Kraftisried | 5 | | | | | | >20-50 [c] | |
| Plattling | 6.5 | | | | | | >20-50 [c] | |
| Oberding | 3.5 | | | | | | >20-50 [c] | |
| TBA GZM Lyss | 7 | | | | | | >20-50 [c] | |
| ARA Lyss | | | | | | | >20-50 [c] | |
| Rosenheim | 8.2 [c] | 0.26 | | 170-230 [d] | | | 30 | |
| Rapp-Kutzenhausen | 5.6 | 0.3 | | 68.3 | 5.74 | | | |
| Ingolstadt | 9.5 [c] | 0.81 | | 45.8 [c] | 6.48 | | | |
| Waldsassen | 3.4 | 0.2 | | 132.3 | 8.47 | 7 | 12.9 | |
| Ampfing | 5.3 | 0.4 | 12 | 102.4 | 10.3 | | 21.8 | |
| Altmannstein | 2.22 | 0.64 | | | | | | |
| Langenzenn | 3.84 | 0.61 | | 78.1 [d] | | | 15 | |
| Seefeld | 3.18 | | 1 | 141.5 [d] | | | | |
| Radeburg | 3.6 | 0.26 | | 78 | 3.61 | | 25 | <0.20 |
| Bad Zwischenahn | 3.19 | 0.26 | | 52.75 | 1.68 | | ~20 [c] | 0.3 |
| Deuz | 3.35 | 0.31 | 4 | 82.2 | 7.6 | 4 | ~20 [c] | 0.4 |
| Bruchmühlen | 3.23 | 0.32 | 15 | 93.41 | 8.9 | 15 | ~20 [c] | 0.35 |
| Weisstal SBR | 4.8 | 0.78 | | 66.75 | 5.91 | | ~20 [c] | 0.2 |
| Huntlosen | 5.95 | 0.07 | | n.d. | | | ~20 [c] | |
| Hettstedt | 6.8 | 0.33 | 5 | 89.6 [d] | | | ~20 [c] | 0.35 |
| Spenge | 3.12 | 0.31 | | 109.7 | | | ~20 [c] | 0.35 |

**Table 36:** Abundances of the genus *Nitrospira* (relative to all bacteria) and *Nitrospira* Cluster I and II (each relative to all *Nitrospira*) as obtained via FISH, CLSM and digital image analysis (daime)

For each sample the Volume fraction (Vol.fraction (%)), Standard error (Std.error), Variation coefficient (Var.coefficient) and Congruency, as given by daime, can be seen.

[a] not quantified but assumed based on qualitative FISH-results (only Cluster I or II detected and no "other Ntpsa")

