Taxonomic and functional diversity of nitrifying biofilm communities in biofilters of different recirculating aquaculture systems

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List of Abbreviations

AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
AOM	ammonia oxidizing microorganisms
AMO	ammonia monooxygenase
amoA	ammonia monooxygenase subunit α
anammox	anaerobic ammonium oxidation
bp	base pair
BLAST	basic local alignment search tool
BR	brackish
cDNA	copy-DNA
comammox	complete ammonia oxidizer
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DIN	dissolved inorganic nitrogen
DNA	deoxyribonucleic acid
DOC	dissolved organic matter
EM	electron microscopy
EPS	extracellular polymeric substances
eq.	equation
fig.	figure
FISH	fluorescence *in situ* hybridization
FISH-MAR	fluorescence *in situ* hybridization-microautoradiography
FR	freshwater
GH	glycoside hydrolase
HAO	hydroxylamine reductase
HMM	hidden Markov model
HPLC	high performance liquid chromatography
ICM	intracytoplasmic membrane
MA	marine
N	nitrogen
NAP	periplasmic nitrate reductase
NAR	respiratory nitrate reductase
NGS	next generation sequencing
NIR	nitrite reductase
NMDS	non-metric multidimensional scaling
NOB	nitrite oxidizing bacteria
NOR	nitric oxide reductase
NOSZ	nitrous oxide reductase
nr	non-redundant

Ns.	Nitrospira
Nsm.	Nitrosomonas
Nssc.	Nitrosococcus
Nssp.	Nitrosospira
Nt.	Nitrotoga
NXR	nitrite oxidoreductase
nxrB	nitrite oxidoreductase subunit β
ORF	open reading frame
OTU	operational taxonomic unit
P	phosphate
PAO	polyphosphate accumulating organism
PCR	polymerase chain reaction
POC	particulate organic matter
PSU	practical salinity unit
RAS	recirculating aquaculture system
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
SBBR	sequencing batch bioreactor
sp.	species
spp.	species pluralis
TAN	total ammonia nitrogen
TEM	transmission electron microscopy
TNN	total nitrite nitrogen
WWTP	wastewater treatment plant

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1 Summary

Recirculating aquaculture systems (RAS) offer an ecological and efficient way of fish farming with potentially minimal environmental impact. The removal of excreted toxic ammonia and nitrite is carried out by nitrifying chemolithoautotrophic microorganisms. Nitrification takes place in biological filters, inside these biofilters ammonia and nitrite oxidizing bacteria (AOB and NOB) colonize biocarriers, forming dense biofilms together with heterotrophic bacteria. In this study, biofilters of three different RAS operated with either freshwater, brackish or marine process water, have been investigated with the goal of characterizing and comparing the microbial biofilm communities. Three biofilters at each RAS have been sampled. RNA and DNA extracted from biofilms was used as the template for amplicon sequencing using Illumina MiSeq. Sequencing the V4 region of the 16S rRNA gene revealed a high microbial diversity with on average 1000-2500 operational taxonomic units (OTUs) found in each sample. The highest diversity was identified in the brackish samples. A low shared richness of 332 (RNA) and 270 (DNA) OTUs was found among the three plants, indicating different community structures and hence, site specific microbial communities. In all three RAS *Proteobacteria* was the predominant phylum, which contributed to 24–77 % of the total reads, followed by members of the phyla *Bacteroidetes*, *Planctomycetes*, *Chloroflexi* and *Nitrospirae*. Although on the genus level >50 % of all OTUs was assigned to uncultured bacteria, 674 known bacterial genera were identified in total. 85 and 76 genera were found to be shared between all samples in the RNA and DNA pools, respectively. Among these core-taxa, the nitrifying bacteria *Nitrosomonas* and *Nitrospira* as well as heterotrophs such as *Rhodobacter*, *Planctomyces* and *Lewinella* were frequently found.

Comprehensive analysis of nitrifiers revealed site specific AOB and NOB communities at each RAS, mainly composed of uncultured representatives of the genera *Nitrosomonas* and *Nitrospira*. Amplicon sequencing of the *amoA* gene of β-proteobacterial AOB and the *nxrB* gene of *Nitrospira*-like NOB using Illumina MiSeq confirmed the results of the 16S rRNA amplicon approach by revealing a co-existence of different, closely related uncultured members of *Nitrosomonas* and *Nitrospira* in the biofilms. This strongly indicates that several potentially novel AOB and NOB species are present in RAS biofilters.

A metagenomic approach using Illumina NextSeq revealed putative genes encoding for all necessary enzymes involved in nitrification and denitrification. Moreover, taxonomic assignment of nitrogen removal genes suggested that members of the identified predominant phyla (*Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Nitrospirae*) act as key players in nitrogen removal processes inside the biofilms. Furthermore, the metagenomic data sets gave evidence for the presence of comammox in the biofilms of the freshwater and brackish samples.

Batch-experiments designed to investigate the impact of the substrate concentration, nitrate and salt stress on nitrification activity with colonized biocarriers derived from three different RAS showed distinctly different, characteristic physiological properties of each nitrifying community. The freshwater AOB showed the highest tolerance to rising ammonium concentrations among the samples, while the freshwater NOB were sensitive to increased nitrite levels. In contrast to the freshwater NOB, the brackish and marine NOB were more tolerant to nitrite

related stress. The AOB communities were shown to be highly tolerant towards nitrate, while the NOB slowed down their nitrite oxidation activity in dependence on increasing nitrate concentrations. Salt tolerance tests showed that nitrification performance of freshwater nitrifiers was drastically reduced with increasing salinity, while the brackish and marine nitrifiers showed a high robustness and maintained nitrification activity in a broad range of salt concentrations.

Competitive *in situ* studies via FISH-MAR with NOB enrichment cultures originally inoculated with biofilm material derived from a freshwater RAS showed that both, *Nitrotoga* and *Nitrospira*, were metabolically active when incubated in medium with either pH 6.4 or 7.2 at 14 °C under short-term conditions. Nevertheless, it was assumed that in the long-term, under conditions with a slightly acidic pH-value in combination with low temperature, *Nitrotoga* can outcompete *Nitrospira*, which is of importance for the biofiltration performance in RAS operated under such conditions.

To conclude, the data obtained in this study provided a deep insight into the microbial community compositions and structures as well as their potential in nitrogen removal processes in biofilms. With the knowledge gained of the physiological plasticity of nitrifiers inhabiting the biofilms, this data is useful for RAS operators and may facilitate optimization of system operation strategies.

Zusammenfassung

In Kreislaufsystemen (Recirculating aquaculture system, RAS) wird das Haltungswasser über Wasseraufbereitung gereinigt und in die Haltungsbecken zurückgeleitet. RAS zeichnen sich durch einen geringen Wasserverbrauch aus und ermöglichen eine ökologische und umweltfreundliche Fischzucht. Von den Fischen ausgeschiedenes toxisches Ammoniak wird von nitrifizierenden chemolithoautotrophen Mikroorganismen entfernt. Dieser Nitrifikationsprozess findet in Biofiltern statt, in denen Ammoniak und Nitrit oxidierende Bakterien (AOB und NOB) zusammen mit heterotrophen Bakterien auf Trägermaterialen (Füllkörper) Biofilme bilden.

In dieser Studie wurden Biofilter aus drei unterschiedlichen RAS beprobt, die entweder mit Süßwasser, Brackwasser oder Salzwasser betrieben werden, mit dem Ziel, die mikrobiellen Gemeinschaften zu charakterisieren und zu vergleichen. An jeder RAS wurden jeweils drei Biofilter beprobt und aus dem Biofilm extrahierte RNA und DNA als Template für eine Amplicon-Sequenzierung mittels Illumina MiSeq eingesetzt. Die Sequenzierung der V4-Region des 16S rRNA Gens erzielte eine hohe bakterielle Diversität mit durchschnittlich 1000-2500 Operational Taxonomic Units (OTUs), wobei die höchste Diversität in den Brackwasser-Proben vorherrschte. Es wurden lediglich 332 (RNA) und 270 (DNA) gemeinsame OTUs gefunden, was auf Habitat-spezifische bakterielle Gemeinschaften hindeutet. In allen drei RAS waren *Proteobacteria* am häufigsten vertreten (24–77 % der Gesamt-Sequenzen), gefolgt von Vertretern der Phyla *Bacteroidetes*, *Planctomycetes*, *Chloroflexi* und *Nitrospirae*. Die Mehrheit der OTUs auf Gattungs-Ebene (>50 %) wurde unkultivierten Bakterien zugeordnet. Innerhalb der 674 identifizierten bekannten Gattungen waren 85 (RNA) und 76 (DNA) als gemeinsame Vertreter in allen Proben zu finden. Unter diesen „Core-Taxa“ waren die nitrifizierenden Bakterien der Gattungen *Nitrosomonas* und *Nitrospira* und Heterotrophe wie *Rhodobacter*, *Planctomyces* und *Lewinella* häufig vertreten. Die umfassende Diversitäts-Analyse der Nitrifikanten wies für jede RAS eine Habitat-spezifische AOB- und NOB-Gemeinschaft auf, welche überwiegend aus unkultivierten Vertretern von *Nitrosomonas* und *Nitrospira* bestand. Die weiterführende Amplicon-Sequenzierung der *amoA* und *nxrB* Gene erwies eine Co-Existenz von unterschiedlichen, nahverwandten und unkultivierten Vertretern von *Nitrosomonas* und *Nitrospira*, was bestätigt, dass in den Biofilmen viele neue AOB- und NOB-Spezies zu finden sind.

Die Metagenom-Sequenzierung mittels Illumina NextSeq zeigte, dass putative Gene, die für alle notwendigen Enzyme der Nitrifikation und Denitrifikation kodieren, im Biofilm vorhanden sind. Darüber hinaus konnte Vertretern der identifizierten vorherrschenden Phyla (*Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Nitrospirae*) eine potentielle Schlüssel-Rolle im Prozess der Stickstoffentfernung im Biofilm zugeschrieben werden. Desweiteren erwiesen die metagenomischen Daten das Vorkommen von potentiellen comammox Organismen in den Biofilmen der Süßwasser und Brackwasser RAS.

Substrat-, Nitrat- und Salz-Toleranztests mit bewachsenen Füllkörpern aus den drei RAS zeigten unterschiedliche, charakteristische Eigenschaften der Nitrifikanten. Die Süßwasser-AOB erwiesen die höchste Toleranz gegenüber steigenden Ammoniumkonzentrationen, wohingegen die Süßwasser-NOB sensitiv gegenüber erhöhten Nitritkonzentrationen waren. Im Gegensatz zu

den Süßwasser-NOB waren die Brackwasser- und Salzwasser-NOB jedoch toleranter gegenüber Nitrit. Die AOB-Gemeinschaften erwiesen sich als sehr unempfindlich gegenüber Nitrat, während die NOB in Abhängigkeit von der Nitratkonzentration ihre Nitritoxidation herabsetzten. Salztoleranztests zeigten eine signifikante Reduzierung der Nitrifikationsleistung der Süßwasser-Nitrifikanten mit zunehmender Salinität, wohingegen die Brack- und Salzwasser-Nitrifikanten sich als sehr robust herausstellten und die Nitrifikation in einem breiten Spektrum an Salinitäten aufrecht erhielten.

Kompetitive Experimente via FISH-MAR mit einer NOB-Anreicherungskultur, die ursprünglich mit Biofilm-Material aus einer Süßwasser-RAS angeimpft wurde, zeigten, dass beide NOB, *Nitrotoga* und *Nitrospira*, in Medium mit pH 6.4 und 7.2 bei 14 °C metabolisch aktiv sind. Dennoch wurde angenommen, dass auf längere Sicht, unter Bedingungen mit einem leicht saurem pH-Wert und niedriger Temperatur, *Nitrotoga Nitrospira* überwachsen kann.

Die Daten dieser Studie geben einen tiefen Einblick in die Struktur und Diversität mikrobieller Gemeinschaften und deren potentielle Funktionen im Prozess der Stickstoffentfernung im Biofilm. Mit dem gelernten Wissen über die physiologische Plastizität der im Biofilm lebenden Nitrifikanten können die Daten helfen, den Biofiltrations-Prozess in RAS zu optimieren.

2 Introduction

2.1 Recirculating aquaculture systems

The worldwide expansion of the human population and the demand for protein sources in the human diet has led to a strong decline of the oceans' fish populations. Many fish populations are now considered overfished. Thus, the growing demand for fish protein can only be covered by culturing fish and other edible aquatic animals on industrial scale in aquaculture. Aquaculture is among the fastest growing animal food producing sectors (Crab *et al.* 2007). According to the Food and Agriculture Organization (FAO), the sector has grown at an average rate of 8.9 % since the 1970s, compared to 1.2 % for capture fisheries and 2.8 % for meat production (FAO 2004). The total aquaculture production was almost 1.3 million tons in the EU countries in 2008, which amounts for 2.4 % of the world's production (FAO 2010). Today, the sector accounts for almost half of the total fish supply (FAO 2014). Traditional aquaculture systems include open ponds, on- and off-shore cage culture systems as well as flow-through-systems in freshwater and marine environments. The production process generates substantial amounts of polluted effluents, containing uneaten feed and feces (Read *et al.* 2003). Discharges into the aquatic environment contain nutrients, organic and inorganic compounds (ammonium, phosphorus, dissolved or particulate organic carbon) (Piedrahita 2003; Sugita *et al.* 2005), which can cause environmental deterioration. Moreover, fish that escape from aquaculture may harm wild fish populations through competition and inter-breeding or by spreading pathogens (Thompson *et al.* 2002). Consequently, the intensive development of the aquaculture industry in the last decades has caused considerable environmental impacts. If it is performed without adequate controlling, it can cause environmental degradation as a result of the eutrophication of water bodies, landscape modification, and changes in biodiversity (Tovar *et al.* 2000).

To overcome these problems, recirculating aquaculture systems (RAS) provide an alternative way of fish breeding, in which water is recirculated between the culture and water treatment stages (Rosenthal *et al.* 1986). RAS enable a year-round controlled fish production in isolation of the surroundings. Combined with the re-use of water and nutrient cycling, these systems help provide a solution to some of the above-mentioned issues. Hence, RAS provide production systems with minimum ecological impact and make intensive fish production compatible with environmental sustainability (Van Rijn 2013). One of the advantages of reduced water consumption is that it makes the RAS technology of particular importance in regions with limited access to water. Moreover, the application of these systems enables the production of a diverse range of seafood in close proximity to the markets and thereby reduces CO_2 emissions associated with food transport (Schneider *et al.* 2006). Today, several species of fish and other small aquatic animals (e.g. African catfish, eel and trout as major freshwater species and turbot, sea bass and sole as major marine species) are produced in RAS (Martins *et al.* 2010). However, the on-shore or land-based production of fish in recirculating aquaculture plants is only slowly increasing since the introduction of this technology in the 1980s (Martins *et al.* 2010). This can be attributed mainly to the high initial capital investments and running costs and the

intensive management (Schneider *et al.* 2006). However, the possibility to maintain constant water quality via RAS may contribute to an improved welfare (Roque d'Orbcastel *et al.* 2009), providing a strong impetus for further research and improvements of the RAS technology.

2.1.1 Nitrogen compounds in RAS

A fish body typically contains 65 to 70 % protein (Hecht 2001). In addition, unlike terrestrial animals that use carbohydrates and lipids mostly, fish use proteins for energy production. Therefore, fish protein requirement is in general three to four fold higher than that of mammals (Hepher 1988). A large fraction of the nitrogen input from protein rich fish feed is not converted into biomass and ends in the rearing water. In general, the fate of nitrogen in aquaculture can be regarded as follows: Part of the feed remains uneaten in the system (1.4 %), consumed feed is partly converted into fish biomass (47.8 %) and partially excreted as ammonium as the end product of protein metabolism via the gills (49.8 %) or as faeces (<1 %) (Handy *et al.* 1993).

All these facts contribute to the high nitrogen levels in aquaculture water. The equilibrium of free ammonia and ammonium in water depends on the pH and the temperature. The sum of both forms is called total ammonia nitrogen (TAN). Although, both forms may be toxic to aquatic organisms, the unionized free ammonia is more toxic owing to the uncharged character enabling the passage of biological membranes more easily than for the ammonium ion. Ammonia can cause a range of damages to aquatic animals e.g. damage of gill epithelium and osmoregulatory stress (Camargo *et al.* 2006). The tolerable level of ammonia for cultured fish ranges in dependence on the species from 0.025–1.5 $\mathrm{mg\,l^{-1}}$ (Chen *et al.* 2006). Therefore, in RAS the common way to remove ammonium from the water is the application of microbial biofiltration in which chemolithoautotrophic nitrifying microbes oxidize ammonia via nitrite to nitrate. The sum of nitrite and free nitrous acid is total nitrite nitrogen (TNN). The equilibrium depends on pH and temperature as well. Nitrite, however, is toxic to aquatic organisms and can cause severe damage, mainly by the production of methemoglobin causing hypoxia (Bradberry *et al.* 1994). Nitrate, the end product of nitrification, is far less toxic than ammonia and nitrite. However, comparable to nitrite, nitrate can block oxygen transporting pigments in blood plasma. Camargo *et al.* (2006) stated that the nitrate concentration in marine aquaculture should not exceed 20 $\mathrm{mg\,l^{-1}}$ (1.4 mM), although values differ between cultured species. To avoid nitrate accumulation, process water in RAS is exchanged or alternatively led to denitrification reactors in which nitrate is anaerobically reduced to atmospheric nitrogen via denitrifying microbes.

2.1.2 Biofiltration in RAS

In RAS water is reused after undergoing treatment (Rosenthal *et al.* 1986). In comparison to traditional aquaculture systems such as flow-through-systems and open ponds, in RAS the waste is concentrated using filter techniques with the advantages of a reduction in and control of wastewater effluents. The treatment methods that are applied in RAS are based on physical, chemical and biological processes. In the physical unit, solid contaminants are removed by

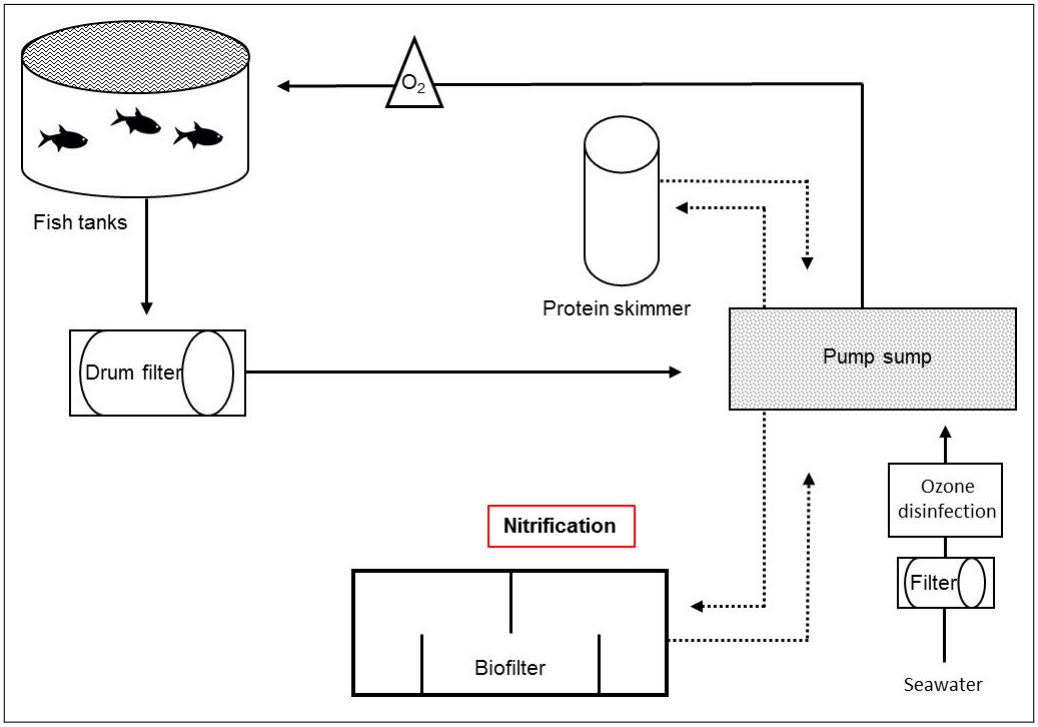


Figure 1: Schematic overview of a simplified set-up of RAS. Particulate waste is removed from the rearing water before it flows back to the pump sump. The protein skimmer, biofiltration units and oxygen device are all operated in by-passes from the pump sump.

sedimentation or mechanical filtration. Chemical contaminants are removed mainly via ozonation disinfectant treatment. However, the most important processes in aquaculture wastewater treatment are biological. The microbiological detoxification of ammonia via nitrification is the major biofiltration process in RAS. Nitrification takes place in special compartments, the biofilters. Different types of biofilters are used, most common types are trickling, fixed film or moving-bed filters. Fig. 1 shows an example of a simplified set-up of RAS using moving-bed biofilters.

The biofilters are designed to immobilize bacteria on carrier materials such as sand, stones, plastic or ceramic beads (biocarriers). This technique has the advantage that the bacteria are not washed-out, as happens in suspended growth systems. Heterotrophic and nitrifying microbes introduced by the process water colonize the carrier materials and form dense biofilms. The biofilters create optimal conditions for the nitrifying bacteria to grow and nitrification takes place mainly inside the biofilters, although it is said that 30 % of the TAN removal happens by passive nitrification on surfaces within the system (Losordo *et al.* 2000). In the present study all experiments are based on RAS operated with moving-bed biofilters as the compartment of nitrification. These moving-bed biofilters are filled with plastic biocarriers made of high density polyethylen (HDPE), which are moved continuously by aerators or stirring (fig. 2 shows a moving-bed biofilter, which has been sampled in this study). The movement provides sufficient oxygen, essential for the aerobic nitrification process and an even substrate distribution is ensured. Moreover, the movement prevents the biocarriers from clogging due to strong shear forces and promotes rejuvenation of the biofilms. Due to their low density, the

Figure 2: Moving-bed biofilter filled with HDPE-biocarriers.

biocarriers float beneath the surface. Nitrifiers together with heterotrophic bacteria colonize the protected area of these biocarriers and form dense biofilms, which were used as basic material for all experiments performed in this study. Fig. 3 shows exemplary a HDPE-biocarrier of type BCN009, which have been used in this study.

2.2 Chemolithoautotrophic nitrifiers

Chemolithoautotrophic nitrifiers mediate the crucial step of ammonia removal in RAS biofilters. Classically, the process of nitrification is divided in two steps: the oxidation of ammonia to nitrite mediated by ammonia oxidizing bacteria or archaea (AOB/AOA), followed by the oxidation of nitrite to nitrate mediated by nitrite oxidizing bacteria (NOB). Hence, nitrification is a synthrophic process, where ammonia oxidizing microorganisms (AOM) supply the substrate of NOB, namely nitrite. This in turn prevents the accumulation of toxic nitrite in the surrounding. As they are chemolithoautotrophic organisms, nitrifiers gain their energy via the oxidation of inorganic nitrogen compounds and they can derive all cellular carbon from the fixation of CO_2, which is mostly done via the Calvin-Cycle. Ammonia and nitrite oxidizers build very specialized groups, which are not closely phyolgenetically related. Both groups are widely distributed in man-made and natural habitats and the nitrification process occupies a central position in the biogeochemical N-cycle since nitrifiers are the only organisms able to convert the most reduced form of nitrogen (NH_4^+) to the most oxidized form (NO_3^-). The nitrification process has several direct and indirect implications for engineered and natural ecosystems. On the one hand, nitrification increases the loss of soil nitrogen due to leaching of nitrate and is consequently not beneficial in agricultural soils. On the other hand, it is highly desirable for the treatment of wastewaters for the efficient removal of ammonium (Painter 1986).

Figure 3: HDPE-biocarrier of type BCN009 overgrown with biofilm. Nitrifying and heterotrophic bacteria form biofilms in the protected area of the biocarrier. The photograph was kindly provided by Dr. Sandra Off.

2.2.1 Ammonia oxidizers

Phylogeny and distribution of AOM Ammonia oxidizing bacteria and archaea are responsible for the first and rate limiting step in nitrification, the conversion of ammonia to nitrite. According to comparative 16S rRNA and *amoA* gene sequence based phylogenetic analysis, all known AOB are found among two lineages within the β- and γ-class of *Proteobacteria* (Purkhold *et al.* 2000). The genera *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a monophyletic group within the β-*Proteobacteria* (Woese *et al.* 1984). Despite their morphological differences based on ultrastructure criteria, the latter three genera revealed a very close relationship on the basis of 16S rRNA phylogeny and Head *et al.* (1993) suggested combining them into a single genus since they form a monophyletic clade. However, until now the proper phylogenetic classification of these three genera has not been definitively confirmed. Besides these, two marine species of the genus *Nitrosococcus* cluster together in the γ-*Proteobacteria* class (Woese *et al.* 1985). Until recently nitrification was thought to be restricted to the domain of bacteria, however, the capability of ammonia oxidation was extended to the Archaea domain with the discovery of ammonia oxidizing archaea (Könneke *et al.* 2005). All identified AOA are *Thaumarcheota* and carry *amoA* genes, distantly related to those of AOB (Schleper *et al.* 2005).

The β-proteobacterial AOB are widely distributed in engineered and natural habitats. Members of the genus *Nitrosomonas* have so far been subdivided into eight different phylogenetic clusters on the basis of their 16S rRNA gene and *amoA* gene sequences as well as ecophysiological characteristics (Purkhold *et al.* 2000; Koops *et al.* 2001). These known phylogenetic clusters of the nitrosomonads represent organisms with different distribution patterns and are found among diverse environments such as limnic and saline aquatic habitats, sewage and soils (Norton 2011). Since members of the *Nitrosospira* lineage have been frequently found in

different soils (e.g. agricultural, grassland, turfgrass systems), *Nitrosospira*-like bacteria are thought to be the dominant AOB in soils (Daims *et al.* 2010). However, representatives of the four phylogenetic clusters have also been identified in aquatic habitats and sediments as well as occasionally in wastewaters (summarized in Norton 2011). To date, the γ-proteobacterial AOB only encompass the genus *Nitrosococcus* which representatives are found to be strictly halophilic and hence, distributed only in saline ecosystems with particular appearance in the world's oceans (Norton 2011; Ward *et al.* 2002). Since their discovery, molecular surveys on the basis of the archaeal *amoA* gene revealed that AOA are widely distributed throughout the world's oceans and soils, and they may have a high impact on ammonia oxidation in these habitats (Francis *et al.* 2005; Leininger *et al.* 2006; Prosser *et al.* 2008).

Physiology of ammonia oxidation The ammonia oxidation to nitrite is a two-step process catalyzed by two key enzymes, the membrane-bound ammonia monooxygenase (AMO) and the periplasmic hydroxylamine oxidoreductase (HAO). In the first step the AMO oxidizes ammonia to the intermediate hydroxylamine (eq.1). In the second step the HAO further oxidizes hydroxylamine to nitrite (eq.2).

$$NH_3 + O_2 + 2H^+ + 2e^- \overset{AMO}{\rightarrow} NH_2OH \qquad (\Delta\ G^{\circ\prime}\ \text{-120 kJ mol}^{-1}) \tag{1}$$

$$NH_2OH + H_2O \overset{HAO}{\rightarrow} HNO_2 + 4H^+ + 4e^- \qquad (\Delta\ G^{\circ\prime}\ \text{-114 kJ mol}^{-1}) \tag{2}$$

AOM derive their energy via the oxidation of ammonia to nitrite and carbon via autotrophic fixation of CO_2. However, these organisms have been shown to be metabolically more versatile. For members of *Nitrosomonas* it has been shown that they are not restricted to CO_2 as a carbon source, instead heterotrophic growth with other organic substrates including amino acids (Clark *et al.* 1967) and fructose as well as pyruvate as the sole carbon source (Hommes *et al.* 2003) has been detected. Furthermore, it was shown for *Nsm. eutropha* and *Nsm. europaea* that they were able to simultaneously nitrify and denitrify under mixotrophic conditions (presence of ammonia and suitable organic substrates) and oxygen limitation by using nitrite in addition to oxygen as an electron acceptor (Bock *et al.* 1995). Moreover, both species were capable to denitrify with H_2 as an electron donor and nitrite as an electron acceptor under anoxic conditions (Stüven *et al.* 1992; Bock *et al.* 1995). A further growth advantageous fact for some *Nitrosomonas*-like AOB is the existence of ureases, which enable these bacteria to use urea as substrate by its hydrolization to ammonia and CO_2 (Koops *et al.* 1991).

2.2.2 Nitrite oxidizers

Phylogeny and distribution of nitrite oxidizers Nitrite oxidizing bacteria catalyze the second step of nitrification, the oxidation of nitrite to nitrate. Nitrite oxidizers are phylogenetically more heterogeneous than AOB. All identified NOB belong to one of the six genera *Nitrobacter*, *Nitrospira*, *Nitrococcus*, *Nitrospina*, *Nitrotoga* and *Nitrolancea*. The first described

nitrite oxidizing bacterium was *Nitrobacter* (Winogradsky 1892), which species belong to the *α-Proteobacteria* class. *Nitrobacter*-like NOB are found in different aquatic and terrestrial environments including alkaline soda lakes (Sorokin *et al.* 1998). The genera *Nitrocooccus* and *Nitrospina* are members of the *γ-Proteobacteria* and *δ-Proteobacteria* class, respectively (Watson *et al.* 1971; Teske *et al.* 1994). Both genera contain only few taxonomically described species, which were isolated from seawater and have never been detected outside of saline environments (Daims *et al.* 2010). *Nitrospina gracilis* seems to be the most abundant and wide spread nitrite oxidizer in marine systems and is of central importance to the oceanic nitrogen cycle (Lücker *et al.* 2013). On the basis of 16S rRNA phylogenetic analysis, the novel phylum *Nitrospinae* was proposed recently (Spieck *et al.* 2014).

The genus *Nitrospira* represents the most diverse group of NOB. This genus encompasses *Nitrospira* species, isolated from a broad variety of environments such as seawater, water heating systems, activated sludge and geothermal springs. To date, the different species of *Nitrospira* have been subdivided into six phylogenetic lineages on the basis of their distribution patterns and ecophysiological traits (Daims *et al.* 2001a). In addition, several uncultured *Nitrospira*-like NOB have been found in freshwater, seawater, wastewater and terrestrial habitats (Pester *et al.* 2014). The phylogenetic assemblage of the genera *Nitrospira* together with *Thermodesulfovibrio*, *Magnetobacterium* and *Leptospirillum* has been combined in the phylum *Nitrospiraea* (Ehrich *et al.* 1995). Cold-adapted NOB Candidatus *Nitrotoga arctica* was originally isolated from permafrost-affected soils in Siberia (Alawi *et al.* 2007) and is so far the only described species of this genus. Since its discovery it has been associated with nitrite oxidation in cold and low substrate environments such as deglaciated or periglacial soils (Sattin *et al.* 2009; Schmidt *et al.* 2009), neutral groundwater seep or inside drinking water filters (Roden *et al.* 2012; White *et al.* 2012). Recent studies indicate that *Nitrotoga* may be more wide spread than previously assumed and even may act as a key nitrite oxidizer in wastewater treatment (Alawi *et al.* 2009; Lücker *et al.* 2015). Recently, a novel nitrite oxidizer, *Nitrolancea hollandica*, was isolated from a moderate thermophilic bioreactor and phylogenetically classified into the phylum *Chloroflexi* (Sorokin *et al.* 2012). In addition to the chemolithoautotrophic NOB a phototrophic nitrite oxidizing bacterium (*Thiocapsa* KS1) has been detected in the oceans. However, this organism uses light as an energy source and nitrite only as an electron donor for biosynthesis (Griffin *et al.* 2007).

Physiology of nitrite oxidation Nitrite oxidizers derive their energy from the oxidation of nitrite to nitrate (eq.3). This process is catalyzed by the nitrite oxidoreductase (NXR), a membrane-bound enzyme that oxidizes nitrite to nitrate by using water as source of oxygen.

$$NO_2^- + H_2O \overset{NXR}{\rightarrow} NO_3^- + 2H^+ + 2e^- \qquad (\Delta\ G^{\circ\prime}\ \text{-76 kJ mol}^{-1}) \tag{3}$$

Two NXR types are found among the NOB with the substrate binding subunit NxrA being located in the periplasmic space in *Nitrospira*, *Nitrotoga* and *Nitrospina*, but in the cytoplasma in *Nitrobacter*, *Nitrococcus* and *Nitrolancea*.

Like the AOB, not all NOB are restricted to a chemolithotrophic lifestyle. At least some members of the genera *Nitrobacter* and *Nitrospira* have been shown to exhibit a relatively high metabolic versatility. When no nitrite is available, *Nitrobacter* is able to grow completely heterotrophically with simple organic substrates such as pyruvate and acetate (Bock 1976). For *Nitrospira marina* it has been shown that growth was enhanced under mixotrophic conditions with nitrite and pyruvate, compared to conditions with nitrite solely (Watson *et al.* 1986). In addition, *Nitrobacter* and some *Nitrospira* are able to denitrify in the absence of oxygen by the reduction of nitrate with electrons derived from H_2 or from organic compounds (Bock *et al.* 1988; Ehrich *et al.* 1995). Recently, growth by aerobic oxidation of H_2 and thus, the first chemolithautotrophic lifestyle of a nitrifier outside the nitrogen cycle, was reported for *Nitrospira moscoviensis* (Koch *et al.* 2015). During the writing process of this study, the present dogma of nitrification as a two-step reaction by two separated groups of organisms, has been broken by the unexpected discovery of comammox (complete ammonia oxidizer) *Nitrospira*-like bacteria capable to perform the complete ammonia oxidation to nitrate (Daims *et al.* 2015; van Kessel *et al.* 2015).

2.3 Bacteria in RAS biofilters

Each compartment in RAS provides its own habitat for specific microbial inhabitants. Besides the fish tank and the fish gut itself, the biofilters, and if implemented, denitrification reactors are large reservoirs of microbes. In biofilters, biocarriers provide an optimal surface for settling, and hence, bacteria in the rearing water can segregate and form biofilms. Although biofilters are the place of aerobic nitrification, they also integrate other aerobic and anaerobic microbial processes due to the composition of diverse heterotrophic bacteria together with autotrophic nitrifying bacteria in the biofilms. Heterotrophic growth is enhanced by easily accessible carbon sources accumulating from uneaten fish feed and faecal matter. Main groups of bacteria identified in biofilters of freshwater and marine RAS were members of *Proteobacteria*, *Nitrospirae*, *Bacteroidetes*, *Chloroflexi* and *Verrucomicrobia* as summarized in the review of Schreier *et al.* (2010). Among the biological wastewater treatment processes in RAS, nitrification is the most important. For a better understanding of the sensitive nitrification process, analyzes of the nitrifying community compositions are mandatory. With this regard, molecular surveys about nitrifying microorganisms have been undertaken in the past and nitrification in RAS is mainly associated with members of the genera *Nitrosomonas* and *Nitrospira*. However, AOA have recently been identified in high abundances in biofilters of aquaria and of marine RAS (Sauder *et al.* 2011; Brown *et al.* 2013). The predominance of AOA over AOB was credited to their physiological advantage to grow at low substrate concentrations where AOB are outcompeted (Sauder *et al.* 2011).

Besides nitrification, some RAS also involve denitrification reactors in the wastewater treatment process. Denitrification is an anaerobic process catalyzed mainly by facultatively anaerobic heterotrophic microorganisms. Organic compounds are used as electron donors by these bacteria for the reduction of NO_3^- and NO_2^- to NO, N_2O and N_2 (Zumft 1997). Denitrification

is a very heterogeneous process and perfomed by diverse bacteria. Typical denitrifiers identified in denitrification biofilters in RAS were e.g. *Rhodobacter* sp., *Planctomyces* sp., *Pseudomonas* and *Comomonas* spp. (reviewed by Schreier *et al.* 2010). It is highly probable that denitrifiers inhabit not only specific anaerobic denitrification reactors but also the biofilms grown on biocarriers in nitrification biofilters. This is due to the fact that the biofilms themselves develop microanaerobic zones in the deeper layers and in combination with high nitrate load and organic waste released by feed off-cut, heterotrophic denitrification can be enhanced. In anaerobic parts of marine RAS, such as sludge digesters, autotrophic denitrification by members of the genera *Desulfobrio*, *Dethiosulfobrio*, *Fusibacter* and *Bacteroidetes* was also reported (Cytryn *et al.* 2003). In this case, sulfate of the seawater is part of a sulfur based oxidation/reduction cycle where microorganisms oxidize organic waste to reduce sulfate to sulfide, which is used for sulfide-dependent denitrification to produce N_2 (Schreier *et al.* 2010).

Recent studies about N-removal bacteria in RAS also involved organisms capable to perform anaerobic ammonium oxidation (anammox). Via this shortcut of the biogeochemical nitrogen cycle, ammonium and nitrite are directly transformed to N_2 with nitrate as a by-product of the reaction (Jetten *et al.* 1999). Since the discovery of anammox, research focuses on the application of this process in wastewater treatment. The classical wastewater treatment via nitrification and denitrification is costly due to high energy costs for aeration of the nitrification reactors and the supply with organic carbon sources to maintain denitrification. In this context, the application of anammox is a promising way with reduced costs since no organic carbon has to be added and energy for aeration can be reduced. Key anammox organisms are found among the phylum *Planctomycetes*. Unique to the anammox *Planctomycetes* is the presence of the anammoxosome, a cell compartment harbouring the enzyme hydrazine oxidoreductase, catalyzing the conversion of hydrazin to dinitrogen. Anammox is successfully applied in industrial wastewater treatment systems as part of the elimination of dissolved inorganic nitrogen (DIN). Although, anammox activities have been measured in denitrification reactors of marine RAS and microorganisms with the capability to perform the reaction have been identified in freshwater and marine RAS biofilters (Tal *et al.* 2003; Lahav *et al.* 2009; van Kessel *et al.* 2010), aerobic nitrification is still the most commonly used process in RAS.

Moreover, studies about microbial community composition in RAS biofilters focused on the detection of potential fish pathogenic bacteria and such pathogens e.g. *Vibrio*, *Erwinia*, *Coxiella* and *Aeromonas* spp. have been identified among others (King *et al.* 2004; Schneider *et al.* 2007; Wietz *et al.* 2009; Michaud *et al.* 2009; Martins *et al.* 2013). RAS biofilters allow for accumulation of pathogens within the protective biofilms. This could result in infection of fish and ultimately in the fish consumer. Antibiotic treatment against pathogens is often used in RAS but as a consequence resistances rise (Smith 2008). Alternatively, UV-irradiation or ozonation are performed as disease controlling disinfection measure (Summerfelt 2003). In addition, recent approaches have focused on the application of probiotic bacteria in biofilters that may produce growth inhibitors, or quench cell-to-cell communication that allows for settling within biofilms and hence, give protection against pathogens (Defoirdt *et al.* 2008; Kesarcodi-Watson

et al. 2008).

Developing industrial-like approaches for large scale production of diversified fingerlings and very large units for fish breeding is the current trend in on-shore aquaculture. As stated in the review of Blancheton *et al.* (2013), proper process control in large-scale RAS needs to include quality assurance in addition to management of biotechnical and economic aspects. Hence, determining the development of bacterial populations and microbial management strategies are very important and have not received sufficient attention so far. Accordingly, the authors suggested that all key biological mechanisms involved in wastewater treatment in RAS urgently need to be better understood. This statement strengthens the importance to expand our knowledge of microbial community structures and functions in RAS biofilters. In addition, gaining thorough knowledge of factors controlling the sensitive process of nitrification can help optimize operation strategies and enhancement of N-removal performance in RAS.

2.4 Aim of this study

Biofilters are important parts in RAS and widely used for the removal of toxic ammonia and other metabolic waste products. Nevertheless, our knowledge of the community composition of microbes inhabiting the attached biofilms and their functional dynamics is incomplete. In this study, microbial communities derived from biofilters of three different RAS, located in Germany, have been investigated. These RAS are operated with either freshwater, brackish or marine process water. The aim of this study was to comprehensively characterize and compare the microbial communities and their potential functions inside the biofilms. As they mediate the crucial step of ammonia removal, emphasis was placed on studying abundance, diversity and physiology of nitrifiers. Different next generation sequencing (NGS) approaches as well as batch-experiments with colonized biocarriers were performed in this study, as described in the following paragraph (fig. 4 gives a schematic overview of main experiments).

2.4.1 Microbial community characterization

Closer investigation of microbial communities in RAS biofilters has only recently been initiated by the use of molecular identification tools such as restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and 16S rRNA based clone libraries (Tal *et al.* 2003; Sugita *et al.* 2005; Foesel *et al.* 2008; Michaud *et al.* 2009). However, these methods tend to underestimate the microbial diversity due to low throughput and resolution. The application of NGS methods, though, which can generate millions of sequence reads, provides deep insights into microbial diversity, species composition and their potential functions in the biofilms. Against this background, different NGS approaches were applied in this study with the goal of gaining highly resolved data enabling an extensive characterization of microbial communities and putative nitrogen removal processes in RAS biofilters.

16S rRNA amplicon sequencing PCR generated 16S rRNA gene amplicons were sequenced on the Illumina MiSeq platform. This approach was applied to samples collected

from biofilters of the three RAS facilities. At each RAS, three biofilters have been sampled and thus, replicated comparison across nine biofilters was performed. Main research topics of this approach were: (1) Taxonomic classification of microbial communities. The microbial communities were taxonomically classified to draw conclusions on their potential physiological roles in biofilms. (2) Site specific versus shared microbial taxa among the different RAS. By hypothesizing that microbial communities differ among biofilters of the different RAS facilities, focus was set on the identification and quantification of RAS site specific versus shared core-taxa across the biofilters. (3) Diversity and abundance of nitrifiers in biofilms. Since nitrifying microbes play the crucial role of ammonia removal in biofilters the focus was set on the characterization and comparison of nitrifying communities among the different biofilters. Ammonia and nitrite oxidizers build very specialized groups with typical representatives inhabiting certain environments and hence, it was hypothesized that in RAS site specific AOB and NOB communities are found.

Amplicon sequencing of *amoA* and *nxrB* genes After identifying the genera *Nitrosomonas* and *Nitrospira* as the most abundant nitrifiers in the biofilms via 16S rRNA analysis, the same samples have been undertaken hightroughput sequencing of nitrification relevant functional genes *amoA* and *nxrB* by using Illumina MiSeq. These genes code for the α-subunit of AMO and β-subunit of NXR, respectively and are very useful phylogenetic and functional marker genes for β-proteobacterial AOB and *Nitrospira*-like NOB (Daims *et al.* 2010). Since biofilms offer microhabitats due to spatial heterogeneity it was hypothesized that a high diversity of closely related representatives of β-proteobacterial AOB and *Nitrospira*-like NOB exists in these environments. The goal of this approach was to gain highly resolved data sets used for fine-scale phylogenetic microdiversity analysis of the predominating AOB and *Nitrospira*-like NOB inhabiting the biofilms.

Metagenome sequencing A metagenomic approach was chosen to compare microbial structures and potential functions in the biofilms. This was done by shotgun sequencing on the Illumina NextSeq platform with biofilm material obtained from one biofilter each in the freshwater, brackish and marine RAS. The main focus was set on the identification and quantification of genes encoding for enzymes potentially involved in major N-removal and carbon degradation processes. In addition, it was verified if the recently discovered comammox is found among the biofilms.

2.4.2 Nitrification performance in dependence on RAS operational parameters

Besides the sequencing based approaches, batch-experiments with colonized biocarriers were performed in this study with the goal to physiologically characterize the AOB and NOB communities from the three RAS. Both groups have low specific growth rates (Keen *et al.* 1987; Bock *et al.* 1991) and the process of nitrification is highly sensitive to environmental factors such as temperature, substrate concentration and availability, pH, dissolved oxygen concentration

and inhibitory substances (Sharma *et al.* 1977; Antoniou *et al.* 1990). In RAS, sudden nitrite peaks as well as the accumulation of nitrate as the end product of nitrification are reported frequently. All these factors can influence the nitrification performance in biofilters negatively with consequences for the well-being of fish and the resulting economical loss for RAS operators. Nowadays, salt is also considered as a common stress factor in wastewater treatment processes, as input streams can contain high salt concentrations. For RAS, the salinity is an important factor, as in times of deficiency in hydric resources associated to global warming, the operation of marine and brackish RAS in coastal regions gains increasing attention. However, published studies about the effect of salt on nitrification are difficult to compare and show very contradictory results. Apparently, it is very important to understand the impact of changing salt concentrations on the nitrification activity with the goal to maintain an efficient removal capacity in saline wastewaters. Considering all these facts, the aim of this study was to investigate the physiological plasticity of nitrifiers inhabiting the biofilters in RAS in dependence on different operational parameters. Therefore, batch-experiments with nitrifying biofilms derived from RAS operated at different salinities were performed to elucidate (1) the impact of the substrate concentration on ammonia and nitrite oxidation, (2) if the presence of nitrate affects the nitrification performance, and (3) to study the consequences of acute salt stress on nitrifying activities.

2.4.3 NOB niche differentiation in a RAS biofilter

In addition to the sequence based approaches in this study, a cultivation dependent approach was performed. In 2010, about three years before starting with experimental work in the course of the present study, an unexpected strong appearance of *Nitrotoga*-like NOB has been observed in biofilters of a cold freshwater RAS for the production of rainbow trouts (Off 2012). This was surprising since the occurrence of *Nitrotoga* in noteworthy amounts in RAS biofilters has not been reported before, instead members of *Nitrospira* were thought to be the predominant NOB. By hypothesizing that the slightly acidic pH-value (6.8) and the low temperature (13 °C) of the process water favors growth of *Nitrotoga* over *Nitrospira*, NOB enrichment cultures were used for *in situ* experiments to investigate competitive factors between species of *Nitrotoga* and *Nitrospira*. The goal was to find possible explanations which factors could have led to the strong occurrence of *Nitrotoga* in the freshwater RAS in 2010.

