Characterization of nitrifying bacteria in marine recirculation aquaculture systems with regard to process optimization

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Sabine Keuter

aus Nordhorn

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LIST OF ABBREVIATIONS

AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
BC	biocarrier
bp	base pairs
DAPI	4',6-Diamidin-2-phenylindol
DGGE	denaturing gradient gel electrophoresis
DIN	dissolved inorganic nitrogen
EM	electron microscopy
FISH	fluorescence *in situ* hybridization
HDPE	high density polyethylene
NOB	nitrite oxidizing bacteria
PCR	polymerase chain reaction
PP	polypropylene
RAS	recirculation aquaculture system
RFLP	restriction fragment length polymorphism
TAN	total ammonia nitrogen
TNN	total nitrite nitrogen

SUMMARY

The aim of this study was to gain new insights into the process of nitrification in moving-bed biofilters of marine recirculation aquaculture systems (RAS) and the bacteria involved, with special emphasis on the possible causes of malfunction of the crucial conversion of ammonia to nitrate. Three marine RAS with in total five moving-bed biofilters were made available for the investigations by project partners.

Nitrospira was identified as the major autotrophic nitrite oxidizer (NOB) in one of the biofilter systems (Ecomares in Büsum) as revealed by fatty acids analyses. One of at least two representatives of this genus present in the biofilter could be isolated successfully and further characterized. The isolate Ecomares strain 2.1 exhibited high tolerances against high substrate and product concentrations. It can grow mixotrophically and grows remarkably well in culture. The high similarity of its 16S rRNA sequence to a sequence derived from a marine RAS in Israel let us assume that this strain is characteristic for this habitat. This assumption was corroborated by further findings of this species (with up to 100 % sequence similarities on 16S rRNA basis) in the other two investigated aquaculture facilities, the Marifarm in Strande, and the GMA in Büsum.

The facility of the GMA comprises three modules identical in construction. The biofilters of these modules were started consecutively in summer 2009 with varying inocula (mineral salts, fish feed, slightly colonized biocarriers). Activity tests discriminating between AOB (ammonia oxidizing bacteria) and NOB conversion potentials mirrored the increase of nitrogen load during initial operation of biofilters and can be correlated to specific operational events (e.g. feeding stoppages), though definite biofilter specific removal rates could not be extrapolated. After a generally long lag-phase NOB activities eventually exceeded AOB potentials by far and showed less fluctuation during the progress. A clear advantage of fish feed inoculum over mineral salt to start a biofilter could not be inferred. High initial and continually increasing N input, however, might accelerate start-up periods.

The nitrifying populations, as identified by 16 rRNA gene sequences obtained by PCR, differed only slightly between the biofilters. Dominant AOB were *Nitrosomonas*-like bacteria, one of the sequences found was identical to a sequence derived of the before mentioned RAS in Israel. The nitrite oxidizers were more diverse on the genus level (*Nitrospira*, *Nitrobacter*, *Nitrotoga),* but *Nitrospira* was assumed to be the most dominant NOB, since it was detected by PCR for the whole sampling period and found in huge colonies by electron microscopy of ultrathin sections of biofilm from the carriers.

SUMMARY

Quick colonization of virgin plastic biocarriers is desirable to minimize biofilter activation time and to reduce the risk of accumulation of toxic ammonia or nitrite. Plastics contain a range of additives which migrate into the surrounding water. The effect of these substances on marine nitrifiers was investigated in this study. The presence of all plastics tested (HDPE, LDPE, PP, with different admixtures) in the test media reduced nitrification potentials of AOB and NOB. Degrees of inhibition differed between the materials. The results of blank tests showed that the inhibiting substances must be volatile.

High concentrations of nitrate in marine RAS were also deemed potentially inhibiting. Pure cultures, enrichment cultures as well as colonized biocarriers were exposed to nitrate concentrations up to 200 mM. Nitrite oxidation decreased with increasing nitrate concentration, this effect was especially observed in activity tests with biocarriers from systems with low nitrate concentrations. *Nitrobacter* turned out to be less sensitive towards nitrate than *Nitrospira* strains. Inhibition of ammonia oxidation was neither observed for biocarriers nor for cultures.