| Treatment plant | Genus *Nitrospira* | | | | Cluster I *Nitrospira* | | | | Cluster II *Nitrospira* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Vol. fraction (%) | Std.error | Var. coefficient | Congruency (%) | Vol. fraction (%) | Std.error | Var. coefficient | Congruency (%) | Vol. fraction (%) | Std.error | Var .coefficient | Congruency (%) |
| Kraftisried | 4.0 | 1.2 | 159.8 | 91 | 14.9 | 2.8 | 100.1 | 92 | 85.2 | 3.5 | 22 | 93 |
| Plattling | 3.3 | 0.3 | 46.6 | 96 | 37.8 | 3.5 | 50.4 | 97 | 64.3 | 5.8 | 48.3 | 65 |
| Oberding | 3.4 | 0.2 | 35.6 | 98 | 22.6 | 3.3 | 76.6 | 66 | 77.6 | 4.1 | 28.3 | 79 |
| TBA GZM Lyss | 4.8 | 0.9 | 97.5 | 80 | 80.6 | 6.6 | 35.8 | 88 | 12.4 | 4.0 | 174.2 | 85 |
| ARA Lyss | 1.2 | 1.3 | 446.6 | 79 | 100 [a] | | | | 0 [a] | | | |
| Rosenheim | 8.4 | 2.2 | 139.6 | 99 | 4.6 | 2.8 | 268.1 | 74 | 62.3 | 10.2 | 73.1 | 84 |
| Rapp-Kutzenhausen | 0 | | | | | | | | | | | |
| Ingolstadt | 16.5 | 1.9 | 62.2 | 98 | 81.9 | 6.2 | 40.8 | 88 | 3.3 | 1.8 | 290.9 | 74 |
| Waldsassen | 3.4 | 0.9 | 150.7 | 97 | 100 [a] | | | | 0 [a] | | | |
| Ampfing | 3.1 | 1.1 | 181.8 | 97 | 0 [a] | | | | 92.6 | 2.7 | 15.5 | 79 |
| Altmannstein | 0.1 | 0.1 | 292.7 | 97 | 88.5 | 10.5 | 35.5 | 83 | 13.6 | 9.2 | 202.9 | 64 |
| Langenzenn | 0 | | | | | | | | | | | |
| Seefeld | 1.6 | 0.2 | 85.1 | 95 | 100 [a] | | | | 0 [a] | | | |
| Radeburg | 2.8 | 0.8 | 153 | 95 | 100 [a] | | | | 0 [a] | | | |
| Bad Zwischenahn | 0 | | | | | | | | | | | |
| Deuz | 14.1 | 5.0 | 182.1 | 80 | 61.7 | 5.5 | 46.5 | 89 | 31.9 | 5.9 | 96.2 | 78 |
| Bruchmühlen | 4.4 | 1.1 | 133.5 | 95 | 100 [a] | 0 | | | 0 [a] | | | |
| Weisstal SBR | 3.3 | 0.7 | 123.5 | 97 | 83.9 | 2.6 | 15.8 | 87 | 9.6 | 2.5 | 130.7 | 54 |
| Huntlosen | 1.3 | 0.6 | 247.2 | 97 | 53.2 | 7.1 | 71.9 | 81 | 36.9 | 8.0 | 117 | 91 |
| Hettstedt | 2.3 | 0.4 | 83.9 | 82 | 80.4 | 2.2 | 14.7 | 92 | 3.3 | 2.5 | 400.6 | 95 |
| Spenge | 0.2 | 0.1 | 135.3 | 92 | 100 [a] | 0 | | | 0 [a] | | | |

**Zusammenfassung**

Nitrifikation is ein wesentlicher Bestandteil der Stickstoffentfernung aus Abwasser und wird vor allem den zwei funktionellen Gruppen der Ammonium-oxidierenden Bakterien (AOB) und Nitrit-oxidierenden Bakterien (NOB) zugeschrieben. Vor einiger Zeit konnte die Oxidation von Ammonium auch bei Vertretern der Archeen festgestellt werden (Ammonium-oxidierende Archeen (AOA)), deren Rolle bei der Abwasserreinigung aber noch unbekannt ist. Da Nitrifikation in Kläranlagen häufig Störungen unterworfen ist, ist sie Gegenstand zahlreicher Untersuchungen. Dabei wird oft Bezug auf das ökologische Konzept der funktionellen Redundanz genommen, welches besagt, dass ein Prozess stabiler ist wenn er von mehreren verschiedenen Arten ausgeführt wird, da diese unterschiedlich auf sich ändernde Umweltbedingungen reagieren können und somit die Wahrscheinlichkeit steigt, dass manche der Arten die Reaktion selbst noch unter nachteiligen Umweltbedingungen durchführen.

In Rahmen dieser Studie wurden 21 Kläranlagen, die sich in Aufbau, Operationsmodus und Zusammensetzung des Zuflusses unterschieden, mithilfe von FISH auf ihre Diversität an AOB und NOB sowie auf die Häufigkeit von Bakterien der Gattung *Nitrospira* untersucht. Darüber hinaus wurden innerhalb der Gattung *Nitrospira* die jeweiligen Abundanzen von Cluster I- und Cluster II-Nitrospiren ermittelt. Anschließend sollte untersucht werden ob und inwiefern Diversität und Abundanz der nitrifizierenden Bakterien durch kläranlagen-spezifische Parameter beeinflusst werden.