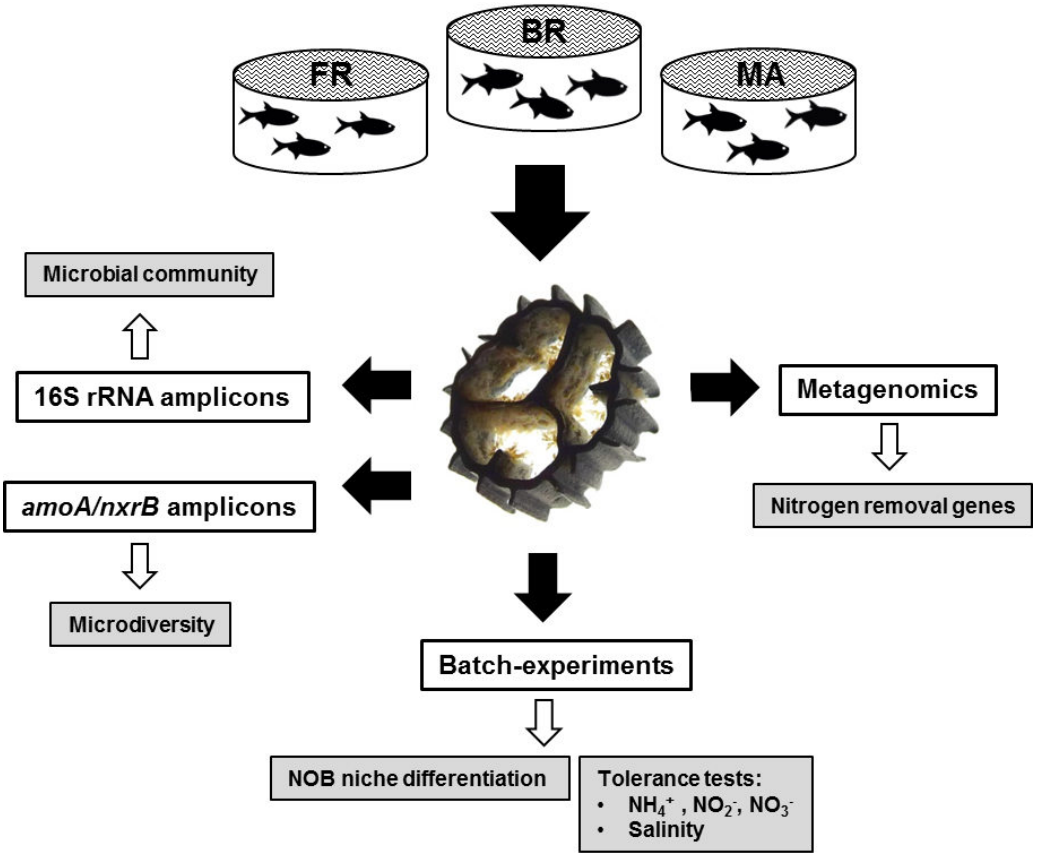


Figure 4: Overview of main experiments performed in this study. Colonized biocarriers were derived from biofilters of three RAS operated with either freshwater (FR), brackish (BR) or marine (MA) process water. Biofilm material was harvested from the biocarriers and the microbial communities characterized via NGS approaches in combination with batch-experiments, as described in detail in the text.

3 Material and methods

3.1 Description of the aquaculture plants

Three different recirculating aquaculture systems (RAS), which are located in Germany, have been investigated in this study. Experiments were done with biocarriers derived from different biofilters of the respective plant. The biofilters were all operated as moving bed-reactors and inside these the biocarriers were moved continuously by aerators arranged circularly at the bottom of the tank. The oxygen concentration in the RAS was kept at 9–10 $mg\,l^{-1}$ and the pH-value at about 7.0. Physiochemical parameters of the different modules were examined in the process water regularly as described in section 3.8.3. Mean physicochemical and operational data are listed in tables S1 and S2 in the appendix. Main characteristics of the sampled modules for all experiments performed in this study are listed in table 1.

Table 1: Main characteristics of the modules of three different RAS, whose biofilters have been sampled for the experiments performed in this study. If available, physicochemical data are given with standard deviation calculated from the beginning of a rearing cycle until the day of sampling. Parameters were determined in April 2014. FR: Freshwater; BR: Brackish; MA: Marine.

RAS	Module	PSU	Temp.[°C]	Fish species	Operating time [yrs.]
	C	-	22 ±0.6	Pikeperch	3.5
	D	-	22 ±0.7	Pikeperch	3.5
FR	F[1)]	-	22 ±1.0	Pikeperch	3.5
	TR	-	12 ±0.9	Rainbow trout	1.5
	AQ1	4	19 ±3.8	Rainbow trout	1.5
BR	GMA	4	19 ±0.3	Pikeperch, perch, Maraena whitefish	1.5
	RMB[2)]	5	19 ±0.7	Maraena whitefish, Perch	1.5
	M1[1)]	24 ±2.8	17 ±0.6	Turbot, sea bass, Atlantic salmon	9
MA	M3	26 ±1.5	17 ±0.5	Turbut, sea bass, Atlantic salmon	9
	M5[3)]	25 ±1.7	18 ±0.3	Turbut, sea bass, Atlantic salmon	9

[1)] Connected to a denitrification reactor

[2)] Larvae breeding module

[3)] The biofilter is in operation only occasionally

Freshwater RAS The freshwater plant (FR) is located in North-East Germany amid the Mecklenburg-West Pomerania lake-district and is operated with groundwater. Biofilters of

modules C, D, F and TR have been sampled. The two identical modules C and D have a total volume of 70–73 m^3, the volume of the fish tanks is 2.7 m^3 and of the biofilters 25-30 m^3. Identical modules E and F are connected to a denitrification reactor and have a total volume of 95–100 m^3 with 4.9 m^3 in the fish tanks and 35–40 m^3 biofilter volume (note that only module F was sampled). Biofilters are filled with HDPE-biocarriers (4–5 m^3 in C & D; 6–7 m^3 in E & F) of type BCN 009 (surface of 497 m^2/m^3, Stöhr, Marktrodach, Germany). These modules are continuously in operation since 2012 and stocked with pikeperch (*Sander lucioperca*). The large module TR for the production of rainbow trout (*Oncorhynchus mykiss*) has a total volume of 600 m^3 with 350 m^3 production volume and it was initially put into operation in 2008. Module TR is operated with two moving-bed biofilters, which are filled with two types of HDPE-biocarriers (BCN009: surface of 497 m^2/m^3 and KLL012: surface of 704 m^2/m^3, Stöhr, Marktrodach, Germany). For simplification, only biocarriers of type BCN009 were chosen for all experiments regarding to this study.

Brackish RAS The brackish RAS (BR) is situated in North-East Germany close to the Baltic-Sea. Process water is derived from Darss-Zingst Bodden chain (mean salinity 3-5 PSU). Parallel modules GMA and GMB contain a total volume of 50 m^3 each, consisting of ten fish tanks (3 m^3 each) connected to moving-bed biofilters (15 m^3), which are filled with about 5 m^3 HDPE-biocarriers of type KLL012 (surface of 704 m^2/m^3, Stöhr, Marktrodach, Germany) (note that only module GMA was sampled). Both modules are continuously in operation since 2013 and stocked alternately with pikeperch (*Sander lucioperca*), maraena whitefish (*Coregonus maraena*) or perch (*Perca fluviatilis*). Module AQ1 consists of ten aquaria (300 L each) connected to a biofilter (0.8 m^3), which is filled with 0.5 m^3 biocarriers of type KLL012. AQ1 is in operation only occasionally. During time of sampling it was low stocked with rainbow trout (*Oncorhynchus mykiss*). Module RMB consists of six tanks (550 L each) connected to a biofilter (4 m^3), which is filled with about 1.5 m^3 HDPE-biocarriers of type KLL012. RMB is in operation since 2013 as larvae breeding module for maraena whitefish and perch with occasional periods without fish stocking.

Marine RAS The marine RAS (MA) is located in North Germany and is operated with North-Sea surface water. The process water is maintained at an average salinity of 25-30 PSU. Samples were taken from the moving-bed biofilters of three independent recirculation modules modules (M1, M3, M5). All three modules are in operation since 2005. The total volume of the two identical modules M1 and M3 is about 45 m^3 with 25 m^3 in the fish tanks and 4.5 m^3 in the biofilter, each. M1 is connected to a denitrification reactor. Module M5 consists of several aquaria (9 m^3) connected to a biofilter (4.5 m^3), amounting to a total volume of 29 m^3. Each biofilter is filled with about4 m^3 (60 % filling level) HDPE-biocarriers of type 2H-BCN 012 KL (surface of 859 m^2/m^3, GEA 2H Water Technologies, Wettringen, Germany). M1, M3 and M5 were stocked alternately with turbot (*Psetta maxima*), sea bass (*Dicentrarchus labrax*) or Atlantic salmon (*Salmo salar*). M5 is in operation only occasionally. During time of sampling it was low stocked with fish but has been undergone long periods without stocking before.

3.2 Used enrichment cultures

Three years old enrichment culture 'Aquakultur HW' (Off 2012), which was initially inoculated with densely covered biocarriers derived from a biofilter of the freshwater RAS (module TR), was used as pre-culture for *in situ* activity studies of *Nitrotoga* and *Nitrospira* performed in this study as described in section 3.5.8. The presence of *Nitrospira* and *Nitrotoga* in the culture was checked via genus-specific PCR (see section 3.5.4).

3.3 Mineral salts media for cultivation and batch-experiments

All cultivation and physiological experiments of AOB and NOB were done in autotrophic mineral salts medium with defined volumes of 50–150 ml in 300 ml Erlenmeyer flasks, if not noted otherwise. Medium for freshwater and marine derived nitrifiers was prepared in case of the AOB after the protocols of (Hankinson *et al.* 1984) and (Watson *et al.* 1971) with slight modifications and in case of the NOB as described in (Spieck *et al.* 2011). The composition of the different AOB and NOB mineral salts media used, is listed in table 2 (AOB) and table 3 (NOB). Ammonium or nitrite as the sole energy source was added to the desired concentration and pH-value adjusted before autoclaving. Marine medium was prepared with 70 % seawater derived from the North-Sea with an original salinity of 27 PSU (water was taken close to the island of Helgoland, Germany).

3.4 Physiological tests with biofilm inhabiting nitrifiers

3.4.1 Nitrification potential test

Determination of nitrification potential of biofilm inhabiting AOB and NOB derived from different RAS, was done in batch-tests under laboratory conditions. Biocarriers were sampled of the respective biofilter, transferred into 1 L plastic bottles covered with 2/3 biofilter water and shipped via overnight express on cool packs. After arrival, five to ten biocarriers were counted into 50 ml sterile mineral salts AOB or NOB medium (medium I and III for samples derived from the freshwater and brackish RAS and medium II and IV for samples derived from the marine RAS). The medium was supplied with 1 mM ammonium and nitrite, respectively, as substrate. Flasks were incubated at 22 °C on a shaker (150 rpm) for 8 h. Samples (1 ml) were taken hourly and nitrite and nitrate analyzed via HPLC as described below (section 3.8.1). Tests were done in duplicates and mean values were used for the calculation of turnover rates. The conversion of total ammonia nitrogen (TAN) to nitrite and total nitrite nitrogen (NO_2-N) to nitrate was defined as turnover rate of AOB and NOB, respectively. Hence, the turnover rates were analyzed by the consumption of ammonia via AOB and nitrite via NOB within eight hours. Since the stoichiometric conversion of ammonia over nitrite to nitrate is 1:1:1, the sum of nitrite and nitrate served as substrate conversion of AOB. To determine the nitrification potential turnover rates were calculated in reference to the deployed amount of biocarriers used in the batch-test. To draw conclusions on the nitrification performance of AOB and NOB on-site, the calculated values were projected to the amount of biocarriers in the biofilter.

Table 2: Mineral salts medium for the cultivation of freshwater and marine AOB

(a) Medium I: freshwater AOB

Trace element solution 10x	
$MnSO_4$ x H_20	0.0338 g
H_3BO_3	0.0494 g
$ZnSO_4$ x 7 H_2O	0.0431 g
$(NH_4)_6$ Mo_7O_{24}	0.0371 g
$FeSO_4$ x 7 H_2O	0.973 g
$CuSO_4$ x 7 H_2O	0.025 g
Disssolve in HCl	(0.01M)
Aqua dest.	ad 1000 ml
Stock solution 10x	
KH_2PO_4	0.54 g
KCl	0.74 g
$MgSO_4$ x 7 H_2O	0.49 g
NaCl	5.84 g
Aqua dest.	ad 1000 ml
Medium	
NH_4Cl	0.5–5 mM
Trace element solution 10x	1 ml
Stock solution 10x	100 ml
$CaCl_2$	0.5 mM
$CaCO_3$	5 g
Aqua dest.	900 ml

(b) Medium II: marine AOB

Trace element solution 10x	
Na_2MoO_4 x 2 H_2O	0.025 g
$MnCl_2$ x 4 H_2O	0.050 g
$CoCl_4$ x 6 H_2O	0.0005 g
$ZnSO_4$ x 7 H_2O	0.025 g
$CuSO_4$ x 5 H_2O	0.006 g
Aqua dest.	ad 1000 ml
Medium	
NH_4Cl	0.5–5 mM
Trace element solution 10x	1 ml
KH_2PO_4	0.054 g
$FeSO_4$ x 7 H_2O	0.001 g
$CaCl_2$	0.5 mM
$CaCO_3$	5 g
Seawater	700 ml
Aqua dest.	300 ml

Table 3: Mineral salts medium for the cultivation of freshwater and marine NOB

(a) Medium III: freshwater NOB

Trace element solution 10x	
$MnSO_4$ x H_2O	0.0338 g
H_3BO_3	0.0494 g
$ZnSO_4$ x 7 H_2O	0.0431 g
$(NH_4)_6$ Mo_7O_{24}	0.0371 g
$FeSO_4$ x 7 H_2O	0.973 g
$CuSO_4$ x 7 H_2O	0.025 g
Disssolve in HCl	(0.01M)
Aqua dest.	ad 1000 ml
Stock solution 10x	
$CaCO_3$	0.07 g
NaCl	5.0 g
$MgSO_4$ x 7 H_2O	0.5 g
KH_2PO_4	1.5 g
Aqua dest.	ad 1000 ml
Medium	
$NaNO_2$	0.5–5 mM
Trace element solution 10x	1.0 ml
Stock solution 10x	100 ml
Aqua dest.	900 ml
pH 8.4	

(b) Medium IV: marine NOB

Trace element solution 10x	
Na_2MoO_4 x 2 H_2O	0.025 g
$MnCl_2$ x 4 H_2O	0.050 g
$CoCl_4$ x 6 H_2O	0.0005 g
$ZnSO_4$ x 7 H_2O	0.025 g
$CuSO_4$ x 5 H_2O	0.006 g
Aqua dest.	ad 1000 ml
Stock solution 10x	
$CaCl_2$	0.05 g
$MgSO_4$ x 7 H_2O	1.0 g
$FeSO_4$ x 7 H_2O	0.01 g
KH_2PO_4	0.017 g
Seawater	700 ml
Aqua dest.	300 ml
Medium	
$NaNO_2$	0.5–5 mM
Trace element solution 10x	1.0 ml
Stock solution 10x	100 ml
Seawater	600 ml
Aqua dest.	300 ml
pH 6.4-7.0	

3.4.2 Tolerance tests

To test the nitrification performance of AOB and NOB in dependence on different RAS relevant operational parameters short-term batch-experiments with fresh (overnight shipping) biofilter derived biocarriers were done. Five to ten biocarriers were added into 100 ml basic medium supplemented with 1 mM substrate if not mentioned otherwise. Experiments with samples derived from the freshwater and brackish plants were performed in medium I (AOB) and medium III (NOB) and the marine RAS derived samples in medium II (AOB) and medium IV (NOB). Substrate tolerance tests were performed with following concentrations of either NH_4Cl or $NaNO_2$: 0.5, 2, 5, 10, 25 mM. Nitrate tolerance tests were done by the addition of nitrate in concentrations of 0, 0.5, 3, 9 and 15 mM to the batches. For salt tolerance tests the salinity was adjusted by the percentage addition of North-Sea water revealing an original salinity of 27 PSU and thereby 5, 10, 15 and 27 PSU was adjusted and compared to batches with no North-Sea water added. To simplify matters, the samples which were not supplied with North-Sea water were defined as 0 PSU batches, even if they contained $0.5\,g\,l^{-1}$ NaCl provided by the stock solution of medium I and II. Flasks were incubated at 22 °C on a shaker (150 rpm) and samples taken regularly (three to five times per day) until substrate depletion and analyzed via HPLC. AOB samples from the substrate as well as the nitrate tolerance tests were analyzed using a photometer as described below (section 3.8.2). All tests were done in duplicates and mean values were used for the calculation of ammonia and nitrite oxidation rates, which were determined as substrate conversion per hour during exponential growth phase, if not mentioned otherwise. The percent inhibition of oxidation activity refers to the maximum oxidation rate observed at the control batches.

3.5 Molecular methods

3.5.1 Sample preparation and cell harvesting from biocarriers

Biocarriers were sampled from the running biofilters with a strainer. Subsequently, ten biocarriers were transferred into a sterile 50 ml Nuclease-free falcon tube and either stored on dry ice or for the purpose of RNA extraction covered with RNAlater® (RNAlater® Stabilization solution, Thermo Fisher Scientific Inc., Waltham, MA). Samples for DNA extraction were stored at -80 °C and samples for RNA extraction covered with RNAlater® solution and stored at 4 °C until further proceeding. Before cell harvesting DNA samples were covered with 10 ml sterile 0.9 % (2.5 % in case of the marine samples) NaCl solution. DNA and RNA samples were then spiked with 2 g sand particles (0.2 µm Ø and 10 µm Ø) and tubes were shaken horizontally on a vortexer at 4 °C for 2 h. Subsequently, biofilm containing supernatant was collected into a fresh falcon tube and centrifuged at 13.000 rpm for 15 min at 4 °C. Supernatant was discarded and the pellet was shock frozen in liquid nitrogen and stored at -80 °C until DNA or RNA extraction, respectively.

3.5.2 DNA extraction

DNA was extracted with the PowerSoil® DNA isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) after manufacturer's instruction with slight modifications: Before cell disruption via vortexing (step 5) Proteinase K ($1\,mg\,ml^{-1}$), Lysozyme ($4\,mg\,ml^{-1}$) and RNase A ($1\,mg\,ml^{-1}$) were added to the samples. The vortexing step was extended to 30 min at 37 °C. Finally the DNA concentration was determined by using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc, Waltham, MA). DNA was stored at -20 °C until further proceeding.

3.5.3 RNA extraction

RNA was extracted by the use of the PowerBiofilm® RNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) after manufacturer's instructions. Yielded RNA was further treated with DNase I to remove residual DNA (RQ1 RNase-Free DNase, Promega Corporation, Madison, WI) at 37 °C for 30 min, following an DNase I inactivation step at 65 °C for 10 min. The RNA was verified on a 1.2 % formaldehyde-agarose gel, running at 120 V for 70 min as described elsewhere (Haack *et al.* 2016). RNA concentration was defined spectrophotometrically on a Microplate reader (Synergy HT Multi-Detection Microplate Reader, BioTek Instruments GmbH, Bad Friedrichshall, Germany). RNA was stored at -80 °C until further proceeding.

3.5.4 Polymerase chain reaction

To proof the presence of *Nitrospira* and *Nitrotoga* in the culture used for FISH-MAR (see section 3.2) genus-specific PCR was done with primers targeting the 16S rRNA gene of *Nitrospira* (27f/Nsp1158r) and of *Nitrotoga* (200f/840r). PCR reactions were performed by the use of DreamTaq DNA Polymerase (Thermo Scientific Inc., Waltham, MA) after manufacturer's instructions and cycling was done in a gradient cycler (T100 Thermal Cycler, Bio-Rad Laboratories Inc., Berkeley, CA). Results of the PCR were verified on a 0.8 % agarose gel. Positive PCR products were purified with Geneaid Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) and subsequently sequenced using the Sanger method. Resulted nucleotide sequences were quality checked using BioEdit (Hall 1999) and analyzed with BLAST (Altschul *et al.* 1990). All primers used in this study are listed in table 4.

3.5.5 Preparative PCR for amplicon sequencing on Illumina MiSeq

Prior to amplicon sequencing a preparative PCR was done with specific primers F515 and R806, targeting the V4 region of the 16S rRNA gene (Caporaso *et al.* 2011). In addition, for the detection of nitrification relevant functional genes, primers targeting the bacterial *amoA* gene (*amoA*-1F/*amoA*-2R) and the *Nitrospira*-like *nxrB* gene (nxrB169f/nxrB638r) have been used (Rotthauwe *et al.* 1997; Pester *et al.* 2014). In addition to the specific priming regions, these primers are complementary to Illumina forward, reverse and multiplex sequencing primers. Table 4 lists the primers used for the amplicon sequencing approaches. Preparative PCR was done by using proofreading high fidelity polymerase (Phusion Flash High-Fidelity PCR Master

Table 4: Primers used in this study. [1)]Primers were modified for Illumina sequencing as described in Caporaso *et al.* (2011).

Primer	Sequence 5'-3'	Reference
F515	GTGCCAGCMGCCGCGGTAA	[1)]Caporaso *et al.* (2011)
R806	GGACTACHVGGGTWTCTAAT	[1)]Caporaso *et al.* (2011)
amoA-1F	GGGGTTTCTACTGGTGGT	[1)]Rotthauwe *et al.* (1997)
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	[1)]Rotthauwe *et al.* (1997)
nxrB169f	TACATGTGGTGGAACA	[1)]Pester *et al.* (2014)
nxrB638r	CGGTTCTGGTCRATCA	[1)]Pester *et al.* (2014)
Arch-amoAF	TTTCTACTGGTGGT	Francis *et al.* (2005)
Arch-amoAR	GCGGCCATCCATCTATATGT	Francis *et al.* (2005)
27f	AGAGTTTGATCMTGGCTCAG	Lane (1991)
Nsp1158R	CCCGTTMTCCTGGGCAG	Maixner *et al.* (2006)
200f	CTCGCGTTTTCGGAGCGG	Alawi (2007)
840r	CTAAGGAAGTCTCCTCCC	Alawi (2007)

Mix, Thermo Scientific Inc., Waltham, MA) with 10 µl 2x Phusion Flash Master Mix, 0.5 µM Primer Forward and Primer Reverse and H_2O added to a final volume of 20 µl for each reaction. Primer annealing temperature was calculated by using Tm Calculator (Thermo Scientific Inc, Waltham, MA). 10 ng of DNA or 1 µl of First-Strand cDNA (see section 3.5.6) was used as template. PCR-cycling was done after the following instructions: an initial denaturing step at 98 °C for 10 sec, 30 cycles of 98 °C for 2 sec, annealing for 5 sec, followed by 72 °C for 10 sec and a final elongation step at 72 °C for 1 min. PCR products were analyzed on a 2 % agarose gel and the expected amplicon sized product subsequently extracted from the gel. Purification was done by the use of MinElute® Gel Extraction Kit (QIAGEN, Hilden, Germany).

3.5.6 First-Strand cDNA synthesis

First-Strand copy-DNA (cDNA) was synthesized by the use of GoScript™ Reverse Transcription System (Promega Corporation, Madison, WI). 500 ng of RNA was mixed with 0.5 µg Random Primer and Nuclease-free water added to a final volume of 5 µl. The reaction was incubated for 5 min at 70 °C and immediately chilled on ice. The reverse transcription mix was prepared by combining 4 µl GoScript™ 5x Reaction Buffer, 2 mM $MgCl_2$, 0.5 mM PCR Nucleotide Mix, 20 u Recombinant RNasin® Ribonuclease Inhibitor, 1 µl GoScript™ Reverse Transcriptase and Nuclease-free water added to a final volume of 15 µl. The reverse transcription mix was added to the 5 µl RNA and primer mix on ice. To start the annealing reaction the tubes were transferred on a 25 °C heat block and incubated for 5 min. The extension reaction took place at 42 °C for 1 h. Finally, the Reverse Transcriptase was inactivated at 70 °C for 15 min. The First-strand cDNA containing, heat-inactivated Reverse Transcriptase reaction mix was stored at -20 °C until further proceeding. 1 µl of Reverse Transcriptase reaction mix was subsequently used as template for PCR amplification.

3.5.7 Fluorescence *in situ* Hybridization

Identification of different AOB and NOB inside the biofilm was done via Fluorescence *in situ* Hybridization (FISH). Biofilm material was harvested as explained in section 3.5.1. Subsequently, the obtained pellet was treated by paraformaldehyde (PFA) fixation as described by Amann *et al.* (1995). FISH was performed according to the protocol of Manz *et al.* (1992). All used oligonucleotide probes and their respective competitors were applied with a final working concentration of 5 pmol. Following hybridization, cells were stained with DAPI (4',6-diamidino-2-phenylindole) and embedded in Citifluor prior to microscopic observation. Table 5 lists the FISH probes used in this study. Probe conferred fluorescence was recorded on confocal scanning microscope Leica LSM SP8 (Leica Microsystems, Wetzlar, Germany). Digital image analysis was carried out by the use of software package LAS AF Lite 2.6 (Leica Microsystems CMS GmbH, Wetzlar, Germany).

Table 5: Oligonucleotide probes used in this study.

Probe	Sequence 5'-3'	Reference
EUB 338	GCTGCCTCCCGTAGGAGT	Amann *et al.* (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	Daims *et al.* (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Daims *et al.* (1999)
Nsp712	CGCCTTCGCCACCGGCCTTCC	Daims *et al.* (2001a)
Comp-Nsp712	CGCCTTCGCCACCGGTGTTCC	Daims *et al.* (2001a)
Ntspa662	GGAATTCCGCGCTCCTCT	Daims *et al.* (2001a)
Comp-Ntspa662	GGAATTCCGCTCTCCTCT	Daims *et al.* (2001a)
Ntoga122	TCCGGGTACGTTCCGATAT	Lücker *et al.* (2015)
c1Ntoga122	TCWGGGTACGTTCCGATAT	Lücker *et al.* (2015)
Nso190	CGATCCCCTGCTTTTCTCC	Mobarry *et al.* (1996)
Nso1225	CGCGATTGTATTACGTGTGA	Mobarry *et al.* (1996)
NEU	CCCCTCTGCTGCACTCTA	Mobarry *et al.* (1996)

3.5.8 Fluorescence *in situ* Hybridization-Microautoradiography

Fluorescence *in situ* Hybridization-Microautoradiography (FISH-MAR) was done in order to observe differences in the active autotrophic CO_2-uptake of co-cultivated cells of *Nitrospira* and *Nitrotoga* under two distinct pH-values. Enrichment culture 'Aquakultur HW' (see section 3.2) containing representatives of *Nitrospira* and *Nitrotoga* was used as pre-culture. Cells were transferred into 150 ml medium III and incubated at 17 °C without agitation in the dark. Nitrite consumption was tested regularly via the Griess-Ilosvay spot test (Schmidt *et al.* 1994) and sterile substrate solution was added in case of consumption. After three to five times of repeated feeding cells were used for FISH-MAR. Before starting, the nitrite concentration was determined and in case of consumption 5 ml of sample aliquots were transferred into sterile 100 ml glass serum bottles containing NOB medium III with a pH-value adjusted before autoclaving to

6.4 or 7.2. All experiments were performed in duplicates and PFA-fixed biomass harvested from the starter culture was used as dead control. To demonstrate that CO_2-uptake is nitrite dependent, a control sample without the addition of $NaNO_2$ was included to the set-up. To start the incubation 0.3 mM $NaNO_2$ was added and each bottle was supplemented with 10 µCi [^{14}C] H_2CO_3 and sealed air-tight with rubber stoppers. Incubation was performed at 14 °C and after 6 h stopped by harvesting the biomass (13000 rpm, 5 min, 4 °C). PFA-fixation of biomass was done after the protocol of Amann *et al.* (1995) and FISH-MAR was carried out as described by Lee *et al.* (1999) with an exposure time of seven days. Probes used in this experiment were Nsp772 mixed with Nspa662 and Ntoga122 to visualize cells of *Nitrospira* and *Nitrotoga* (see table 5). Probe conferred fluorescence visualization and digital image analysis was done as described above (section 3.5.7).

3.6 Transmission electron microscopy

Visualization of cells was performed via transmission electron microscopy (TEM). For this, cells were mechanically removed from the biocarriers (see section 3.5.1) and fixed in 2.5 % (v/v) glutaraldehyde for 1.5 h followed by an overnight incubation in 2 % (w/v) osmium tetroxide. Fixed cells were embedded in Spurr (Spurr 1969) as described in Spieck *et al.* (2011). Ultrathin sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963). Micrographs were taken at Zeiss model Leo 906E with a CCD camera model 794 (Carl Zeiss, Jena, Germany).

3.7 Next generation sequencing

A comparative study of biofilm microbial communities inhabiting biocarriers was done by the use of different next generation sequencing (NGS) approaches. Biofilters of different modules at three different RAS, which mainly differ in the salinity of the process water, were sampled. Sampling, sample preparation and subsequent DNA and RNA extraction of biofilm material was performed as described above (sections 3.5.1, 3.5.2, 3.5.3). For main characteristics of the sampled modules refer to table 1.

3.7.1 Amplicon sequencing on Illumina MiSeq

16S rRNA sequencing of cDNA and DNA generated amplicons was carried out on the Illumina MiSeq platform at the sequencing facility of Heinrich Pette Institute (HPI, Hamburg, Germany). Samples were amplified with V4 region targeting primerset F515/R806 as described in section 3.5.5 and barcode labeled amplicons were subsequently sequenced via Illumina MiSeq producing a sequencing depth of 1.5 million paired-end reads per sample and 2 x 150 bp read length. The method of barcode labeled 16S rRNA amplicon sequencing on the Illumina MiSeq platform is described in detail in the studies of Caporaso *et al.* (Caporaso *et al.* 2011, 2012). cDNA as well as DNA samples were chosen to study the microbial community composition on the long-term state (DNA) and to recognize short-term changes within the biofilm (cDNA).

In addition, microdiversity analysis of main groups of AOB and NOB was done via amplicon sequencing of the β-proteobacterial *amoA* gene encoding for the α-subunit of ammonia monooxygenase (AMO) as well as the *Nitrospira*-like *nxrB* gene encoding for the β-subunit of nitrite oxidoreductase (NXR), by using the Illumina MiSeq platform at Göttingen Genomics Laboratory (G2L, University of Göttingen, Göttingen, Germany). Prior to sequencing DNA samples were PCR amplified with *amoA*- or *nxrB*-specific primers with Illumina overhang adaptors as described in section 3.5.5. By using the Nextera® XT Index Kit (Illumina Inc., San Diego, CA) a subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adaptors. Libraries were normalized and pooled and sequenced on the MiSeq system generating 2 x 300 bp paired-end reads.

3.7.2 Metagenome sequencing on Illumina NextSeq

For a metagenomic approach biofilm material derived from a single biofilter out of three different RAS was used as template. The same sample material was used for the metagenomic analysis as used for the amplicon studies described above. The sampled biofilters and the respective DNA concentration after extraction of genomic DNA are listed in table 6. Equimolar amounts of DNA from each sample was sent to the sequencing facility of Max Planck Institute for Evolutionary Biology (Plön, Germany) for Illumina sequencing using the NextSeq platform with the Nextseq High-Output V2-Kit (300 cycles) (Illumina Inc., San Diego, CA). The three samples were multiplexed into a single NextSeq run. Finally, Illumina paired-end reads with a length of 2 x 150 bp were generated. Data processing and downstream bioinformatic analyzes of the metagenomic data sets (as described in sections 3.9.2-3.9.4) were performed in collaboration with Malik Alawi from Heinrich Pette Institute and Simon Güllert from University of Hamburg.

Table 6: Sampling sites and quantity/quality data of extracted DNA used for metagenome sequencing on Illumina NextSeq. Concentration and purity was measured by NanoDrop 2000 spectrophotometer without fluorescent dye.

Sample name	Sampling site	ng µl^{-1}	ratio 260/280
FR	Biofilter module C, freshwater RAS	22.8	1.81
BR	Biofilter module GMA, brackish RAS	29.1	1.78
MA	Biofilter module M3, marine RAS	24.3	1.80

3.8 Analytical methods

3.8.1 High performance liquid chromatography

Nitrite and nitrate were quantified by high performance liquid chromatography (HPLC) via ion pair chromatography with LiChrospher® RP-18 column (125 x 4 mm, Merck KGaA, Germany) and UV detection in an automated system (Hitachi LaChrom Elite®, VWR International GmbH, Darmstadt, Germany). Data acquisition and processing was done with the integrated software EZChrom Elite® 3.3.2.

3.8.2 Photometrical determination of ammonia

Quantification of ammonia was performed with a phthaldialdehyde (OPA)-based method described by Corbin (1984). This method based on the indirect detection of ammonia ions by the reaction of β-mercaptoethanol and OPA to the derivate 1-(2-hydroxyethyl)-thioisoindol. Measurements were done photometrically at 420 nm.

3.8.3 Determination of physicochemical parameters at the RAS

Ammonium, nitrite and nitrate in the process water of the RAS were quantified via bulb test (LCK 339, LCK 341, LCK 304, Hach, Düsseldorf, Germany) and analyzed photometrically (DR 2800 spectrophotomter, Hach, Düsseldorf, Germany). Temperature, pH-value and oxygen concentration were monitored permanently via installed probes.

3.9 Data processing and phylogenetic analysis

3.9.1 Processing and analysis of data sets generated by amplicon sequencing on Illumina MiSeq

Generated 16S rRNA gene and *amoA*/*nxrB* gene data sets were processed with USEARCH version 8.0.1623 (Edgar 2010) as follows: Paired end reads were merged and quality filtered. Filtering included the removal of reads shorter than 200 bp. Processed sequences of all samples were joined and clustered in operational taxonomic units (OTUs) at 3 % (16S rRNA amplicons) and at 1 % (*amoA*/*nxrB* gene amplicons) genetic dissimilarity, respectively, using the UPARSE algorithm implemented in USEARCH (Edgar 2013). A *denovo* chimera removal was included in the clustering step. Afterwards, putative chimeric sequences were removed using UCHIME (Edgar *et al.* 2011) in reference mode with the most recent SILVA database (Silva SSURef 123 NR) as reference data set. Subsequently, processed sequences were remapped on OTU sequences to obtain an OTU table. To determine taxonomy, the representative sequence of each OTU was classified by BLAST alignment (Camacho *et al.* 2009) against the most recent SILVA database. All non-bacterial OTUs were removed. The described processing and bioinformatic analysis of Illumina MiSeq generated data was performed in collaboration with Dr. Bernd Wemheuer from Göttingen Genomics Laboratory. Following that, the generated OTU tables were used for downstream analysis. Relative abundances of bacterial taxa were determined as percent of the total reads for each sample. OTUs assigned to AOB and NOB were extracted from the 16S rRNA amplicon data set and the NCBI BLAST tool (Altschul *et al.* 1990) was used for identifying the closest matches for the taxonomic assignment of uncultured nitrifiers. For determination of α-diversity in the amplicon sequencing generated data sets Simpson's Diversity Index (D), Shannon Index (H) and Shannon's equitability (EH) have been applied to normalized data sets as described elsewhere (Simpson 1949; Mulder *et al.* 2004; Ramette 2007). Assessment of phylogenetic relatedness between different communities was done by a non-metric multidimensional scaling (NMDS) approach, based on Bray-Curtis similarities. These statistical analyzes were conducted by using the program package PAST version 2.17 (Hammer

et al. 2001). Determination of unique and shared OTUs of amplicon sequencing generated data sets was done via the COUNTIF-function in the software Excel (Microsoft Office Professional Plus 2010).

3.9.2 Assembly and gene prediction of metagenomic data obtained after sequencing on Illumina NextSeq

Trimmomatic version 0.35 (Bolger *et al.* 2014) was employed to remove adapters and low quality (phred quality score <30) bases from the 3' ends of sequence reads. Reads shorter than 100 bp were removed subsequently. The assembly was done via MEGAHIT version 1.0.3-29 (Li *et al.* 2014) using the programs preset parameter set 'meta-sensitive'. Quality of assemblies was estimated through Assemblathon 2 (Bradnam *et al.* 2013). Prodigal version 2.6.1 was applied to find open reading frames (ORFs) on the assembled contigs of the samples (Hyatt *et al.* 2012). The predicted ORFs were then used for following functional analyzes.

3.9.3 Taxonomic analysis of metagenomic data

16S and 18S rRNA sequences of the three metagenomic data sets were extracted and uploaded to the web-based tool SILVA NGS for quick taxonomic profiling (Quast *et al.* 2013). Archaea were not identified with this method and therefore not considered in following downstream analyzes. In addition, taxonomic profiling of generated contigs was performed with the software AMPHORA2 by using the universal marker set of 31 bacterial genes (Wu *et al.* 2012).

3.9.4 Analysis of genes related to nitrogen removal and organic breakdown

Predicted ORFs were searched against hidden Markov models (HMM) (Eddy 1995) of selected nitrogen removal and organic breakdown genes extracted from Pfam (Finn *et al.* 2016), FUNgenes (Fish *et al.* 2013) and carbohydrate-active enzyme (CAZy) (Lombard *et al.* 2014) databases. Table S4 lists the set of HMMs used for this approach. HMM search was done using HMMER (http://hmmer.org/) via the 'hmmsearch' tool. Subsequently, predicted ORFs of interest were extracted from the data set and used for analyzing nitrogen removal/organic breakdown genes and their taxonomic assignment. This was done by using DIAMOND (Buchfink *et al.* 2015) for the alignment of protein sequences against NCBI non-redundant (nr) database with E-value of 1 x 10^{-5}. Results of the alignments were imported into MEGAN6 (Huson *et al.* 2016) using default parameters to employ KEGG pathways (Kanehisa *et al.* 2016) and taxonomy of nitrogen removal/organic breakdown bacteria via the lowest common ancestor (LCA) algorithm (KEGG database: status July 2016).

3.9.5 Phylogenetic trees

Reads of interest were extracted from the data sets and imported into MEGA6 software (Tamura *et al.* 2013). ORFs derived from the metagenomic data sets with putative comammox *amoA* genes were compared by MUSCLE alignment (Edgar 2004) based on amino acid sequences.

The phylogenetic tree was generated by the Maximum Likelihood method with 1000 bootstraps replications. OTUs achieved via amplicon sequencing of *amoA* and *nxrB* genes were compared by using multiple sequence alignment package T-coffee version 11.00.8 (Notredame *et al.* 2000). Nucleic acid sequences were preferred over deduced amino acid sequences for the alignment because of their higher phylogenetic resolution. Phylogenetic trees were generated by the Maximum Likelihood method with 1000 bootstraps replication.

4 Results

In this study a comprehensive characterization of biofilm microbial communities derived from biofilters at three different RAS (freshwater: FR, brackish: BR, marine: MA) was done. Biocarriers were sampled and biofilm inhabiting microbes analyzed via NGS sequencing approaches by using Illumina MiSeq and NextSeq platforms. In addition, via EM and FISH cells were visualized in the biofilms. Emphasize was set on nitrifiers and their physiological characteristics with respect to RAS relevant operational parameters were analyzed in batch-experiments. For a detailed description of the sampled modules refer to section 3.1. Mean physicochemical data were examined in the process water of the different modules from the beginning of a rearing cycle until the day of sampling for the experiments regarding this study (between three to six months), as listed in tables S1 and S2 in the appendix.

4.1 16S rRNA amplicon sequencing

Analysis and comparison of microbial biofilm communities derived from three RAS was performed via 16S rRNA amplicon sequencing using Illumina MiSeq as described in section 3.7.1. For this approach samples were taken from the biofilters of the following modules: FR: C, D, F; BR: AQ1, GMA, RMB; MA: M1, M3, M5. To address the question how the total and active microbial communities differ and which part of the total microbial community is metabolically active at a given time point, reverse transcribed RNA (cDNA) and DNA generated amplicons were sequenced.

4.1.1 α-diversity

After data processing and quality trimming, between 200.000 to 1.8 Mio reads could be identified in total and further classified into 1000 to 2000 OTUs per sample. To describe the α-diversity within the different samples (the diversity of the community within one site or one sample, the number of species and their proportion within one sampling site), different α-diversity indices were applied on normalized data sets as described in 3.9.1. This showed that all samples reached a high species richness, which was reflected in the Simpson's Index of Diversity (1-D) tending towards 1 in all samples. The brackish samples AQ1, GMA and RMB exhibited the highest number of species of all samples within the RNA and DNA pool. This was further confirmed by the Shannon Index (H), which predicted highest values for the brackish samples. Considering the calculated Evenness (EH), which ranges from 0.03-0.1, it becomes apparent that the different taxa within the samples are numerically unevenly distributed. Table 7 shows the number of reads and OTUs of each sample as well as α-diversity indices applied for RNA and DNA pools.

4.1.2 β-diversity

To document shifts in the membership or structure of communities that may occur between sample categories or environmental categories, phylogenetic β-diversity methods are very use-

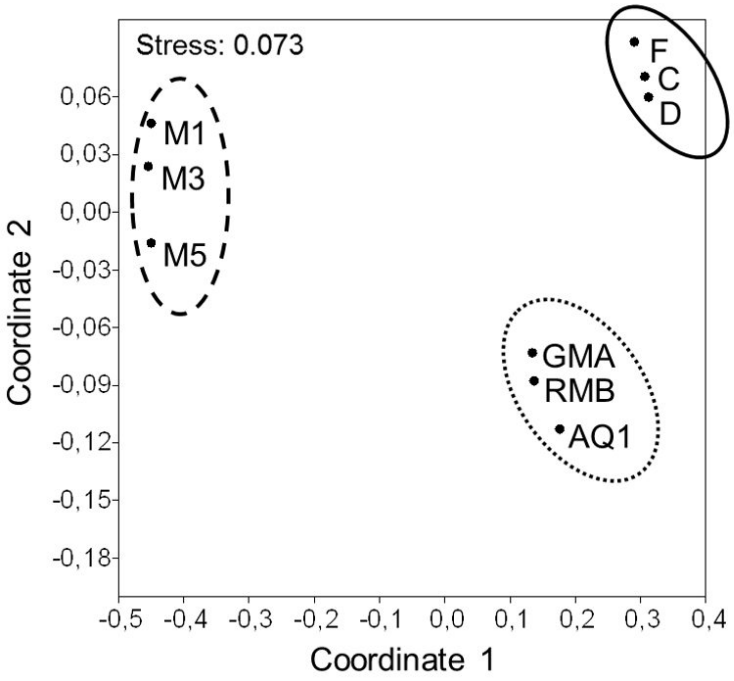
Table 7: Number of obtained reads and α-diversity indices determined in the RNA and DNA derived 16S rRNA amplicon sequencing data of the microbial communities in the freshwater (C, D, F), brackish (AQ1, GMA, RMB) and marine (M1, M3, M5) biofilter samples. Data were normalized before calculation of indices. R: RNA derived sequences, D: DNA derived sequences.

				Richness estimate	Diversity	Evenness
	Sample	# of reads	# of OTUs (S)	Simpson (1-D)	Shannon (H)	EH=$e^{\frac{H}{S}}$
FR	C_R	646,247	1,427	0.96	4.78	0.07
	C_D	835,543	1,435	0.98	5.01	0.10
	D_R	1,070,115	1,411	0.97	4.60	0.07
	D_D	266,044	1,435	0.97	4.72	0.08
	F_R	726,343	1,442	0.96	4.55	0.05
	F_D	337,457	1,425	0.95	4.40	0.05
BR	AQ1_R	1,826,510	2,144	0.98	5.34	0.09
	AQ1_D	578,067	2,094	0.98	5.23	0.08
	GMA_R	1,421,165	2,028	0.97	5.15	0.08
	GMA_D	575,487	2,113	0.99	5.53	0.11
	RMB_R	1,256,388	2,238	0.98	5.49	0.10
	RMB_D	596,525	2,225	0.97	4.95	0.06
MA	M1_R	651,323	1,202	0.89	3.76	0.03
	M1_D	1,061,498	1,263	0.89	3.71	0.03
	M3_R	452,155	1,266	0.96	4.47	0.07
	M3_D	406,972	1,289	0.91	3.80	0.03
	M5_R	381,885	1,337	0.93	4.03	0.04
	M5_D	199,452	1,406	0.93	4.08	0.04

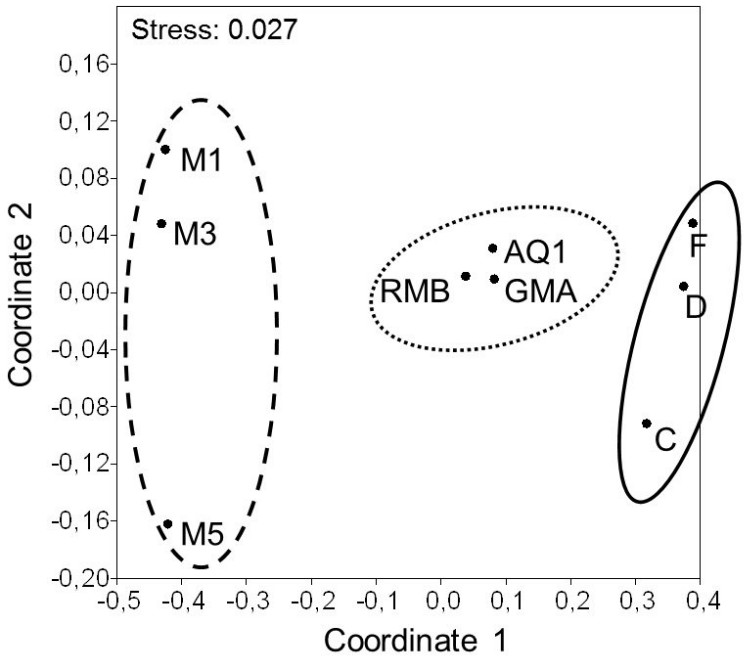
ful. β-diversity is a measure of the degree of similarity between pairs of samples (in this case communities). Accordingly, to assess the phylogenetic relatedness between the different communities from the three RAS, non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities was applied. The resulting 2-D ordination plots revealed that all three samples from one sampling site cluster densely together as shown in fig. 5. RNA as well as DNA samples from each RAS clustered and were separated from the other RAS samples in a two-dimensional scale, which indicates different microbial communities in the three investigated plants and hence, spatial heterogeneity. Moreover, it became visible that the freshwater and the brackish fraction were in closer proximity to each other than to the marine samples which were located on the opposing side in the 2-D plot. The NMDS analysis further revealed dissimilarities between samples from the same sampling site, particularly visible in the DNA pool of sample M5 and sample C. These samples were located slightly separated from the other two samples from the same RAS (fig. 5b). In contrast to the DNA pool, the RNA pool did not reveal conspicuous dissimilarities. Instead, all three samples from the same site were tightly clustered (fig. 5a), indicating that differences in the community structures between the RNA and DNA pools exist. To visualize unique and shared OTUs among the samples from three RAS, Venn-diagrams based on the RNA and DNA pools of the merged samples were constructed (fig. 6). The results confirmed the observed distribution patterns in the NMDS plots. Hence, most of the OTUs were unique for the sampling site. In addition, the brackish and freshwater samples had twice as many shared OTUs than the brackish and marine samples with respect to the RNA and DNA pools. Least of all shared OTUs were found between the marine and freshwater samples.