In activity tests with biocarriers (at pH 5.3 and 7.5) from a RAS system that temporarily run at pH 6, the difference of 2 pH units reduced the potential activities of NOB by about 36 % and of AOB by about 57 %. After incubation without substrates at pH 7.5 for 6 days, nitrite oxidation rates had increase considerably. Ammonia oxidation rates, in contrast, were even lower. Here, especially substrate deprivation as effect of low pH is discussed.

For the reactivation of biofilters after idle phases or to start new biofilters, the use of already colonized biocarriers (BC) is of advantage. Residual nitrifying potentials of bacteria on biocarriers were measured after 3, 6 and 9 months of dry or wet storage at 4 and 17 °C. The BC stored dry at 17 °C lost their nitrifying potentials after 3 months storage, whereas the ones stored at 4 °C could still be reactivated after 9 months. The BC stored in water still showed high instantaneous nitrifying potentials as measured by activity tests. However, AOB seem to lose their nitrification potentials to a higher extend than NOB. The highest residual activities of both nitrifiers were obtained after storage in water at 4 °C.

ZUSAMMENFASSUNG

Verschiedene Aspekte der Nitrifikation in Bewegtbett-Filtern von marinen Kreislaufanlagen wurden mit dem Ziel untersucht, eventuelle Ursachen für beeinträchtigte Nitrifikationsleistungen zu erkennen und durch erweitertes Wissen über Abläufe in Biofiltern sowie beteiligter Mikroorganismen den Prozess zu optimieren. Für die Untersuchungen standen drei marine Anlagen mit insgesamt fünf Bewegtbett-Biofiltern (moving bed) zur Verfügung.

Als dominierendes NOB (Nitrit oxidierendes Bakterium) in einer dieser Anlagen (Ecomares in Büsum) wurde *Nitrospira* identifiziert. Einer von mindestens zwei Vertretern dieser Gattung in den Biofiltern konnte isoliert und physiologisch und morphologisch charakterisiert werden. Dieser *Nitrospira*, Ecomares strain 2.1 genannt, zeigte sich als sehr tolerant gegenüber hohen Ammonium-, Nitrit- und Nitratkonzentrationen. Wie sein einziger bisher isolierter mariner Verwandter aus der Gattung *Nitrospira*, *N. marina*, kann Ecomares 2.1 mixotroph wachsen und lässt sich ausgesprochen gut in Kultur führen, im Gegensatz zu *N.marina*. Die große Ähnlichkeit seiner 16S rRNA Sequenz zu einer *Nitrospira*- Sequenz, die aus einer marinen Kreislaufanlage in Israel stammt, lässt vermuten, dass diese Art charakteristisch für das Habitat ist. Weitere Funde dieser Art (mit bis zu 100 % Ähnlichkeit auf 16S rRNA Sequenzebene) in den zwei anderen untersuchten Anlagen, der Marifarm in Strande und der GMA in Büsum, stützen diese Vermutung.

Die Anlage der GMA besteht aus drei baugleichen Modulen mit jeweils einem moving-bed Biofilter, die ab Sommer 2009 nacheinander in Betrieb genommen wurden. Die Biofilter wurden dabei unterschiedlich angeimpft (mit Ammoniumchlorid und Natriumnitrit, Futter und bereits bewachsene Füllkörper) und die Entwicklungen der nitrifizierenden Aktivitäten wurden mit der Zunahme an eingebrachtem Stickstoff (Futter), den Konzentrationen von Ammonium, Nitrit und Nitrat im Biofilterwasser, pH, Temperatur, gelöstem Sauerstoff und Salinität sowie anlagen- und hälterungsbedingten Ereignissen verglichen.

Die Anlaufzeiten dauerten insgesamt extrem lang (> 6 Monate), wobei sich die potentiellen Nitrifikationsleistungen der AOB (Ammoniak oxidierende Bakterien) früher und schneller entwickelten als die der NOB. Nach ca. 250 Tagen aber zeigten die NOB im Labor weitaus höhere Aktivitäten als die AOB. Die Aktivitäten von AOB und NOB korrelierten mit der Zunahme des Stickstoffeintrages in Form von Futter. Es konnte nicht geklärt werden, welche Art der Beimpfung von Vorteil war, jedoch scheinen hohe und kontinuierlich ansteigende Stickstoffgaben die Entwicklung der Nitrifizierer zu fördern.