Vertreter des *N. oligotropha*-Clusters sowie des *Nitrospira* Clusters I konnten in fast allen Anlagen detektiert werden, was einerseits eine hohe Anpassungsfähigkeit dieser Bakterien an verschiedene Bedingungen bedeuten könnte, sowie andererseits das Auftreten mehrerer unterschiedlicher an verschiedene Bedingungen angepasste Arten oder Populationen innerhalb der Cluster. Wurden nur „Sequencing Batch Reaktoren“ (SBRs) für die Analyse herangezogen, war ein Zusammenhang zwischen dem Vorkommen halophiler bzw. –toleranter AOB und erhöhten C- und N-Konzentrationen sowie erhöhter Leitfähigkeit erkennbar. Dieser Trend stimmt mit bisherigen Annahmen überein, denen zufolge diese AOB vornehmlich in nährstoffreichen Habitaten vorkommen und zudem eine hohe Salztoleranz aufweisen. Manche halophile bzw. –tolerante AOB scheinen sogar erhöhte Salinität zum Wachstum zu benötigen. Dieser Zusammenhang sollte dennoch kritisch betrachtet werden, da er nur unter Ausschluss der konventionellen Anlagen aus der Analyse gefunden werden kann. Während die Vergleichbarkeit von C- und N-Konzentrationen zwischen den zwei

unterschiedlichen Anlagentypen fraglich ist, wurde die Leitfähigkeit jedoch als vergleichbar eingestuft.

Unter Berücksichtigung beider Anlagentypen konnte ein leichter Trend gesehen werden, wonach die Abundanz von Cluster I-Nitrospiren mit zunehmender Leitfähigkeit abnimmt während die Abundanz von Cluster II-Nitrospiren zunimmt. Um diesen Trend zu bestätigen sollten jedoch weitere Anlagen untersucht werden.

Für eine Untersuchung der Diversität wurde die Anzahl der verschiedenen pro Probe detektierten AOB- oder/und NOB-Cluster herangezogen. Interessanterweise konnten signifikante Korrelationen zwischen zunehmender Diversität und zum einen steigender Temperatur, zum anderen einer steigenden Konzentration an Biomasse (Trockensubstanz) festgestellt werden. Eine Korrelation zwischen Diversität und der Konzentration an Biomasse ist jedoch fraglich, da die Konzentration an Trockensubstanz über die Menge an abgezogenem Übersschlussschlamm aktiv reguliert werden kann, was Interpretationen nur eingeschränkt zulässt. Zumindest für SBRs konnte eine signifikante Korrelation zwischen der zugeführten C-Konzentration und der Biomassekonzentration festgestellt werden, die als gesteigerte Produktivität bei erhöhter Nährstoffzufuhr interpretiert werden kann. Diese erhöhte Produktivität kann wiederum in Zusammenhang mit erhöhter Diversität gesetzt werden da eine höhere Verfügbarkeit an Ressourcen das Überleben mehrerer unterschiedlicher Arten ermöglichen könnte.

In manchen Proben wurde die Anwesenheit bisher noch unbekannter Vertreter der Gattung *Nitrospira* vermutet. Diese Annahme konnte jedoch bisher durch eine Analyse von 16S rDNA Sequenzen nicht bestätigt werden. Die vermeintlich noch unbekannten Nitrospiren wurden oft gemeinsam mit halophilen bzw. –toleranten AOB detektiert und ihr Vorkommen scheint wie das halophiler bzw. –toleranter AOB mit erhöhten C- und N-Konzentrationen zu korrelieren, vorausgesetzt, dass nur SBRs in der Analyse berücksichtigt werden.

Im Großen und Ganzen konnten Auftreten und Abundanz von Nitrifikanten anhand der beobachteten Parameter nicht zur Genüge erklärt werden.

Vermeintliche ammonium-oxidierende Archeen der Gruppe *Crenarchaeota* konnten mittels PCR in drei Proben nachgewiesen werden. CARD-FISH gab jedoch keine positiven Signale in diesen Proben, was möglicherweise auf eine extrem niedrige Abundanz und geringes Wachstum dieser AOA zurückgeführt werden kann. Dies wiederum könnte als Indiz für eine untergeordete Rolle dieser Prokaryoten in der Abwasserreinigung gewertet werden.

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