4.1.3 Phylogenetic diversity

Phylogenetic classification on phylum level revealed similar patterns for RNA and DNA derived samples. Five phyla emerged as the most abundant in all samples: *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes* and *Nitrospirae*. To a lower amount but still above the cut off-level of 0.5 % of the total reads, members of *Verrucomicrobia* and *Gemmatimonadetes* appeared in all samples. Fig. 7 shows the percent distribution of the different bacterial phyla among the nine samples in the RNA and DNA pools. In accordance with their abundance, *Proteobacteria* and *Bacteroidetes* were defined as predominant and *Chloroflexi*, *Planctomycetes* as well as *Nitrospirae* as dominant phyla. In most of the samples *Proteobacteria* was the predominant phylum, followed by *Bacteroidetes*, which members contributed to 24–70 % and 9–33 %, respectively, of the total reads. However, freshwater sample F and marine sample M5, which modules were in case of the former connected to a denitrification reactor and in case of the latter only low stocked during time of sampling, were an exception with *Bacteroidetes* identified as the predominant phylum. Members of the *Chloroflexi* phylum accounted for 10–30 % of the total reads and were in general more abundant in the freshwater samples compared to the two other RAS. Nevertheless, the number of *Chloroflexi* reads was strikingly high in marine sample M5 (27 %). In all samples 2–28 % could be affiliated with representatives of the *Planctomycetes* phylum, whereat this group contributed to a higher degree in the three brackish samples than

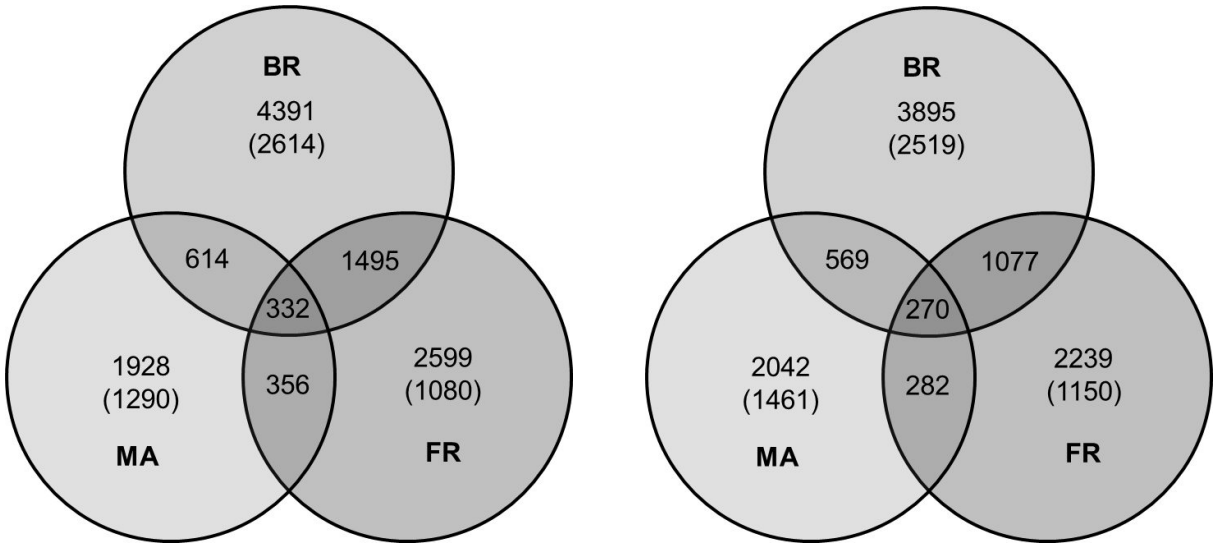


(a)



(b)

Figure 5: 2-D plots of non-metric multidimensional scaling (NMDS) approaches based on Bray-Curtis similarities of the RNA and DNA 16S rRNA amplicon sequencing data based on the total OTUs for each sample. (a) Distribution of RNA OTUs, (b) of DNA OTUs. Samples from the same sampling site are marked by circles as follows: FR (black), BR (dotted), MA (broken line).



(a) (b)

Figure 6: Venn-diagram showing the number of unique and shared OTUs, representing the microbial communities at the freshwater (FR), brackish (BR) and marine (MA) RAS. Note that OTUs of the three samples derived from the same RAS were merged. The number in each circle indicates the total number of OTUs and the number in brackets shows the unique OTUs for each sampling site. Values in overlapping regions indicate numbers of shared OTUs. The diagrams were constructed based on OTUs derived from the (a) RNA and (b) DNA pools.

in the other two RAS (10–28 %). In contrast to that, *Gemmatimonadetes* constituted 1–10 % of the total reads with highest occurrence in the freshwater samples. Moreover, members of *Verrucomicrobia* were identified with particular frequency in the freshwater samples and in the DNA pool of marine sample M5. *Nitrospirae* accounted for 2–25 % among the samples with strongest occurrence in the three marine samples. *Actinobacteria* and *Acidobacteria* contributed to 0.04–5 % and were somewhat less represented in the marine samples.

Taken together, the same dominating phyla were found in all investigated RAS with *Proteobacteria* and *Bacteroidetes* as predominating groups, followed by *Plantomycetes*, *Nitrospirae* and *Chloroflexi*. Since the phylum *Nitrospirae* only contains one class (*Nitrospira*), the four other phyla were further resolved on class level (see fig. S1 in the appendix). In accordance with the analysis on phylum level, RNA and DNA pool derived microbial communities revealed very similar results on class level distribution. Within the *Proteobacteria* the three major classes α-, β- and δ-*Proteobacteria* appeared in all samples. In the brackish and marine samples γ-*Proteobacteria* emerged as the fourth dominant group. *Bacteroidetes* were mainly composed of *Sphingobacteriia*, *Cytophagia* and *Flavobacteria* class members and the majority of organisms belonged to *Sphingobacteria* in all samples. Within the *Planctomycetes* the two classes *Planctomycetacia* and OM190 subdivision represented the main fraction. The class level resolution of the *Chloroflexi* phylum showed a high diversity with mainly members of *Anaerolinea*, *Caldilinea*, *Chloroflexia*, *Thermomicrobia* as well as unclassified *Chloroflexi* being unevenly distributed among the samples from the three RAS.

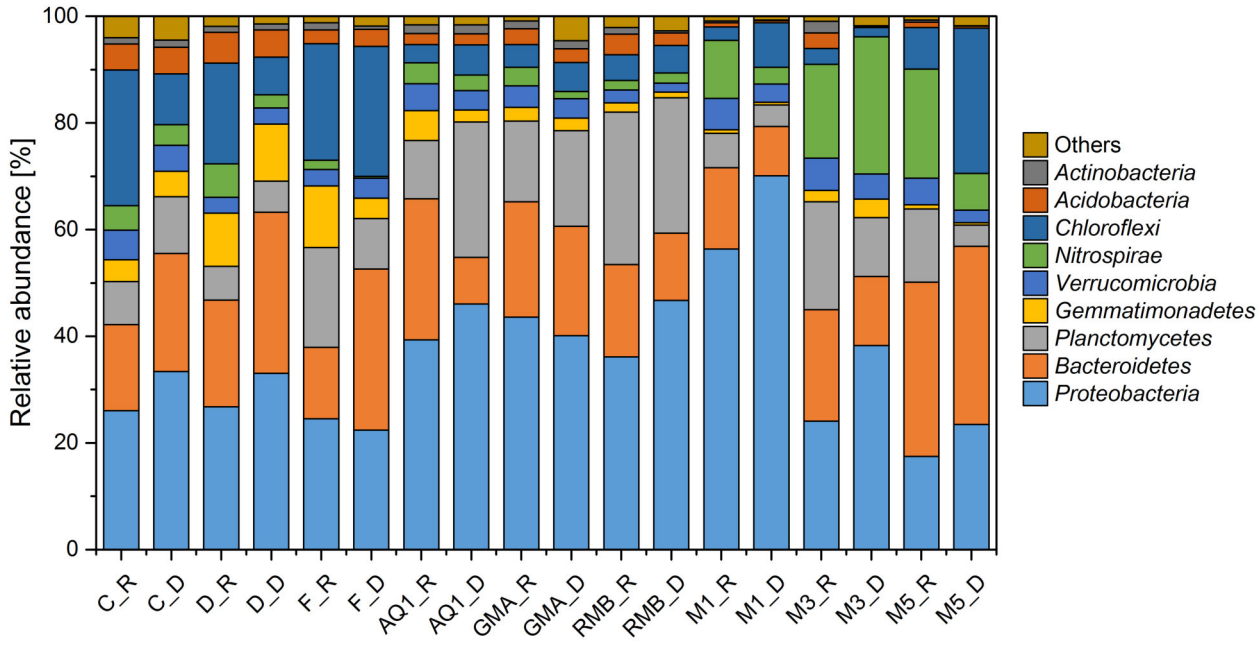


Figure 7: Relative abundance of different phyla identified in the RNA (R) and DNA (D) derived 16S rRNA amplicon sequencing data of the freshwater, brackish and marine biofilter samples. Others: subdominant phyla represented by <0.5 % of the total reads. FR: C, D, F; BR: AQ1, GMA, RMB; MA: M1, M3, M5.

4.1.4 Frequently observed taxa

The taxonomic classification revealed that 674 genus level OTUs were identified in total. However, a large fraction of OTUs could not be classified until genus level and was clustered into the group of uncultured or unassigned bacteria (average in the merged RNA and DNA pools: FR: 55 % ±5.7; BR: 51 % ±4.8; MA: 30 % ±10.2). Fig. 8 shows the distribution of different genera among the nine biofilter samples. Among the known genera 85 and 76 were found to be shared between all nine samples in the RNA and DNA pools, respectively (see table S3 in the appendix). Accordingly, these shared genera were defined as core-taxa of the three RAS. However, the majority of shared genera appeared with <0.5 % of the total reads and only very few genera were found of higher abundance in the RNA and DNA data sets. Hence, within the core-community only six genera were found with particular frequency in at least one biofilter at each sampling site in the RNA and DNA pool (minimum 0.5 % of the total reads). These six genera were accordingly defined as frequent core-taxa, representing the most abundant and metabolically active core-community members. The nitrifying genera *Nitrosomonas* and *Nitrospira* as well as members of the heterotrophic genera *Planctomyces*, *Blastopirellula*, *Nannocystis* and *Lewinella* were present among the frequent core-taxa, with the latter accounting for 14.7 % in the DNA pool of M5, which was only low stocked with fish during time of sampling. Furthermore, *Marinobacter* and *Muricauda* appeared frequently in the brackish and marine samples and *Variovorax*, *Rhodobacter* as well as *Gemmatimonas*, in the freshwater and brackish samples as depicted in fig. 8.

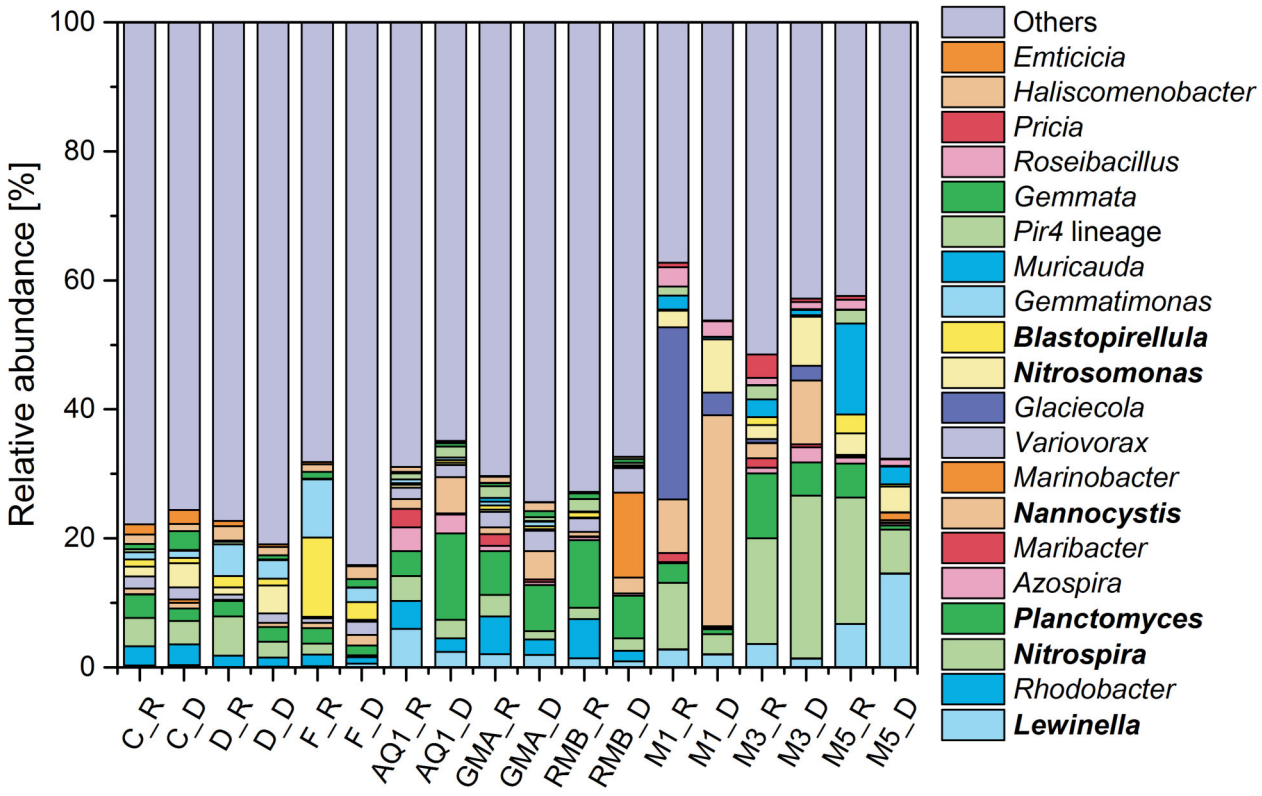


Figure 8: Distribution of bacterial genera among the nine investigated biofilters in the RNA (R) and DNA (D) pools after 16S rRNA amplicon sequencing. OTUs not classified down to genus level and genera of low abundance (<0.5 % of the total reads) were grouped and defined as 'others'. In the legend, six genera are marked in bold type, representing core-taxa which were frequently identified in the RNA and DNA pools in at least one biofilter of each RAS.

Table 8: Number of OTUs classified to AOB and NOB and the contribution of nitrifiers to the total reads in the RNA (R) and DNA (D) pools after 16S rRNA amplicon sequencing. The nine samples were derived from biofilters at a freshwater, brackish and marine RAS.

		# OTUs AOB	#OTUs NOB	% nitrifiers	% AOB	% NOB
FR	C_R	23	12	7.38	2.78	4.59
	C_D	27	19	8.72	4.81	3.91
	D_R	25	24	13.79	7.50	6.29
	D_D	19	13	9.25	6.75	2.50
	F_R	34	18	4.24	2.46	1.77
	F_D	17	7	1.31	1.01	0.30
BR	AQ1_R	46	33	6.87	2.92	3.95
	AQ1_D	42	33	5.21	2.30	2.91
	GMA_R	46	38	16.93	13.47	3.47
	GMA_D	33	27	3.81	2.50	1.32
	RMB_R	39	29	8.85	7.04	1.81
	RMB_D	35	28	6.18	4.27	1.92
MA	M1_R	26	15	13.59	2.71	10.88
	M1_D	25	13	11.67	8.50	3.18
	M3_R	16	14	19.93	2.36	17.58
	M3_D	31	21	33.49	7.81	25.68
	M5_R	27	24	23.93	3.48	20.45
	M5_D	19	14	10.97	4.07	6.91

4.1.5 Nitrifying communities

Community structure To characterize the nitrifying community composition, sequences assigned to AOB and NOB were extracted from the data set and analyzed separately. In total 46 OTUs were found conferring to AOB and 38 to NOB with most OTUs appearing in the brackish samples. The nitrifiers contributed on average to 7.4 % and 7.9 % of the total reads in the freshwater and brackish and even to 18.9 % in the marine samples. Table 8 shows the number of OTUs as well as the percentage of nitrifiers to the total reads for each sample. The average ratio between AOB and NOB was almost equal in the freshwater samples with 1.5:1 (RNA) and 1.1:1 (DNA). A higher proportion of AOB with 1.5:1 (RNA) and 2.3:1 (DNA) was found in the brackish samples. In contrast, in the marine samples the ratio was 0.7:1 (RNA) and 0.2:1 (DNA) revealing a higher abundance of NOB than AOB in these biofilms.

NMDS analysis based on Bray-Curtis similarities of either the AOB or the NOB OTUs showed that a clear separation was only visible for the marine samples in the RNA as well as in the DNA pool, while the freshwater and brackish samples were arranged in close proximity in the 2-D plot (fig. 9). The ordination plots further indicated variability in the communities within the samples from one sampling site. Hence, freshwater sample F, derived from a module connected to a denitrification reactor, was located separately from the other two samples in the plot for both, AOB and NOB. A similar pattern was observed in marine sample M5 for the AOB fraction, but only in the DNA pool, which on the other hand indicated differences in the

nitrifying community composition between the RNA and DNA pool (fig. 9b).

Species composition of AOB and NOB Taxonomic assignment of AOB and NOB OTUs resulted to the vast majority in unknown ammonia/nitrite oxidizing bacteria of the genera *Nitrosomonas* and *Nitrospira*. To further classify the unknown members, reads were extracted from the data set and the closest taxonomically described species was searched via manual sequence alignment against the NCBI database. To simplify matters, OTUs mapping the same closest taxonomically described species, but with different similarities to the 16S rRNA gene were merged, resulting in ten AOB and eight NOB groups (shown in fig. 10 depicted as heatmap). Same groups of nitrifiers appeared in the RNA and DNA pools, indicating the metabolic activity of these bacteria. A habitat specific community of AOB and NOB was observed at each RAS, composed of one predominant group accompanied by at least one less abundant and several subdominant groups with low abundances (<1 % of the total reads), as described in the following paragraphs.

AOB In the freshwater samples 97–99 % of the total AOB reads were assigned to uncultured ammonia oxidizing bacteria with *Nitrosomonas communis* Nm2 as the closest relative. Several subdominant AOB groups were found with few reads of e.g. relatives of *Nsm. urea* and moderately halophilic *Nsm. aestuarii*. Representatives of the genus *Nitrosospira* appeared in the sample derived from module C (0.16 % in the RNA pool) with *Nitrosospira multiformis* and *Nitrosovibrio tenuis* as the nearest described organisms, revealing sequence similarities of 95–99 % (due to difficulties in the 16S rRNA phylogeny of the *Nitrosospira* lineage, all OTUs allocated to *Nitrosospira* and *Nitrosovibrio* were merged and defined as *Nitrosospira*-like AOB).

The highest number of AOB OTUs was found in the brackish samples. 54–93 % of the total AOB reads were classified to uncultured members of the genus *Nitrosomonas* with *Nsm. urea* as the closest relative. *Nitrosococcus oceani*-like AOB from the *γ-Proteobacteria* phylum emerged as the second group and accounted even for >50 % in sample RMB. Furthermore, relatives of *Nsm.* sp. Nm59 were found, with highest contribution in sample AQ1 (>25 %). Among the subdominant groups relatives of limnic *Nsm. communis* Nm2, *Nsm.* sp. AL2121 together with marine *Nsm. aestuarii* and *Nsm.* sp. NS20 as well as members of the genus *Nitrosospira* were identified in all brackish samples.

The AOB community in the marine samples consisted of a predominant group of uncultured ammonia oxidizing bacteria with *Nitrosomonas aestuarii* as the closest relative, which contributed to 57–77 % of the total AOB reads. A second group of abundant OTUs was found, which had *Nsm.* sp. NS20 as closest phylotype, contributing to 21–42 %. In all marine samples members of the genus *Nitrosospira* were found among the subdominant groups.

NOB In the freshwater samples >99 % of the total NOB reads were classified to different uncultured bacteria belonging to the genus *Nitrospira*, which had *Ns. defluvii* as closest relative. Besides this, few subdominant groups were found, which revealed *Ns. moscoviensis*, Cand. *Ns. inopinata* and marine Cand. *Ns. salsa* as the closest known relatives.

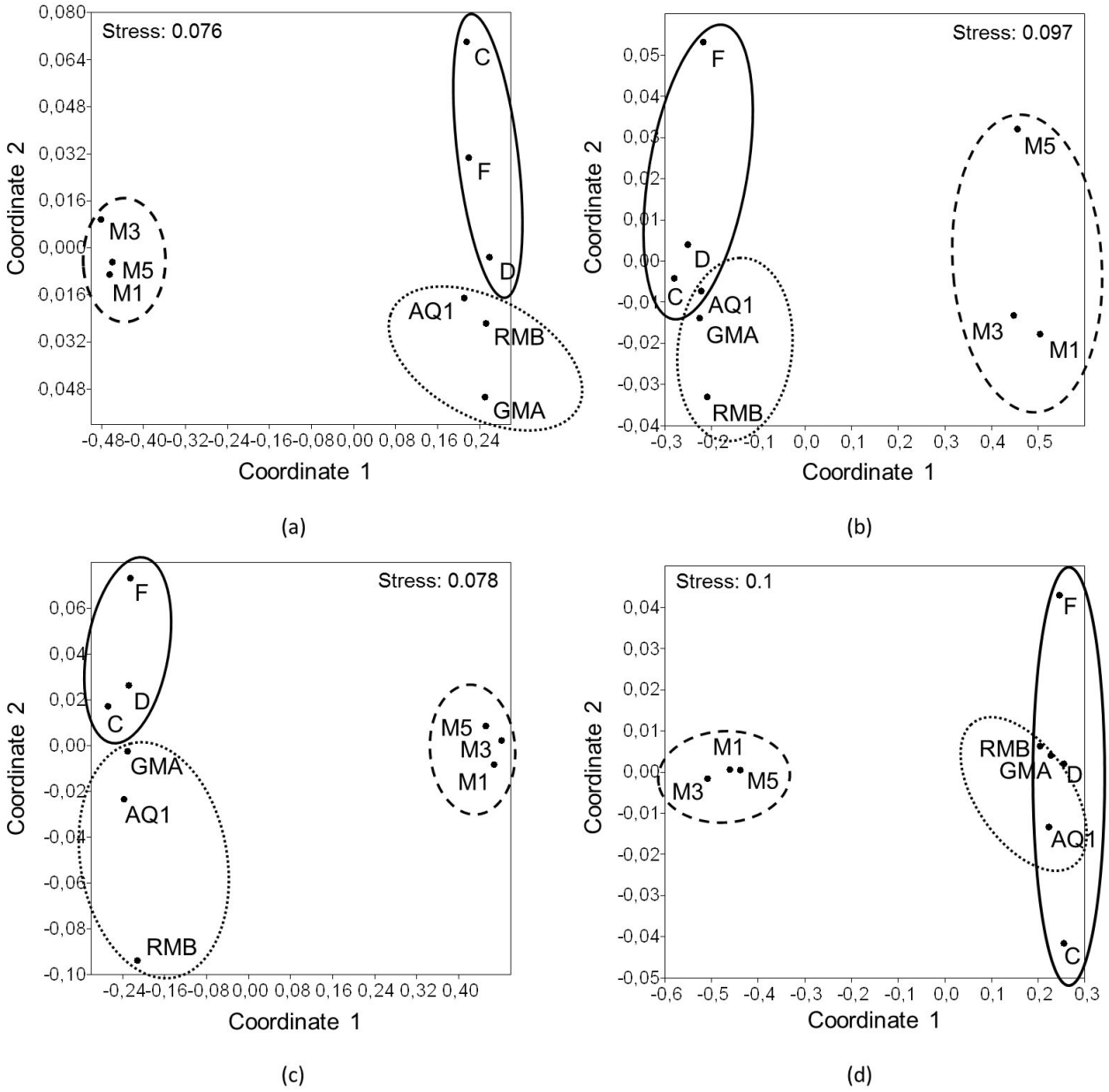


Figure 9: Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the AOB and NOB OTUs extracted from the 16S rRNA amplicon sequencing data of the nine samples derived from three RAS. FR: C, D, F; BR: AQ1, GMA, RMB; MA: M1, M3, M5. (a) Based on OTUs representing the AOB of the RNA pool and (b) of the DNA pool. (c) Based on OTUs representing the NOB of the RNA pool and (d) of the DNA pool. Samples from one sampling site are marked by circles as follows: FR (black), BR (dotted), MA (broken line).

	Nsm. aestuarii (98%)	*Nsm. cryotolerans* (97%)	*Nsm.* sp. NS20 (98%)	*Nssc. oceani* (92%)	*Nssp.* spp. (95-99%)	*Nsm. ureae* (98%)	*Nsm.* sp. Nm59 (97%)	*Nsm. communis* Nm2 (96%)	*Nsm.* sp. Is79A3 (98%)	*Nsm.* sp. AL212 (98%)	*Ns. marina* Nb-295 (98%)	*Ns.* sp. Ecomares 2.1 (98%)	Cand. *Ns. salsa* clone Cb18 (96-99%)	*Npn.* sp. clone Cb12 (94%)	*Ns. defluvii* (99-100%)	Cand. *Ns. inopinata* ENR4 (94-99%)	*Ns. moscoviensis* SBR1024 (96-99%)	Cand. *Nt. arctica* (98-99%)
C_R	0.06	0.00	0.06	0.00	0.16	0.02	0.57	99.14	0.00	0.00	0.00	0.00	0.06	0.00	99.89	0.00	0.05	0.00
C_D	0.02	0.00	0.01	0.00	0.03	0.00	0.01	99.94	0.00	0.00	0.00	0.00	0.05	0.00	99.88	0.00	0.07	0.00
D_R	0.08	0.00	0.03	0.00	0.00	0.06	0.10	99.72	0.00	0.00	0.00	0.00	0.08	0.00	99.76	0.01	0.15	0.00
D_D	0.03	0.00	0.00	0.00	0.00	0.00	0.00	99.97	0.00	0.00	0.00	0.00	0.19	0.00	99.53	0.00	0.28	0.00
F_R	0.20	0.00	0.30	0.65	0.00	0.50	0.75	97.61	0.00	0.00	0.01	0.00	0.18	0.00	99.59	0.07	0.16	0.00
F_D	0.50	0.00	0.17	0.00	0.00	0.17	0.08	99.09	0.00	0.00	0.00	0.00	0.97	0.00	98.58	0.00	0.44	0.00
AQ1_R	0.22	0.00	0.23	7.49	0.19	59.68	31.64	0.14	0.03	0.38	0.15	0.44	0.08	0.00	97.07	0.77	1.49	0.00
AQ1_D	0.52	0.00	0.21	18.44	0.28	53.81	26.19	0.25	0.00	0.31	0.12	0.52	0.17	0.00	95.48	1.13	2.59	0.00
GMA_R	0.21	0.00	0.79	4.40	0.23	93.85	0.27	0.20	0.00	0.06	0.17	0.09	0.09	0.00	97.42	0.63	1.57	0.03
GMA_D	0.51	0.00	1.01	15.62	0.15	81.40	0.20	1.11	0.00	0.00	0.08	0.48	0.40	0.00	95.97	1.44	1.62	0.00
RMB_R	0.15	0.00	0.44	50.28	0.10	35.05	13.80	0.17	0.00	0.02	1.25	1.96	0.50	0.00	29.20	26.55	40.54	0.01
RMB_D	3.17	0.00	0.67	55.55	0.16	34.35	5.35	0.63	0.00	0.12	1.99	4.80	2.12	0.00	16.98	32.80	41.31	0.00
M1_R	62.13	0.01	36.90	0.29	0.65	0.00	0.00	0.01	0.01	0.00	0.00	0.00	99.98	0.01	0.01	0.00	0.00	0.00
M1_D	77.57	0.02	21.69	0.10	0.42	0.00	0.00	0.01	0.19	0.00	0.00	0.00	99.96	0.01	0.04	0.00	0.00	0.00
M3_R	57.36	0.01	42.07	0.00	0.47	0.01	0.00	0.01	0.08	0.00	0.00	0.00	99.99	0.00	0.01	0.00	0.00	0.00
M3_D	66.13	0.04	32.28	0.00	0.80	0.00	0.00	0.04	0.69	0.00	0.00	0.00	99.98	0.00	0.02	0.00	0.00	0.00
M5_R	61.04	0.05	38.13	0.00	0.68	0.02	0.00	0.01	0.06	0.01	0.00	0.00	99.98	0.00	0.02	0.00	0.00	0.00
M5_D	63.87	0.01	35.57	0.01	0.47	0.00	0.00	0.06	0.01	0.00	0.00	0.00	99.91	0.02	0.06	0.01	0.00	0.00
	AOB										NOB							

Figure 10: Distribution of AOB and NOB in the RNA (R) and DNA (D) pools of the RAS derived samples after 16S rRNA amplicon sequencing illustrated as heatmap. OTUs with the same closest taxonomically described species were grouped, resulting in (left) ten AOB and (right) eight NOB groups. Similarities of the 16S rRNA gene to the closest known species are depicted in brackets. The numbers in the graphic indicate the relative abundance in percent of OTUs affiliated with the respective group of AOB or NOB, in green being the most and in red the less frequently observed group.

In the brackish samples most OTUs were assigned to uncultured nitrite oxidizing bacteria with *Ns. defluvii*, *Ns. inopinata* and *Ns. moscoviensis* as the closest relatives. These three groups were differently distributed among the three investigated brackish biofilter samples, whereat *Ns. defluvii*-like NOB revealed >95 % of the total NOB reads in AQ1 and GMA but maximum 29 % in RMB. As a consequence, the contribution of *Ns. moscoviensis*- and *Ns. inopinata*-like NOB was higher in RMB (40 % and 26 %). Besides the three strongly represented groups, relatives of marine *Nitrospira* such as *Ns. marina* Nb-295, *Ns.* sp. Ecomares 2.1 and *Ns. salsa* build the subdominant groups in the brackish samples, whereat the contribution of these marine groups was slightly higher in RMB. In the RNA pool few reads of cold-adapted NOB Cand. *Nitrotoga arctica* were detected (maximum 0.03 %).

In the marine samples >99 % of the total NOB reads were assigned to uncultured *Nitrospira* bacteria with *Ns. salsa* as the closest relative. Among the subdominant groups, uncultured bacteria with *Nitrospina* sp. as well as limnic *Ns. defluvii* as closest described species appeared in some samples.

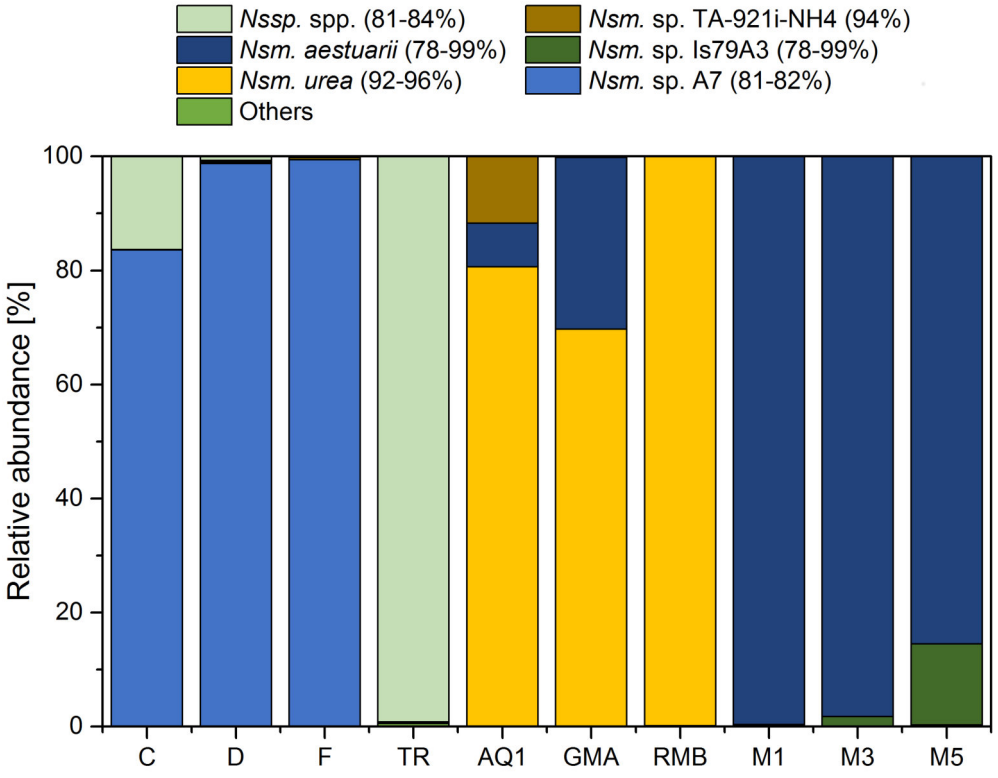
In general, the other known NOB genera *Nitrotoga*, *Nitrospina*, *Nitrolancea* and *Nitrobacter* were either not identified or in case of the former two with very few reads (<0.05 %) and with occasional appearance only.

4.2 Amplicon sequencing of phylogenetic marker genes *amoA* and *nxrB*

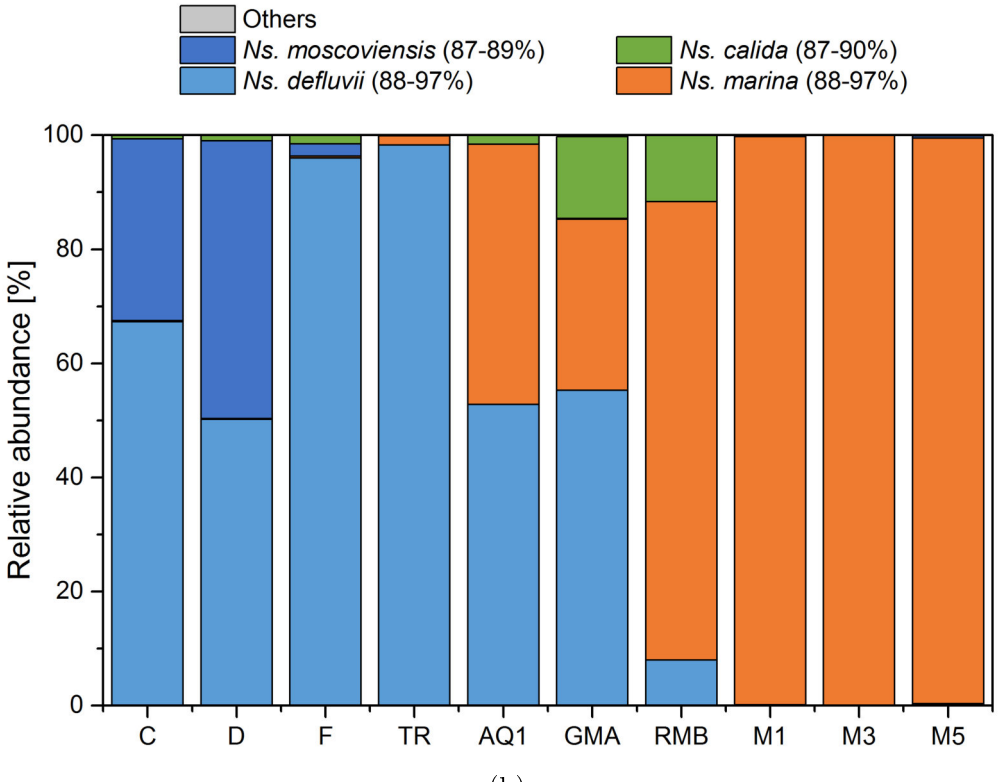
In addition to the phylogenetic analysis based on the 16S rRNA gene, an amplicon sequencing approach was chosen targeting phylogenetic and functional genes for ammonia oxidation (*amoA*) of β-proteobacterial AOB and nitrite oxidation (*nxrB*) of *Nitrospira*-like NOB (described in section 3.7.1). Initial screening PCR showed that archaeal *amoA* amplification resulted in weak PCR products or no product at all (data not shown). Hence, focus was set on the study of bacterial *amoA* genes. Samples for this approach were taken as following: FR: C, D, F, TR; BR: AQ1, GMA, RMB; MA: M1, M3, M5. In total 3291-84655 *amoA* gene sequences were assigned to 7-35 OTUs and 1274-17902 *nxrB* gene sequences assigned to 10-43 OTUs (table 9). The vast majority of OTUs matched with environmental clones of β-proteobacterial AOB and *Nitrospira*-like NOB originally identified in different habitats, revealing that these RAS derived biofilms harbor several potentially novel nitrifying species. For simplification, OTUs sharing the same closest taxonomically described species but with different similarities were grouped. Fig. 11 shows the distribution of AOB and *Nitrospira*-like NOB identified in the different samples. A habitat specific AOB and NOB community was found, as described in the following paragraphs.

4.2.1 AOB diversity

In samples C, D and F derived from the freshwater RAS, 15 OTUs were identified in total. The majority of reads (83–99 % of the total reads) was assigned to environmental clones with



(a)



(b)

Figure 11: Distribution of (a) β-proteobacterial AOB and (b) *Nitrospira*-like NOB in the biofilter samples derived from three RAS (FR: C, D, F, TR; BR: AQ1, GMA, RMB; MA: M1, M3, M5) after *amoA* gene and *nxrB* gene amplicon sequencing using Illumina MiSeq. Note that OTUs assigned to the same closest taxonomically described species but with different similarities to the *amoA* and *nxrB* gene, respectively, were grouped. Similarities are depicted in brackets.

Table 9: Number of reads and number of OTUs obtained after amplicon sequencing of *amoA* and *nxrB* genes by using Illumina MiSeq. The ten samples were derived from biofilters of three RAS (FR: C, D, F, TR; BR: AQ1, GMA, RMB; MA: M1, M3, M5).

	amoA		*nxrB*	
	# reads	# OTUs	# reads	# OTUs
C	84,655	15	6,848	23
D	67,766	15	13,084	28
F	50,613	8	3,794	16
TR	24,635	11	6,130	26
AQ	56,277	18	1,274	16
GMA	20,269	12	16,769	43
RMB	3,291	7	17,902	31
M1	72,658	24	5,615	10
M3	50,627	31	12,911	10
M5	54,548	35	13,201	13

Nitrosomonas sp. A7 as the closest relative (81–82 % similarity to the *amoA* gene). Sample origins of the environmental clones were mainly freshwater aquaria, wastewater treatment reactors as well as lake sediments. A second group of environmental clones was found in the freshwater samples with *Nitrosospira* spp. as closest relatives (81–82 % similarity; all members of the lineage *Nitrosospira* were merged and defined as *Nitrosospira*-like AOB). The environmental clones have been originally derived from different freshwater and brackish habitats such as rivers, lakes and estuary sediments. *Nitrosospira*-like AOB constituted 0.2–0.4 % in D and F, and even 16 % in C. In sample TR, which was stocked with rainbow trout and operated at a colder temperature compared to the other modules, the vast majority of reads was affiliated with *Nitrosospira*-like AOB (>99 %).

In the brackish samples most of the 18 OTUs in total clustered with *Nitrosomonas urea* as closest relative (92–96 % similarity), contributing to 70–99 %. The different *Nsm. urea*-like environmental clones have been originally derived from habitats such as river sediments and estuary water. In addition, few OTUs affiliated with environmental clones, which showed *Nsm. aestuarii* isolate SF_AOB_C09 or *Nsm.* sp. TA-921i-NH4 as closest phylotypes, were found in the brackish samples. Compared to samples AQ1 and GMA, RMB revealed less reads in total and only seven OTUs, which had *Nsm. urea* Nm10 as closest relative.

With 35 OTUs in total, the marine biofilter samples revealed the highest number of OTUs, while most of the reads (98–99 %) were assigned to different environmental clones with *Nsm. aestuarii* isolate SF_AOB_C09 as the closest related AOB species (93–99 % similarity). These environmental clones have been originally isolated from salt-water aquaria, salt-marsh sediment or aquaculture wastewater. Unlike the 16S rRNA amplicon analysis, no sequences of *Nitrosospira*-like AOB *amoA* genes were identified in the marine samples. In sample M5 six unique OTUs were found and affiliated with an aquarium biofilter derived environmental clone. The closest relative of this clone was *Nsm.* sp. Is79A3 (78 % similarity), which has been origi-

nally isolated from a freshwater lake (Accession: CP002876.1).

4.2.2 *Nitrospira* diversity

In the freshwater samples 28 OTUs were identified in total and the majority of reads (50–95 % of the total reads) was assigned to environmental clone *Nitrospira* sp. HKA-B3, which originates from a main municipal WWTP (Pester *et al.* 2014). The closest taxonomically described species was *Ns. defluvii* with sequence similarities to the *nxrB* gene ranging from 93–97 %. 31 % of all NOB reads in sample C, 48 % in sample D and 2 % in sample F were found to belong to relatives of *Ns. moscoviennsis* (88 % similarity). Sample TR, which was derived from the trout module, revealed 26 OTUs, all belonging to *Ns. defluvii*-like environmental clones.

With up to 43 *nxrB* OTUs, the three brackish biofilms revealed the highest microdiversity of nitrite oxidizing bacteria. In samples GMA and AQ1 two major groups were identified in the ratio of 1:1, which consisted of environmental clones with either *Nitrospira marina* or *Nitrospira defluvii* as closest relatives. The similarities to the *nxrB* gene ranged for both from 89–97 %. Besides these, few reads of two further groups were found, which belong to relatives of *Ns. moscoviensis* NSP M-1 and to environmental clones originating from Gorjachinsk geothermal spring with *Ns. calida* as closest described species. The latter group constituted 15 % of the total reads in GMA. Compared to the other brackish samples, the distribution of OTUs was slightly different in RMB. Hence, the majority of reads in RMB (80 %) was assigned to *Ns. marina*-like nitrite oxidizers, 11 % and 9 % to environmental clones with *Ns. calida* or *Ns. defluvii* as closest relatives, respectively.

The three marine samples revealed 13 OTUs in total, while the vast majority of reads (>99 %) was assigned to *Ns.* marina strain ATCC 43039 (Pester *et al.* 2014) with similarities ranging from 88–89 %. Only very few reads (<0.3 %) were found in the three samples assigned to others such as *Ns.* Ecomares 2.1 or relatives of *Ns. defluvii*.

4.3 Metagenomic analysis

In order to compare microbial structure and potential functions in the biofilms, shotgun metagenomic sequencing using Illumina NextSeq was applied on three samples derived from biofilters at a freshwater (FR), brackish (BR) and marine (MA) RAS as described in section 3.7.2. For characteristics of the sampled modules at the three RAS refer to section 3.1. The metagenomic data were employed (1) to estimate the phylogenetic diversity via marker genes, (2) to investigate the occurrence and abundance of key enzymes of nitrogen removal and carbon breakdown, (3) to elucidate the potential functions of dominant taxa in nitrogen removal processes and (4) to find evidence of complete ammonia oxidizers (comammox) in the biofilms. Before starting with the comprehensive analysis, data were pre-screened by using the SILVA NGS platform and taxonomic profiling on the basis of 16S rRNA genes indicated that archaea might be under-represented in the biofilms (data not shown). Therefore, all downstream analyzes were restricted to the Bacteria domain. Processing and downstream analysis of generated metagenomic data sets is described in section 3.9.

Table 10: Statistics report of assembly and gene prediction of the metagenomic data sets after sequencing on Illumina NextSeq. Samples were derived from biofilters at a freshwater, brackish and marine RAS.

	FR	BR	MA
Assembly statistics			
# contigs (bp)	2,004,409	3,115,645	1,499,160
Largest contig (bp)	378,311	165,440	466,515
Total bases assembled (bp)	1,874,257,931	2,541,572,205	1,479,983,842
N50 contig size (bp)	1,312	1,054	1,533
GC (%)	55.3	55.5	52.0
Gene prediction			
# ORFs	3,107,831	4,440,847	2,378,766
# Full length ORFs	720,726	846,301	593,653

4.3.1 Assembly and gene prediction

De novo assembly via MEGAHIT resulted in 1.9 Gb (FR), 2.5 Gb (BR) and 1.5 Gb (MA) assembled contigs, producing 720,726 (FR), 856,302 (BR) and 593,653 (MA) full length ORFs. The quality of the provided assemblies was estimated through Assemblathon 2 for a general statistics estimation (see table 10 for assembly and gene prediction statistics).

4.3.2 Phylogenetic analysis by using marker genes

The assembled metagenomic data sets were used to estimate the bacterial phylogenetic diversity. This was done by using the software AMPHORA2 which uses 31 genes of conserved bacterial proteins as phylogenetic markers (described in section 3.9.3). In total 14360 (FR), 18429 (BR) and 11474 (MA) marker genes were identified in the three samples. The classification revealed that the majority of marker genes was affiliated with *Proteobacteria* (FR: 46 %, BR: 44 %, MA: 47 %), followed by *Bacteroidetes* (FR: 17 %, BR: 20 %, MA: 22 %) and *Planctomycetes* (FR: 6 %, BR: 10 %, MA: 12 %). *Verrucomicrobia*, *Actinobacteria*, *Acidobacteria* and *Chloroflexi* each contributed to 2–4 %. Members of *Nitrospirae* phylum represented 1.3–2.7 % of the sequences in all three systems. Fig. 12 shows the phylogenetic assessment on phylum level based on marker gene analysis for the three samples.