Obwohl Temperaturen, Fischarten und die Art des Futters zwischen einem Modul im Gegensatz zu den beiden anderen variierten, war die Zusammensetzung der nitrifizierenden Bakterien äußerst ähnlich. *Nitrosomonas*-ähnliche Bakterien waren die dominierenden Ammoniak-Oxidierer. In elektronenmikroskopischen Aufnahmen des Biofilms auf den Füllkörpern wurden lediglich *Nitrospira*-ähnliche Zellen als Nitrit-Oxidierer erkannt, obwohl durch PCR-basierte Methoden auch 16S rRNA-Gensequenzen von *Nitrobacter* und *Nitrotoga* vorlagen.

Während der Startphase eines Biofilters sollen sich die nitrifizierenden Bakterien möglichst schnell die dafür vorgesehen Kunststoff-Füllkörper besiedeln. Kunststoffe enthalten jedoch eine große Anzahl an Additiven, die in das umgebene Medium migrieren. Der Effekt dieser Substanzen auf die Nitrifikation wurde untersucht. Im Beisein aller getesteten Kunststoffe (HDPE, LDPE, PP, mit verschiedenen Beimischungen aus Talkum, Glasfasern und Ruß) verlangsamte sich sowohl die Ammoniak- als auch die Nitritoxidation, der Grad der Inhibition war dabei unterschiedlich. Die Ergebnisse von Versuchen mit Medien, in denen sich vorher Kunststoffe befanden, deuten an, dass die inhibierenden Substanzen flüchtig sein müssen.

Hohe Nitratwerte in Marikulturanlagen wurden ebenfalls als möglicherweise inhibierend erachtet, diese scheinen jedoch auf die Substratoxidation von AOB (auf Füllköpern und Kulturen) keine Auswirkung zu haben. Die Nitritoxidation von NOB Kulturen wurden von hohen, jedoch für Anlagen irrelevanten Konzentrationen herabgesetzt. Die Aktivitäten von NOB auf Füllkörpern wurden schon bei niedrigen Nitratkonzentrationen von 1,5 mM beeinträchtigt.

Niedrige pH Werte in der Anlage Ecomares über einen Zeitraum von 2 Monaten hatten nur eine vorrübergehende Herabsetzung der Aktivitäten von NOB auf Füllkörpern bewirkt. AOB schienen sich dagegen langsamer zu erholen, was wahrscheinlich an der verlängerten Hungerphase durch das verschobene Gleichgewicht zwischen Ammoniak (Substrat der AOB) und Ammonium gelegen hat.

Die Nutzung von bereits besiedelten Füllkörpern ist von Vorteil für die Reaktivierung von Biofilter nach Ruhephasen oder für den Start komplett neuer Biofilter. Restaktivitäten von AOB und NOB auf Füllkörpern wurden nach langer Lagerung (3, 6 und 9 Monate, trocken oder in Wasser, bei 4 und 17 °C) gemessen. Die trocken bei 17 °C gelagerten Füllkörper verloren ihre Nitrifikationspotentiale nach 3 Monaten Lagerung, während die bei 4° C gelagerten auch nach 9 Monaten wieder reaktiviert werden konnten. Die in Wasser gelagerten Füllkörper zeigten in Aktivitätstests noch sehr hohe Nitrifikationspotentiale ohne lag-Phasen, wobei sich die Aktivitätsraten der AOB mit der Lagerungszeit im höheren Maße verringerten als die NOB. Die höchsten Restaktivitäten zeigten AOB und NOB nach Lagerung in Wasser/Meerwasser bei 4 °C.

CHAPTER I

Introduction

RECIRCULATION AQUACULTURE SYSTEMS

The need to reduce fishing pressure on the declining fish stocks in our oceans is inevitable (Pauly *et al.*, 1998), especially with regard to the worldwide increasing seafood consumption (FAO, 2010). With the remarkable growth of the world´s aquaculture production of averagely 6.6 % in the last 40 years (FAO, 2010) the seafood industry seems to be well prepared. However, a half of the global aquaculture production consists of fresh water species, which cannot compensate for the dwindling marine fish stocks. In Western Europe, as much as 86 % of the aquacultural products are marine species. The 7 % growth of Western European aquaculture production before 2000, however, stagnates at a rate of 1.5 % since then (Rana, 2007). Europe´s (EU countries) total aquaculture production in 2008 was almost 1.3 million tons, which amounts for 2.4 % of the world´s production (FAO, 2010).