4.3.3 Presence and abundance of predicted functional enzymes based on HMMs

Due to the higher proportion of heterotrophic than autotrophic nitrifying bacteria found in the biofilms via 16S rRNA amplicon sequencing, it was of interest to quantify the enzymes responsible for autotrophic nitrification and heterotrophic denitrification as well as biomass degradation in the metagenomic data sets. To address this question, the assembled metagenomic data were analyzed with respect to the predicted functional enzymes involved in nitrification (ammonia monooxygenase: AMO; hydroxylamine reductase: HAO; nitrite oxidoreductase: NXR), denitrification (nitrate reductase: NAR; nitrite reductase: NIR; nitrous oxide reductase: NOSZ;

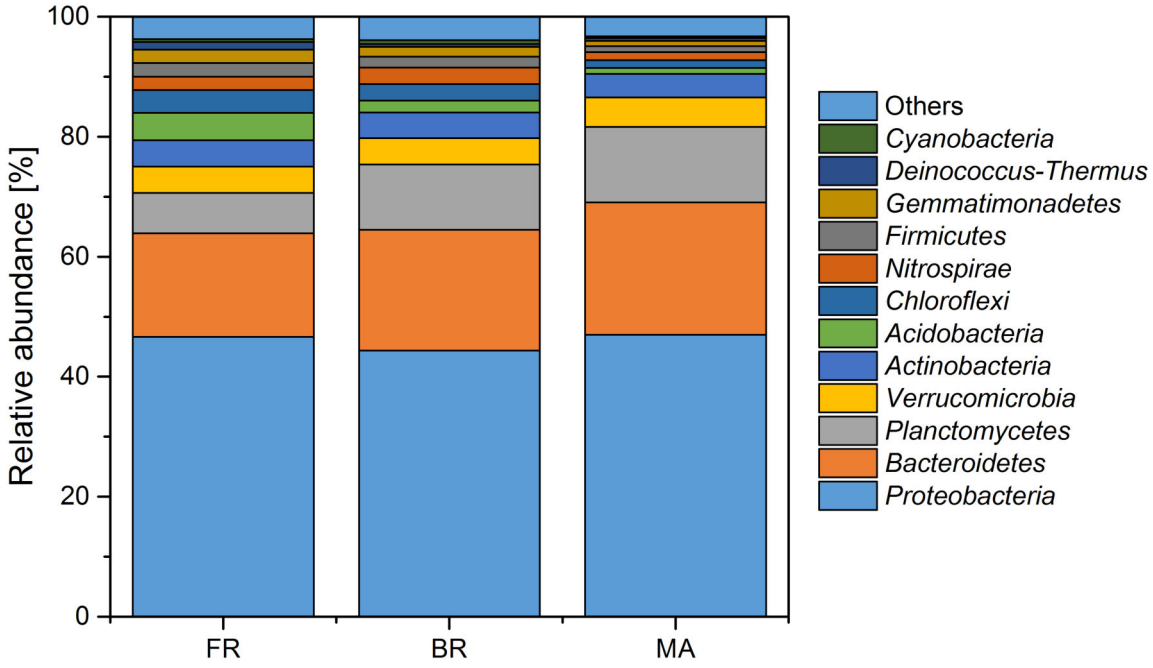


Figure 12: Phylogenetic assessment of the three assembled metagenomic data sets based on AMPHORA2 marker gene analysis. The bars show the marker gene affiliation with bacterial phyla of the samples FR, BR, MA. Others: phyla represented by <1 % of the total marker gene sequences.

nitric oxide reductase: NOR) and organic matter degradation (glycoside hydrolase: GH). For this approach, available profile hidden Markov models (HMMs) covering the respective enzyme families were used as described in section 3.9.4. See fig. 13 for HMMs used for the detection of specific putative enzymes.

Putative genes encoding for nitrification and denitrification enzymes were identified within the predicted genes via the HMM-based approach, whereat most HMMs were found with <50 hits in all three samples (fig. 13a). Regarding to the ammonia oxidation as the first step in nitrification, the total number of AMO hits was 18 (FR), 19 (BR) and 28 (MA) (summarized hits of AMO enzyme family related HMMs). Archaeal AMO was not identified via HMM-based search in the samples (HMM 'amoA_Arch'). For HAO, as the second enzyme in ammonia oxidation, the used HMMs ('Cytochrome_C554', 'Multi-haem_cyto') predicted a higher abundance than for AMO with 64 (FR), 96 (BR) and 117 (MA) hits. For nitrite oxidation as second step in nitrification, few hits were found for HMM 'nxrB' (FR: 14; BR: 12; MA: 15). In contrast, HMMs for the molybdopterine dinucleotide binding domain and molybdopterine oxidoreductase ('molydop_ binding' and 'molybdopterine'), which have been additionally used for the detection of potential genes encoding for NXR, were found with >100 and >250 hits. These putative genes encoding for NXR were more abundant in FR and MA compared to BR (fig. 13a). However, it has to be taken into account that these HMMs match protein families which contain enzymes involved in several distinct metabolic pathways and not only nitrification, but also denitrification as well as nitrate and nitrite ammonification and therefore, they were not considered for quantification of genes encoding for nitrite oxidoreductase enzymes involved in

nitrification.

All HMMs for denitrification enzymes matched with the three samples, but in low abundances. The HMM for nitrite reductase ('nirA') revealed the highest abundance in all three samples (about 50 hits).

Since glycoside hydrolase enzyme families encompass a large set of enzymes involved in breakdown or modification of polysaccharides, HMMs for different GH enzyme families, according to CAZy database, were used as marker for carbohydrate degradation. Within the set of used HMMs matching different GHs most hits were found for GH23 (>350), GH3 (>200), GH43 and GH5 (>50), indicating a high abundance of heterotrophic bacteria involved in carbon degradation and turnover in the biofilms (fig. 13b).

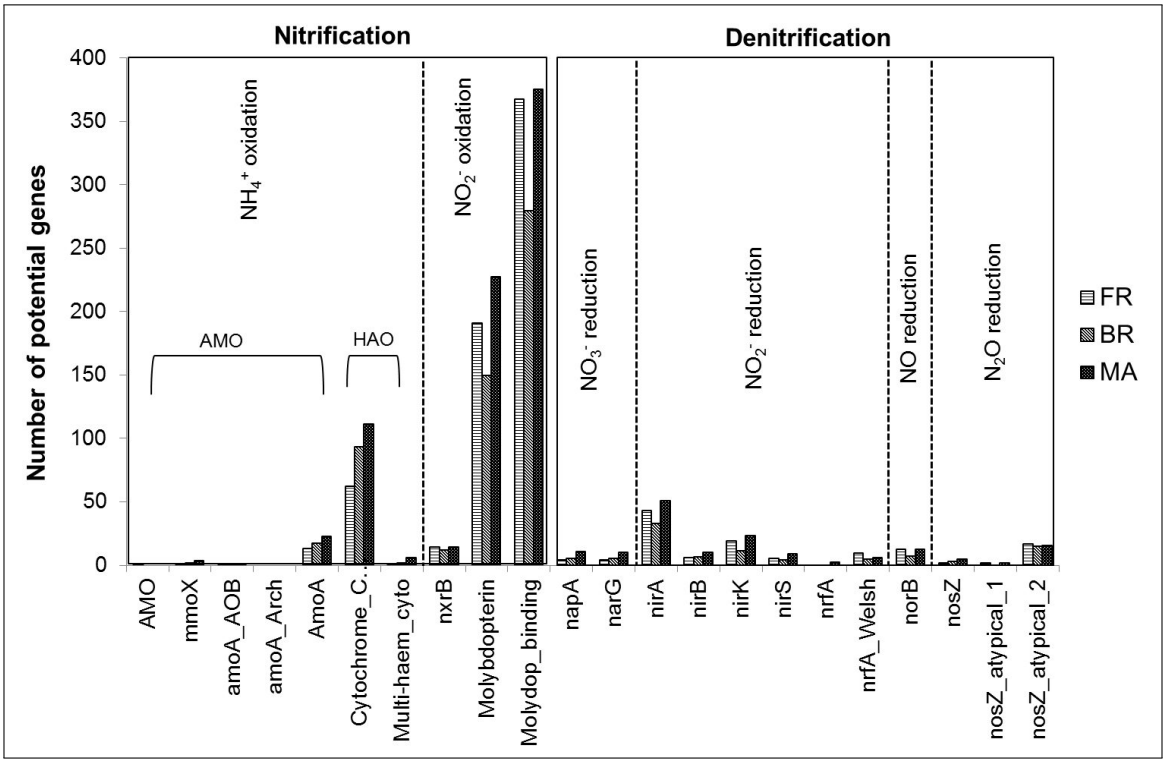
4.3.4 Analysis of functional genes related to nitrogen removal

Subsequently to the HMM-based search for predicted functional enzymes involved in nitrogen removal, the respective ORFs with hits to putative genes with relevance for nitrification and denitrification were further analyzed by comparing with NCBI non-redundant (nr) database (described in section 3.9.4). KEGG pathway reconstruction resulted in 301 (FR), 308 (BR) and 264 (MA) putative nitrogen metabolism enzymes (table 11). For nitrification, the abundance of mapped *amo* sequences was relatively low in the three samples (<1 % of all N-removal genes). In addition, the *hao* gene was only slightly more abundant than *amo* in all samples (<3 %). *Nxr* was not identified in the automatically annotated set of N-metabolism genes via KEGG classification. For denitrification, the abundance of several functional genes, such as *nar*, *nir* and *nor* was similar in all three samples and higher than that of the nitrification related genes.

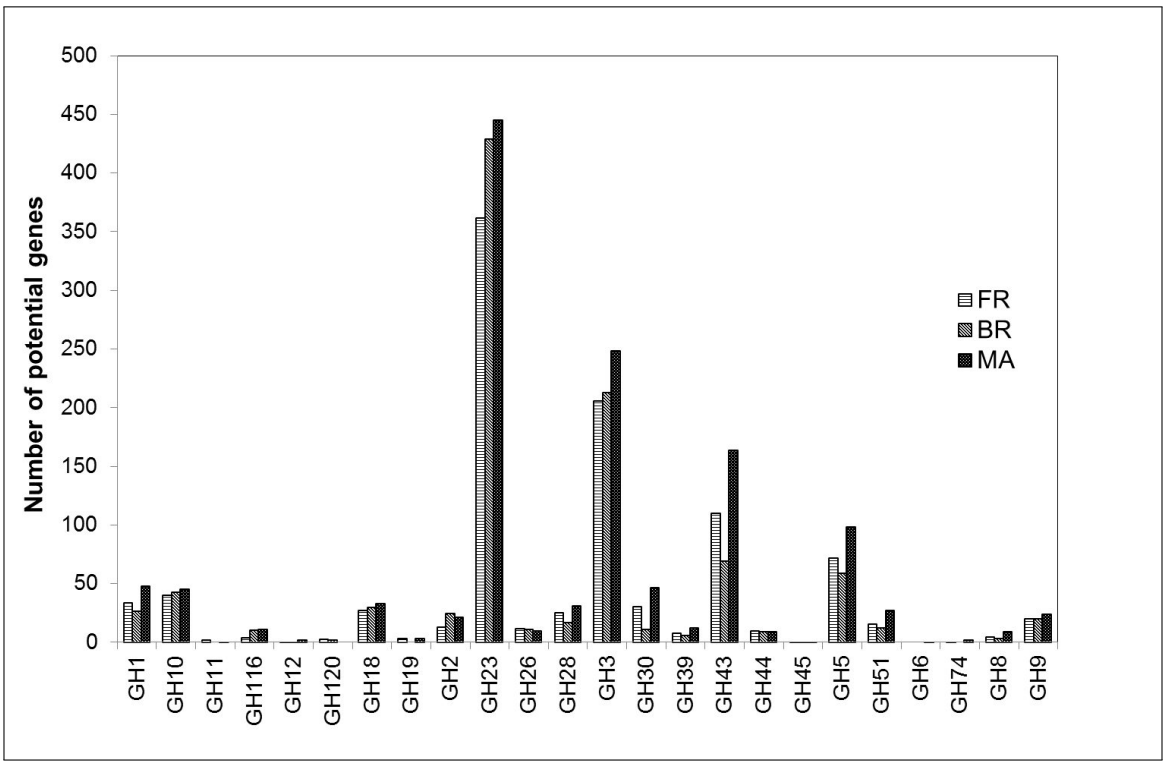
Furthermore, the ORF annotation revealed that *Proteobacteria* was the predominant phylum in all three samples, followed by *Planctomycetes*, *Bacteroidetes*, *Actinobacteria* and *Nitrospirae* (fig. 14a), indicating that members of these phyla are the key players in nitrogen removal processes in the biofilms. The genus level classification showed that *Nitrosomonas* and *Nitrospira* represented the known nitrifying genera, with the latter accounting for 11 % and 12 % in FR and BR, respectively, and for 2 % in MA. Several dominant known denitrifying taxa, such as *Rhodopirellula*, *Haliscomenobacter*, *Hyphomicrobium* and *Lewinella*, which have also been identified among the core-taxa via 16S rRNA amplicon sequencing in this study (see section 4.1.4), were found in all samples (fig. 14b).

4.3.5 Phylogenetic classification of GHs

ORFs with hits to putative genes encoding for GHs, as analyzed via HMM search, were compared with NCBI nr database for phylogenetic classification. This revealed that *Proteobacteria* and *Bacteroidetes* were the predominant phyla in the three samples, contributing to 30–44 % of the total GH sequences in each sample (fig. 15a). On genus level most sequences matched to different genera which were found with low abundance (<1 %), together accounting for approximately 62–74 % in each sample. Among the different genera e.g. *Nitrosomonas*, *Nitrospira*, *Lewinella* and *Gemmatimonas* contributed to >1 % of the total GH sequences (fig. 15b).

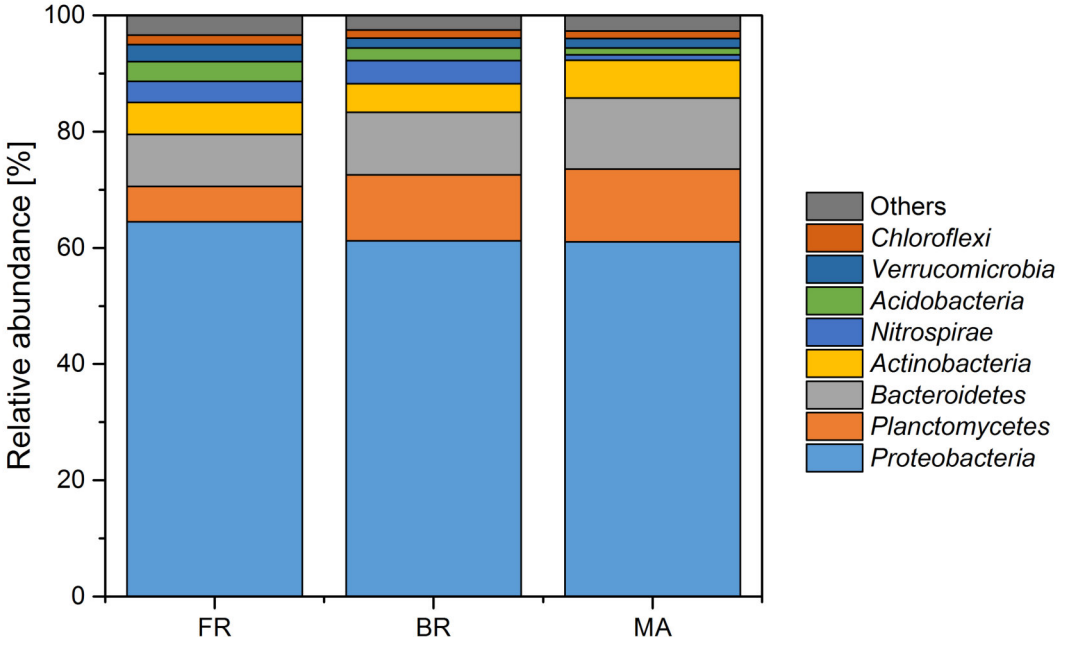


(a)

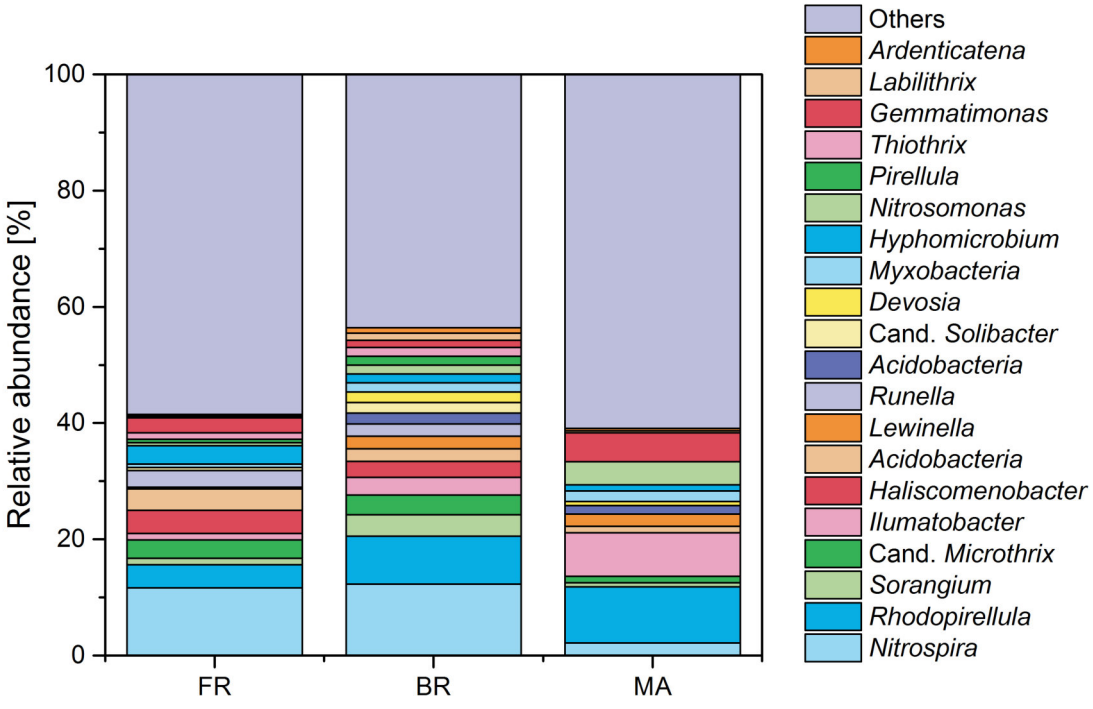


(b)

Figure 13: Abundance of potential enzymes involved in (a) nitrification, denitrification and (b) organic matter degradation via glycoside hydrolases in the metagenomic data sets of samples FR, BR and MA, assessed by using HMMs. The number of potential enzymes was normalized to 1 Gb of assembled DNA. Note that names of the different HMMs used are depicted in the y-axis.

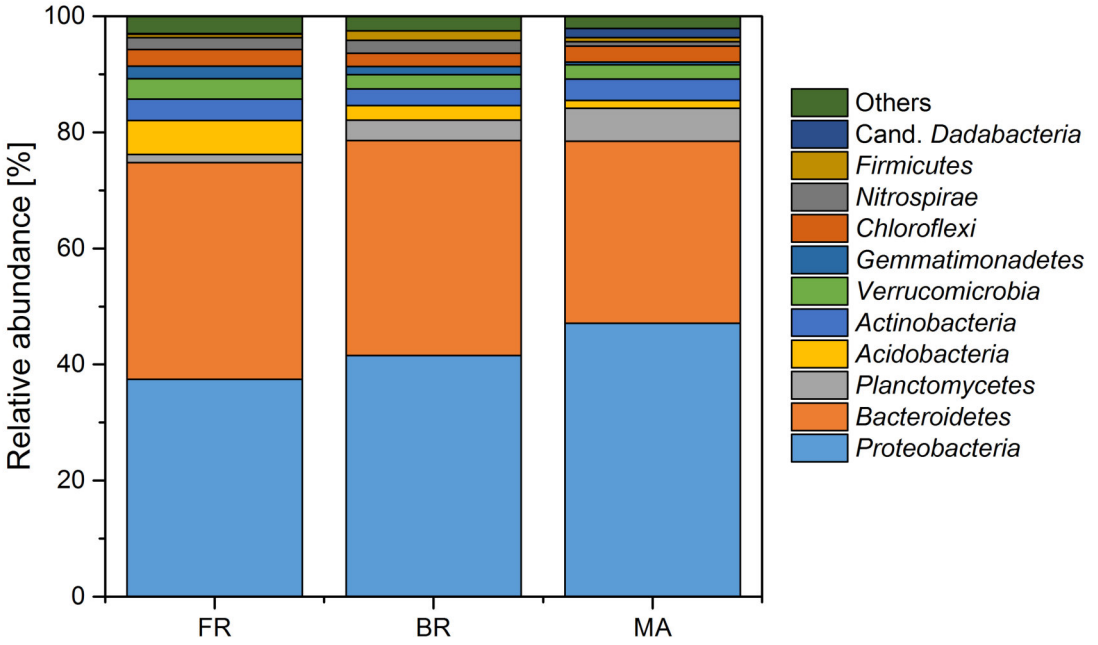


(a)

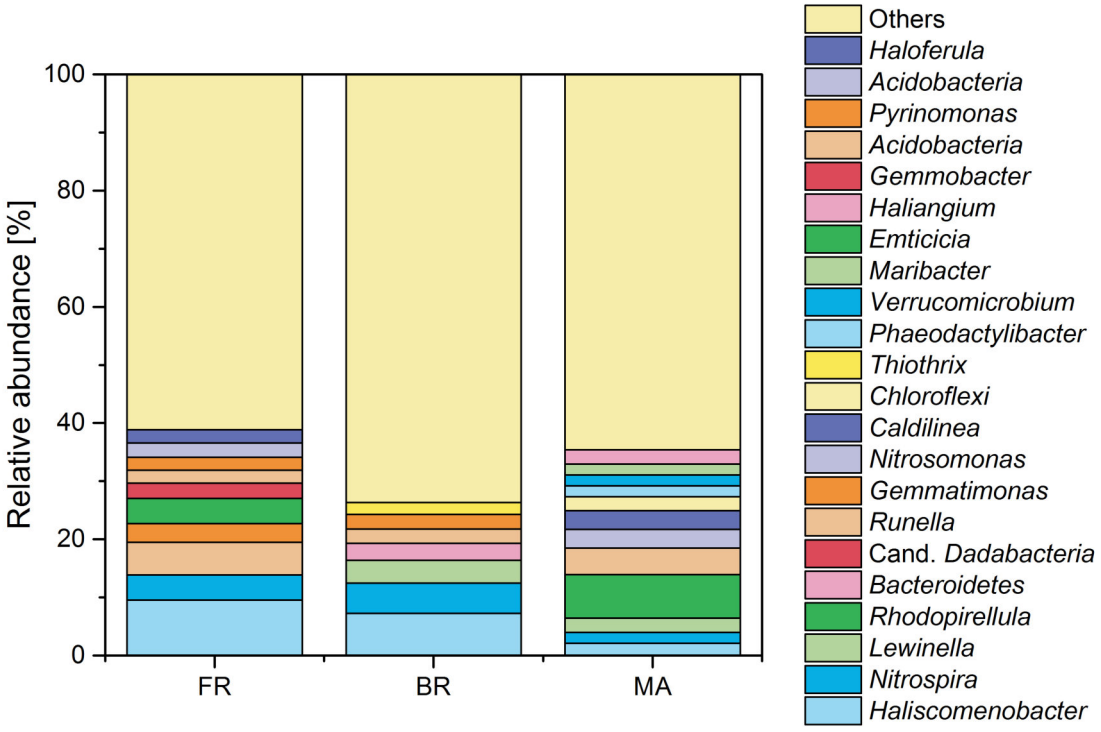


(b)

Figure 14: Relative abundance of (a) phyla and (b) genera identified in the freshwater, brackish and marine RAS biofilter samples, based on predicted ORFs related to nitrogen removal identified via HMM-based search. Taxonomic classification was done in MEGAN using the LCA algorithm. Others: phyla and genera, respectively, with low abundance (<1 %) in the data sets.



(a)



(b)

Figure 15: Relative abundance of (a) phyla and (b) genera identified in the freshwater, brackish and marine RAS biofilter samples, based on predicted ORFs encoding for different GHs identified via HMM-based search. Taxonomic classification was done in MEGAN using the LCA algorithm. Others: phyla and genera, respectively, with low abundance (<1 %) in the data sets.

Table 11: Number of metagenomic reads which mapped to enzymes involved in nitrogen removal in samples FR, BR and MA, analyzed via KEGG classification.

KEGG no.		FR	BR	MA
K1000910	**Nitrogen metabolism**	**301**	**308**	**264**
K10535	hydroxylamine dehydrogenase [EC:1.7.2.6]	2	5	7
K10944	methane/ammonia monooxygenase subunit A [EC:1.14.18.3 1.14.99.39]	3	1	1
K00372	assimilatory nitrate reductase catalytic subunit [EC:1.7.99.4]	100	91	76
K00376	nitrous-oxide reductase [EC:1.7.2.4]	40	49	31
K00371	nitrate reductase beta subunit [EC:1.7.99.4]	23	27	21
K00368	nitrite reductase (NO-forming) [EC:1.7.2.1]	31	24	33
K04561	nitric oxide reductase subunit B [EC:1.7.2.5]	24	19	19
K00362	nitrite reductase (NADH) large subunit [EC:1.7.1.15]	12	17	13
K00370	nitrate reductase alpha subunit [EC:1.7.99.4]	10	17	15
K02567	periplasmic nitrate reductase NapA [EC:1.7.99.4]	8	16	18
K00367	ferredoxin-nitrate reductase [EC:1.7.7.2]	13	15	9
K03385	nitrite reductase (cytochrome c-552) [EC:1.7.2.2]	18	14	12
K00366	ferredoxin-nitrite reductase [EC:1.7.7.1]	14	13	9

4.3.6 Metagenomic evidence for the presence of comammox

In order to investigate comammox in the biofilms, ORF annotation against NCBI nr database was performed and two putative comammox *amoA* genes were identified in the freshwater and the brackish biofilm (FR-ORF: k99_314926_2; BR-ORF: k99_2655270_12). In FR the level of similarity was 99 % to the corresponding *amoA* gene of NOB Cand. *Nitrospira nitrosa* and in BR 92 % to the *amoA* gene of Cand. *Ns. inopinata*. In addition, a phylogenetic tree was constructed, which shows the phylogenetic relationship between different *amoA* and *pmoA* genes (fig. 16). In FR two further ORFs were affiliated with *amoA* genes from AOB with 97 % similarity to the *amoA* gene of *Nitrosomonas* sp. (ORF: k99_6984_2; k99_894424_2). In contrast to FR and BR, in MA no commamox *amoA* gene was identified, instead one ORF was affiliated with the *amoA* gene of *Nitrosomonas* sp. with 97 % similarity (ORF: k99_1384322_1). In addition to the ORF-based search, contigs were screened against a characteristic set of published *Nitrospira*-like *amoA* nucleotide sequences using BLAST (Accession: LN885086.1, CZPZ01000032.1, CZQA00000000.1). In FR two reads mapped to the *amoA* gene sequence of *Ns. nitrosa* with 96 % and 80 % similarity. In BR nine reads were found mapping to the *amoA* gene sequence of *Ns. inopinata* with 81–97 % similarity. In accordance with the ORF-based search, no *Nitrospira*-like *amoA* gene was identified in MA. The results of the BLAST search are listed in table S5.

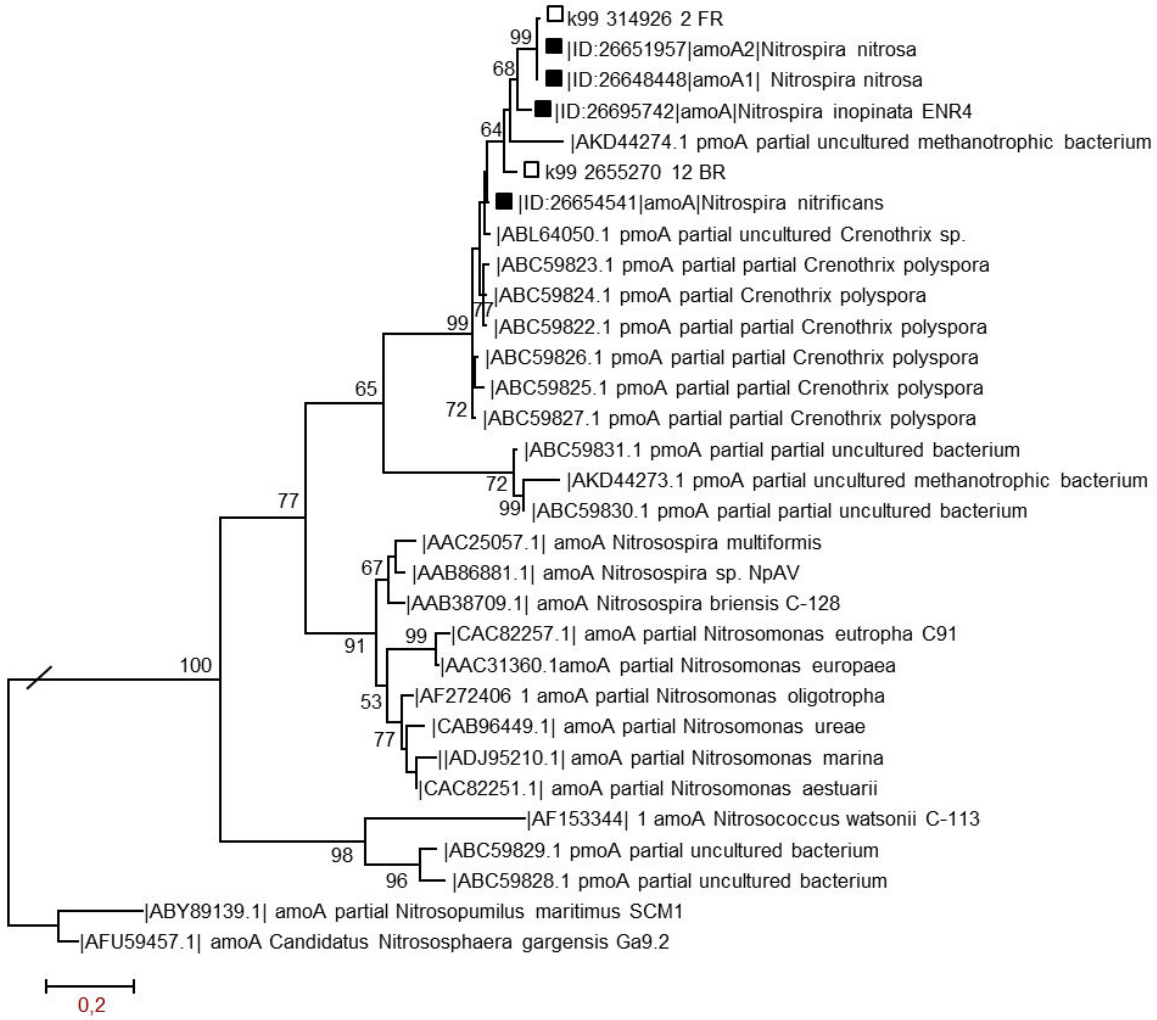


Figure 16: Phylogenetic tree of *amoA* and *pmoA* gene sequences. White squares indicate the putative *amoA* genes obtained from the metagenomic data set of samples FR and BR. Black squares indicate *Nitrospira*-like comammox *amoA* genes discovered by Daims *et al.* (2015) and van Kessel *et al.* (2015). Archaeal *amoA* gene sequence of *Nitrososphaera gargensis* was defined as outgroup. The phylogenetic tree was generated by the Maximum Likelihood method with 1000 bootstrap replications. Bootstrap values >50 % are indicated at the branches.

4.4 Visualization of biofilm inhabiting nitrifiers

To visualize cells within the biofilm, electron microscopy (EM) was performed (see section 3.6) with samples derived from the brackish and freshwater biofilters. The biofilm samples revealed microcolonies of *Nitrosomonas*-like AOB and *Nitrospira*-like NOB growing in close vicinity to each other, indicating their syntrophic association (fig. 17a). As visible in fig. 17a, two types of *Nitrosomonas*-like AOB were found in the brackish biofilm, with loosely arranged colonies and in contrast, tightly packed colonies surrounded by distinctly visible extracellular polymeric substances (EPS). Cells of *Nitrosomonas* showed the characteristic peripheral arrangement of intracytoplasmic membranes (ICM) (fig. 17b). *Nitrospira* microcolonies consisted of short, slightly curved cells, which were tightly packed in EPS. Black granular indicated polyphospate accumulations (fig. 17c). A second NOB was found in brackish sample GMA with few cells of *Nitrotoga*, identifiable due to their irregular shape and the extremely wide periplasmic space (fig. 17d). However, only very few, scattered *Nitrotoga*-like cells were detected via EM, while *Nitrospira* microcolonies were widely distributed in the biofilm of GMA. In the freshwater biofilter derived samples (C, D, F) a co-existence of *Nitrosomonas*- and *Nitrosospira*-like AOB was identified. In contrast, in freshwater module TR cells of *Nitrosospira*-like AOB were predominant in the biofilm and detected in high densities, strongly confirming the results of the *amoA* amplicon sequencing. Cells of *Nitrosospira* showed a slightly curved shape and a lack of ICMs (fig. 17e). Neither typical cells of other NOB genera such as *Nitrobacter* nor AOA were detected in the samples.

In addition to EM, specific molecular identification via FISH (see section 3.5.7) was done with biofilm samples derived from the brackish RAS (module GMA). FISH visualization revealed cells distributed across the biofilm matrix, which hybridized with *Nitrosomonas* as well as *Nitrospira* genus-specific probes (probe-mix NSO190-1225 and probe-mix Nspa712-662) (fig. 18a). Loosely arranged colonies of *Nitrosomonas*-like AOB were situated in close vicinity to cells of *Nitrospira*-like NOB, surrounded by heterotrophic microbes (fig. 18b). Cells of *Nitrospira*-like NOB were typically arranged in small, densely packed microcolonies (fig. 18c).

4.5 Determination of nitrification potential

To assess the nitrification performance in the biofilters, nitrification potential tests were carried out with biocarriers derived from modules C, D, F (FR), AQ1, GMA, RMB (BR) and M1, M3, M5 (MA), and substrate consumption was observed over eight hours (described in section 3.4.1). For correlation, the tests were performed soon after sampling for the NGS approaches. Nitrification potential was determined as the conversion of total ammonia nitrogen (TAN) mediated via AOB and of total nitrite nitrogen (NO_2-N) via NOB. The calculated turnover rates of TAN and NO_2-N are listed in table 12. In general, the TAN turnover rates were similar among the samples. In contrast, the NOB showed different oxidation activities and the highest NO_2-N turnover rates were found in marine samples M1 and M3. Module M5 was low stocked with fish during time of sampling and hence, very low residual nitrifying activities were

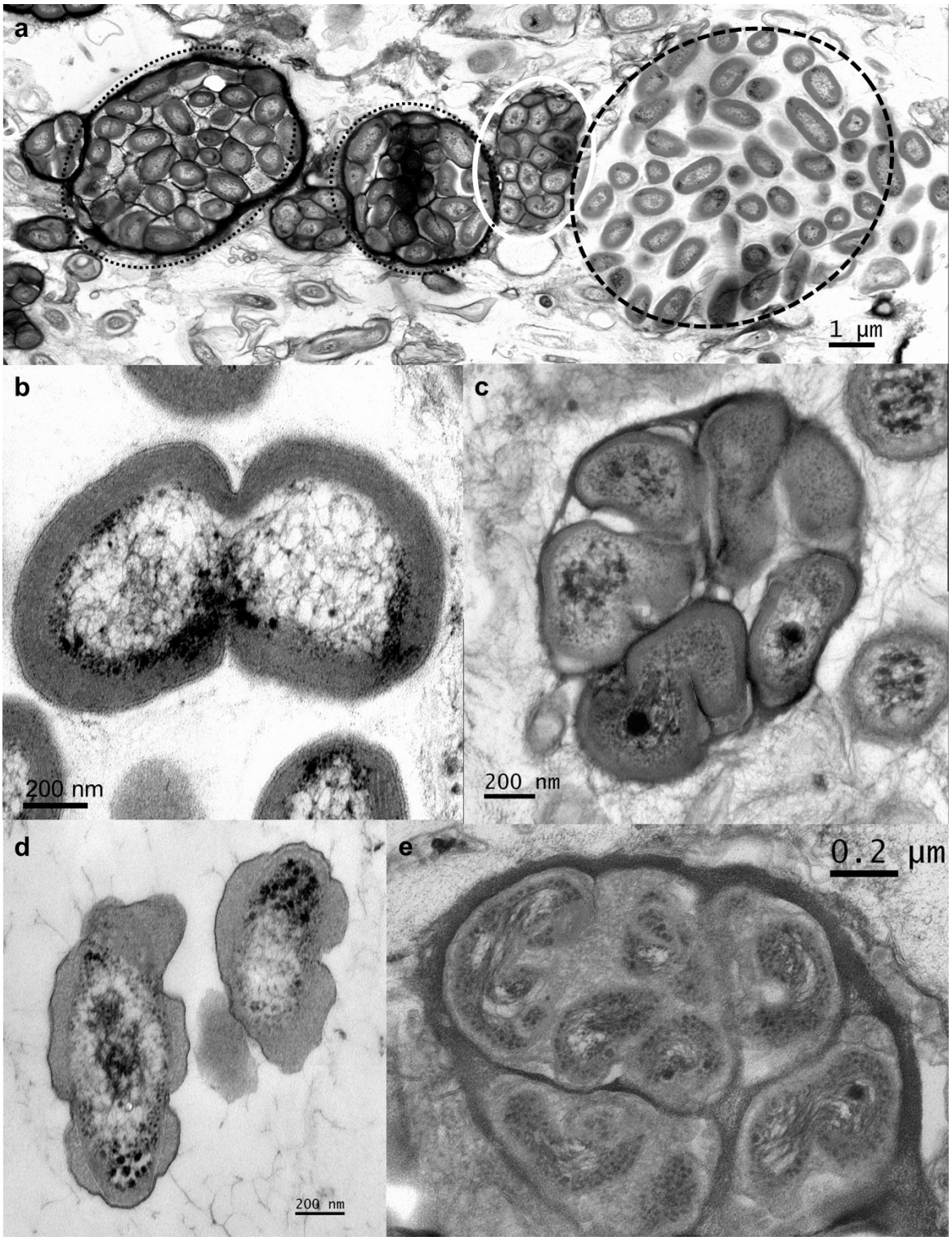
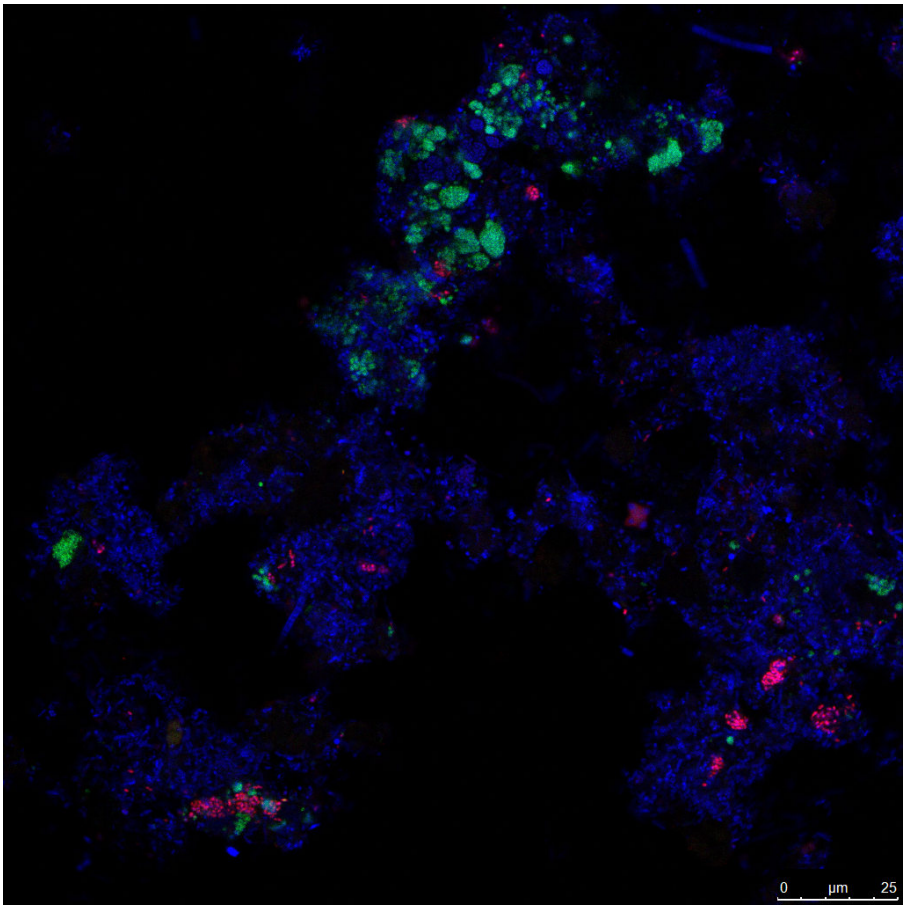


Figure 17: EM visualization of nitrifying biofilms derived from biocarriers. (a) Overview of the nitrifiers inside the biofilm. Two types of *Nitrosomonas*-like AOB were found (colonies marked with black circles, either broken or dotted line) in close vicinity to a microcolony of *Nitrospira* (white line). (b) Microcolony of *Nitrosomonas* showing the ICMs and (c) of *Nitrospira* in detail. (d) Typical ultrastructure of *Nitrotoga* cells, revealing an extremely wide periplasmic space. The biocarrier was derived from brackish module GMA. (e) Detailed section of typical *Nitrosospira* cells, identified in freshwater module TR.



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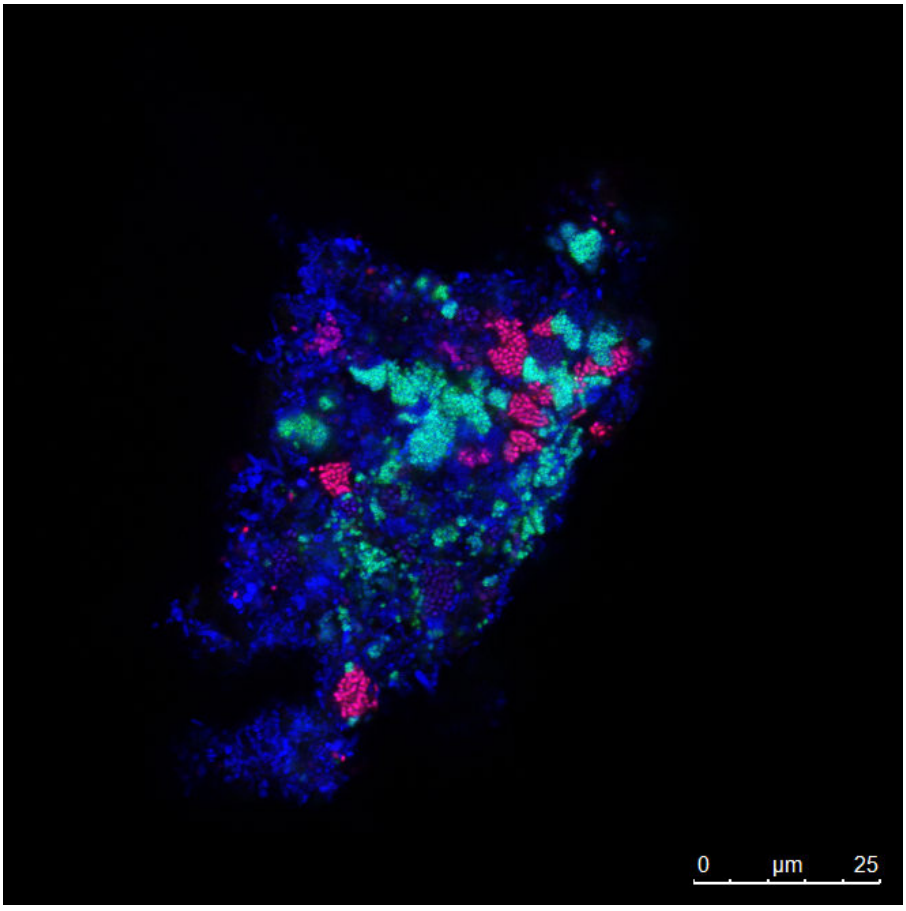

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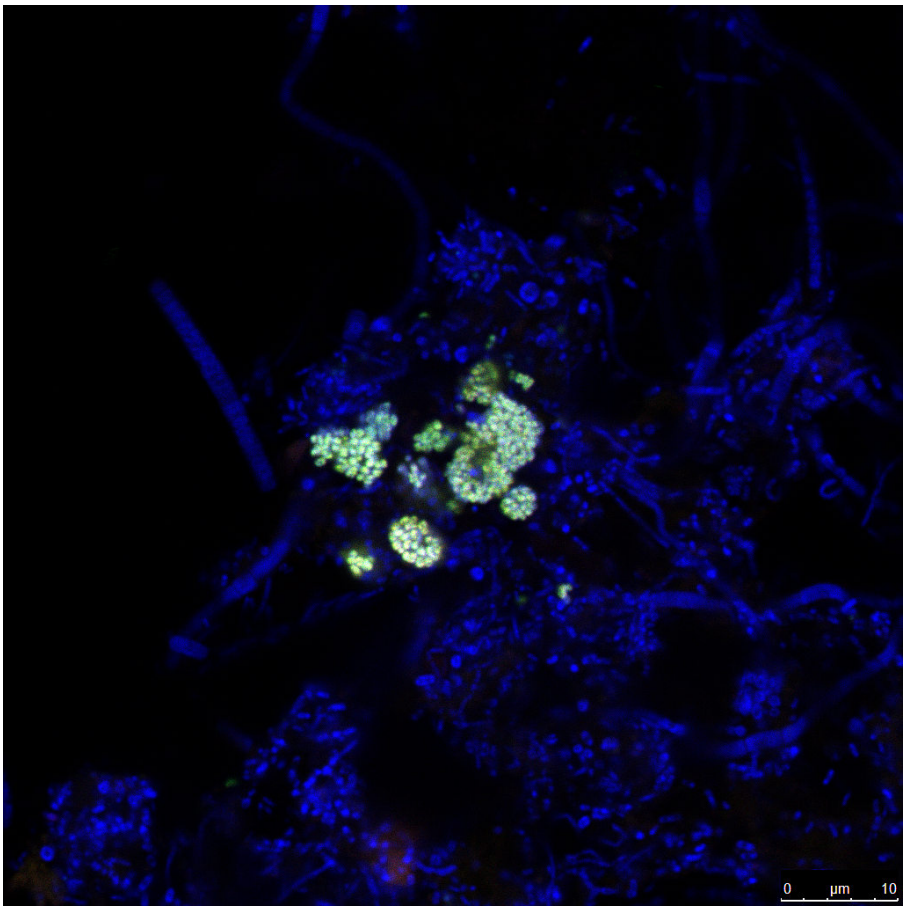


(c)

Figure 18: Confocal micrographs of FISH-stained cells within the biofilm matrix. The biofilm material was derived from brackish module GMA. AOB cells of the genus *Nitrosomonas* hybridized with probe-mix NSO190-1225-CY3 (pink), *Nitrospira*-like NOB with probe-mix Nspa712-662-FLUOS (green) and eubacterial cells with probe-mix EUB338-CY5 (blue). (a) Arrangement of *Nitrosomonas* and *Nitrospira* cells within the biofilm matrix, (b) colonies of *Nitrosomonas* and densely aggregated colonies of *Nitrospira*, (c) detail of *Nitrospira* micro-colonies.

observed. In the marine samples, the NOB activities were three to five times higher than that of the AOB. In the freshwater and brackish samples though, TAN and NO_2-N turnover rates were similar.

Table 12: Potential nitrification rates in the biofilters of different RAS. Tests were performed with biocarriers derived from modules C, D, F (FR), AQ1, GMA, RMB (BR) and M1, M3, M5 (MA). Turnover rates refer to the conversion of total ammonia nitrogen (TAN) via AOB and total nitrite nitrogen (NO_2-N) via NOB within eight hours.

	C	D	F	AQ1	GMA	RMB	M1	M3	M5
TAN [$g\,h^{-1}\,m^{-3}$]	5.4	4.8	3.3	5.5	5.0	2.3	5.9	4.4	0.2
NO_2-N [$g\,h^{-1}\,m^{-3}$]	7.0	6.2	2.9	4.0	5.8	3.5	15.5	14.1	1.1

4.6 Tolerance tests of biofilm inhabiting nitrifiers

Colonized biocarriers derived from biofilters of three different RAS were tested in batch-experiments regarding the influence of various substrate and nitrate concentrations as well as salt stress on the nitrifying activity. Tests were performed separately for AOB and NOB as described in section 3.4.2. For all tests, modules with a similar running time and a high stocking density were chosen. For detailed information about the RAS refer to section 3.1 and for mean physicochemical parameters see table S1 in the appendix.

4.6.1 Substrate tolerance test

To determine the tolerance towards the substrate concentration of nitrifying communities colonizing biocarriers from different RAS, substrate tolerance tests were performed. The influence of various substrate concentrations (0.5–25 mM) on the maximum ammonia and nitrite oxidation rate, respectively, was tested. Biocarriers were taken from biofilters of modules C (FR), GMA (BR) and M1 (MA).

AOB The test demonstrated that the tolerances towards the ammonia concentration differ among the AOB communities derived from the different RAS, as visible in fig. 19a, which shows the ammonia oxidation rates of the AOB communities in dependence on the substrate concentration. Freshwater sample C exhibited a rising ammonia oxidation activity with increasing substrate concentration and showed the maximum ammonia oxidation rate ($95\,\mu M\,h^{-1}$) when exposed to 10 mM substrate. However, in the presence of 25 mM the activity rapidly decreased by 80 % compared to the maximum oxidation rate. Brackish sample GMA showed a markedly higher sensitivity to elevated ammonium concentrations in comparison to the freshwater sample. The maximum ammonia oxidation rate ($38\,\mu M\,h^{-1}$) was observed in the presence of 2 mM substrate and with increasing ammonium concentration the oxidation rate decreased continuously. When exposed to 25 mM substrate, ammonia oxidation was no longer detectable. Marine sample M1 revealed a rising ammonia oxidation activity with increasing substrate concentration

and the maximum oxidation rate (216 µM h^{-1}) was reached when exposed to 5 mM substrate. Higher concentrations led to a slight decrease. However, still about 90 % of the maximum oxidation rate was maintained in the presence of 10 mM ammonium and thus, the difference was only minor. Comparable to the freshwater sample, in the presence of 25 mM the activity decreased by 70 %.

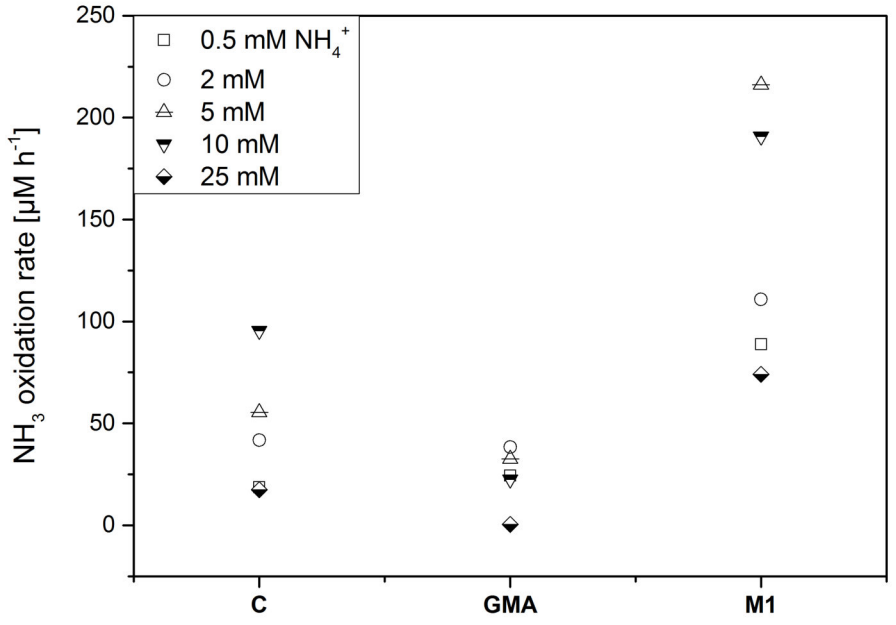
NOB The NOB populations revealed similar responses towards nitrite concentrations of up to 5 mM as depicted in fig. 19b, which shows the nitrite oxidation rates of NOB communities obtained in the presence of various nitrite concentrations. In freshwater sample C the maximum nitrite oxidation rate (36 µM h^{-1}) was observed at the batches supplied with 2 mM substrate. In brackish sample GMA as well as in marine sample M1 maximum nitrite oxidation (GMA: 31 µM h^{-1}; M1: 215 µM h^{-1}) was achieved in the presence of 5 mM nitrite. However, in all three samples the differences in the nitrite oxidation rates between the batches supplied with 2 mM and 5 mM substrate were only minor (5–10 % deviation). However, rising substrate concentrations caused a reduction in nitrite oxidation activity in all three samples and in the presence of 25 mM, the highest concentration tested, the activity decreased by 92 % in the freshwater, 71 % in the brackish and 73 % in the marine samples compared to the maximum oxidation rate. Hence, the brackish and marine NOB communities showed a slightly higher tolerance to high nitrite concentrations than the freshwater communities.

4.6.2 Nitrate tolerance test

To elucidate if nitrate, as the endproduct of nitrification, affects the nitrification performance in RAS biofilters, nitrate tolerance tests of AOB and NOB colonizing biocarriers derived from three different RAS were conducted via batch-experiments. Samples were taken as follows: FR: C, F; BR: GMA; MA: M1, and the impact of different nitrate concentrations (0.5–15 mM) on the ammonia and nitrite oxidation rate was analyzed for each of the samples. To address the question if denitrification reactors influence the nitrate tolerance of nitrifiers in biofilters, samples derived from freshwater modules C and F were tested with the latter module being connected to a denitrification reactor. Nitrate tolerance test of marine AOB was not performed during this survey.

AOB The AOB tolerance test showed that neither the freshwater nor the brackish communities were affected in the presence of nitrate. The ammonia oxidation rate did not decrease in dependence on increasing nitrate concentrations and the achieved oxidation rates remained similar in all batches. Thus, the AOB communities of the two different RAS seem to be highly tolerant to nitrate under the tested conditions. 1 mM ammonia was fully oxidized after 24 h in all batches (see fig. S4 in the appendix).

NOB In contrast to the AOB, the NOB tolerance test revealed a continuous reduction in the nitrite oxidation activity with rising nitrate concentrations starting from 0.5 mM in all samples. The initially added 1 mM nitrite was fully oxidized after 24 h of incubation in the control



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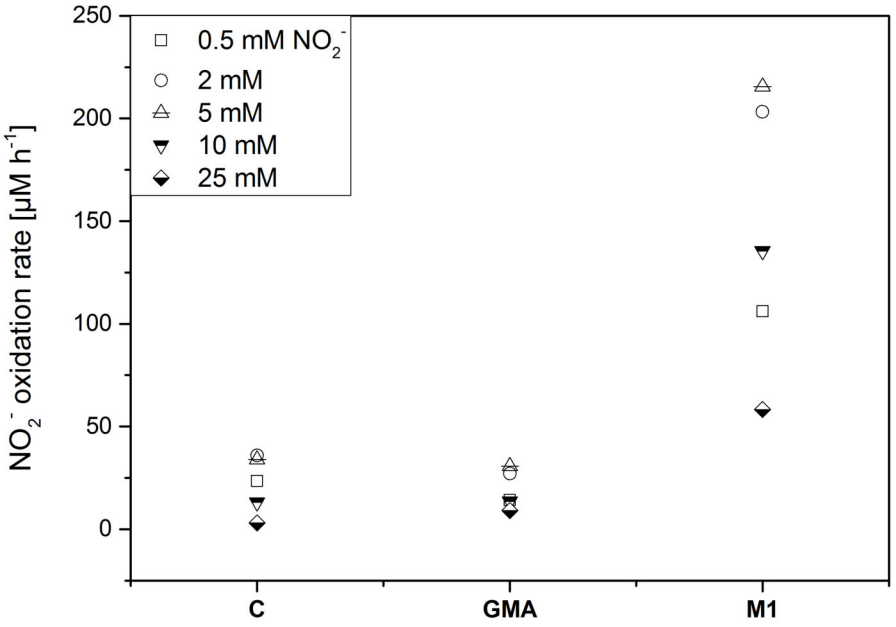


(b)

Figure 19: (a) Ammonia oxidation rates of AOB and (b) nitrite oxidation rates of NOB in dependence on various substrate concentrations. Tests were performed via batch-experiments inoculated with colonized biocarriers derived from different RAS. Oxidation rates were calculated as substrate consumption per hour during exponential growth phase. FR: C; BR: GMA; MA: M1.

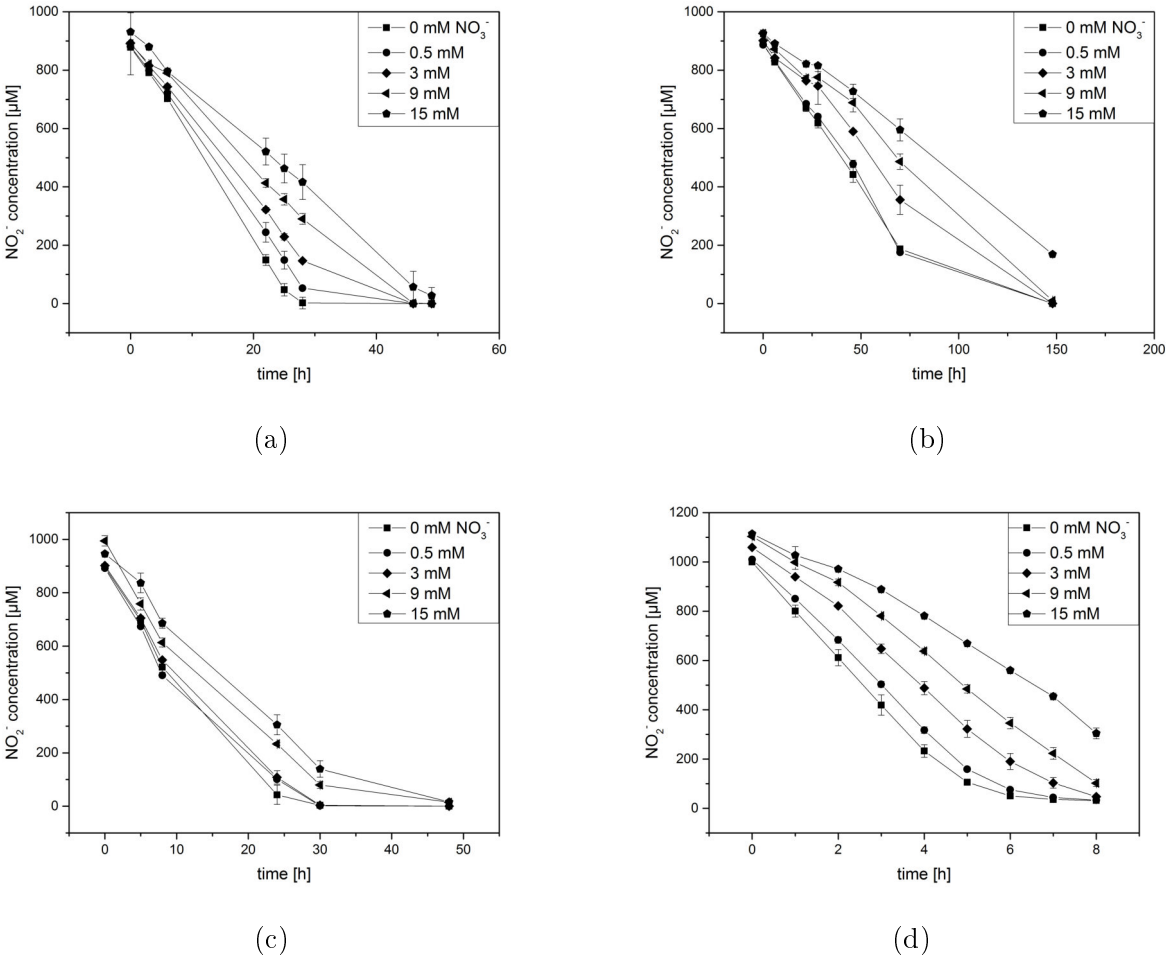


Figure 20: Nitrite oxidation in dependence on various nitrate concentrations of biofilm inhabiting NOB communities derived from biofilters of different RAS. Tests were performed via batch-experiments inoculated with colonized biocarriers. Samples were taken from biofilters of freshwater modules (a) C and (b) F, with the latter being connected to a denitrification reactor, (c) brackish module GMA and (d) marine module M1. Error bars indicate standard deviation calculated from duplicate samples.

samples, but still detectable in the nitrate spiked batches. In comparison to the control, in which the maximum nitrite oxidation rate was determined, activities slowed down with rising nitrate concentrations as shown in fig. 20 for the different samples. The percent inhibition of the maximum nitrite oxidation rate in dependence on the nitrate concentration was similar between the freshwater, brackish and marine samples (table 13). The sample derived from freshwater module C revealed a slightly higher tolerance towards all tested nitrate concentrations in comparison to the sample from module F, which was connected to a denitrification reactor.

4.6.3 Salinity tolerance test

In order to analyze the influence of salt stress on nitrification performance, batch-experiments with colonized biocarriers derived from biofilters of three different RAS were performed. The three RAS differ principally in the salinity of the process water. Mean salinity during sampling

Table 13: (Left) Maximum nitrite oxidation rates of biofilm inhabiting NOB communities determined via batch-experiments with colonized biocarriers. (Right) Percent inhibition of the maximum nitrite oxidation rate in dependence on the nitrate concentration. Biocarriers were derived from biofilters of modules C and F (FR), module GMA (BR) and module M1 (MA).

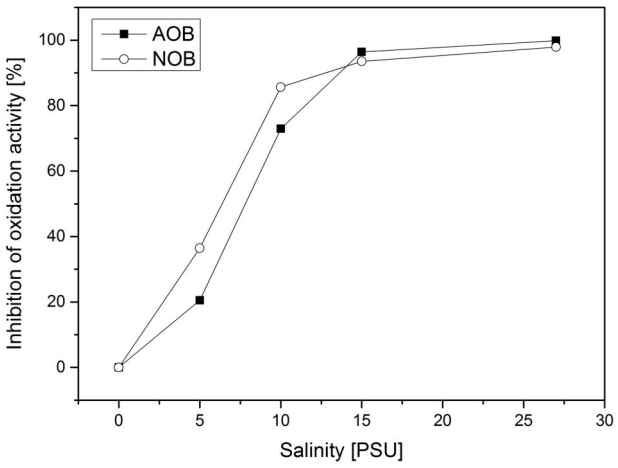
	NO_2^- [µM h^{-1}]	NO_3^-	[mM]	0.5	3	9	15
C	33.6	C	[%]	4.3	12.6	32.3	44.9
F	10.5	F	[%]	12.6	21.4	46.4	58.2
GMA	34.4	GMA	[%]	6.1	6.0	40.2	37.8
M1	156.7	M1	[%]	5.9	19.3	33.3	52.7

was 0 PSU at the freshwater, 4-5 PSU at the brackish and 24-26 PSU at the marine RAS. Biofilters of modules C (FR), GMA (BR) and M1 (MA) have been sampled and the impact of different salinities (0-27 PSU) on the maximum ammonia and nitrite oxidation rate was analyzed for each sample.

The tests showed that the AOB and NOB of freshwater module C revealed a gradual decrease in ammonia and nitrite oxidizing activity with rising salt concentrations. Hence, the maximum ammonia and nitrite oxidation rate was observed at the 0 PSU batches (AOB: 30 µM h^{-1}; NOB: 35 µM h^{-1}). At 15 PSU more than 95 % of the nitrification activity was lost and at 27 PSU, the highest salinity tested, no ammonia oxidation and only 2 % residual nitrite oxidation was detected. Thus, AOB and NOB derived from the freshwater RAS revealed very similar responses to elevated salt concentrations. Figure 21a shows the percent inhibition of ammonia and nitrite oxidation rates of freshwater AOB and NOB caused by salinity.

In brackish sample GMA the maximum ammonia and nitrite oxidation rate (AOB: 50 µM h^{-1}; NOB: 71 µM h^{-1}) was observed in the 0 PSU batches. Hence, there was a slight stimulation on the nitrifying activity when reducing the native salinity (4-5 PSU). However, the difference to the 5 PSU batches was minor with a decrease in ammonia and nitrite oxidation activity of only 5 %. Higher salt loadings led to a continuous reduction of ammonia and nitrite oxidation with similar patterns for AOB and NOB as shown in fig. 21b.

The marine AOB and NOB samples derived from module M1 revealed the maximum oxidation rate (AOB: 114 µM h^{-1}; NOB: 198 µM h^{-1}) in the 15 PSU batches. In the presence of 27 PSU (closest to the native salinity of 24-26 PSU), oxidation of both groups was inhibited by 10 %. Hence, likewise to the brackish sample, a slight stimulation on the nitrifying activity occurred when reducing the native salinity. However, further reduction of the salinity resulted in a continuous decrease in oxidation rates (fig. 21c). Nevertheless, the marine nitrifiers showed a high robustness to the reduction in salinity. Even at 5 PSU still 85 % (AOB) and 88 % (NOB) of the maximum activity was maintained. In the 0 PSU batches, the NOB had the continuing ability to perform well while retaining 84 % activity. In contrast, the AOB were less tolerant with revealing a decrease in ammonia oxidation of 77 %.



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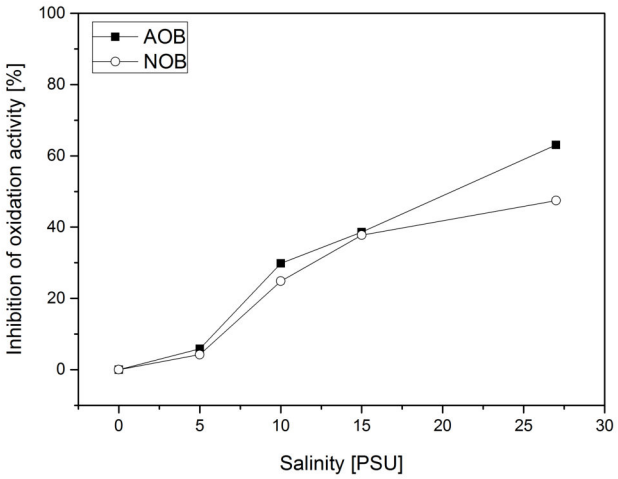

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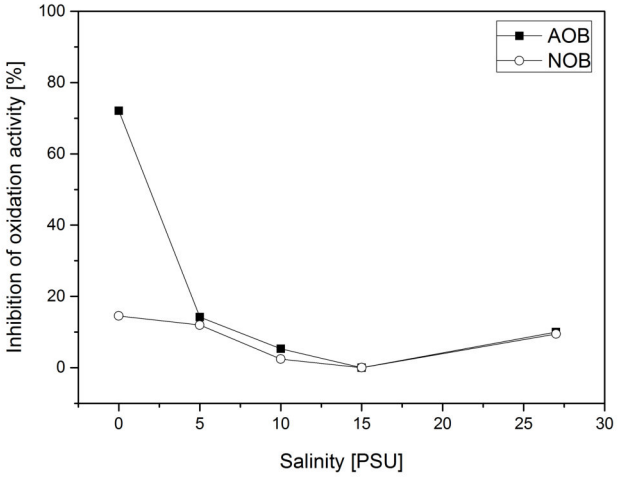


(c)

Figure 21: Percent inhibition of the maximum ammonia oxidation rates of AOB and the maximum nitrite oxidation rates of NOB caused by salinity. Tests were performed via batch-experiments inoculated with colonized biocarriers derived from different RAS. Samples were taken from (a) freshwater module C, (b) brackish module GMA and (c) marine module M1.

4.7 Co-existence of *Nitrotoga* and *Nitrospira* in a RAS biofilter

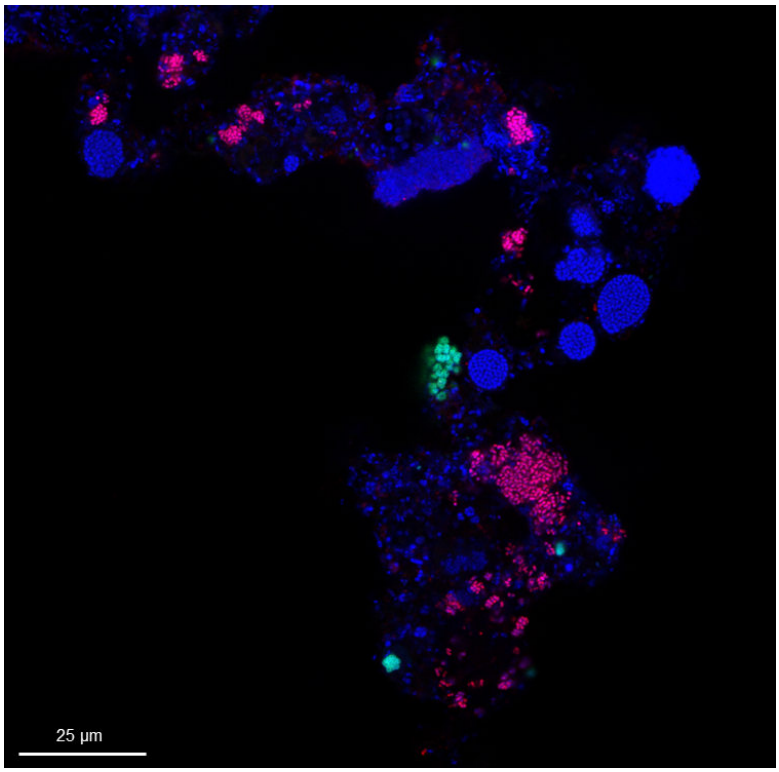
In addition to the sequence based approaches for the characterization of nitrifiers in RAS biofilters, a cultivation dependent approach was performed in this study. In 2010, about three years before starting with experimental work in the course of the present study, an unexpected strong occurrence of *Nitrotoga*-like NOB has been observed in biofilters of a cold freshwater RAS for the production of rainbow trouts (module TR). PCR-based community characterization of the biofilm material revealed a co-existence of *Nitrotoga*- and *Nitrospira*-like nitrite oxidizers (Off 2012; Hüpeden *et al.* 2016). To address the question wether one of the nitrite oxidizers dominated the biofilters, semi-quantitative FISH analysis of biofilm material was conducted. In addition, competitive *in situ* studies with NOB enrichment cultures were performed as described in the following paragraph.

4.7.1 Cell visualization via FISH

PFA-fixed biofilm material derived from the biofilter sampled in 2010 was used for semi-quantitative analysis of *Nitrotoga* and *Nitrospira* via FISH as described in section 3.5.7. FISH visualization revealed a high level of cells in large colonies, which hybridized with *Nitrotoga* genus-specific probe Ntoga122 and a lower abundance of cells, which hybridized with *Nitrospira* genus-specific probe-mix Nspa712-662 (fig. 22a). Coccoid cells of *Nitrotoga* were clearly different from densely aggregated cells of *Nitrospira* and formed large amoeba-like colonies (fig. 22b).

4.7.2 Chemolithoautotrophic activity of *Nitrotoga* and *Nitrospira*

Motivated by the finding of a strong occurrence of *Nitrotoga* colonies in the RAS derived biofilm, the question arose if this NOB is metabolically more active under the RAS predominant conditions compared to *Nitrospira*. Therefore, NOB enrichment culture ('Aquakultur HW'), which contained both nitrifiers as initially verified by genus-specific PCR (Off 2012), was used for competitive FISH-MAR experiments and thus, nitrite depended chemolitoautotrophic carbon fixation was monitored on the single cell level (described in section 3.5.8). To test the hypothesis that *Nitrotoga* favors a slightly acidic pH-value in combination with a low temperature and a low substrate concentration over *Nitrospira*, the enrichment culture was inoculated in NOB medium with either pH 6.4 or 7.2 containing 0.3 mM nitrite as substrate and incubated at 14 °C. The results of this approach indicated that *Nitrotoga* and *Nitrospira* both incorporated carbon from $[^{14}C]H_2CO_3$ in the tested media. Hence, both NOB were metabolically active under the two different conditions. Figure 23 shows the *in situ* chemolitoautotrophic activity of *Nitrotoga* and *Nitrospira* visualized by FISH-stained cells.



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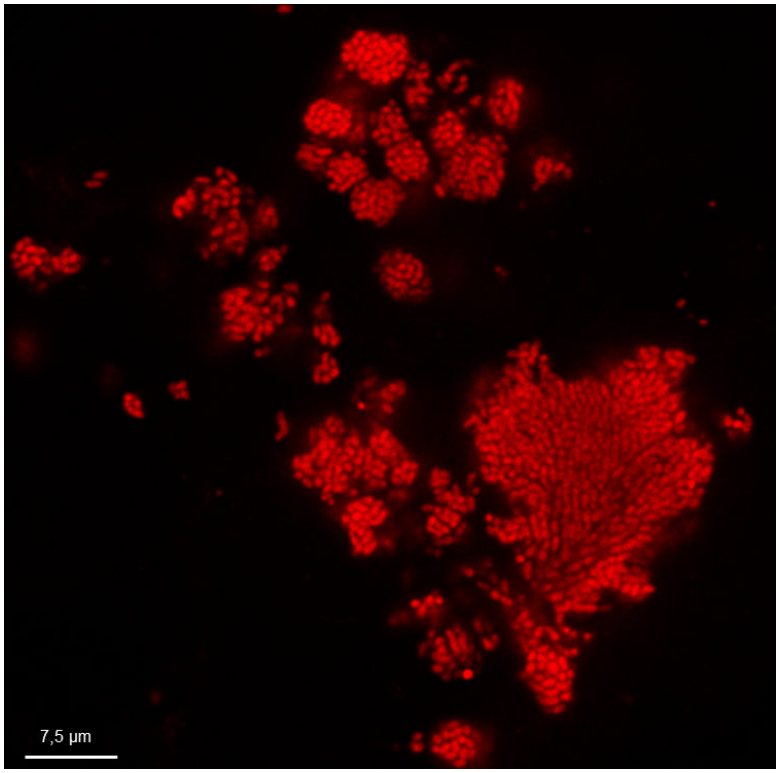
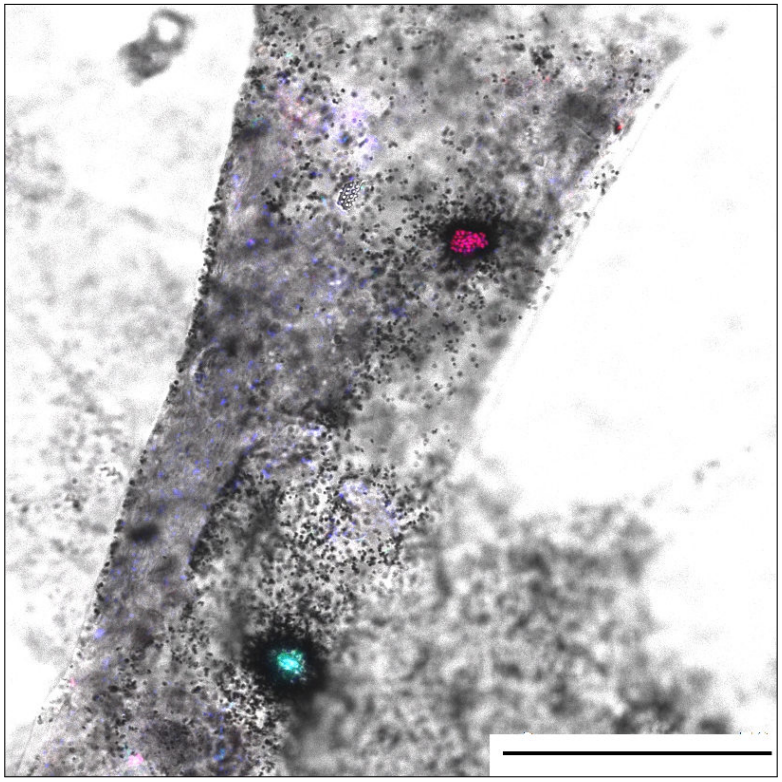


(b)

Figure 22: Confocal micrographs of FISH-stained, three months old biofilm material using genus-specific probes. (a) Dense biofilm revealing a high number of Ntoga122 stained *Nitrotoga* clusters (pink) and fewer Nspa712-662 stained microcolonies of *Nitrospira* (green). EUB338 stained cells are colored in blue. (b) Section of a large *Nitrotoga* colony stained with probe Ntoga122. Note that figures were taken from article Hüpeden *et al.* (2016) with slight modifications. Copyright© 2016, American Society for Microbiology.



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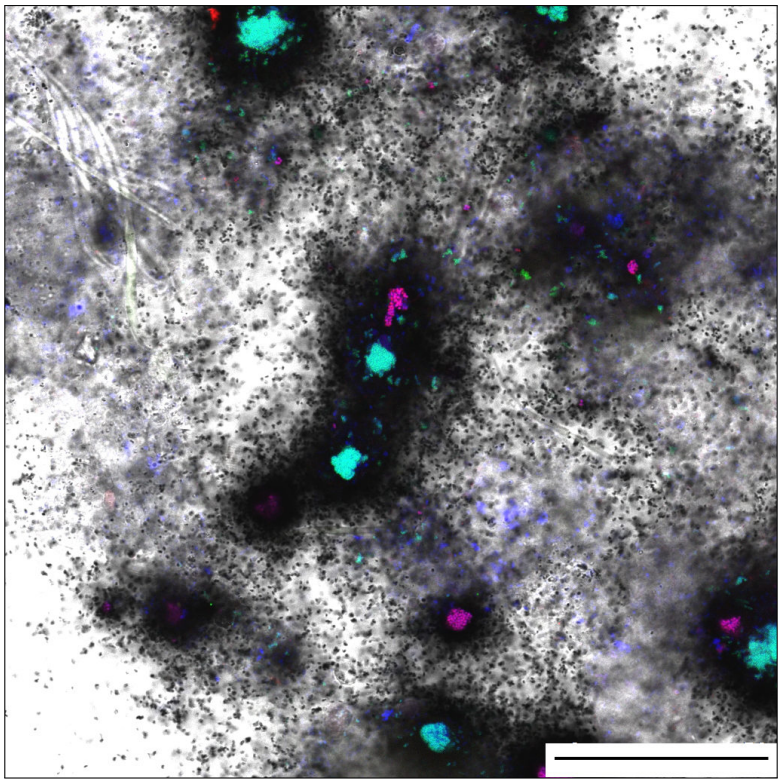


(b)

Figure 23: Confocal micrographs of FISH-stained *Nitrotoga* and *Nitrospira* cells with corresponding MAR signal. The images show the overlay FISH and MAR signals from the (a) pH 6.4 and (b) pH 7.2 batches inoculated with cells of the enrichment culture 'Aquakultur HW', incubated with 0.3 mM nitrite at 14 °C for 6 h. Cells hybridized to *Nitrotoga* genus-specific probe Ntoga122 (pink), to *Nitrospira* genus-specific probe-mix Nspa712-662 (green) and to EUB338 (blue). Scale bars represent 50 µm.

5 Discussion

5.1 Analysis of microbial diversity in RAS derived biofilms via 16S rRNA amplicon sequencing

5.1.1 General assessment of bacterial diversity

So far, most studies addressing the microbial community composition in RAS were based on 16S rRNA-fingerprinting methods such as denaturing gel gradient electrophoresis (DGGE) and clone libraries. Here, the comprehensive analysis of microbial biofilm communities in biofilters of three different RAS by high-throughput Illumina MiSeq sequencing the V4 region of 16S rRNA genes, demonstrated a high diversity at the 97 % cut-off level. On average 1662 (±166) OTUs were found in the freshwater, 2690 (±95) OTUs in the brackish and 1227 (±43) OTUs in the marine samples in the RNA pools and 1346 (±211), 2268 (±58) and 1240 (±125) OTUs in the DNA pools, respectively (mean numbers of OTUs of the three biofilter samples at each sampling site). Interestingly, the highest diversity was found in the brackish samples, in contrast to other investigations where the brackish sites were less diverse than freshwater and marine sites (Wang *et al.* 2012). The estimated species richness identified in the samples of the present study was around one order of magnitude higher than those found in previous studies about RAS biofilters by using conventional 16S rRNA-fingerprinting methods (Tal *et al.* 2003; Sugita *et al.* 2005; Michaud *et al.* 2009; Martins *et al.* 2013). Microbial community studies of wastewater treatment systems as revealed by high throughput sequencing methods gain increasing attention, but published data sets are still scarce. However, the high diversity identified here, was also reported in the study of Yun-Jie *et al.* (2015), in which the authors analyzed biofilm samples derived from a marine RAS by a 16S rRNA 454 amplicon sequencing approach. In similar habitats such as WWTP derived activated sludge, the identification of 2455 bacterial OTUs was reported by Ye *et al.* (2013) and 3034 OTUs were found in suspended and 1451 OTUs in attached activated sludge samples by Kwon *et al.* (2010). Finally, in the present study a high microbial diversity with about 1000 site specific OTUs and only 270 shared OTUs among the three aquaculture plants (in the merged DNA pools) was identified, indicating distinct communities at the three different RAS. In accordance with the assumption stated by Ye *et al.* (2013) it is concluded that likewise to activated sludge, RAS biofilter derived biofilms are highly species-rich ecosystems, which are more complex than seawater (Qian *et al.* 2011) and comparable to soil (Roesch *et al.* 2007).

5.1.2 Differences between total and active microbial communities

The comparison of the normalized RNA and DNA derived data sets revealed that in most of the samples the RNA and DNA pools achieved similar numbers of OTUs. In other studies comparing RNA and DNA derived microbial community structures, the RNA pool was less diverse (Baldrian *et al.* 2012). However, overestimation and fidelity of amplicon sequencing generated data is strongly discussed and a conflicting issue of recent research activities. The

bias potentially generated during PCR amplification and library preparation is proposed as one factor controlling the species richness (Lee *et al.* 2012; Schirmer *et al.* 2015). Hence, the abundance of RNA reads observed in this study might be overestimated and caused during cDNA synthesis and amplification of the second strand DNA. Nevertheless, the taxonomic classification on phylum and class level showed that the distribution patterns for the RNA and DNA derived communities from one sample were similar. Moreover, on genus level active microbial populations obtained by RNA analysis and the DNA derived populations largely overlapped and among the abundant OTUs very few appeared exclusively either in the RNA or DNA pool. On the other hand, few OTUs were highly enriched in the RNA pool but showed low abundance or even absence in the DNA pool for some samples. These data showed that DNA sequencing may miss a significant and relevant part of microbial communities and that species with low abundance can be highly active in the biofilm.

5.1.3 Evaluation of abundant bacterial phyla

Based on the results of the 16S rRNA amplicon sequencing, the taxonomic classification on phylum level revealed that *Proteobacteria* and *Bacteroidetes* were the predominant bacterial phyla in all samples, contributing to 24–70 % and 90–33 %, respectively, of the total reads. *Proteobacteria* followed by *Bacteroidetes* have been identified as the prevalent phyla in other investigations of full-scale RAS biofilters, both freshwater and marine (Sugita *et al.* 2005; Wietz *et al.* 2009; Martins *et al.* 2013; Yun-Jie *et al.* 2015; Huang *et al.* 2016), Additionally, the dominance of *Planctomycetes*, *Chloroflexi* and *Nitrospirae* followed by *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* found in the present study, coincides with recent analyzes about RAS biofiters performed via NGS approaches (Yun-Jie *et al.* 2015; Huang *et al.* 2016). The high fraction of *Proteobacteria* in wastewater treatment systems is not surprising since this physiologically heterogeneous phylum is widespread in aquatic environments and plays an important role in nutrient cycling processes and mineralization of organic compounds (Kersters *et al.* 2006). Moreover, the majority of AOB is found among the β-proteobacterial class. However, the proportion of β-proteobacterial AOB found in this study was low (1–13 % of the total reads). Accordingly, it is assumed that a large part of *Proteobacteria* is rather involved in heterotrophic processes such as biomass degradation and denitrification inside the biofilms. Most reads within the *Bacteroidetes* (previously *Cytophaga-Flavobacter-Bacteroides*) were affiliated with the bacterial classes *Sphingobacteria*, *Flavobacteria* and *Cytophagia*. Members of these three classes are well known for their contribution on carbohydrate degradation and in particular *Flavobacteria* and *Cytophagia* are counted among the predominant organisms in aquatic habitats which are responsible for the consumption of organic material (Kirchman 2002). Therefore, members of the *Bacteroidetes* are expected to strongly contribute to the degradation and consumption of organic matter in the investigated biofilms.

Members of the phylum *Planctomycetes* occurred in the different samples (4–20 %), which is noteworthy due to their heterogeneous physiology. *Planctomycetes* are found ubiquitously throughout the environment including freshwater, marine and soil habitats (Fuerst *et al.* 2011)

and sequences are just as frequently observed as in samples derived from wastewaters (Chouari *et al.* 2003). Moreover, *Planctomycetes* were found as common component of marine snow and of bacterial communities involved in blooms of marine diatoms (Delong *et al.* 1993; Morris *et al.* 2006). Thus, likewise to *Proteobacteria* and *Bacteroidetes*, members of *Planctomycetes* may play an important role in carbon-turnover in RAS habitats. Beyond that, this bacterial group harbors species capable of performing the anaerobic anammox reaction (Jetten *et al.* 1999). Anammox capable *Planctomycetes* are widely distributed in freshwater and marine habitats and are major contributors to the global nitrogen-cycle (Kuypers *et al.* 2003, 2005; Jetten 2008). In engineered systems the anammox process is of great significance for the remediation of nitrogen-rich wastewater and full-scale plants using this anaerobic process for treating municipal wastewaters are in operation (van der Star *et al.* 2007). Linked to aquaculture the presence of potential anammox *Planctomycetes* in RAS systems was reported previously (Tal *et al.* 2003; Lahav *et al.* 2009; van Kessel *et al.* 2010). However, even if the presence of anammox bacteria is likely in the anoxic zones of the here investigated biofilms, the performance of the anammox reaction and the relevance in N-removal is questionable. Although the anammox process has attractive advantages over the traditional nitrification-denitrification pathway, it is rather suitable in other compartments like the biofilter, such as in anaerobic sludge digesters, since the process depends on a high ammonium content, a low C:N ratio and a low oxygen concentration (Jetten *et al.* 2001).

The phylum *Nitrospirae*, which harbors the autotrophic nitrifying species of the genus *Nitrospira*, was represented differentially in the biofilms of the three RAS. In the freshwater and brackish samples members of this group contributed on average to 4 % and 3 %, respectively, of the total reads. In the marine samples they were conspicuously abundant with contributing to up to 33 %. Information about the abundance of *Nitrospirae* in other studies about RAS biofilters, as achieved via application of NGS methods, are inconsistently and are restricted to marine systems. Therefore, *Nitrospirae* phylum members were reported to contribute to 6 % in different sections of marine biofilters (Yun-Jie *et al.* 2015), to 1–7 % in submerged biofilters and even to 17 % in fluidized sand filters (Huang *et al.* 2016).

A noticeable abundant phylum found in this study was *Chloroflexi* with highest proportion in the freshwater biofilter samples (on average 22 % and 13 % in the RNA and DNA pools). Members of this group are known for their mesophilic to thermophilic lifestyles and they are ubiquitous in natural and man-made habitats (Yamada *et al.* 2009). Filamentous *Chloroflexi* are frequently found in activated sludge of WWTP, which provides a habitat with moderate to slightly elevated temperatures (Björnsson *et al.* 2002; Yamada *et al.* 2005; Kragelund *et al.* 2008). Previous NGS studies reported on the occurrence of *Chloroflexi* among the dominant phyla in marine RAS biofilters with temperatures of 14–26 °C (Yun-Jie *et al.* 2015; Huang *et al.* 2016), supporting the results found in the present study. These data lead to the assumption that RAS biofilters might provide a favorable environment for these bacteria. Filamentous members of the phylum *Chloroflexi* found in WWTP have been associated with the degradation of organic compounds derived from dead biomass. Moreover, studies via MAR-FISH revealed that

uncultured *Chloroflexi* might play an important role in the degradation of microbial products in autotrophic nitrifying biofilms and membrane bioreactors (Kindaichi *et al.* 2004; Okabe *et al.* 2005; Miura *et al.* 2007; Kindaichi *et al.* 2012). Kindaichi *et al.* (2012) studied the substrate uptake patterns of uncultured *Chloroflexi* bacteria present in an anammox reactor and the results indicated that *Chloroflexi* scavenge organic compounds derived from the dead biomass and metabolites of anammox cells. The authors concluded that they might prevent the accumulation of organic waste products in the biofilm. Miura *et al.* (2007) stated that due to the capability of *Chloroflexi* bacteria to degrade soluble microbial products, it is possible that they might even be involved in the reduction of membrane fouling potential. However, due to the lack of cultured representatives the basic ecophysiology of *Chloroflexi* is still largely unknown and their role in the RAS derived biofilms remains unclear. Keeping in mind that only recently a novel NOB (*Nitrolancea hollandica*) was isolated from a nitrifying reactor operating at 37 °C (Sorokin *et al.* 2012) and unexpectedly phylogenetically assigned to the *Chloroflexi* phylum, it is likely that among this phylum bacteria with metabolic versatility and physiological features, other than only heterotrophic degradation of organic compounds, can be expected to be found in the future.

Finally, is has to be mentioned that the abundant phyla contain bacterial groups potentially capable to perform denitrification. Denitrification is a widespread feature among the prokaryotes and it is not restricted to a special microbial group, instead denitrifiers are phylogenetically an extremely heterogeneous group (Daims *et al.* 2010) and thus can be found among different phyla. Most denitrifiers are not obligate anaerobes, which enables them to thrive in aerated moving-bed biofilters in RAS. They can respire oxygen as electron acceptor and alternatively switch to denitrification when the oxygen partial pressure falls below a certain threshold. As micro-anaerobic zones evolve in the deeper parts inside the biofilm, the combination with high nitrate/nitrite load and organic carbon sources (derived from uneaten feed or fish waste), promotes heterotrophic denitrification in freshwater and marine RAS (van Rijn *et al.* 2006; Schreier *et al.* 2010). On genus level many potential denitrifiers were found e.g. *Pseudomonas* spp., *Comamonas* spp., *Marinobacter* spp., *Hyphomicrobium* spp. and *Rhodobacter* spp., indicating that the RAS biofilter derived biofilms provide a favorable environment for these microbes.

5.1.4 Heterotrophs versus nitrifiers in biofilms

The results obtained by the 16S rRNA amplicon sequencing revealed that the nitrifiers contributed to 1–13 % (FR), 4–16 % (BR) and 11–33 % (MA) of the total RNA and DNA reads. Therefore, heterotrophic bacteria represent the vast majority of bacteria inhabiting the biofilms. On the one hand, the biofilters provide ammonia released mainly by the fish gills for chemolithoautotrophic growth of syntrophic nitrifiers. On the other hand, organic matter released by fish waste and uneaten food as well as products derived from microbial growth and decay, supply easily accessible substrates for heterotrophs. Even if coarse organic waste is removed via filters, dissolved organic carbon (DOC) as well as small sized particulate organic carbon (POC) retains in the rearing water, supporting growth of heterotrophs. Moreover, the heterotrophic

bacteria profit from their short generation times of about 2.7 h compared to the long generation times of AOB (16 to 189 h) and NOB (18 to 69 h) (Belser 1984). Due to their growth advantages, heterotrophs can easily overgrow nitrifiers. To keep the nitrification performance in the biofilters, the organic matter content should be kept low as discussed in the following section 5.1.5. The struggle for oxygen and space between nitrifiers and heterotrophs in the biofilms is a continuous process. However, evidence exists that a moderate outer layer of heterotrophic bacteria can protect the nitrifiers against flow detachment and grazing (Blancheton *et al.* 2013).