In the recent past, marine aquaculture produced rather negative connotations due to a range of environmental issues involved. Examples are the threat of wild stocks by escapees (biological pollution), the excessive use of antibiotics or other pharmaceutical products, the use of fish as feed (depletion of wild stocks), the occupation and degradation of coastal and marine habitats (e.g. mangrove destruction, near-shore aquaculture) and pollution by discharge of effluents.

In recirculation aquaculture systems (RAS) water is reused after undergoing treatment (Rosenthal *et al.*, 1986). This reduces water exchange rates to 1-10 % daily and leads to an enormous water use reduction per kg fish produced. The waste is concentrated by filter techniques, that way waste water effluents are reduced and controlled in contrast to open systems like net cages or flow-through systems. Apart from that, the technology offers further advantages: controlled cultivation conditions year-round even for exotic species, proximity to markets (less energy for transports), less energy costs in high temperature systems, no competition for space in coastal waters, no escapees.

But still, the on-shore or land-based production of fish in recirculation aquaculture plants especially for the grow-out of marine species in Europe is increasing only slowly after the introduction of this technology in the 1970´s (Timmons *et al.*, 2001; Martins *et al.*, 2010). This is contributed mainly to the high investment and running costs and the intensive management (Schneider *et al.,* 2006).

Legal environmental constraints (consumption of water and wastewater release) and decreasing allocation of offshore farm concessions (Ojeda, 2010), on the other hand, might help to push aquaculture production towards RAS technologies and to oppose the stagnating development of the cultivation of marine species in Europe. The increasing demand for high quality fingerlings (and to some extend marine ornamentals) is likewise expected to promote marine or brackish RAS technology, since here extreme oligotrophic water conditions are required (Martins *et al.*, 2010).

NITROGENIC WASTE IN AQUACULTURE

Fish feed is generally rich in proteins, and a great fraction of the nitrogen input in form of fish feed is not converted to biomass but ends up in the rearing water. Estimations of percentages of assimilation, retention and excretion of N as well as the excreted compounds (ammonia, urea, creatine etc.) vary immensely; Handy and Poxton (1993) presumed that "52-95 % of any N added to the system as food will ultimately pollute the environment", whereas 50 % is commonly used as a rule of thumb. Fig 1.1 depicts the routes of N in a low food wastage scenario.

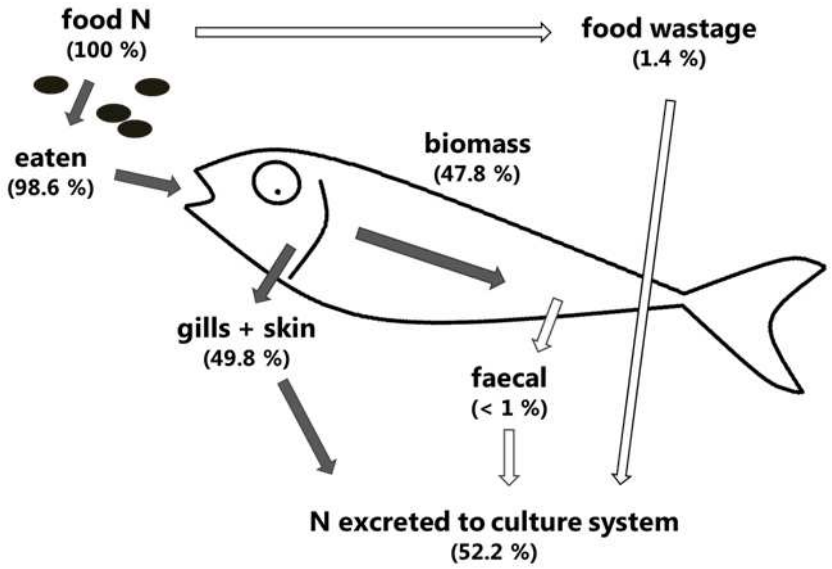

Fig. 1.1: Routes of nitrogen pollution in mariculture with low food wastage (1.4 %) (redrafted from Handy and Poxton, 1993).