5.1.5 Identification of core-taxa and their potential physiological role in biofilms

Taxonomic classification has shown that >50 % of the total reads were not identified on genus level, since most microbes have not been cultured and not described taxonomically. In accordance with that, other studies have also found a large fraction of unclassified bacteria in RAS biofilters (Michaud *et al.* 2009; Martins *et al.* 2013; Huang *et al.* 2016). Among the known genera, a core-community of the RAS biofilter samples was identified in this study, with 85 and 76 shared genera in the RNA and DNA pools, respectively. Only six genera of the core-community were detected frequently (>0.5 % of the total reads in at least one sample at each sampling site) among the nine biofilm samples and subsequently defined as frequent core-taxa.

Core-taxa have been identified in other habitats such as anaerobic digesters and activated sludge systems (Rivière *et al.* 2009; Saunders *et al.* 2016), but so far not for RAS biofilters. One the one hand, the identification of shared core-taxa in RAS biofilters can help to identify microbes that govern central system functions and on the other hand, core-taxa may indicate system failures. Potential physiological roles and functions in the biofilms of the most frequent core-taxa are discussed in the following.

The biofilters in RAS are the compartments of nitrification, as the most important part of the microbial N-removal process, and hence, a high presence of nitrifying species is desirable. Accordingly, the genera *Nitrosomonas* and *Nitrospira*, containing most of the so far known nitrifiers, appeared with 0.2–8 % and 0.3–25 % of the total reads on genus level among the shared core-community. However, heterotrophic bacteria represent the vast majority of bacteria inhabiting the biofilms. One dominating heterotrophic core-taxon was *Lewinella*, appearing with up to 14 % among the most frequent genera in all samples. *Lewinella* belongs to the *Bacteroidetes* phylum and the *Saprospiraceae* family, whose members are found in marine and freshwater aquatic environments, but also frequently found with high abundances in activated sludge (Nielsen *et al.* 2009). The authors from the latter publication showed that *Saprospiraceae* generally constitute a substantial proportion of the microbial community in different Danish activated sludge plants with 1–12 % of the total community biovolume. Due to the ability for hydrolysis and utilization of complex carbon sources, members of this family are likely important in the breakdown of complex organic compounds in environments such as biofilms and activated sludge (McIlroy *et al.* 2014). It is remarkable that in marine sample M5, which was only low stocked during time of sampling and has undertaken non-operation modes before, representatives of the genus *Lewinella* were more abundant (7 % and 15 % of the total reads in

the RNA and DNA pool) than in the the other modules. This fact might reflect the conditions inside the biofilter of module M5 and indicates that temporarily non-operation phases can lead to a shift in microbial community composition with heterotrophic degraders of dead biomass overgrowing more fastidious organisms such as nitrifiers. This is in agreement with previous studies on activated sludge pilot plants, in which it was shown that transient substrate overlaods and oxygen deficiency caused an increase in the proliferation of the *Saprospiraceae* member *Haliscomenobacter hydrossi* (Pernelle *et al.* 2001; Gaval *et al.* 2003). It is likely that the high fraction of *Lewinella* found here, reflects the key player in the degradation of carbohydrates derived from fish excrement and feed off-cut as well as dead biomass inside the investigated biofilms. However, since heterotrophs overgrow nitrifiers in the presence of high organic matter load, it is important to maintain a low C:N ratio in the rearing water to obtain good nitrification performance inside RAS biofiters (Zhu *et al.* 2001). Accordingly, *Lewinella* might serve as indicator organism in the biofilms with a strong occurrence reflecting a high C:N ratio.

Besides *Lewinella*, members of the heterotrophic genus *Nannocystis* occurred frequently in all samples with a strong contribution to 33 % of the total reads in sample M1. As a member of the *Myxobacteria*, *Nannocystis* is associated with the production of off-flavors in RAS (Auffret *et al.* 2013), which are strongly disadvantageous for the performance of fish production in aquaculture plants. The earthy taste of aquatic animals is the common type of off-flavors in aquaculture and is caused by the production of the terpenoid compounds geosmin and 2-methylisoborneol (MIB) by *Cyanobacteria*, several genera of fungi and *Myxobacteria* (Rurangwa *et al.* 2015). In contrast to M3 and M5, module M1 was connected to a denitrification reactor. Growth promoting conditions in the anaerobic denitrification reactor such as a high C-load might be a possible reason for the abundance of *Nannocystis* species in M1.

In contrast to *Nannocycstis*, the occurrence of *Gemmatimonas* sp., which was found among the core-taxa shared between the freshwater and brackish samples, could be of advantage for the biological wastewater treatment in the plants. Some representatives of this genus such as *Gemmatimonas aurantiarca*, which has been isolated from a wastewater treating reactor, are namely reported as polyphosphate accumulating organisms (PAO) (Zhang *et al.* 2003). Phosphorous (P) can contribute to eutrophication and thus, the removal of P is of importance in wastewater treatment systems. PAO are key players in biological P-limitation processes in WWTPs and the application of microbial P-removal processes in RAS is subject of current research (Barak *et al.* 2000).

In addition, bacteria with potential probiotic function for the well-being of the kept fish were found among the frequent core-taxa. Thus, the abundant genera *Marinobacter*, *Muricauda*, *Rhodobacter* and *Variovorax* were identified as potential probiotics with aquaculture relevant functions. Members of the genera *Marinobacter* and *Rhodobacter* have been proven to enhance the growth performance and activate the innate immune response of different fish species (Amin *et al.* 2012; Chiu *et al.* 2014; Montero *et al.* 2014; Grotkjær *et al.* 2016). The prevention against fish pathogens such as *Vibrio* was also reported due to the activity of *Muricauda* and several members of the *Roseobacter*-clade (D'Alvise *et al.* 2010; Chiu *et al.* 2014; Kuo *et al.* 2015;

Grotkjær *et al.* 2016), in this study represented by *Phaeobacter*. Another notable organism with potential probiotic function was *Variovorax* sp., since many strains of this bacterium are associated with important catabolic processes including the degradation of toxic and/or complex chemical compounds (Satola *et al.* 2013). Moreover, *Variovorax paradoxus* has been isolated from a septic tank connected to RAS and was capable to grow with MIB or geosmin as sole energy source (Guttman *et al.* 2012) and thus, it might play an important role in the degradation of unfavorable off-flavors in RAS.

It has to be mentioned that among the core-community many heterotrophic bacteria harbor the potential or are well known to perform denitrification such as members of the genera *Planctomyces*, *Azospira*, *Marinobacter* and *Rhodobacter*. Moreover, the capability to denitrify under microaerophilic conditions is a known fact for some nitrifying species of *Nitrosomonas* and *Nitrospira* (Daims *et al.* 2010). Since micro-anaerobic zones evolve in the deeper parts inside the biofilms, denitrification occurs likely in RAS biofilms and could play a crucial role in the N-removal process, in particular when no denitrification reactor is implemented.

To conclude, among the core-taxa in all three investigated RAS, the nitrifying genera *Nitrosomonas* and *Nitrospira* occurred along with heterotrophs, representing degraders of organic waste products but also potential denitrifiers and PAOs as well as bacteria with potential probiotic functions for the well-being of the reared fish. Moreover, the identification of a core-community in RAS biofilters can be of importance for aquaculture operators since the recognition of a predominance of certain heterotrophic members in the biofilms, such as *Lewinella*, might serve as indication for a high C:N ratio, which may result in a reduced nitrification efficiency. An interesting question for future investigations should be if members of the heterotrophic core-community in the biofilms play a broader role in stimulating growth and activity of nitrifiers. For certain heterotrophs it is known that they enhance nitrification rates in *Nitrosomonas* and *Nitrobacter* bioreactors (Sedlacek *et al.* 2016), and it is conceivable that in complex habitats such as RAS biofilms the interplay between heterotrophs and nitrifiers leads to the enhancement of nitrification performance.

5.1.6 Abundant taxa in the RNA derived communities

Comparison of RNA and DNA derived community composition on genus level revealed that some genera only appeared in the RNA pool to strikingly high numbers, which indicates that these organisms were metabolically active during time of sampling. This was noticeable in freshwater sample F where *Blastpirellula* (phylum *Planctomycetes*) accounted for 12 % of the total reads in the RNA pool, but only for 2.8 % in the DNA pool. In addition, in marine sample M1 representatives of the genus *Glaciecola* (phylum *Proteobacteria*) contributed to 28 % in the RNA pool, while only 3.5 % could be detected in the DNA pool. Hence, the environmental conditions in the biofilters dominant to that given moment during time of sampling, have led to a shift in the microbial community composition. Changes in the operational as well as physicochemical parameters in the RAS could have provided advantageous conditions for the species which were represented strongly in the RNA pools.

5.1.7 Nitrifying communities

Species composition of nitrifying communities in RAS biofilters The results of the 16S rRNA amplicon sequencing approach showed that a site specific AOB and NOB community at each RAS occurred. The majority of reads was affiliated with unknown members of *Nitrosomonas* and *Nitrospira*. The comprehensive phylogenetic analysis to determine the closest taxonomically described species revealed a co-existence of different *Nitrosomonas*-like AOB and *Nitrospira*-like NOB in all biofilter samples with one representative being the predominant ammonia or nitrite oxidizer at each RAS, respectively. The analysis showed similar patterns for the RNA and DNA derived communities, which led to the conclusion that the identified organisms reflect the metabolic active guild of nitrifiers inside the biofilms. In accordance, FISH and EM visualization of the biofilm material revealed typical colonies of *Nitrosomonas*-like AOB and *Nitrospira*-like NOB growing in close vicinity to each other, demonstrating their syntrophic association.

AOB In the freshwater biofilms, organisms with 96 % sequence similarity to the 16S rRNA gene of *Nsm. communis* Nm2 build the predominant AOB group (>97 % of the total AOB reads). *Nsm. communis* has been originally isolated from soil (Koops *et al.* 1991) and classified to *Nitrosomonas* cluster 8 (Purkhold *et al.* 2003), whose members have diverse ecophysiological characteristics and environmental sources (Norton 2011). So far, the appearance of *Nitrosomonas* cluster 8 members in RAS has not been reported yet. In other studies about freshwater RAS biofilters, *Nitrosomonas* cluster 6-like AOB related to *Nsm. oligotropha*, *Nsm. urea* and *Nsm. aestuarii* have been identified (Pedersen *et al.* 2009; van Kessel *et al.* 2010).

The results further showed that the highest AOB diversity was found in the brackish samples. Co-existing limnic and marine species of *Nitrosomonas*-like AOB were identified, indicating that brackish biofilters with a native salinity of 4-5 PSU provide an environment for both physiological types. The results revealed an organism with 98 % sequence similarity to *Nsm. urea* Nm10 as the predominant AOB in the brackish samples. *Nsm. urea*, as a member of cluster 6A, has been originally isolated from soil and is mainly found in terrestrial and limnic habitats, and cultures do not require saline medium for growth (Koops *et al.* 1991, 2001). However, reports about the occurrence of *Nsm. urea* in saline systems exist (Purkhold *et al.* 2003) and the predominance in the brackish biofilters found in this study further indicates the halotolerant character of this AOB. Besides *Nitrosomonas*, an organism with 92 % sequence similarity to γ-proteobacterial *Nitrosococcus oceani* was found among the most abundant AOB, with highest occurrence in RMB (>50 % in the RNA and DNA pool). This finding was unexpected since the organism is described as obligately halophilic with high salt requirements and so far, it has not been detected in engineered systems (Koops *et al.* 2001).

The AOB community analysis of the marine samples showed that relatives of *Nsm. aestuarii* (98 % similarity) and *Nsm.* sp. NS20 (98 % similarity) were the dominating ammonia oxidizers, occurring in the ratio 1.5:1. Both species have been originally isolated from saline water and sediments and classified to *Nitrosomonas* cluster 6B (Koops *et al.* 1991). In accordance with

the present study, the predominance of *Nsm. aestuarii*-like AOB was reported in biofilters of a marine RAS cultivating shrimps (Brown *et al.* 2013). In addition, the occurrence of other *Nitrosomonas*-like AOB with relatedness to known marine species such as *Nsm. marina*, *Nsm.* sp. Nm22, *Nsm.* sp. Nm143, *Nssc. mobilis* and *Nsm. cryotolerans* has been reported for these systems (Tal *et al.* 2003; Foesel *et al.* 2008; Brown *et al.* 2013; Yun-Jie *et al.* 2015).

The data further revealed that *Nitrosospira*-like AOB contributed to maximum 0.8 % in all three plants. This group of nitrosomonads is widely distributed in soils and marine habitats (Freitag *et al.* 2006) and was found with low abundances in engineered systems such as WWTPs and freshwater RAS (Itoi *et al.* 2006; van Kessel *et al.* 2010). Their contribution in ammonia removal in wastewater treatment though, is still unclear. The presence of AOA in aquarium or RAS biofilters, however, was reported recently (Sauder *et al.* 2011; Brown *et al.* 2013) and growth of AOA at relatively low ammonia concentrations has been demonstrated for enriched and isolated representatives (Nicol *et al.* 2011; Sauder *et al.* 2012). Moreover, low ammonia concentration has been proposed as niche differentiating factor determining the dominance of AOA over AOB in WWTPs (Sauder *et al.* 2012). Nevertheless, AOA were not detected via 16S rRNA amplicon sequencing in the present study. Amplification with an archaeal *amoA* primerset was inconsistent and revealed positive products only for some of the freshwater and brackish samples (data not shown), but further downstream procedures for amplicon sequencing on Illumina MiSeq failed. It has to be taken under consideration that AOA might be present in the investigated biofilters but were not noticed with the molecular methods used, due to issues such as primer biases and sequencing errors. However, the fact that typical cells of AOA were not detected via EM and that PCR and 16S rRNA sequencing gave no clear results led to the assumption that these organisms might rather play a minor role in the investigated biofilters.

NOB The results of the freshwater samples demonstrated that mainly uncultured bacteria with *Ns. defluvii* as closest relative (99 % similarity to the 16S rRNA gene) dominated the biofilms, revealing >99 % of the total NOB reads. The residual 1 % was found to belong to relatives of *Ns. mosocviensis* and Cand. *Ns. salsa*. In accordance with this study, the presence of *Ns. defluvii*- and *Ns. moscoviensis*- like NOB was previously reported on biofilters in freshwater RAS (Hovanec *et al.* 1998; Sugita *et al.* 2005; Itoi *et al.* 2006). Interestingly, only in the RNA pool, relatives of recently discovered Cand. *Ns. inopinata* ENR4 were detected in the samples of modules D and F. This NOB was reported as organism capable to perform the whole two-step nitrification in one cell (van Kessel *et al.* 2015; Daims *et al.* 2015). However, the very low abundance (maximum 0.1 %) indicates that this organism seems to play a minor role in ammonia and nitrite oxidation in the investigated freshwater biofilters.

In line with the brackish AOB species composition deciphered in this study, the brackish NOB community revealed co-existing limnic and marine species. Nitrite oxidizers with *Ns. defluvii* as the closest relative (99 % similarity) represented the prevailing group in samples AQ1 and GMA (contributing to 95–97 %). Relatives of *Ns. moscoviensis* (96–99 % similarity), *Ns. inopinata* (94–99 % similarity) as well as marine *Nitrospira* such as *Ns. salsa*, *Ns.* strain Ecomares 2.1 and *Ns. marina* Nb-295 (96–98 % similarity) were further identified with

low abundance (<3 %) in AQ1 and GMA. The predominance of *Ns. defluvii*-like NOB in the brackish biofilters indicates that this bacterium is capable to cope with and thrive well at the prevailing salinity of 4-5 PSU. Interestingly, the NOB community composition in RMB was in general different to that of AQ1 and GMA with a higher contribution of *Ns. inopinata*-like and *Ns. moscoviensis*-like NOB but a lower contribution of *Ns. defluvii*-like NOB. In accordance with that, the AOB community in RMB revealed a slightly different composition than that of the other modules in the same RAS, as discussed above. RMB was operated as larvae rearing system with irregular operating durations and interim non-operation phases. Moreover, experiments with the fish larvae with frequent temperature changes and shifting day night cycles were performed. The differences in the environmental conditions might have caused conditions favorable for the mentioned AOB and NOB and led to the differences in the community composition compared to AQ1 and GMA.

In GMA and RMB very few reads of an organism with 98 % similarity to the 16S rRNA gene of cold-adapted Cand. *Nitrotoga arctica* were detectable (maximum 0.03 %). The occurrence of this NOB in wastewater treatment systems was so far only reported in few studies (Alawi *et al.* 2007; Lücker *et al.* 2015; Hüpeden *et al.* 2016). As part of this study, *Nt. arctica* was found as predominant NOB in the biofilter of a cold-water operated freshwater RAS (see section 5.6). However, in modules GMA and RMB the contribution of *Nt. arctica* was minor and only visible in the RNA pool. This in turn might reflect that this NOB was active during time of sampling as a response to short-term changes in environmental conditions in the biofilter.

The data of the marine samples revealed relatives of *Ns. salsa* (96–99 % similarity) as the predominant group in these biofilms (>99 %). *Ns. salsa* belongs to *Nitrospira marina*-like NOB in cluster IV and has been originally isolated from North-Sea water (Haaijer *et al.* 2013). Hence, this finding is not surprising since the process water at the marine RAS investigated in this study, is charged from the North-Sea. In addition to *Ns. marina*-like bacteria, very few reads of *Nitrospina* sp. clone Cb12 relatives were found in the RNA and DNA pool of the marine samples (maximum 0.02 %). In line with this finding, few reads of *Nitrospina*-like NOB have also been detected in other marine RAS biofilters (Huang *et al.* 2016).

In general, the analysis of the nitrifying communities of the freshwater and brackish samples furthermore showed for both, the AOB and NOB guild, that among the subdominant groups few OTUs appeared, which clustered to halophilic nitrifiers as closest taxonomically described species, and on the other hand species known from freshwater habitats were found in the marine samples. This suggests that these organisms might not be restricted to saline and limnic environments, respectively, or that they remain in low abundances as opportunists in the biofilms.

To conclude, the analysis on species level indicated that the predominant *Nitrosomonas*-like AOB and *Nitrospira*-like NOB differ among the sampling sites and moreover, it was shown that different species co-exist in these environments. The dominance of certain groups of AOB and NOB might be controlled by environmental parameters such as the salinity, temperature, substrate concentration and the pH-value. Accordingly, the co-existence and distribution of

distinct species with probable different ecophysiologies found in this study, reflect AOB and NOB niche differentiation within the biofilms. A diverse nitrifying community with co-existing species can be advantageous for the biofiltration performance in RAS, since diverse nitrifiers with different ecophysiologies may react more flexible to changes in RAS operational conditions.

Ratio between AOB and NOB The 16S rRNA amplicon data showed that the ratio between AOB and NOB was different in the three RAS. The marine samples revealed on average around five times more NOB than AOB reads. The high proportion of NOB in the marine samples was also visible on phylum level whereat members of *Nitrospirae* contributed to up to 33 % of the total reads. A higher NOB than AOB population was identified previously in freshwater bioreactors inoculated with nitrifying sludge (Gonzalez-Silva *et al.* 2016) and in a WWTP (Figuerola *et al.* 2010), while the latter authors stated that a dominance of NOB over AOB appeared to correlate with good nitrification. Indeed, the results of the nitrification potential tests obtained in the present study revealed that the nitrite turnover rates of NOB in the marine biofilters of modules M1 and M3, were around two to three times higher than in the freshwater and brackish biofilters (where the ratio between AOB and NOB was almost equal, or as in case of the freshwater samples, slightly more shifted for the benefit of the AOB). In contrast to that, the nitrification potential tests showed that the ammonia turnover rates between the samples from the three RAS were similar, independently from the AOB/NOB ratio. Thus, these findings lead to the assumption that the marine NOB feature beneficial physiological capabilities in comparison to the predominant freshwater and brackish nitrite oxidizers. Cultured representatives of marine *Nitrospira* have been proven to use simple organic substrates, such as pyruvate for carbon assimilation and also as energy source in addition to CO_2 and nitrite (Watson *et al.* 1986; Keuter *et al.* 2011). This mixotrophic lifestyle offers a growth advantageous feature compared to a solely autotrophic lifestyle and provides a plausible explanation for the significantly higher occurrence and nitrite oxidation activity of *Nitrospira*-like NOB in the marine RAS found in this study.

What drives abundance and activity of nitrifiers in RAS biofilters? The 16S rRNA amplicon sequencing results showed that samples derived from freshwater module F and marine module M5 had less AOB and NOB reads in total than the other modules of the same sampling site. The contribution of the AOB and NOB to the total reads was on average 10–50 % lower compared to the other modules of the same RAS. The dissimilarities were further confirmed via NMDS plots of the extracted AOB and NOB OTUs. Possible reasons for the differences between modules at one site may be found in the RAS prevalent operational conditions. M5 was only occasionally in operation and low stocked during time of sampling. The turnover rates of AOB and NOB determined via nitrification potential test (see section 5.4) were 96 % and 93 % lower compared to M1 and M3, which in contrast, have been continuously operated. Hence, the turnover rates clearly reflected the metabolic activity of the nitrifiers. Accordingly, the nitrifying biofilms on the biocarriers of M5 were probably not well established due to occasional non-operation periods or operation without or with low fish stocking. Without ammonium

input excreted by the fish, the nitrifiers might rapidly be overgrown by heterotrophic bacteria. However, these facts may not explain the low abundance of nitrifiers and the lower turnover rates (AOB: 36 % and NOB: 56 %) in sample F compared to C and D, since module F was continuously operated and fully stocked with fish comparable to the other modules (see table S2 in the appendix). In contrast to C and D, F was coupled to a denitrification reactor, which represents the main difference between these modules. In accordance with that, the nitrate concentration in F was lower (79 mg l^{-1}) than in C and D (135 mg l^{-1}; 150 mg l^{-1}). Considering the nitrate sensitivity of NOB identified in this study (see section 4.6.2), the lower nitrate concentration in F should have had a rather beneficial effect on the nitrite oxidation activity. However, the lower abundance and reduced nitrifying activity of AOB and NOB in F could be explained by the predominance or absence of certain heterotrophic bacteria in the denitrification reactor, which might overgrow or stimulate growth of nitrifiers when the process water is fed back into the biofilter via recirculation.

***Nitrosomonas* and *Nitrospira* as the predominant genera in RAS biofilters** The microbial community analysis in this study revealed that the vast majority of nitrifiers belongs to the genera *Nitrosomonas* and *Nitrospira*, which is in accordance with several previous studies about RAS biofilters (Sugita *et al.* 2005; Itoi *et al.* 2006; Foesel *et al.* 2008; Keuter *et al.* 2011; Kruse *et al.* 2013; Yun-Jie *et al.* 2015). Both genera enclose several species classified into different *Nitrosomonas* clusters and *Nitrospira* sublineages with distinct physiological features, allowing them to occupy diverse ecological niches. Furthermore, members of both genera have been shown to exhibit a relatively high metabolic versatility such as the capability to use simple organic substrates in addition to ammonia and nitrite, respectively. As discovered in recent years, in particular *Nitrospira* possesses diverse metabolic pathways as some are even capable of growth on H_2 and formate, indicating that nitrite oxidation may not be the primary lifestyle (Koch *et al.* 2015). Additionally, it has been shown experimentally that *Nitrospira* is able to hydrolyze urea and cyanate to ammonia and thereby initiating nitrification through reciprocal feeding with AOM (Koch *et al.* 2015; Palatinszky *et al.* 2015). Only recently, the unexpected discovery of comammox *Nitrospira* (Daims *et al.* 2015; van Kessel *et al.* 2015) has caused a breakthrough in the scientific community. As a consequence, *Nitrospira* spp. are highly flexible and the features of reciprocal feeding and the metabolic versatility, including the participation in different nitrogen cycle processes as well as the capability to perform the whole step of nitrification, are likely the key factors for the ubiquity and high diversity of *Nitrospira* in natural and engineered ecosystems such as RAS.

5.2 Phylogenetic analysis of AOB and *Nitrospira*-like NOB via *amoA* and *nxrB* gene amplicon sequencing

The analysis of the 16S rRNA amplicon sequencing data demonstrated that the biofilter derived biofilms of the three RAS were dominated by β-proteobacterial AOB and *Nitrospira*-like NOB. As a consequence, amplicon sequencing of the phylogenetic and functional marker genes for

ammonia oxidation (*amoA*) of β-proteobacterial AOB and nitrite oxidation (*nxrB*) of *Nitrospira*-like NOB was performed. In accordance with the 16S rRNA amplicon sequencing data, the results of this approach showed that at each RAS a characteristic nitrifying community was found and that the majority of OTUs was affiliated with different uncultured bacteria of the genera *Nitrosomonas* and *Nitrospira*. The similarities to the *amoA* and *nxrB* gene sequences of the closest taxonomically described species were low and ranged mostly from 80–96 %, suggesting that these OTUs represent novel nitrifying species. In general, the identified *amoA*/*nxrB* phylotypes mostly matched with those identified via 16S rRNA based phylogenetic analysis before.

5.2.1 β-proteobacterial AOB populations

The results of the *amoA* amplicon sequencing showed that the majority of *amoA* OTUs was phylogenetically affiliated with uncultured members of the genus *Nitrosomonas*. Comprehensive taxonomic classification, though, showed that the different environmental clones found, shared the same closest relative with different similarities to the *amoA* gene. This represents a microdiversity among the *Nitrosomonas* species.

In the freshwater biofilms of samples C, D and F most of the environmental clones were assigned to distantly related *Nsm.* sp. A7 (81–82 % similarity to the *amoA* gene). The strain *Nsm.* sp. A7 (Accession: KF194201.1) has been originally isolated from a SBBR (sequencing batch bioreactor) and reveals *Nsm. eutropha* as closest taxonomically described species (95 % similarity). *Nsm. eutropha* belongs to *Nitrosomonas* cluster 7 and members of this cluster have been isolated from a wide variety of environments, including sewage, wastewaters and freshwater habitats (Norton 2011). In contrast to the results of the *amoA* gene sequencing, the 16S rRNA amplicon sequencing data revealed *Nsm. communis*-like bacteria as the predominant AOB in samples C, D and F (section 4.1.5). However, both species were considered eutrophic (Norton 2011). The fact that the freshwater AOB communities in this study revealed a high tolerance to raised ammonia concentrations as verified in the substrate tolerance test (section 4.6.1), supports the finding of AOB related to eutrophic *Nitrosomonas* species dominating the freshwater biofilms. Besides *Nitrosomonas*, members of the genus *Nitrosospira* were found in the freshwater samples with particular frequency in sample C (16 %). This coincides with the results of the 16S rRNA amplicon sequencing data where *Nitrosospira* species have been detected in module C. The observed co-existence of members of these two genera correlates well with previous findings of freshwater and marine habitats (Norton 2011). However, members of *Nitrosospira* have only occasionally been detected in wastewater treatment systems or biofilm reactors (Daims *et al.* 2010; Park *et al.* 2002). As a consequence, the finding of *Nitrosospira*-like AOB as the predominant ammonia oxidizers (>99 % of the total reads) in freshwater sample TR was unexpected. In addition to the *amoA* amplicon sequencing analysis, EM visualization of the biofilm confirmed this observation since typical cells of *Nitrosospira* were frequently visible in the biofilm. In contrast, cells and aggregates of *Nitrosomonas*-like AOB could only sporadically be identified in sample TR (see section 4.4). Factors controlling the predominance

of *Nitrosospira* over *Nitrosomonas* may be found in the RAS operational conditions. Module TR for the production of rainbow trout was operated on average at 12 °C. In contrast, the mean temperature at the modules C, D, and F for the production of pikeperch was 22 °C. In the study of Wells *et al.* (2009) the authors showed that *Nitrosospira* can be favored under lower temperatures and higher dissolved oxygen concentrations. Thus, the colder temperature at module TR could have played a key role in controlling the dominance and diversity of AOB lineages.

The analysis of the brackish samples revealed that several environmental clones with 92–96 % similarity to the *amoA* gene of *Nsm. urea* Nm10 (Koops *et al.* 1991) build the predominant group. In addition, relatives of *Nsm. aestuarii* and strain *Nsm.* sp. TA921i occurred in the samples AQ1 and GMA. BLAST analysis of the latter strain (Accession: AF339043.1) showed that *Nsm. aestuarii* represented the closest described *Nitrosomonas* species (97 % similarity). Accordingly, the brackish biofilms were inhabited by limnic *Nsm. urea*- and marine *Nsm. aestuarii*-like AOB, both members of *Nitrosomonas* cluster 6. Compared to AQ1 and GMA, sample RMB showed less *amoA* reads and OTUs in total and revealed a community of almost exclusively *Nsm. urea*-like AOB (>99 %). This fact can be connected to the strong occurrence of *Nitrosococcus oceani* identified via 16S rRNA amplicon sequencing in this study. As γ-proteobacterial AOB, *Nssc. oceani* harbors a distinct α-subunit of the ammonia monooxygenase, which could not be detected with the *amoA* gene targeting primers used for this approach. Keeping the occurrence of *Nssc. oceani* in mind, the brackish modules revealed a community composed of limnic and marine *Nitrosomonas*- and *Nitrosococcus*-like AOB, respectively, indicating that this low salinity habitat (4-5 PSU) provides an environment favorable for a co-existence of the two distinct AOB genera.

In the marine samples relatives of *Nsm. aestuarii* (93–99 % similarity to the *amoA* gene) represented the predominant group (>98 % of the total reads). *Nsm. aestuarii* species are described as obligate halophilic (Koops *et al.* 1991, 2001) and therefore, they might be well adapted to the predominant salinity of 24-26 PSU at the marine RAS. Six OTUs were uniquely detected in sample M5, accounting for 13 %, and strain *Nsm.* sp. Is79A3 was the closest cultured relative (Accession: CP002876.1). This isolate was considered oligotrophic, growing optimally in medium containing low ammonia concentrations of 1–5 mM (Bollmann *et al.* 2013). The occurrence of an AOB adapted to low ammonia concentrations correlates well with the environmental conditions in the biofilter of module M5, since the module was only very low stocked with fish (91 kg) during time of sampling and several non-operation periods have been undertaken before (see table S2 in the appendix). As a consequence, the discharge of ammonia into the biofilters was low and beneath the detection level (<0.1 mg l^{-1}). However, the similarity to the *amoA* gene sequence of *Nsm.* sp Is79A3 was only 78 % and the strain has been originally isolated from a freshwater lake. Hence, these facts indicate that a novel species with similar physiological features such as adaption to low ammonia concentrations, but with additional halotolerance was found in the biofilter of module M5 during low stocked operation periods.

It has to be mentioned that the finding of *Nitrosospira*-like AOB in the brackish and marine

samples via 16S rRNA amplicon sequencing could not be confirmed by the *amoA* sequencing approach. However, problems of the phylogenetic classification of members of the *Nitrosospira* lineage are well known. As Norton (2011) stated, the phylogeny based on the current data set does not support that all nitrosomonads are more closely related to each other than to members of the *Nitrosospira* lineage. Consequently, the proper phylogenetic classification of *Nitrosospira* members remains challenging.

5.2.2 *Nitrospira*-like NOB populations

The data of the *nxrB* amplicon sequencing approach revealed that most of the *nxrB* OTUs clustered with different uncultured members of the genus *Nitrospira*. The comprehensive assignment to the closest described species resulted in four representatives of *Nitrospira*: *Ns. defluvii*, *Ns. moscoviensis*, *Ns. marina* and *Ns. calida*. Several OTUs assigned to environmental clones which had the same closest relative but different similarities to the *nxrB* gene occurred in each sample, reflecting a *Nitrospira* microdiversity in the biofilms.

The data of the freshwater samples revealed mainly OTUs which clustered with environmental clone *Nitrospira* sp. HKA-B3, originally isolated from activated sludge from a municipal WWTP (Pester *et al.* 2014). Analysis of the closest related NOB species resulted in *Ns. defluvii* (89–98 % similarity to the *nxrB* gene), which has been originally isolated from activated sludge (Spieck *et al.* 2006). Distant relatives of *Ns. moscoviensis* (88–89 % similarity) were further identified in the freshwater samples, accounting for 30–48 % in modules C and D. The species *Ns. defluvii* and *Ns. moscoviensis* belong to phylogenetically separate *Nitrospira* sublineages I and II, which are widely distributed in natural habitats. Members of sublineage I and II have been reported to be the main nitrite oxidizers in man-made ecosystems like WWTPs (Daims *et al.* 2001a; Spieck *et al.* 2006; Pester *et al.* 2014). In addition, the dominance of *Ns. defluvii*- or *Ns. moscoviensis*-like NOB in freshwater operated biofilters have been confirmed in several previous studies (Hovanec *et al.* 1998; Sugita *et al.* 2005; Itoi *et al.* 2006; Hüpeden *et al.* 2016).

The results of the brackish samples showed that *Ns. defluvii*- and *Ns. marina*-like NOB dominated the biofilms in co-existence. In samples AQ1 and GMA about 50 % of the environmental clones could be assigned to *Ns. defluvii* as closest relative (88–97 % similarity) and further 50 % to *Ns. marina* strain 43039 (88–97 % similarity) (Pester *et al.* 2014). Accordingly, the brackish biofilters provide an environment favorable for sublineage I and marine sublineage IV *Nitrospira*. The community composition in brackish sample RMB was different to samples AQ1 and GMA with revealing mainly *Nitrospira marina*-like NOB (80 %). The predominance of marine NOB in module RMB in addition to the occurrence of halophilic *Nitrosococcus*-like AOB identified via 16S rRNA amplicon sequencing, can be linked to distinct environmental conditions in module RMB, as discussed above (section 5.1.7). However, in contrast to the results of the 16S rRNA analysis for sample RMB, relatives of *Ns. moscoviensis* and *Ns. inopinata* were not identified based on *nxrB* phylogeny.

The occurrence of few hits assigned to an uncultured *Nitrospira* clone originating from Gorjachinsk geothermal spring (Pester *et al.* 2014) in the freshwater and brackish samples was

unexpected since *Ns. calida* (sublineage IV *Nitrospira*) as the closest related species, has been originally isolated from a geothermal spring and revealed a moderate thermophilic lifestyle (Lebedeva *et al.* 2011). However, the similarity to the *nxrB* gene of *Ns. calida* was low (87–90 %) and no 16S rRNA sequence with affinity to *Ns. calida* was identified in this study. It remains unclear if the organism found belongs to sublineage VI *Nitrospira* which contains so far only thermophilic representatives. In contrast to the 16S rRNA sequencing derived data, comammox *Ns. inopinata* ENR4 was not identified in the freshwater and brackish samples via BLAST analysis of the *nxrB* amplicon sequencing data. However, difficulties in the assignment of comammox *Nitrospira* are discussed below (section 5.3.4).

Furthermore, the results showed that mainly *Ns. marina*-like NOB were present in the marine biofilms (>99 % of the total reads). With several OTUs assigned to *Ns. marina* strain 43039 (sublineage IV *Nitrospira*), which revealed similarities to the *nxrB* gene of 88–89 %, a microdiversity of this nitrite oxidizer was identified. Nevertheless, this finding is in contrast to the 16S rRNA amplicon data, which showed that relatives of Cand. *Ns. salsa* (Haaijer *et al.* 2013) were the predominant NOB in the marine biofilms. However, an explanation for this discrepancy is the fact that so far no *nxrB* gene sequence of *Ns. salsa* was deposited in public databases. It remains unclear whether the 16S rRNA gene and the *nxrB* gene identified in the data set are derived from the same organism.

5.2.3 Niche differentiation of nitrifying species in RAS derived biofilms

The data of the 16S rRNA gene and *amoA*/*nxrB* gene amplicon sequencing approaches revealed that at each sampling site one predominant representative of *Nitrosomonas*-like AOB and *Nitrospira*-like NOB co-exists in the biofilm together with several less abundant members of the two genera. For both, *Nitrosomonas* and *Nitrospira*, it was shown in previous studies that different species co-exist in diverse habitats, which might be possible by niche differentiation. Interestingly, Foesel *et al.* (2008) reported about two co-existing *Nitrosomonas* subpopulations in a biofilm of a marine RAS. As the authors stated, one high affinity/low-activity *Nitrosomonas* population (*Nsm. marina*) may be active under the normally low ammonium concentrations in the investigated RAS, and a second *Nitrosomonas* population (*Nsm.* sp. Nm143) may thrive better during occasional peaks in ammonium concentrations. Moreover, the substrate concentration as determining factor of niche differentiation was reported previously for members of *Nitrospira* as well. Hence, in the study of Maixner *et al.* (2006) it was shown that *Nitrospira* sublineage I and II members settled along nitrite concentration gradients in activated sludge in accordance with their nitrite optima (see sections 5.5.1 and 5.5.2 for a detailed discussion about the substrate concentration as niche differentiation controlling factor of AOB and NOB). Therefore, it is reasonable that the here investigated RAS derived biofilms offer microhabitats due to spatial heterogeneity, and hence, different nitrifying species may be arranged spatially along gradients of e.g. substrate and oxygen. In addition, since operational parameters can fluctuate during RAS operation, the co-existence of AOB and NOB subpopulations representing ecotypes with different ecological adaptions, may be supported in the biofilms, resulting in

niche partitioning.

Moreover, the *amoA*/*nxrB* gene based analysis of the nitrifying communities revealed that read clustering using a nucleotide sequence identity threshold of 99 %, resulted in several species level OTUs (up to 46 OTUs) with high sequence identity to each other (<1–3 % variance), representing closely related organisms. The close phylogenetic relatedness of the OTUs was clearly visible in the constructed phylogenetic trees of *amoA*/*nxrB* gene sequencing generated OTUs, in which closely related OTUs formed dense clusters (see fig. S2 and fig. S3 in the appendix). This indicates a microdiversity of closely related *Nitrosomonas* and *Nitrospira*, respectively. In accordance with the present study, Gruber-Dorninger *et al.* (2014) identified a *Nitrospira* microdiversity with even 121 highly similar species level OTUs in activated sludge via 454 pyrosequencing of the *nxrB* gene. Functional differences of these closely related *Nitrospira* regarding preferred nitrite concentration, formate utilization and a spatial arrangement in the biofilm, have additionally been reported in that study. Together, these data highlight the enormous amount of apparently co-existing nitrifying organisms in engineered ecosystems such as WWTPs and RAS.

Nevertheless, it has to be mentioned that the genomic copy number of *amoA* and *nxrB* genes varies. E.g. in *Nitrospira defluvii* two *nxrB* copies have been identified and six in *Ns. marina*, and all copies from the same species are phylogenetically closely related (Pester *et al.* 2014). Therefore, it can not be excluded that highly similar OTUs identified in the present study, represent different *amoA*/*nxrB* copies from the same species. In addition, typical amplicon sequencing errors can cause overestimation of microbial diversity. With this in mind, the analysis pipeline used in this study included several approaches for bias reduction such as sequence quality screening and filtering as well as chimera exclusion (see section 3.9.1). Moreover, OTUs which were represented only by a single read were removed. Hence, OTUs which were represented by only few reads very likely represent rare species, but it can not be excluded that some of these rare OTUs result from sequencing errors.

However, the presence of several closely related members with probable different ecophysiology may have implications for the nitrifying community function. As suggested earlier, a high microdiversity may stabilize ammonia and nitrite oxidation due to a favorable diversity-stability relationship (Yachi *et al.* 1999; Daims *et al.* 2001b; Elmqvist *et al.* 2003; Gruber-Dorninger *et al.* 2014). Furthermore, as Gruber-Dorninger *et al.* (2014) stated, highly diverse but low abundant *Nitrospira* may act as seed bank for compensatory growth after disturbances such as changes in the wastewater composition or phage attack.

In conclusion, the nitrifiers identified in the present study represent several potentially novel *Nitrosomonas* and *Nitrospira* species, which may harbor distinct physiological properties. Intrigued by these findings, batch-experiments with biocarrier inhabiting nitrifiers were performed to investigate how the different communities response to changes in RAS relevant operational parameters. The physiological characterization of biofilm inhabiting AOB and NOB is discussed in section 5.5.

5.3 Metagenomic analysis of microbial biofilm communities in RAS biofilters

5.3.1 Phylogenetic diversity assessed by marker genes

The assessment of bacterial taxonomy via marker gene analysis of the metagenomic data sets showed that within the Bacteria domain *Proteobacteria*, followed by *Bacteroidetes*, *Chloroflexi* and *Nitrospirae* dominated the biofilms in the three investigated biofilters. Similar bacterial phylum level distribution was observed via 16S rRNA amplicon sequencing of the same samples as discussed in section 5.1.3. These results are in line with previous NGS studies about RAS biofilters and similar habitats such as activated sludge (Huang *et al.* 2016; Kwon *et al.* 2010), indicating that the predominance of *Proteobacteria*, *Bacteroidetes*, *Chloroflexi* and *Nitrospirae* is characteristic for those engineered habitats, which provide organic carbon sources for heterotrophic growth together with inorganic electron donors for chemolithoautotrophic growth.

5.3.2 N-removal processes in biofilms

Putative genes involved in the steps of nitrification and denitrification were identified via HMM analysis. Subsequent KEGG analysis of nitrogen removal genes revealed a higher abundance of denitrification genes compared to the relatively low abundance of nitrification genes (*amo* and *hao* genes accounted for <1 % and <3 %, respectively, of the total investigated N-removal genes in the three samples). Putative genes encoding for nitrite oxidoreductase (NXR) as functional marker for nitrite oxidation, have not been detected in the metagenomic data sets via KEGG classification of N-removal genes. Inconsistencies in HMM-based metagenomic analyzes regarding to NXR is a common problem and it may relay to the affiliation of that enzyme with the type II DMSO reductase-like family of molybdopterin-binding enzymes (Lücker *et al.* 2010; Lücker *et al.* 2013). The used HMMs in this study are based on molybdopterin and molybdopterin-binding domains, which are found among a number of molybdopterin-containing oxidoreductases. Hence, the HMMs cover a broad range of enzymes belonging to this family with other activities than nitrite oxidation, making the differentiation between these enzymes via HMM-based analysis difficult. However, the observed ratio of nitrification and denitrification genes is consistent with the taxonomic analysis results of the 16S rRNA amplicon sequencing data in this study. These data indicated a low portion of nitrifiers on the total microbial communities (approximately 10 % of the total reads were affiliated with nitrifiers). One reason for the low abundance of nitrifiers in biofilms might be the high oxygen requirement for nitrification (4.6 g O_2 for 1.0 g NH_4^+-N) (Paredes *et al.* 2007), which is difficult to maintain in biofilms with dense biomass. The gradients of dissolved oxygen concentration in the biofilm matrix may facilitate growth of denitrifiers, as the majority of denitrifiers prefers anoxic environments. In addition, adequate organic carbon, which is necessary for denitrification, is provided by the release of fish waste and microbial biomass.

Taxonomic classification of nitrogen removal genes revealed *Proteobacteria* followed by *Planctomycetes*, *Bacteroidetes* and *Nitrospirae* as the most abundant phyla. The same phyla

have been identified as the most abundant via marker gene analysis of the metagenomic data sets as well as via 16S rRNA amplicon sequencing, suggesting that members of these dominant phyla play the key role in nitrogen removal processes in the biofilms. In addition, the results of the genus level classification of metagenomic data revealed several genera likely involved in nitrification (*Nitrosomonas*, *Nitrospira*) and denitrification (e.g. *Lewinella*, *Haliscomenobacter*) and interestingly, the same genera were found among the frequent core-taxa in the 16S rRNA amplicon sequencing data set (see section 5.1.5).

Furthermore, the taxonomic analysis of nitrogen removal genes showed that *Nitrospira* appeared as the most abundant genus in samples FR (11 %) and BR (12 %), while in MA *Nitrospira* was less abundant (2 %). The high contribution of this NOB to the nitrogen removal bacteria may be explained by the fact that several nitrite oxidizing species are able to denitrify and hence, contain denitrification relevant genes. This indicates that *Nitrospira* may also play significant roles in denitrification processes in the biofilms. The low portion of *Nitrospira* in the marine sample was surprising since the 16S rRNA amplicon sequencing analysis showed conflicting results, with the highest abundance of this NOB identified in the marine biofilter samples. One explanation for the lower abundance of *Nitrospira* found in the metagenome of MA, could be the fact that the marine sample does not contain *Nitrospira*-like *amoA* genes unlike FR and BR, as discussed below in section 5.3.4.

To sum up, the HMM analysis and subsequent KEGG classification of the metagenomic data sets confirmed the hypothesis that biocarriers in RAS biofilters provide an environment suitable for heterotrophic and lithotrophic organisms, performing nitrogen removal processes.

5.3.3 Enzymes involved in carbon breakdown and turnover

The HMM-based analysis of the metagenomic data sets with regard to GHs revealed a high diversity and abundance of putative genes encoding for enzymes belonging to different GH families. GHs are a widely distributed group of enzymes, which hydrolyze the glycosidic bond between two or more carbohydrates and also between a carbohydrate and a non-carbohydrate moiety and they have been used in this study as marker for carbon breakdown. The observation of a high diversity of GH family enzymes combined with their high abundance, suggests that heterotrophic processes such as organic carbohydrate degradation are frequently performed in the biofilms. In this context, the most abundant GH family identified in the three data sets was GH23, which encompasses enzymes such as lysozyme, peptidoglycan lyase as well as chitinase. The presence of these enzymes may be linked to the degradation of bacterial cell walls, indicating the utilization of (dead) bacterial biomass as heterotrophic process in the biofilms. Chitinases though, can be involved in the degradation of zooplankton carapace, which is likely as small crustaceans are feed in larvae modules. However, the identified GH families encompass a variety of hydrolytic enzymes with diverse functions, e.g. endo- and exoglucanases, arabinofuranosidases, endoxylanases, cellobiohydrolases, and xyloglucanases, making these habitats sources for potentially novel enzymes with biotechnological application. The results of the phylogenetic classification of GHs revealed that the majority of genes encoding for these enzymes

is found among the phyla *Proteobacteria* and *Bacteroidetes*. This strengthens the assumption that members of these phyla are responsible for carbon breakdown in the biofilms as discussed before in section 5.1.3. The results of the genus level classification showed very diverse genera appearing in low abundances, indicating that the GH catalyzed processes of organic matter degradation are not mediated by characteristic predominating taxa. The appearance of few reads assigned to GHs of chemolithoautotrophic nitrifying genera such as *Nitrosomonas* and *Nitrospira* are very likely related to their carbon turnover processes necessary for growth and replication.