For simplified operational calculations of nutrient fluxes in RAS it is assumed that protein consists of 16 % N, 80 % of this N is assimilated by the organisms cultivated (fish or shrimp), of these assimilated N 80 % (shrimp: 90 %) is excreted in the form of TAN (total ammonia nitrogen; 90 %) and urea (10 %)

as end product of metabolism (Ebeling *et al.*, 2006). Here, it is also assumed that nitrogen in uneaten feed and feces is removed before breakdown to TAN (ammonification) by mechanical filtration and foam fractioning. Based on these assumptions, an estimate of TAN generated per day in RAS can be calculated as follows (Timmons *et al.*, 2001):

$P_{TAN} = F * PC * 0.092$ (shrimp: 0.144)

(P_{TAN} = total ammonia nitrogen production rate [kg per day]; F = feed rate [kg/day]; PC = protein content in feed [decimal value])

The TAN is mainly excreted by fish and shrimps across the gills (a minor fraction also renal or cutaneous, depending on fish species), the equilibrium of the unionized (NH_3) and the ionized (NH_4^+) ammonia in the rearing water is determined by pH values and temperatures. The unionized form, the free ammonia, is toxic to aquatic organisms, resulting in a range of damages to fish and crustaceans, e.g. damage of gill epithelium, osmoregulatory stress etc. at low concentrations (Camargo *et al.*, 2005, and therein). The most common way to remove accumulating ammonia from the recirculating water is the utilization of aerobic autotrophic nitrification, the microbial oxidation of ammonia to nitrate over nitrite.

The intermediate of aerobic bacterial nitrification, hence, is nitrite. TNN, or total nitrite nitrogen, is the sum of NO_2^- and HNO_2, again pH value and temperature determine the equilibrium. Unlike for ammonia, though, here low levels of pH generate the toxic unionized form nitrite, the HNO_2 or free nitrous acid. The main toxic action of nitrite is the generation of methemoglobin (fish) or methemocyanin (crustaceans), causing hypoxia. In marine aquaculture, elevated salinity concentrations function as protection against nitrite toxicity, as chloride ions compete with nitrite ions for the same uptake mechanism via the gills.

Nitrate, the end product of the nitrification process, is far less toxic. Camargo *et al.* (2005), however, recommended a limit of 20 mg NO_3-N/L (= 1.4 mM) for marine aquaculture, although values differ greatly between species. Like nitrite, nitrate ions can block oxygen-transporting pigments in the blood plasma. Again, toxicity decreases with increasing chloride content.

Apart from the toxicity of DIN for the species cultivated the impact of global aquaculture on the nitrogen cycle is to be taken into account. Being an animal production industry, aquaculture requires nitrogen rich feed and produces nitrogen rich waste (Fig. 1.1). A considerable fraction of commercial fish feed, even for carnivorous fish, is made of vegetable ingredients like wheat, soy, and oil. The ammonia in industrial agricultural fertilizers was produced by the Haber-Bosch process, hence the N

is of atmospheric origin, as is the nitrogen fixed by symbionts of legumes like soybeans. The anthropogenic mobilization of inactive dinitrogen still increases enormously, from 156 Tg N per year (Tg = 10^{12} g) in 1995 to 187 Tg N per year in 2005 (Galloway, 2008). Inorganic fertilizers produced by the Haber-Bosch process provide 80 Tg N per year, symbiotic biofixation adds about 20 Tg (Smil, 1999). Further is ammonia used as fish and shrimp feed supplement (Galloway, 2008). The overall trend of mobilizing inert nitrogen together with the massive combustion of fossil fuels eventually leads to eutrophication and acidification of inland and coastal waters (Vitousek *et al*., 1997).