5.3.4 Complete ammonia oxidizers in RAS biofilters

ORF annotation gave metagenomic evidence for the presence of *Nitrospira* bacteria capable to perform the complete ammonia oxidation to nitrate, in the freshwater and brackish biofilm samples. Only recently, members of the genus *Nitrospira* have been discovered as complete ammonia oxidizers (comammox) and three species have been described: Cand. *Ns. inopinata* ENR4, Cand. *Ns. nitrosa*, Cand. *Ns. nitrificans* (van Kessel *et al.* 2015; Daims *et al.* 2015). So far, further evidence for comammox *Nitrospira* was only reported in few studies about oligotrophic systems such as a drinking water distribution system, a rapid gravity sand filter for the production of drinking water as well as in activated sludge and biofilm from a municipal WWTP (Pinto *et al.* 2015; Palomo *et al.* 2016; Chao *et al.* 2016). These findings indicate that comammox *Nitrospira* are widespread in engineered ecosystems. Corresponding to the hitherto publications, no marine comammox *Nitrospira* has been identified in the present study. Reasons for their absence in marine habitats still remain unclear. In this study, two different *Nitrospira*-like *amoA* genes were identified, with closest similarity to the *amoA* gene of *Ns. nitrosa* in sample FR and of *Ns. inopinata* in sample BR. In accordance with the metagenomic data sets, an organism closely related to *Ns. inopinata* was identified in the same brackish sample (GMA) via 16S rRNA amplicon sequencing (see section 4.1.5). In contrast, in FR, the finding of an organism with sequence similarity to *Ns. nitrosa* could not be confirmed via 16S rRNA sequencing. However, the discoverer of the initial comommox *Nitrospira* stated that these organisms can not be easily distinguished from strictly nitrite oxidizing *Nitrospira* in 16S rRNA-, *nxrA*- or *nxrB*-based phylogeny (Daims *et al.* 2016), explaining the discrepancy observed in the present study.

The finding of comammox *Nitrospira* related to *Ns. nitrosa* in RAS biofilters is not surprising since this organism was initially enriched from a trickling filter connected to an aquaculture system (van Kessel *et al.* 2015). In contrast to that, *Ns. inopinata* was initially enriched from a moderate thermophilic biofilm submerged in hot groundwater and hence, it was not expected to occur in the brackish RAS biofilter. However, relatives of other thermophilic NOB have been identified in the RAS biofilter samples via 16S rRNA amplicon sequencing in this study, suggesting that these organisms seem to be not restricted to thermophilic habitats. Moreover, the similarity of the putative *amoA*-gene in BR to the corresponding *amoA* gene of *Ns. inopinata* was only 92 %. This indicates that the identified organism represents a different species within

the genus *Nitrospira* and may even belong to a novel sublineage and not sublineage II as for the other described comammox *Nitrospira*.

The results of the *nxrB* gene amplicon sequencing in this study showed a broad *Nitrospira* microdiversity in all samples. Hence, the metagenomic identification of *Nitrospira amoA* genes in FR and BR in conjunction with the phylogenetic placement of the *nxrB* genotypes, indicates that comammox and solely nitrite oxidizing *Nitrospira* populations co-exist in the investigated RAS biofilters.

AOB versus *Nitrospira*-like *amoA* genes in biofilms The results of the ORF annotation showed that AOB *amoA* genes were identified in the freshwater and marine, but not in the brackish sample. This seems to be contradictory since a high microdiversity of *Nitrosomonas*-like AOB has been identified in the same brackish sample via *amoA* gene amplicon sequencing. In addition, as analyzed via EM and FISH, typical *Nitrosomonas*-like cells were visible in the biofilms, located in close vicinity to *Nitrospira*-like cells. This strengthens the assumption that AOB play a significant role in ammonia oxidation and that the process is unlikely catalyzed by comammox *Nitrospira* solely. Even if the applied NGS methods are very sensitive, potential errors have to be kept in mind. On the one hand, amplicon generated sequencing data may overestimate the diversity due to biases in initial PCR reactions. On the other hand, if the abundance of AOB *amoA* genes in the sample was low, this gene could have been under-represented in the metagenomic data set of sample BR and thus, it was not found via ORF annotation.

In conclusion, the analysis of the metagenomic data sets together with the *amoA* and *nxrB* gene amplicon sequencing generated data, provide evidence for the co-existence of both ammonia oxidizing types in the freshwater and the brackish biofilters, while in in the marine biofilters AOB seem to be the solely ammonia oxidizing organisms. The metagenomes revealed the functional potential of the novel *Nitrospira*-like *amoA* genes. Nevertheless, for the quantification of AOB and *Nitrospira* ammonia oxidation genes and to verify their function in RAS biofiltration, a RNA-based metatranscriptomic analysis is strongly required.

5.4 Nitrification performance in RAS biofilters

Determination of potential nitrification rates revealed complete nitrification in all operating biofilters but with different turnover rates. Marine module M5 was only low stocked with fish during time of sampling and the nitrifiers revealed only low nitrification rates in the performed nitrification potential tests. This reflects that nitrification performance in biofilters is directly affected by the ammonia concentration and therefore strongly depends on stocking density and feeding intensity. The results of the nitrification potential tests further showed that densely stocked marine modules M1 and M3 revealed the highest nitrite oxidation rates of all investigated biofilter samples. In addition, the NOB turnover rate was two to five times higher than the AOB turnover rate in the marine samples. The high nitrite oxidation rates of the marine NOB populations may be linked to the advantageous mixotrophic lifestyle of *Ns. marina*-like

NOB inhabiting the biofilms as discussed in section 5.1.7. Nevertheless, comparable high nitrite oxidation rates in a freshwater RAS biofilter mediated via *Nitrotoga* sp. were reported previously, indicating that high nitrification performance is not restricted to mixotrophic nitrite oxidizers (Hüpeden *et al.* 2016).

However, the data obtained in the present study implies that the nitrification process in RAS is very sensitive and that it depends on several parameters. Previous studies have shown that further important factors influencing nitrification kinetics in biofilters are e.g. the temperature, pH-value, dissolved oxygen concentration, organic matter content, salinity and turbulence (Chen *et al.* 2006; Crab *et al.* 2007).

5.5 Physiological characterization of biofilm inhabiting nitrifiers

5.5.1 Ammonia tolerance of AOB

Substrate tolerances of biofilm inhabiting AOB communities derived from biofilters of three aquaculture plants have been investigated in this study. The results showed that each sampling site revealed a distinct, specific response of the ammonia oxidizing bacteria to increasing substrate concentrations. In accordance with that, at each RAS a site specific AOB community was found via *amoA* gene amplicon sequencing (see section 4.2.1). The sequencing data further demonstrated that the AOB populations were mainly composed of different representatives of the genus *Nitrosomonas*. As Koops *et al.* (2001) stated, members of the different *Nitrosomonas* clusters represent organisms with different ecophysiological characteristics and distribution patterns and based on that, niche differentiation occurs. Consequently, distinct ammonia tolerances among the different AOB communities could be observed in this study.

The results of the substrate tolerance test showed that the freshwater derived AOB community, which mainly consisted of relatives of strain *Nitrosomonas* sp. A7, emerged as the most tolerant to high ammonia concentrations. BLAST analysis revealed that the closest taxonomically described species of strain *Nsm.* sp. A7 was *Nsm. eutropha*, which belongs to *Nitrosomonas* cluster 7. However, the activity of the freshwater AOB was stimulated with increasing substrate concentration of up to 10 mM where they reached the maximum ammonia oxidation rate. The marine AOB community, which was composed almost exclusively of relatives of *Nsm. aestuarii* (cluster 6B), revealed a slightly less pronounced tolerance to rising substrate levels and they reached the maximum ammonia oxidation rate in the presence of 5 mM substrate. In contrast to that, the brackish AOB with relatives of *Nsm. urea* (cluster 6A) representing the predominant group, were the most sensitive to elevated substrate concentrations and ammonium levels above 2 mM led to a gradual decrease in ammonia oxidation activity. The observed differences in substrate optima and tolerance levels correlate very well with the described substrate affinities for isolated and taxonomically described species of the distinct *Nitrosomonas* clusters. Accordingly, characterized species of *Nitrosomonas* cluster 7 members, such as *Nsm. eutropha*, showed low substrate affinities (Ks-value of 30–61 µM). Marine cluster 6B-like organisms such as *Nsm. aestuarii*, revealed only slightly higher substrate affinities (Ks-

value of 50–52 µM). In contrast to that, *Nsm. urea* as a member of cluster 6A, showed high ammonia affinity (Ks-value of 1.9–4.2 µM) (Stehr 1995; Bollmann *et al.* 2002a; Koops *et al.* 2001). Bollmann *et al.* (2002a) reported that cluster 6A-like AOB revealed the ability to grow best at low substrate concentrations and even 10 mM ammonium was inhibitory. In contrast, in the same study *Nsm. eutropha* (cluster 7) was able to tolerate 600 mM substrate. These data strongly support the observation made in the present study with 10 mM ammonium leading to a reduction and 25 mM to a complete inhibition of ammonia oxidation in the brackish samples, while the freshwater and marine AOB tolerated the raised substrate levels. The data discussed above are all based on cultured isolates of different *Nitrosomonas* species. However, major findings about their substrate affinities could be confirmed for biofilm and activated sludge inhabiting AOB populations (Schramm *et al.* 1999; Limpiyakorn *et al.* 2005; Suwa 1994; Suwa *et al.* 1997), further supporting the results of this study.

Interestingly, besides *Nitrosomonas*-like AOB, members of *Nitrosospira* were identified in freshwater sample C via *amoA* gene amplicon sequencing. Although *Nitrosospira* spp. are not considered as typical ammonia oxidizers in wastewater treatment or other engineered systems, they have been observed occasionally in wastewater treatment systems (Park *et al.* 2002). Members of *Nitrosospira* cluster 3 have even shown a high tolerance towards raised ammonia concentrations (Webster *et al.* 2005). However, the contribution of these organisms to ammonia oxidation at high substrate concentration is unclear since the methods used in this study do not give comparative information about the metabolically active AOB.

5.5.2 Nitrite tolerance of NOB

The results of the substrate tolerance test demonstrated that biofilm inhabiting NOB communities derived from three aquaculture plants were similarly tolerant towards nitrite concentrations from 0.5–5 mM. In comparison with the freshwater samples, the brackish and marine nitrite oxidizers revealed a slightly higher tolerance towards nitrite levels above 5 mM.

Previous studies demonstrated that the nitrite concentration is an important factor in the competition of NOB, indicating that substrate availability may account for the distribution of different NOB in natural and artificial ecosystems (Schramm *et al.* 1999; Nogueira *et al.* 2006; Maixner *et al.* 2006; Blackburne *et al.* 2007; Nowka *et al.* 2015a). Considered as K-strategist with the ability to exploit low amounts of nitrite, *Nitrospira* was identified as the dominating NOB in engineered systems like WWTPs with usually low nitrite concentrations (Daims *et al.* 2010). Accordingly, different members of the genus *Nitrospira* have been identified as predominant NOB in the present study, with a specific subpopulation appearing at each RAS.

The data of the *nxrB* amplicon sequencing analysis revealed that relatives of *Ns. defluvii* and *Ns. moscoviensis* represented the dominant nitrite oxidizing bacteria in the biofilters of the freshwater RAS (see section 4.2.2). Maixner *et al.* (2006) reported that sublineage II *Nitrospira* (*Ns. moscoviensis*) are outcompeted by sublinage I *Nitropira* (*Ns. defluvii)* at high nitrite concentrations. This observation could be confirmed by Nowka *et al.* (2015a) via growth experiments on isolates *Ns. defluvii* A17 and *Ns. lenta* BS10 (sublineage II), in which the

former showed nitrite oxidation activity in the presence of up to 30 mM nitrite, while the latter revealed the upper growth limit already when supplied with 2.5 mM nitrite. In addition, in kinetic experiments *Ns. defluvii* revealed higher nitrite oxidation activities than *Ns. moscoviensis* (Nowka *et al.* 2015a). Therefore, the co-existence of *Ns. defluvii* and *Ns. moscoviensis* found in the freshwater biofilms in the present study is not surprising since both are well suited for the low substrate environment in RAS biofilters, but *Ns. defluvii* seems to be better adapted to a temporary rise in substrate concentration than *Ns. moscoviensis*. Considering this, it is reasonable that the *Ns. defluvii*-like NOB population, detected in the freshwater biofilms, was responsible for nitrite oxidation when exposed to higher substrate concentrations during the substrate tolerance test. However, the results of the tolerance test further showed that nitrite oxidation of the freshwater RAS derived NOB community was reduced by 90 % when exposed to 25 mM substrate.

Contrary to this, in the brackish and marine samples the nitrite oxidation rate was reduced by 70 % in the presence of 25 mM nitrite and thus, the NOB seem to cope better with elevated nitrite concentrations than the freshwater communities. The *nxrB* amplicon sequencing analysis revealed a co-existence of *Ns. defluvii*- and *Ns. marina*-like NOB in the brackish biofilters, while *Ns. marina*-like NOB represented the predominant group in the marine samples (see section 4.2.2). *Ns. marina*-like NOB (sublineage IV) are known to have low nitrite tolerance. Watson *et al.* (1986) reported no further growth of isolate *Ns. marina* 295 at nitrite concentrations above 6 mM. Therefore, when considering the species composition in the brackish and marine biofilms, the observed high nitrite tolerance seems to be unexpected. However, it has to be highlighted that in the study of Keuter *et al.* (2011) a novel marine NOB, *Ns.* strain Ecomares 2.1, was successfully isolated from a marine RAS. As a member of *Nitrospira* sublineage IV, Ecomares 2.1 revealed unexpected eco-physiological features such as a high nitrite tolerance. This observation was later on supported via kinetic experiments in which Ecomares 2.1 exhibited a low substrate affinity, even slightly lower than that of marine *Nitrobacter* sp. 311 and lower than the substrate affinities determined for *Ns. defluvii* and *Ns. moscoviensis* (Boris Nowka unpublished data, Nowka *et al.* 2015a). Consequently, the differences between *Ns. marina* 295 and *Ns.* marina-like strain Ecomares 2.1 reflect that physiological differences may even occur between members within one phylogenetic sublineage of *Nitrospira*. Thus, it is possible that the *Ns. marina*-like nitrite oxidizers detected in the two saline RAS in this study, represent novel species of marine *Nitrospira* with unexpected physiological features, which could explain the observed high nitrite tolerance.

Ammonia and nitrite load in RAS biofilters While it is often assumed that wastewater treatment systems contain high levels of ammonia/ammonium, well-managed systems often maintain a rather low ammonia/ammonium pool. Likewise, RAS are rather low ammonia/ammonium habitats and the modules investigated in this study revealed mean ammonium concentrations of 0.1–0.3 mg l^{-1} (0.006–0.02 mM). Nitrite, as intermediate of the ammonia removal process in RAS, amounted to mean values of 0.2–0.3 mg l^{-1} (0.005–0.006 mM) in the three investigated RAS. With 0.5–25 mM, the tested concentrations of ammonium and nitrite

in the substrate tolerance test were far above the predominant ammonium and nitrite values in the biofilters and are not expected to occur in RAS. In this context, the biofilm inhabiting nitrifiers have shown relatively high substrate tolerances. They were proven to be stable at elevated substrate concentrations in batch-experiments and a significant reduction of the nitrifying activity only occurred when exposed to substrate concentrations of 10 mM and higher. However, the sensitive process of nitrification depends on several factors as discussed above (section 5.4).

5.5.3 Effect of nitrate on the nitrification performance

Nitrification is a syntrophic process between AOB and NOB. Since nitrate as the endproduct of nitrification can accumulate in RAS biofilters, it was of interest to study the impact of nitrate on the nitrification performance for both groups. Despite the importance, not much published data is found regarding the influence of nitrate on ammonia and nitrite oxidation efficiency of AOB and NOB, respectively. The few published studies are restricted to isolates and enrichment cultures of *Nitrosomonas* and *Nitrobacter* or *Nitrospira*.

AOB In the present study the biofilm inhabiting AOB revealed a much higher tolerance towards all tested nitrate concentrations (0.5–15 mM) than the NOB, although they were derived from the same RAS biofilters and exposed to the same environmental conditions. Even when spiked with 15 mM nitrate the ammonia oxidation activity of the AOB communities was not significantly affected. The tolerance of AOB to high nitrate concentrations was observed previously at pure cultures of *Nitrosomonas* (Jones *et al.* 1980; Hunik *et al.* 1992). Recently, Keuter (2012) showed a high nitrate tolerance of biofilm inhabiting AOB derived from marine RAS biofilters, which is in agreement with the findings for the freshwater and brackish RAS derived AOB in this study. In contrast to the observed nitrate tolerances of ammonia oxidizers, Dunfield *et al.* (1995) found a non-competitive inhibition in methane oxidizers in soils at >10 mM nitrate (the enzyme methane monooxygenase is phylogenetically closely related to the ammonia monooxygenase). However, the authors credited the inhibitory effect rather to osmotic stress and pH than directly to nitrate.

In accordance with the biofilm inhabiting AOB investigated in the present study, AOB isolates and enrichment cultures revealed a high nitrate tolerance as mentioned above. Therefore, it is reasonable that not the biofilm itself protects the microorganisms from potential inhibitory substances, as suggested earlier (Allison *et al.* 1993), but rather the insensibility of the enzyme ammonia monooxygenase to nitrate.

NOB The results of the nitrate tolerance tests showed that the NOB communities from all three RAS were similarly sensitive to that ion and the inhibitory effect started already in the presence of 0.5 mM nitrate.

The nitrate sensitivity of NOB was first observed in studies with *Nitrobacter* isolates, in which it was shown that the addition of nitrate caused an extension in the lag-phase and higher

concentrations even resulted in a total inhibition of nitrite oxidation (Aleem *et al.* 1960; Gould *et al.* 1960; Schön 1965). Later on, this effect was confirmed with representatives of the genus *Nitrospira*. However, the observed inhibitory nitrate concentrations for members of *Nitrobacter* were much higher than that for members of *Nitrospira* (Schön 1965; Ehrich *et al.* 1995; Keuter 2012). A plausible explanation for the difference in the nitrate tolerance of these two NOB might rely on the differences in their functional enzyme, the nitrite oxidoreductase. The NXR of *Nitrobacter* is located in the cytoplasm (Sundermeyer *et al.* 1981). In contrast, the NXR of *Nitrospira* is located in the periplasmic space (Spieck *et al.* 1998) and thus more exposed to nitrate ions penetrating the outer membrane. Nevertheless, in accordance with the different substrate affinities as discussed above (section 5.5.2), differences in the nitrate tolerance have been reported among members of different *Nitrospira* sublineages (Nowka *et al.* 2015b; Keuter 2012). However, in this study the different NOB communities were similarly sensitive to nitrate, underlining the physiological differences between isolated species in cultures and environmental communities. In general, nitrate might cause osmotic stress in the cells (Zhang *et al.* 2013) but other influences and factors controlling the nitrate tolerance, still have to be identified (He *et al.* 2010).

The results of the nitrate tolerance test furthermore showed that the NOB communities from freshwater module F were slightly more sensitive to rising nitrate levels compared to the communities from module C derived from the same RAS. Module F was connected to a denitrification reactor to keep the nitrate concentration low. Thus, the NOB in the biofilter were exposed to lower nitrate concentrations than in module C, which was in operation without denitrification step. The mean nitrate concentrations in the freshwater plant were 158 $\pm$16.7 mg l^{-1} (2.5 mM) in module C, and 75 $\pm$20.4 mg l^{-1} (1.2 mM) in module F. Accordingly, the NOB derived from module C revealed a higher tolerance to increased nitrate concentrations in the tolerance tests in comparison to the organisms derived from module F. These findings indicate that the nitrite oxidizing communities inhabiting the biocarriers seem to develop a tolerance towards nitrate with increasing nitrate concentration of the surrounding biofilter water. This effect was previously observed in the study of Keuter (2012), whereat biocarrier inhabiting NOB derived from a marine RAS with lower average nitrate concentrations showed a lower nitrate tolerance compared to organisms derived from a RAS with higher concentrations. These data strongly led to the assumption that adaption plays a key role in the nitrate tolerance of NOB.

Nitrate load in RAS biofilters It is often stated that nitrite oxidation, as the second step of nitrification, seems to be more sensitive to stress factors and harder to establish in biofilters of RAS than the ammonia oxidation step (Schroeder *et al.* 2015). Moreover, interim interruptions in nitrite oxidation during biofiltration in well-established RAS and resulting nitrite peaks, with consequences for the well-being of fish, are reported frequently. The results of the nitrate tolerance tests in this study have shown that nitrate concentration of 0.5 mM already affect the nitrite oxidation of biofilm inhabiting NOB. Hence, if nitrate accumulates in the rearing water it is very likely that thereby the nitrite oxidation is inhibited due to the nitrate sensitivity of NOB. Therefore, if no denitrification reactor is implemented, the nitrate concentration should

be kept low via water exchange to maintain efficient nitrite oxidation inside the biofilters.

5.5.4 Nitrification at different salinities

In salt tolerance batch-experiments with colonized biocarriers, derived from three different RAS, it was shown that the ammonia and nitrite oxidation performance of AOB and NOB, respectively, was similarly affected. Moreover, the nitrifying community at each RAS revealed a characteristic response to changes in the salinity.

The data revealed a gradual decrease in the nitrification activity of the freshwater and brackish nitrifiers with increasing salinity. This inverse relationship between salinity and nitrification rate was reported in several previous studies e.g. of estuary derived AOB isolates (Jones *et al.* 1980; Stehr 1995; Bollmann *et al.* 2002b) as well as sediment and water samples along salinity gradients containing AOB and NOB (Rysgaard *et al.* 1999; Andersson *et al.* 2006; Caffrey *et al.* 2007). In addition, this observation was confirmed via wastewater operated reactor experiments addressing the nitrifying consortium (Windey *et al.* 2005; Moussa *et al.* 2006a,b; Aslan *et al.* 2012; Cortés-Lorenzo *et al.* 2015; Gonzalez-Silva *et al.* 2016).

The results of the salt tolerance test further showed that the brackish nitrifiers revealed their highest nitrification rates in the 0 PSU batches, even though they were derived from biofilters with a salinity of 4-5 PSU in the process water. Likewise, the marine nitrifiers revealed the maximum nitrification activity at 15 PSU, which is lower than the predominant salinity in the RAS (26 PSU). In accordance with that, the highest ammonia oxidation rate of *Nitrosomonas* isolates, derived from brackish estuary sections, was reported in freshwater medium (Stehr 1995; Bollmann *et al.* 2002a). The same effect was recently reported for nitrifiers in batch-reactors, which revealed an increase in nitrification activity when exposed to lower salt concentrations than the native salinity (Gonzalez-Silva *et al.* 2016). Consequently, lowering the salinity can cause a stimulatory effect on nitrification due to the relief in stress.

However, the brackish and marine nitrifiers in this study kept their nitrification performance in a broad spectrum of salinities tested and hence, managed salinity related stress reasonably well. The freshwater communities though, were strongly affected even at low salinity. Similar results have been observed in batch-reactor experiments with non-adapted freshwater biofilm samples, which revealed a decrease in nitrifying activity of 97 % when exposed to 33 PSU (Gonzalez-Silva *et al.* 2016), supporting the results of the present study. Thus, adaption to the salt concentrations predominant at the RAS provides a plausible explanation for the different salt tolerance levels observed. Accordingly, despite the fact that nitrification can be affected by salinity, adaption and acclimatization to salt seems to be a promising way to maintain nitrification in saline wastewaters.

Besides salt adaption, another possible reason for the observed differences in the salt tolerances can be linked to the nitrifying community composition. The results of the *amoA* and *nxrB* gene amplicon sequencing (see section 4.2.1) revealed that the freshwater nitrifiers were dominated by environmental clones distantly related to isolate *Nitrosomonas* sp. A7 and to *Nitrospira defluvii*, while the latter is known as characteristic nitrite oxidizer in freshwater

habitats. The low resistance to salt of the freshwater AOB community seems to be unexpected when correlating with the dominating AOB. *Nsm.* sp. A7 has been originally isolated from a sequencing batch bioreactor (Accession: KF194201.1) and was phylogenetically classified into *Nitrosomonas* cluster 7, which contains halotolerant to halophilic species with high Km-values (Koops *et al.* 2001). Moreover, the results of the substrate tolerance tests in this study revealed that the same freshwater AOB community was highly tolerant to raised substrate concentrations (see section 4.6.1). This implies a halotolerant character when considering that high substrate concentrations can also cause osmotic stress. However, the similarity of the sequences to the *amoA* gene sequence of *Nsm.* sp. A7 was low (81–82 %) and hence, the organisms identified in the freshwater biofilms likely represent novel *Nitrosomonas* species with distinct physiological characteristics. One limitation of this study was that the tolerance tests were performed under short-term incubation. Long-term experiments are necessary to verify if these organisms, such as the novel freshwater *Nitrosomonas*-like AOB, are able to adapt to changes in the salinity.

The nitrifiers of the brackish and marine samples reacted differently to salt stress than that of the freshwater samples, which is likely due to the high abundance of marine species with halotolerant to halophilic character. In the salt tolerance test, the brackish nitrifying communities nicely reflected the halotolerant character with preferred growth in freshwater but revealing tolerance to a broad spectrum of salinities. Accordingly, the amplicon sequencing data showed that the samples consisted mainly of halotolerant *Nitrosomonas urea*-like AOB, which were found in brackish environments before, but do not require marine medium for growth in culture (Koops *et al.* 2001). Interestingly, the brackish NOB guild consists in almost equal amounts of non-halotolerant *Nitrospira* defluvii- together with *Nitrospira* marina-like NOB, while the latter species is well known from saline environments (Watson *et al.* 1971). The composition of organisms closely related to obligate halophilic species *Nitrosomonas aestuarii* and *Nitrospira marina* (Watson *et al.* 1971; Koops *et al.* 2001) in the marine samples, is clearly in accordance with the observed high robustness to salt stress. It displays a halophilic lifestyle with growth in saline environments and the requirement of a certain minimum level of salt for survival (Lay *et al.* 2010). However, the data further showed that the marine NOB were strikingly more resistant to low salt concentrations than the AOB, suggesting a broader physiological flexibility of the NOB and the existence of osmoregulatory mechanisms. Nevertheless, it is well known that salinity causes osmotic stress for the cells, but the strategies behind the tolerances and adaption mechanisms of nitrifiers are still unclear.

All things considered, a characteristic AOB and NOB community was identified at each sampling site, reacting differently to salt stress. The data obtained in this study help to improve the nitrification process in saline environments, which is relevant in wastewater treatment with particular importance for seawater operated RAS.

5.5.5 Conclusion

In this study 16S rRNA as well as *amoA* and *nxrB* gene amplicon sequencing analysis provided a comprehensive insight into the AOB and NOB community compositions in biofilters

of three RAS operated at different salinities. At each RAS a site specific nitrifying community was identified and consequently, characteristic physiological properties of AOB and NOB from each RAS could be revealed via tolerance tests (summarized in table 14). One main finding of this study was that the AOB and NOB communities from one sampling site showed different responses to the tested stress factors, even though they were adapted to the same prevalent conditions at the RAS. This highlights the difficulty of maintaining a high nitrification performance in RAS, as a two-step process. The data demonstrated that the freshwater AOB revealed a high tolerance to rising ammonium concentrations, while the NOB were sensitive to increased nitrite levels. In contrast to the freshwater NOB, the nitrite oxidizers derived from the brackish and marine biofilters were more tolerant to nitrite related stress. Moreover, the AOB communities were highly tolerant to nitrate, while the NOB slowed down their nitrite oxidation activity in dependence on the nitrate concentration. Furthermore, via salt tolerance tests it was shown that the nitrification performance of freshwater nitrifiers was drastically reduced with increasing salinity, while the salt adapted nitrifiers from the brackish and marine RAS showed a high robustness and maintained nitrification in a broad range of salinities.

Finally, the data obtained via the tolerance tests have shown how different the nitrifying populations, derived from biofilters of three RAS, react to the tested stress factors, even though the biofiters provide similar habitats. This indicates the complexity of nitrifying populations inhabiting biofilms and their distinct physiological plasticity. The gained knowledge can be used for optimization of the nitrification process in RAS biofilters.

5.6 Niche differentiation between *Nitrotoga* and *Nitrospira* in a RAS biofilter

Before starting this thesis, a co-occurrence of *Nitrotoga* and *Nitrospira* has been observed in biofilters of a cold freshwater RAS (Off 2012) operated at 13 °C with a slightly acidic pH-value of 6.8 in the rearing water. As part of the present study, the question was investigated which of the two nitrite oxidizers dominated the biofilms and in addition, a cultivation-based approach was chosen to explore competitive factors of these two NOB. The visualization of biofilm inhabiting bacteria via FISH-staining performed in this study, clearly showed that *Nitrotoga* was more abundant than *Nitrospira*. The observed predominance of *Nitrotoga* was surprising since this NOB has not been reported in published studies about RAS biofilters so far. Instead, *Nitrospira*-like NOB seem to represent the dominant nitrite oxidizers in these systems (Itoi *et al.* 2006; Foesel *et al.* 2008; Keuter *et al.* 2011; Kruse *et al.* 2013). However, *Nitrotoga* was reported recently as predominant NOB in different WWTPs (Lücker *et al.* 2015). Since the discovery of *Nitrotoga arctica* in permafrost affected Siberian soils it is well known as cold adapted NOB (Alawi *et al.* 2007), often detected in nutrient-limiting habitats like pristine soils or drinking water (Pradhan *et al.* 2010; White *et al.* 2012). Low substrate concentrations of 0.3 mM nitrite were regarded as optimal for growth of *Nt. arctica* and early enrichment cultures were already inhibited at 1.2 mM (Alawi *et al.* 2007). However, this finding has to be reconsidered, since Lücker *et al.* (2015) detected activity of *Nitrotoga* from wastewater up

Table 14: Summarized results of the performed substrate, nitrate and salt tolerance tests with nitrifying communities, derived from three RAS operated at different salinities, and the respective predominant AOB and NOB population identified via amplicon sequencing of the *amoA*, *nxrB* and 16S rRNA genes, respectively (with sequence similarities to the closest taxonomically described species in brackets). (a) AOB (b) NOB.

(a)

	C (FR)	GMA (BR)	M1 (MA)
NH_4^+ tol.	+++	+	++
NH_4^+ opt.	10 mM	2 mM	5–10 mM
NO_3^- tol.	+++	+++	n.p.
Salinity tol.	-	++	+++
Salinity opt.	0 PSU	0 PSU	15 PSU
amoA	*Nsm.* sp. A7 (81–82 %)	*Nsm. urea* (91–96 %)	*Nsm. aestuarii* (78–99 %)
16S rRNA	*Nsm. communis* (96 %)	*Nsm. urea* (98 %)	*Nsm. aestuarii* (98 %)

(b)

	C (FR)	GMA (BR)	M1 (MA)
NO_2^- tol.	+	++	++
NO_2^- opt.	2–5 mM	5 mM	5 mM
NO_3^- tol.	-	-	-
Salinity tol.	-	++	+++
Salinity opt.	0 PSU	0 PSU	15 PSU
nxrB	*Ns. defluvii* (89–98 %)	*Ns. defluvii*(88–97 %) *Ns. marina* (89–97 %)	*Ns. marina* (89–97 %)
16S rRNA	*Ns. defluvii* (92–100 %)	*Ns. defluvii* (99–100 %)	*Ns. salsa* (96–99 %)

tol.=tolerance; opt.=optimum; n.p.=test was not performed

- highly sensitive; + low tolerant; ++ medium tolerant; +++ highly tolerant

to 10 mM nitrite. The ability of *Nitrotoga* to oxidize nitrite in relatively high concentrations could be a reason for their appearance in WWTPs (Alawi *et al.* 2009; Lücker *et al.* 2015) and aquaculture systems (this study) with elevated nitrite concentrations, although the tolerance level of 30 mM of *Nitrospira defluvii* is much higher (Nowka *et al.* 2015a). As recently shown by Nowka *et al.* (2015b), *Nitrotoga* is able to compete with other NOB at temperatures of 17 °C and below, where activity of *Nitrospira* is drastically reduced. These findings further strengthen the assumption of the importance of *Nitrotoga* in cold environments, such as the RAS operated at 13 °C investigated in this study.

Nt. arctica has been isolated from permafrost soils with a pH-value of 5.6 to 6.5 (Sanders *et al.* 2010). This indicates that slightly acidic conditions might be selective factors for this NOB. Hence, besides the temperature, the pH-value was taken under consideration for the purpose of this study, since most RAS are operated at neutral pH and a possible reason for the predominance of *Nitrotoga* over *Nitrospira* here, could be the slightly acidic pH of 6.8 in the rearing water. Validation of this hypothesis was done by conducting FISH-MAR with enrichment cultures containing *Nitrotoga* and *Nitrospira* derived from the investigated RAS. These activity studies have shown that both NOB were active when incubated at 14 °C in media with pH 6.4 and 7.2. Nevertheless, it should be taken into account that FISH-MAR was done under short term incubation (6h) and that only long term incubation might have led to visible differences in the metabolic activity of both NOB. However, in further experiments *Nitrotoga* derived from this RAS could be successfully highly enriched (>99 %) and separated from *Nitrospira* by the application of a growth medium with a slightly acidic pH-value (personal communication Simone Wegen). Subsequent growth experiments of this highly enriched '*Nitrotoga* sp. HW29' in comparison with *Nitrospira defluvii* A17 clearly showed distinct pH optima for these two NOB (6.8 for *Nt.* sp. HW29 and 7.3 for *Ns. defluvii* A17). These facts indicate the separation of *Nitrospira* and *Nitrotoga* into distinct ecological niches and further strongly confirm the assumption for the relevance of *Nitrotoga* in the investigated RAS running at pH 6.8. (Hüpeden *et al.* 2016). With the adaption to a slightly acidic pH, *Nitrotoga* might be able to occupy distinct habitats, such as in acid-impacted lakes (Percent *et al.* 2008). This fact makes this NOB even more interesting regarding to niche differentiation of NOB. *Nitrotoga* seems not only to be cold-adapted, but additionally moderately acid-tolerant.

The co-existence of *Nitrotoga* and *Nitrospira* in the biofilter of the investigated RAS was regularly monitored via genus-specific PCR in a time series of two years (2010 to 2012) and a continuous presence of both NOB was noticed as described in Hüpeden *et al.* (2016). In further monitoring studies of the biofilter though, *Nitrotoga* was not detectable anymore via genus-specific PCR and FISH from late 2012 until now (personal communication S. Wegen). The sudden disappearance of *Nitrotoga* was strongly associated with the use of disinfectant treatments in the RAS as the negative PCR results started immediately after that treatment. Hence, it was assumed that the disinfectant had led to the extinction of this NOB. However, nitrite oxidation continued in the biofilters as assessed via nitrification potential tests performed by S. Wegen. These facts let to the assumption that *Nitrospira* seems to be more tolerant to

the disinfectant and able to recover more quickly than *Nitrotoga*, which was previously the dominant NOB in the same biofilters.

To conclude, these data implicate the complexity of niche differentiation and population dynamics of different NOB such as *Nitrotoga* and *Nitrospira*. The biofilms provide niches for different co-existing NOB. This and temporal variations in operational parameters (e.g. temperature, pH, nitrite concentration, use of disinfectant) may support the co-existence of different nitrite oxidizers with different ecophysiological adaptions. Considering that seasonal shifts in operational parameters during operation of RAS provide temporally optimal conditions for either group, the co-existence of different NOB might stabilize nitrite oxidation performance in the biofilters with beneficial consequences for fish production.

A Appendix

A.1 Supplementary material

A.1.1 Supplementary tables

Table S1: Mean operational and physicochemical data of the different modules at the three investigated RAS. Physicochemical parameters were examined in the process water of the respective module. Mean values were calculated from the beginning of a rearing cycle until the day of sampling (between three to six months). If available, standard deviation is given below the respective value, otherwise data have been examined during day of sampling. Parameters were determined at the time of sampling for the tolerance tests with nitrifiers.

	C(FR)	GMA(BR)	GMA(BR)[1)]	M1(MA)
Temp [°C]	22.68	18.10	19.50	24.9
	±1.01	n.a.	n.a.	±0.74
pH	7.20	7.10	6.98	7.47
	±0.17	n.a.	n.a.	±0.09
PSU	n.a.	3.86	5.42	25.15
	n.a.	n.a.	n.a.	±1.93
NH_4^+ [mg l^{-1}]	0.13	0.11	0.09	0.27
	±0.05	±0.04	±0.04	±0.12
NO_2^- [mg l^{-1}]	0.29	0.07	0.07	0.55
	±0.09	±0.02	±0.02	±0.23
NO_3^- [mg l^{-1}]	158.52	108.51	213.84	n.a.
	±16.73	±60.99	±45.04	n.a.
Feed [kg d^{-1}]	3.67	1.98	1.44	3.90
	±1.74	±0.62	±0.56	n.a.
Fish [kg]	1900	145.05	154.31	393.45
	n.a.	±54.98	±54.11	n.a.

[1)]Parameter determined at the time of sampling for the salt tolerance tests

[1)]n.a.= Data not available

Table S2: Mean operational and physicochemical data of the different modules at the three investigated RAS. Physicochemical parameters were examined in the process water of the respective module. Mean values were calculated from the beginning of a rearing cycle until the day of sampling (between three to six months). If available, standard deviation is given below the respective value, otherwise data have been examined during day of sampling. Parameters were determined at the time of sampling for the NGS approaches.

	FR				**BR**			**MA**		
	C	D	F	TR	AQ1	GMA	RMB	M1	M3	M5
Temp. [°C]	22.02 ±0.57	22.00 ±0.72	22.21 ±0.96	12.20 ±0.91	18.79 ±3.79	18.95 ±0.31	18.68 ±0.70	16.56 ±0.64	15.89 ±0.52	18.02 ±0.35
pH	7.24 ±0.32	7.18 ±0.27	7.17 ±0.18	6.80 ±0	7.21 ±0.71	7.56 ±0.12	8.07 ±0.37	7.39 ±0.14	7.40 ±0.1	7.62 ±0.12
PSU	n.a.	n.a.	n.a.	n.a.	3.75 n.a.	3.89 n.a	5.02 n.a.	23.77 ±2.78	25.99 ±1.52	24.38 ±1.65
NH_4^+[mg l^{-1}]	0.31 ±0.16	0.29 ±0.12	0.26 ±0.10	0.37 ±0.55	0.51 ±0.27	0.27 ±0.11	0.59 ±0.78	0.28 ±0.14	0.29 ±0.13	0.11 ±0.09
NO_2^- [mg l^{-1}]	0.28 ±0.19	0.19 ±0.12	0.16 ±0.09	0.58 ±0.25	0.91 ±0.75	0.26 ±0.16	0.12 ±0.1	0.76 ±0.25	0.76 ±0.26	0.30 ±0.11
NO_3^- [mg l^{-1}]	135.05 ±28.05	150.46 ±25.54	79.04 ±35.76	60.17 ±11.88	43.65 ±32.43	112.48 ±65.70	71.34 ±33.38	n.a.	n.a.	n.a.
Feed [kg d^{-1}]	5.91 ±3.02	6.90 ±3.84	8.49 ±4.92	112.40 ±34.62	1.74 ±3.46	7.03 ±2.78	0.07 ±0.05	5.40 n.a.	6.70 n.a.	0.85 n.a.
Fish [kg]	1900 n.a	1900 n.a.	5600 n.a.	11646.50 ±2.01	54.63 ±44.49	768.68 ±346.17	1.10 ±0.48	1016 n.a.	3244 n.a.	91 n.a.

[1)]n.a.= Data not available

Table S3: Genus level core-taxa identified in the biofilter samples derived from three RAS after 16S rRNA amplicon sequencing using Illumina MiSeq. 85 and 76 core-taxa were identified in the total RNA (R) and DNA (D) pools. 1=taxon identified, 0=taxon not identified.

Genus	R	D	Genus	R	D	Genus	R	D
Acinetobacter	1	0	*Haliscomenobacter*	1	1	*Pseudaminobacter*	1	1
Altererythrobacter	1	1	*Haloferula*	1	1	*Reichenbachiella*	0	1
Aquicella	1	0	*Halomonas*	1	0	*Rhizobacter*	1	1
Atopococcus	1	0	*Hyphomicrobium*	1	1	*Rhodobacter*	1	1
Aureispira	1	1	*Iamia*	1	0	*Rhodobium*	1	1
Azospira	1	1	*Ideonella*	0	1	*Rhodopirellula*	1	1
Bacteriovorax	1	1	*Ilumatobacter*	1	1	*Rhodovulum*	1	1
Bdellovibrio	1	1	*Leadbetterella*	1	0	*Rickettsia*	1	1
Blastocatella	1	1	*Legionella*	1	1	*Roseibacillus*	1	0
Blastopirellula	1	1	*Lewinella*	1	1	*Roseobacter*	1	1
Bryobacter	1	1	*Limnobacter*	1	0	*Rubrivirga*	1	0
Byssovorax	1	0	*Maribacter*	1	1	*Runella*	0	1
Caldithrix	1	1	*Marinobacter*	1	1	*Sandaracinus*	1	1
Cand. *Alysiosphaera*	1	1	*Mesorhizobium*	1	1	*Sediminibacterium*	0	1
Cand. *Captivus*	1	1	*Muricauda*	1	1	*Shewanella*	1	0
Cand. *Metachlamydia*	1	0	*Mycobacterium*	1	1	SM1A02	1	1
Chlorochromatium	0	1	*Nannocystis*	1	1	*Sorangium*	1	1
Coxiella	1	1	*Nitratireductor*	1	1	*Sphaerotilus*	0	1
Crocinitomix	1	1	*Nitrosomonas*	1	1	*Thiothrix*	1	0
Cryobacterium	1	0	*Nitrospira*	1	1	Truepera	1	1
Cytophaga	0	1	*Novosphingobium*	1	1	*Turneriella*	1	0
Defluviicoccus	1	0	Ochrobactrum	1	1	*Variovorax*	1	1
Defluviimonas	1	1	OM27 clade	1	1	*Woodsholea*	0	1
Devosia	1	1	*Opitutus*	1	1	*Zymomonas*	0	1
Emticicia	0	1	*Paracoccus*	1	1			
Filomicrobium	1	1	*Pedomicrobium*	1	1			
Flavihumibacter	1	0	*Peredibacter*	1	1			
Flavobacterium	1	1	*Phaeobacter*	0	1			
Flexibacter	1	1	*Phaselicystis*	1	1			
Fluviicola	1	1	*Phycisphaera*	1	0			
Frigoribacterium	1	0	*Phyllobacterium*	0	0			
Gemmata	1	1	Pir4 lineage	1	1			
Gemmatimonas	1	1	*Pirellula*	1	1			
Glaciecola	1	1	*Planctomyces*	1	1			
Haliangium	1	1	*Portibacter*	1	1			
Haliea	0	0	*Pricia*	1	1			
Halioglobus	1	0	*Profundibacterium*	1	1			

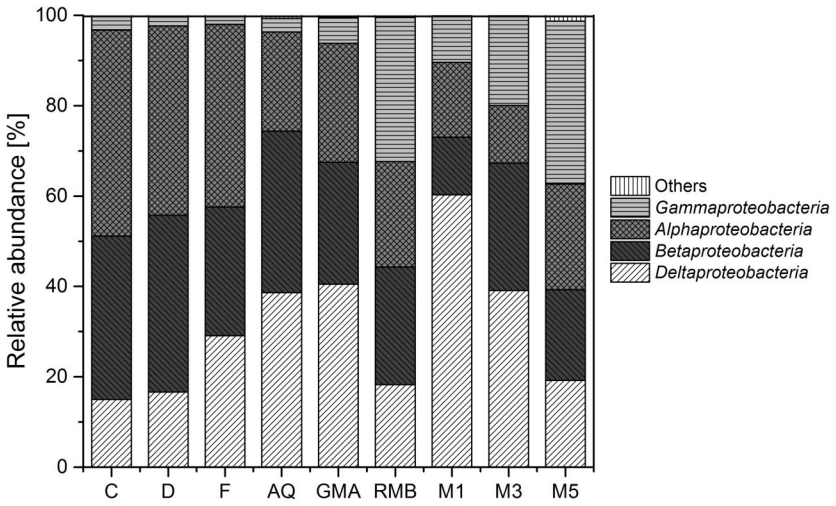
Table S4: Hidden Markov models (HMMs) used in this study for the detection of putative enzymes in the metagenomic data sets.

HMM name	Reference	Putative enzyme/domain	Potential function
AMO (PF02461)	Pfam	putative ammonia monooxygenase	nitrification
amoA_AOB (PF05145)	Pfam	methane monooxygenase	nitrification
Arch_amoA (PF12942)	Pfam	Archaeal ammonia monooxygenase subunit A	nitrification
Multi-haem_cyto (PF13447)	Pfam	Seven times multi-haem cytochrome CxxCH	nitrification
Cytochrome_C554 (PF13435)	Pfam	Cytochrome c554 and c-prime	nitrification
Molybdopterin (PF00384)	Pfam	Molybdopterin oxidoreductase	nitrification
Molydop_binding (PF01568)	Pfam	Molydopterin dinucleotide binding domain	nitrification
amoA_AOB	FUNgenes	putative ammonia monooxygenase	nitrification
amoA_AOA	FUNgenes	putative ammonia monooxygenase	nitrification
pmoA	FUNgenes	methane monooxygenase	nitrification
mmoX	FUNgenes	methane monooxygenase	nitrification
nxrB	FUNgenes	putative nitrite oxidoreductase, beta subunit	nitrification
napA	FUNgenes	periplasmic and membrane-bound nitrate reductase	denitrification
narG	FUNgenes	periplasmic and membrane-bound nitrate reductase	denitrification
nirA	FUNgenes	nitrite reductase	denitrification
nirB	FUNgenes	nitrite reductase	denitrification
nirK	FUNgenes	cytochrome cd1 and copper-containing nitrite reductase	denitrification
nirS	FUNgenes	cytochrome cd1 and copper-containing nitrite reductase	denitrification
norB	FUNgenes	NO reductase	denitrification
nosZ	FUNgenes	nitrous oxide reducaste	denitrification
nosZ_atypical_1	FUNgenes	nitrous oxide reductase	denitrification
nosZ_atypical_2	FUNgenes	nitrous oxide reductase	denitrification
nrfA	FUNgenes	nitrite reductase	denitrification
nrfA_Welsh	FUNgenes	nitrite reductase	denitrification
nifD	FUNgenes	nitrogenase	N-fixation
nifH_new	FUNgenes	nitrogenase	N-fixation
nif	FUNgenes	nitrogenase	N-fixation
GH	CAZy	glycoside hydrolase	carbon breakdown

Table S5: Results of local protein BLAST search of published comammox-*amoA* gene sequences against the assembled metagenomic contigs of samples FR, BR and MA. *AmoA* nucleotide sequences of the described comammox *Nitrospira* Cand. *Ns. inopinata*, Cand. *Ns. nitrificans* and Cand. *Ns. nitrosa* (Daims *et al.* 2015; van Kessel *et al.* 2015) were used.