The fraction of nitrogen mobilized for, and consequently also the amount of dissolved inorganic nitrogen compounds (DIN) produced by marine fish farming, is not comparable to that produced by agriculture, which is one of the major driving forces of coastal eutrophication globally (Tilman, 2001). Nevertheless, it is mandatory to reduce nitrogen waste and effluents from aquaculture production to keep environmental implications as small as possible. An advantage over terrestrial animal production in this respect is that the nitrogen-use of fish and shrimp is more efficient than that of livestock (Dias *et al*., 2010). As water usage in RAS is reduced, no large quantities of diluted waste water are discharged to public receiving waters and concentrated waste can be appropriately disposed or reused. Further can RAS facilities make use of denitrification biofilters to convert nitrate to atmospheric nitrogen.

BIOFILTERS

Different types of biofilters exist for the elimination of TAN in fish farms. The most common are trickling, fixed, and moving bed filters. All biofilters are designed to immobilize bacteria on solid media like sand, stones, plastic beads, foams, fibers etc. This way the bacteria are not washed out, as in suspended-growth systems, and the major part of nitrification occurs within the biofilter, although Losordo and Hobbs (2000) quoted that so called passive nitrification by bacteria on surfaces within the system, other than the biofilter, can account up to 30 %. Additionally, it has been shown that attached cells in biofilms have a higher affinity for ammonia and show quicker recovery after substrate deprivation than planktonic cells (Batchelor *et al*., 1997).

Figure 1.2 shows a simplified system set-up of a RAS with a moving bed biofilter, as it is used by our project partner GMA in Büsum. The biocarriers are kept in motion continuously by aeration or mechanical stirring. This way, the microbial communities in the biofilms on the carriers are provided sufficiently with oxygen and a uniform distribution of substrates is given for aerobic nitrification.

Further, the steady and strong shear forces cause a cleaning effect that prevents the beads from clogging and promotes a rejuvenating of the biofilm. The nitrification rates of such moving bed bioreactors generally range from 0.15 to 1 g TAN per m^2 per day (Losordo and Hobbs, 2000).

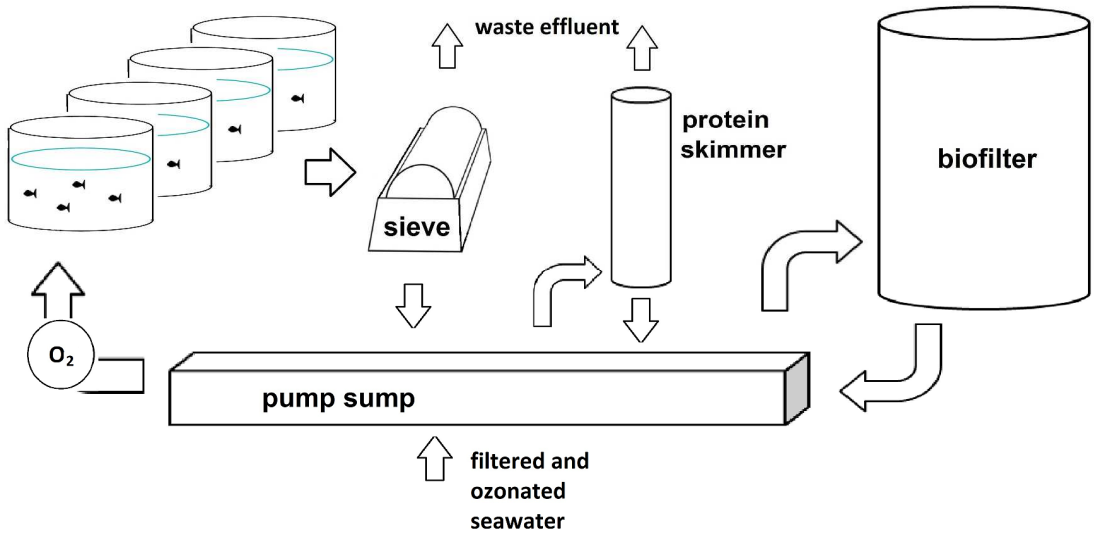

Figure 1.2: Set-up of components of the modules at GMA in Büsum. Three small circuits start and end in the pump sump, to which filtered and ozonated sea water is added. Water going to the rearing tanks is amended with technical oxygen when necessary, the rearing water is sieved before reaching again the pump sump. Waste is extracted from the system with the backwash of the sieve and with the foam of the protein skimmer.