	ORF	Length	Max score	Query coverage [%]	E-value	Max ID [%]
FR						
amoA [Cand. *Ns. nitrosa*]	k99_314926	2214	1408	99	0	96
	k99_625356	868	625	97	8.70E-177	80
BR						
amoA [Cand. *Ns. inopinata*]	k99_2902017	523	707	48	0	97
	k99_2655270	12516	695	98	0	81
	k99_1947079	262	452	31	2.10E-124	97
	k99_1227328	276	431	30	2.74E-118	96
	k99_1804806	265	428	30	3.54E-117	96
	k99_2221079	252	393	28	1.29E-106	95
	k99_250464	226	391	26	4.65E-106	97
	k99_2088385	407	376	30	1.30E-101	92
	k99_1131768	282	93	7	1.47E-16	93

A.1.2 Supplementary figures



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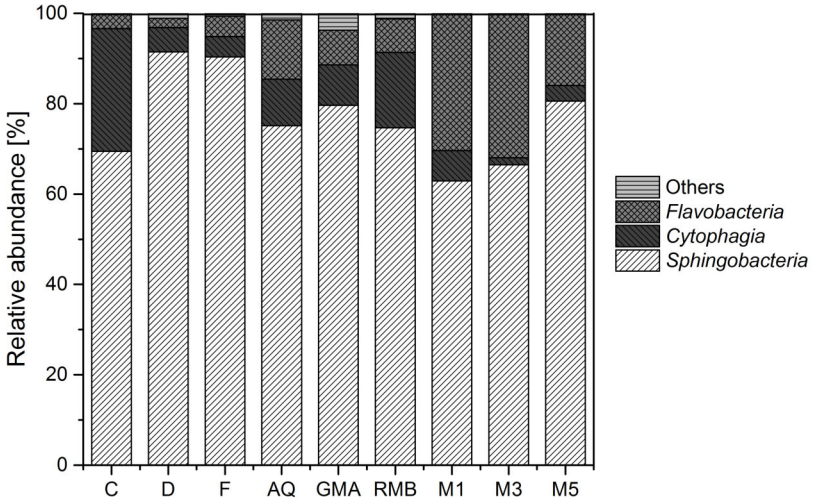

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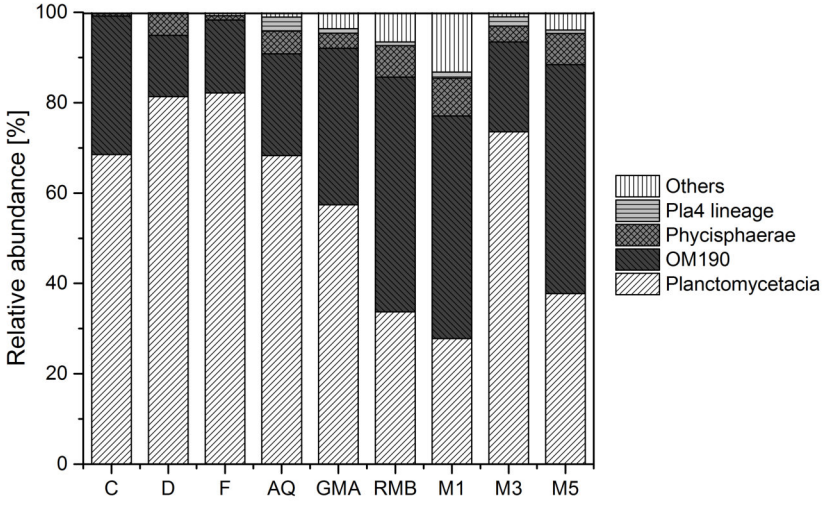

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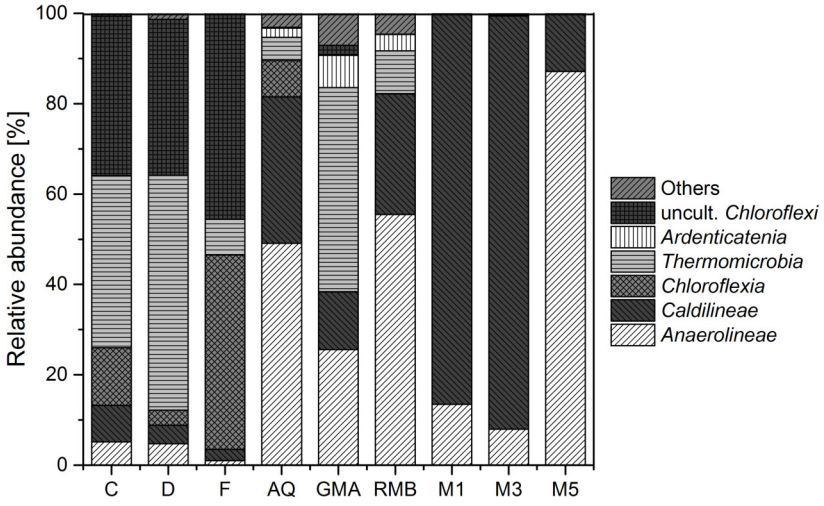
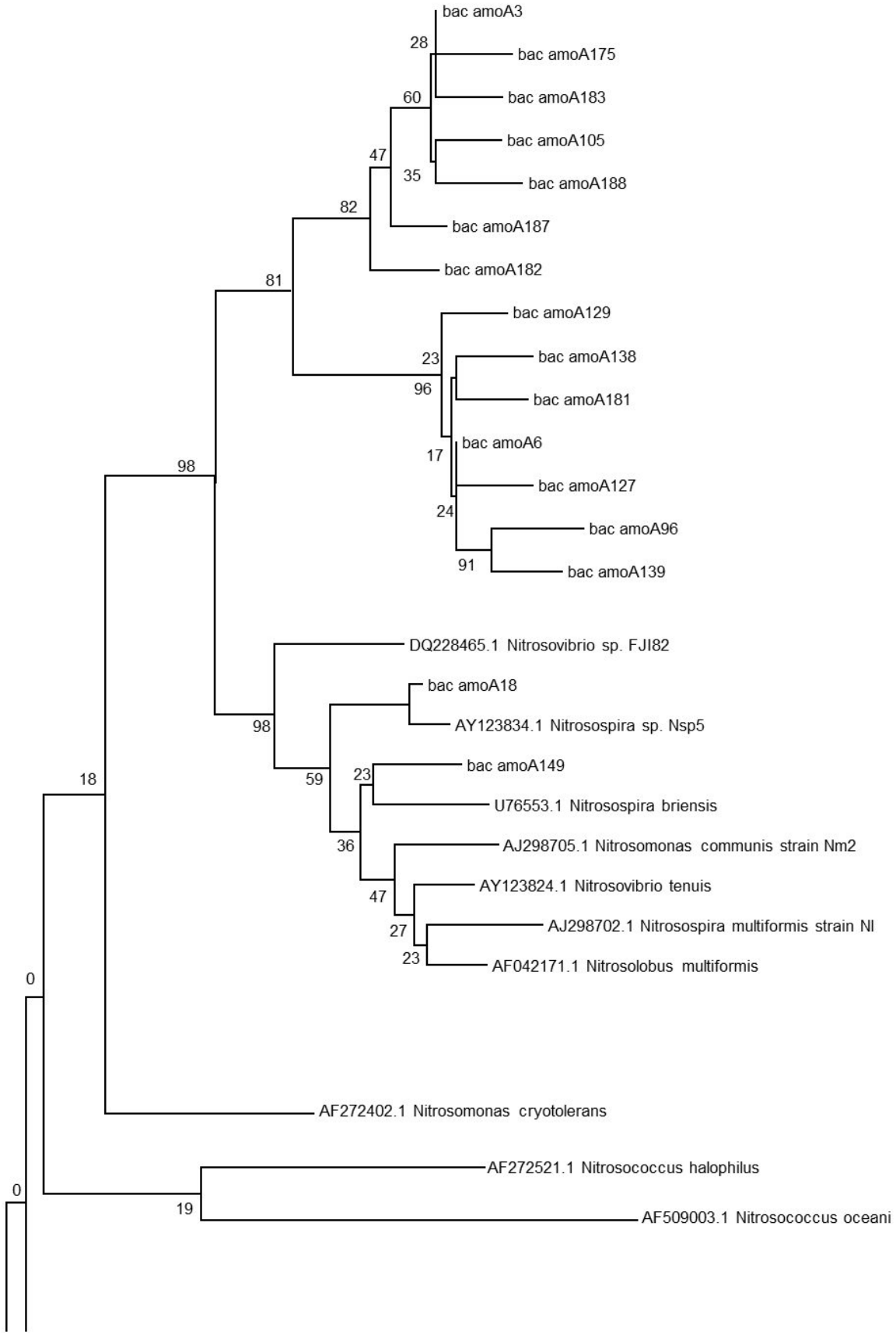


(d)

Figure S1: Relative abundance of the most abundant bacterial classes identified in the biofilter samples after 16S rRNA amplicon sequencing. Classes within the phyla (a) *Proteobacteria*, (b) *Bacteroidetes*, (c) *Planctomycetes* and (d) *Chloroflexi* are shown. The class level distribution shown in the figure is based on DNA generated amplicon data. Others: bacterial classes represented by <0.5 % of the total reads. FR: C, D, F; BR: AQ1, GMA, RMB; MA: M1, M3, M5.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98
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23
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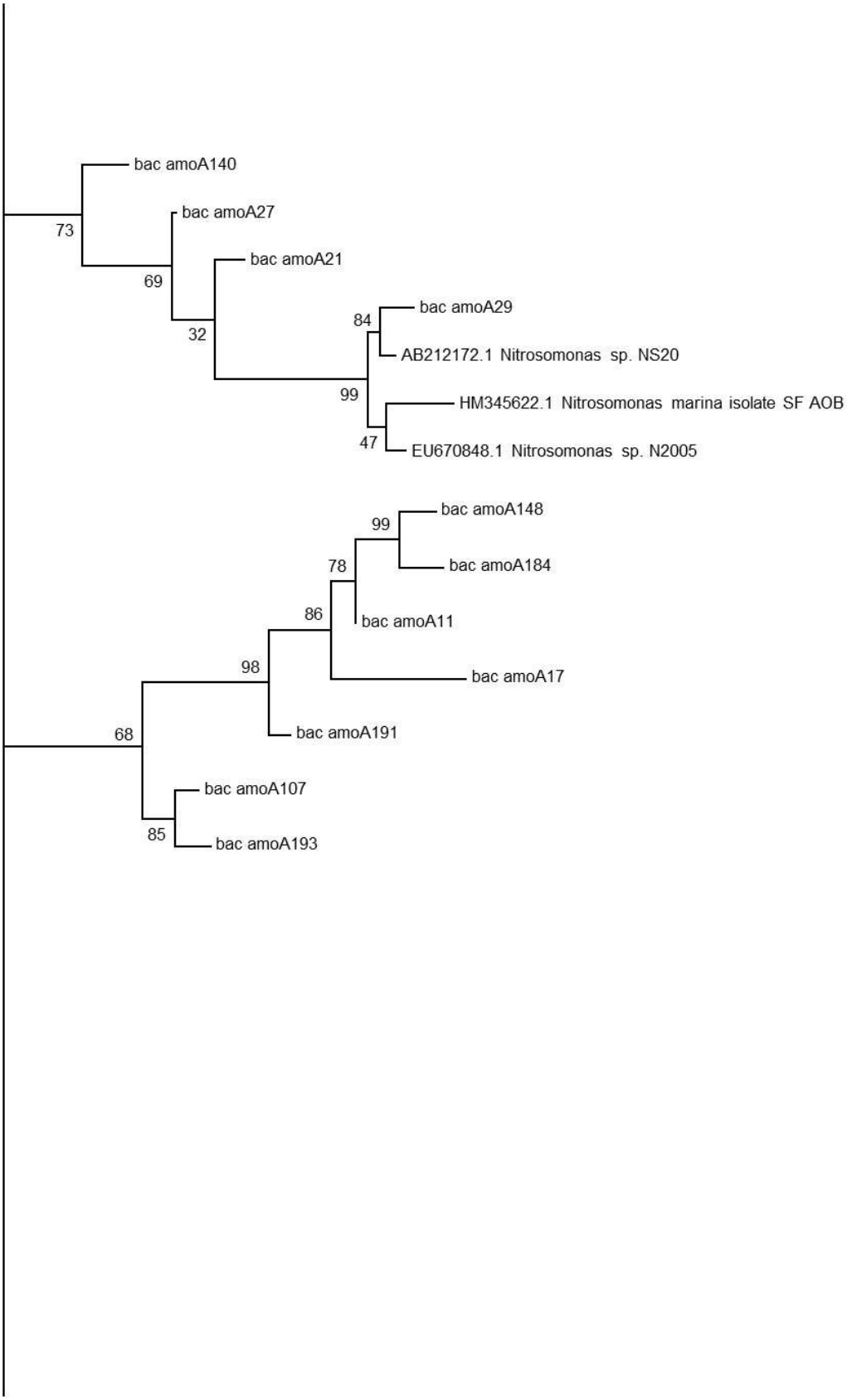
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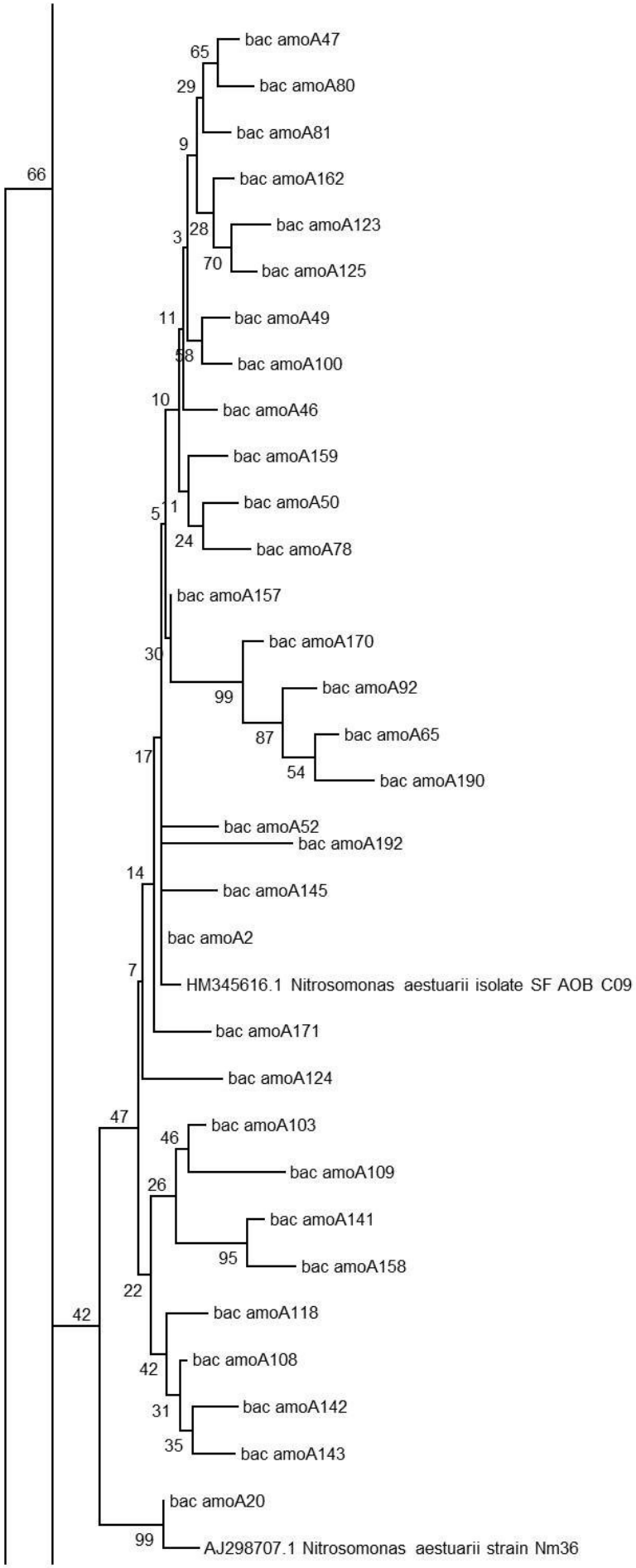
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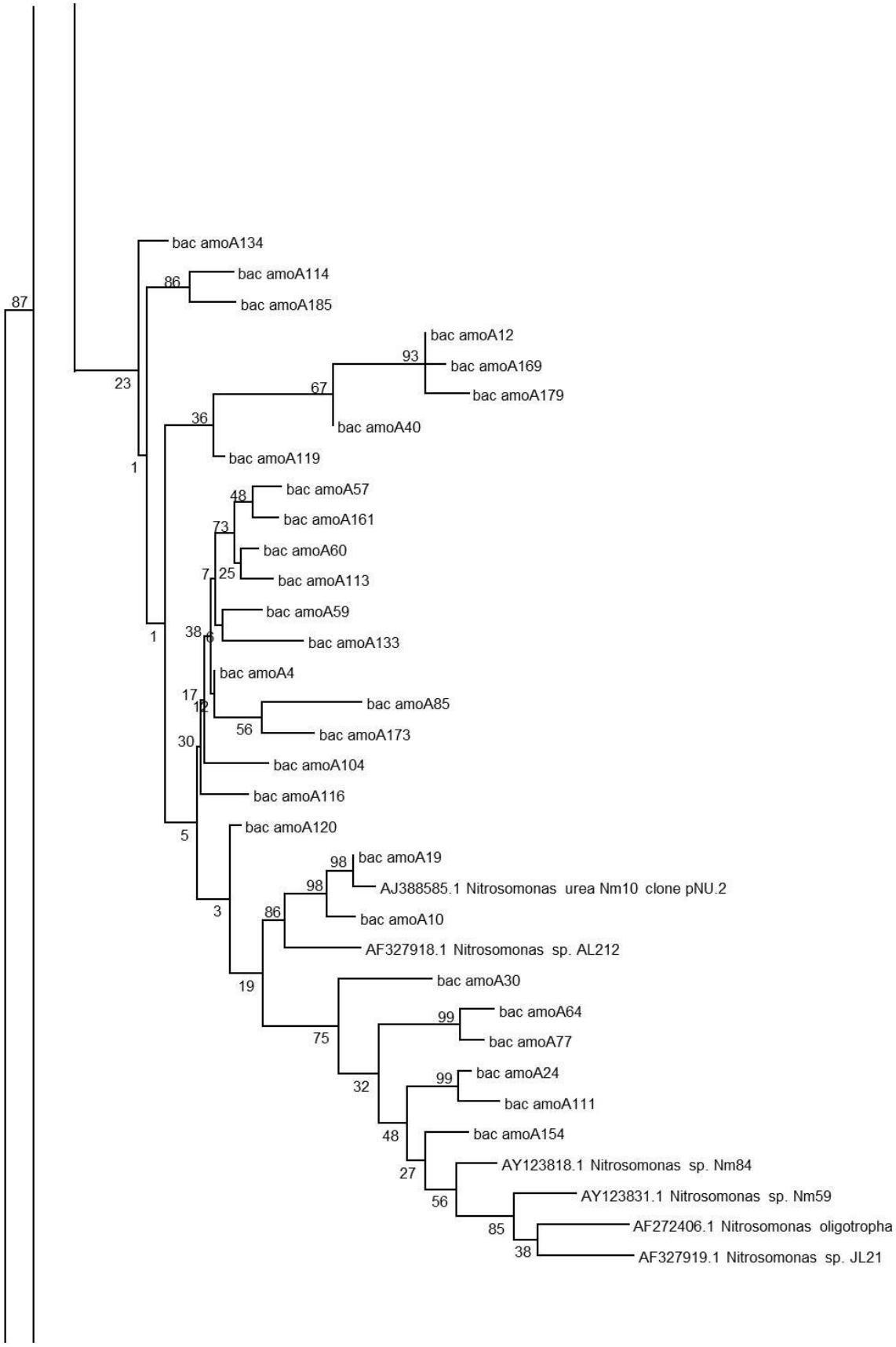
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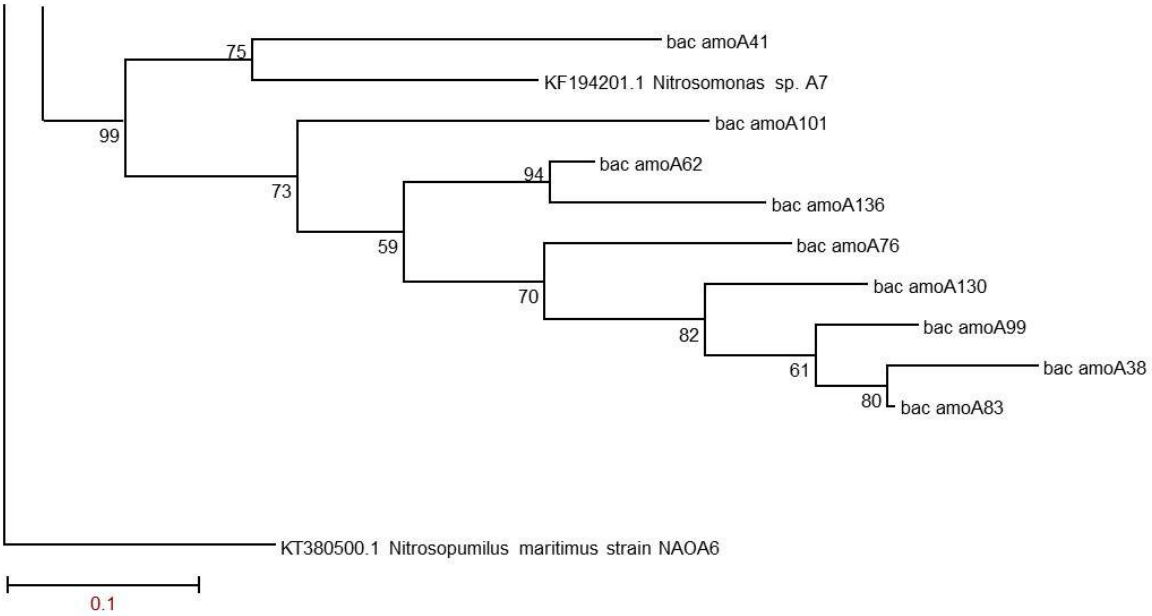
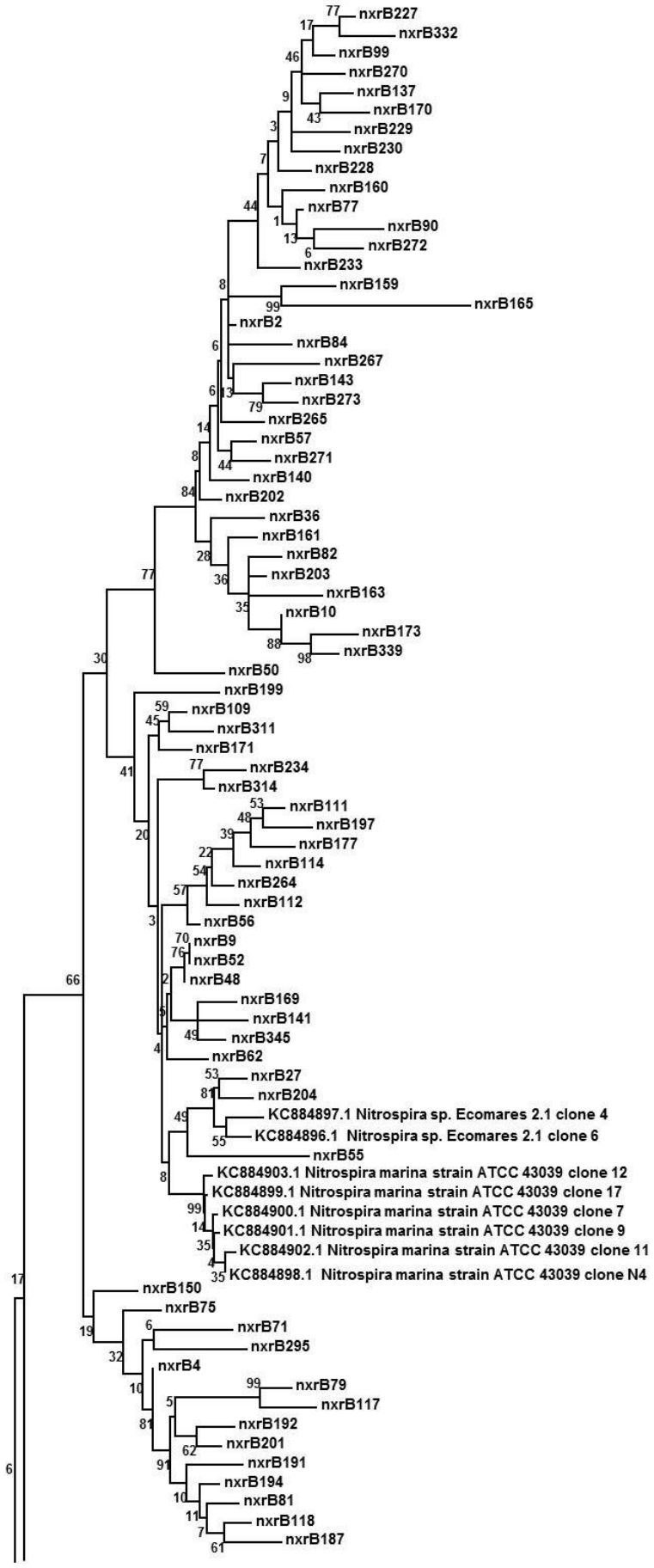


Figure S2: Phylogenetic tree of OTU sequences obtained after amplicon sequencing of β-proteobacterial *amoA* genes using Illumina MiSeq. Samples were derived from biofilters of a freshwater, brackish and marine RAS. The OTU sequences were aligned with *amoA* gene sequences of taxonomically described AOB species deposited at NCBI database. The archaeal *amoA* gene sequence of *Nitrosopumilus maritimus* was defined as outgroup. The phylogenetic tree was generated by the Maximum Likelihood method with 1000 bootstrap replications by using MEGA software as described in section 3.9.5. Bootstrap support is indicated at the branches. Due to the size the phylogenetic tree was split in seven parts (pp. 107-113). bac amoA no. = name of OTU in the data set.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nxrB9
nxrB52
nxrB48
nxrB169
nxrB141
nxrB345
nxrB62
nxrB27
nxrB204
KC884897.1 Nitrospira sp. Ecomares 2.1 clone 4
KC884896.1 Nitrospira sp. Ecomares 2.1 clone 6
nxrB55
KC884903.1 Nitrospira marina strain ATCC 43039 clone 12
KC884899.1 Nitrospira marina strain ATCC 43039 clone 17
KC884900.1 Nitrospira marina strain ATCC 43039 clone 7
KC884901.1 Nitrospira marina strain ATCC 43039 clone 9
KC884902.1 Nitrospira marina strain ATCC 43039 clone 11
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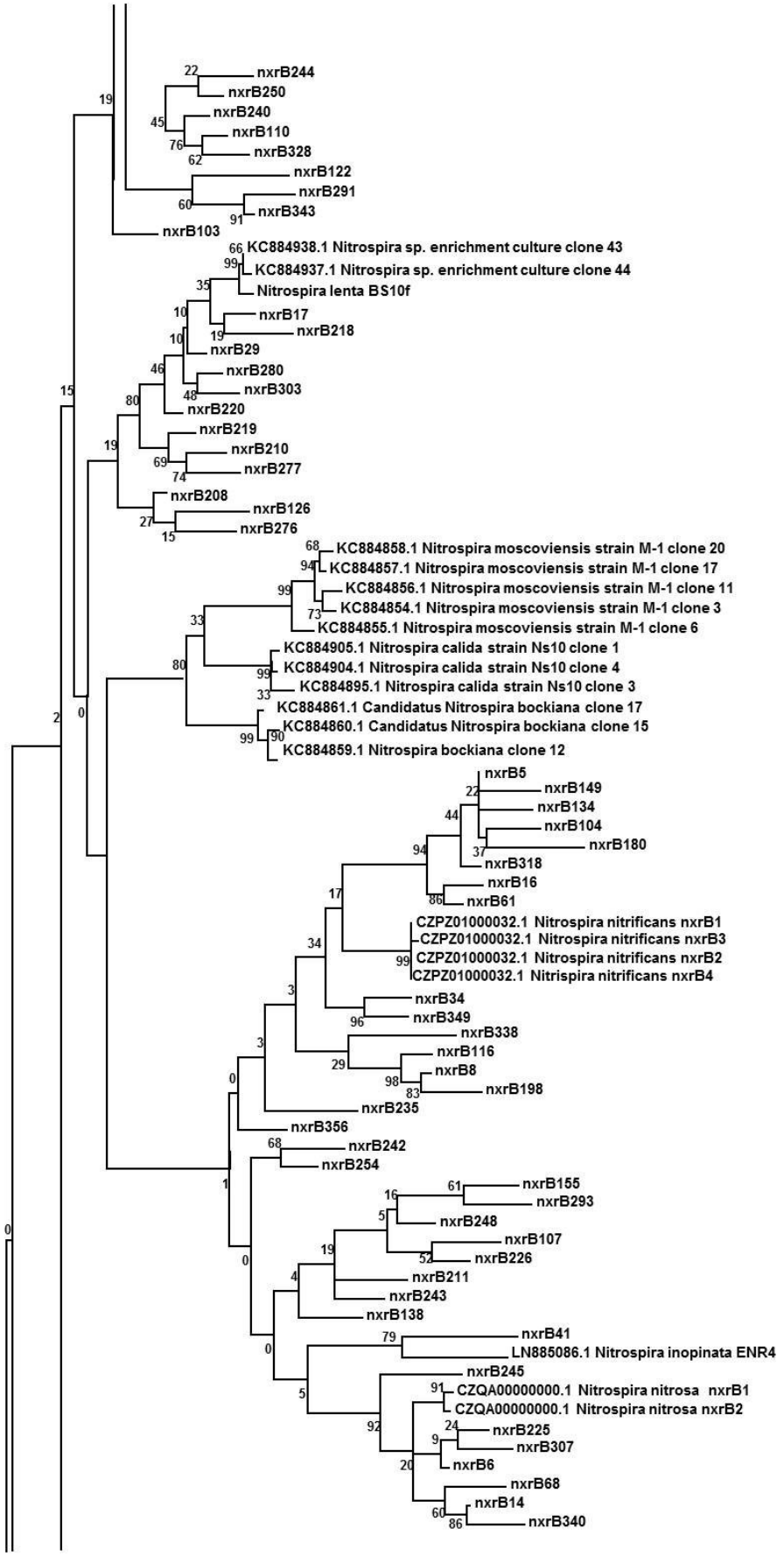
nxrB187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nxrB240
nxrB110
nxrB328
nxrB122
nxrB291
nxrB343
nxrB103
KC884938.1 Nitrospira sp. enrichment culture clone 43
KC884937.1 Nitrospira sp. enrichment culture clone 44
Nitrospira lenta BS10f
nxrB17
nxrB218
nxrB29
nxrB280
nxrB303
nxrB220
nxrB219
nxrB210
nxrB277
nxrB208
nxrB126
nxrB276
KC884858.1 Nitrospira moscoviensis strain M-1 clone 20
KC884857.1 Nitrospira moscoviensis strain M-1 clone 17
KC884856.1 Nitrospira moscoviensis strain M-1 clone 11
KC884854.1 Nitrospira moscoviensis strain M-1 clone 3
KC884855.1 Nitrospira moscoviensis strain M-1 clone 6
KC884905.1 Nitrospira calida strain Ns10 clone 1
KC884904.1 Nitrospira calida strain Ns10 clone 4
KC884895.1 Nitrospira calida strain Ns10 clone 3
KC884861.1 Candidatus Nitrospira bockiana clone 17
KC884860.1 Candidatus Nitrospira bockiana clone 15
KC884859.1 Nitrospira bockiana clone 12
nxrB5
nxrB149
nxrB134
nxrB104
nxrB180
nxrB318
nxrB16
nxrB61
CZPZ01000032.1 Nitrospira nitrificans nxrB1
CZPZ01000032.1 Nitrospira nitrificans nxrB3
CZPZ01000032.1 Nitrospira nitrificans nxrB2
CZPZ01000032.1 Nitrispira nitrificans nxrB4
nxrB34
nxrB349
nxrB338
nxrB116
nxrB8
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nxrB235
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nxrB242
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nxrB155
nxrB293
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nxrB226
nxrB211
nxrB243
nxrB138
nxrB41
LN885086.1 Nitrospira inopinata ENR4
nxrB245
CZQA00000000.1 Nitrospira nitrosa nxrB1
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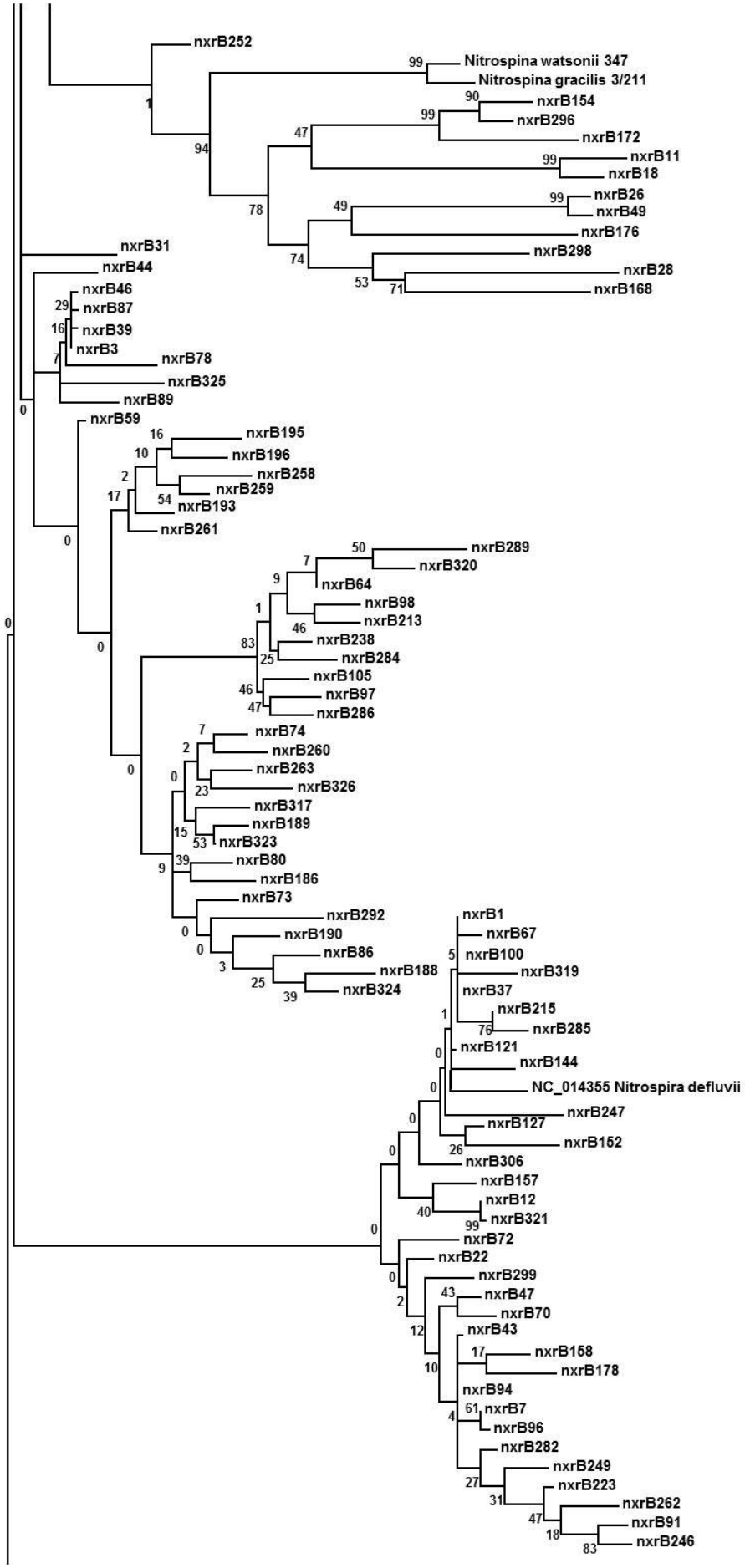
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Nitrospina gracilis 3/211
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nxrB176
nxrB298
nxrB28
nxrB168
nxrB31
nxrB44
nxrB46
nxrB87
nxrB39
nxrB3
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nxrB286
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nxrB186
nxrB73
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nxrB190
nxrB86
nxrB188
nxrB324
nxrB1
nxrB67
nxrB100
nxrB319
nxrB37
nxrB215
nxrB285
nxrB121
nxrB144
NC_014355 Nitrospira defluvii
nxrB247
nxrB127
nxrB152
nxrB306
nxrB157
nxrB12
nxrB321
nxrB72
nxrB22
nxrB299
nxrB47
nxrB70
nxrB43
nxrB158
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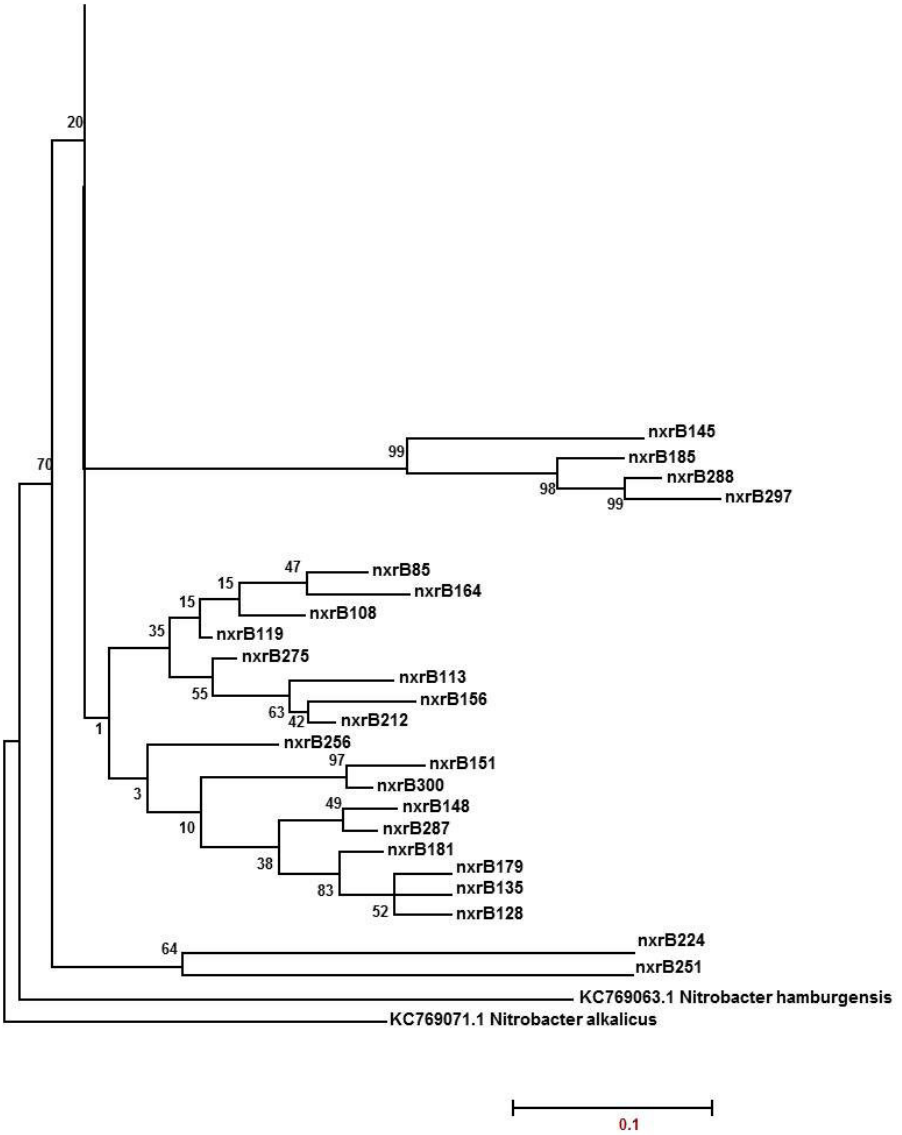
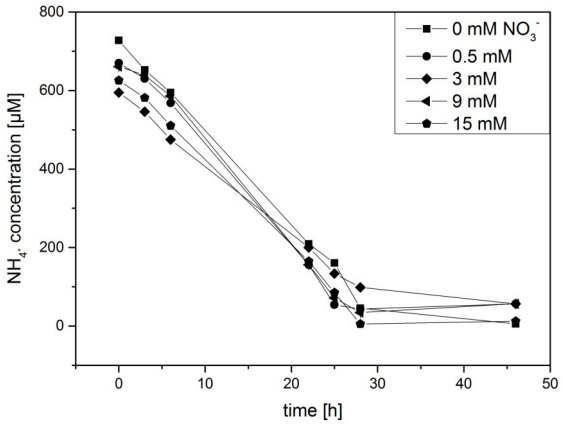
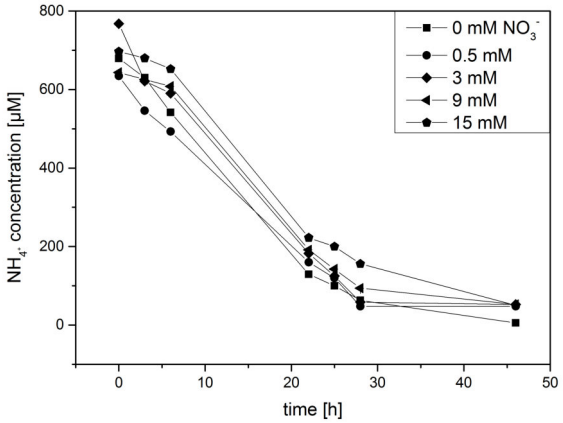


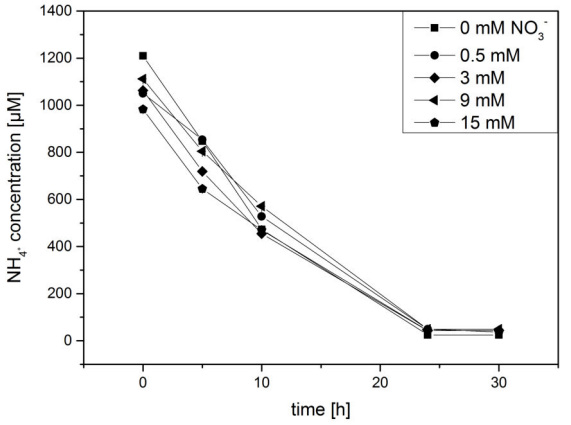
Figure S3: Phylogenetic tree of OTU sequences obtained after amplicon sequencing of *Nitrospira*-like *nxrB* genes using Illumina MiSeq. Samples were derived from biofilters of a freshwater, brackish and marine RAS. The OTU sequences were aligned with *nxrB* gene sequences of taxonomically described *Nitrospira* species deposited at NCBI database. The *nxrB* gene sequences of *Nitrobacter alkalicus*/*Nitrobacter hamburgensis* were defined as outgroups. The phylogenetic tree was generated by the Maximum Likelihood method with 1000 bootstrap replications by using MEGA software as described in section 3.9.5. Bootstrap support is indicated at the branches. Due to the size the phylogenetic tree was split in four parts (pp. 114-117). nxrB no. = name of OTU in the data set.



(a)



(b)



(c)

Figure S4: Ammonia oxidation in dependence on various nitrate concentrations of biofilm inhabiting AOB communities derived from RAS biofilters. Tests were performed via batch-experiments inoculated with colonized biocarriers. The graphs show the ammonia oxidation of samples (a) freshwater C, (b) freshwater F, (c) brackish GMA. Error bars indicate standard deviation calculated from duplicate samples.

List of Publications

Hüpeden, J., S. Wegen, S. Off, S. Lücker, Y. Bedarf, H. Daims, C. Kühn, E. Spieck (2016). "Relative abundance of *Nitrotoga* spp. in a biofilter of a cold-freshwater aquaculture plant appears to be stimulated by slightly acidic pH." *Applied and Environmental Microbiology* 82, pp. 1838–1845.

Hüpeden, J., S. Meyer, S. Lippemeier, E. Spieck (August 2016). "Identification of a core-community in different RAS biofilters and their synergistic effect on nitrifiers–a study to improve the bottleneck of fish larval rearing." Poster presented at the *16th International Symposium on Microbial Ecology*, Montreal, Canada

Hüpeden, J., S. Wegen, S. Lücker, C. Kühn, E. Spieck (August 2015). "Relative abundance of *Nitrotoga* in a biofilter of a freshwater aquaculture plant." Poster presented at the *4th International Conference on Nitrification (and Related Processes)*, Edmonton, Canada

Hüpeden, J., E. Spieck (September 2013). "Characterization of nitrifying communities in brackish recirculation aquaculture systems." Poster presented at the *SAME13 EMBO Conference on Aquatic Microbial Ecology*, Stresa, Italy

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Declaration on Oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg, June 20, 2017

Jennifer Hüpeden

English Language Declaration

I, James Purcell, hereby declare the dissertation "Taxonomic and functional diversity of nitrifying biofilm communities in biofilters of different recirculating aquaculture systems" written by Jennifer Hüpeden to be linguistically sound.

Signed

James Purcell, 18/12/16, London United Kingdom