Many RAS use plastic biocarriers with vast surfaces (squaremeter) per volume (cubic meter) for biofiltration. The challenge here is to design the smallest possible filter (investment costs, space and energy consumption) that still offers the required area for the attachment of nitrifying bacteria for the maximum nitrogen input at the end of a grow-out period.

All investigations in this study are based on moving bed biofilters (further details about the facilities are given below and in the respective chapters) with plastic beads of the type 2H-BCN (recycled high density polyethylene, GEA 2H Water Technology, Wettringen) (Fig. 1.3). These beads are made with 3 % carbon black (to raise UV stability) and have a density of 0.95 $g*cm^{-3}$, thus floating just beneath the surface of sea water. They have a surface of 836 m^2 (type 009) or 859 m^2 (type 012 KLL) per cubic meter. Accordingly, the nitrification efficiency of these filter media would be about 80 to 800 g TAN per m^3 per day. Next to total surface areas, technical sheets of available biocarriers often provide the amount of protected area per biocarrier or cubic meter due to the assumption that growth of the nitrifying biofilm is preferred here. This assumption, however, is controversial according to Suhr and Pedersen (2010). Therefore, nitrifying potentials given in this study are biocarrier-specific or

calculated based on the total area. The biofilter tanks of the study sites (except during start-phases) were filled to about 60 % with biocarriers (neglecting void space).

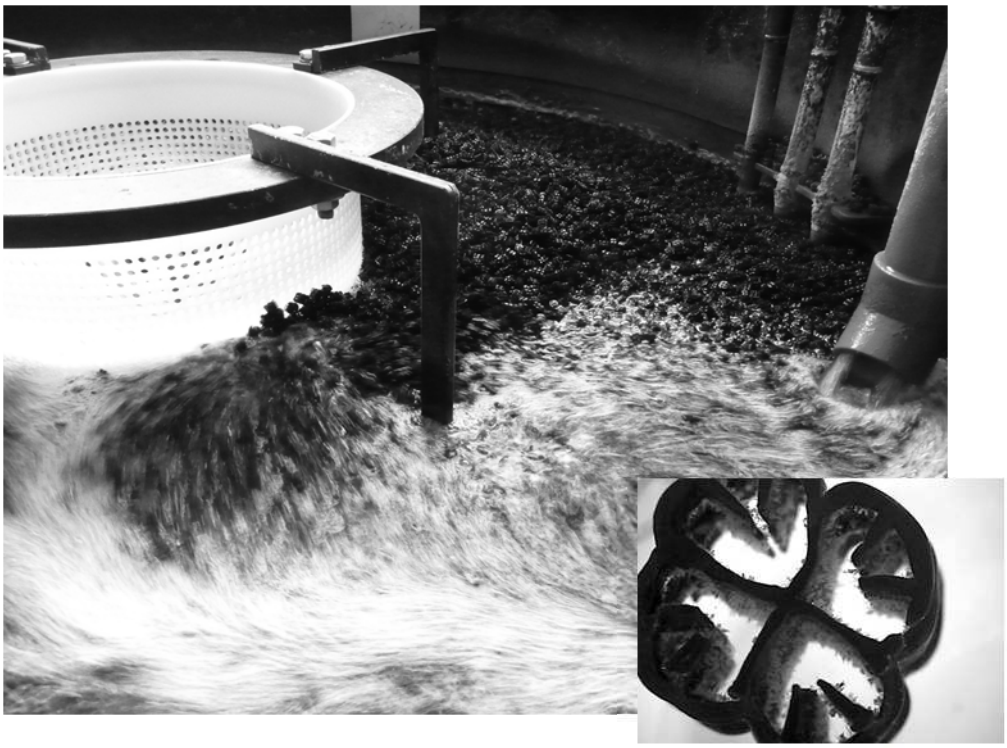

Fig. 1.3: Moving-bed biofilter at the GMA filled with HDPE biocarriers type 2H-BCN 0112 KL. Small picture: BC of the same type with biofilm grown within one year.

BACTERIA INVOLVED IN THE ELIMINATION OF AMMONIA AND NITRITE

Research of microbial processes in engineered systems includes the characterization of the microbial community involved. Comprehensive studies of marine aquaculture systems in this respect were carried out by e.g. Tal *et al*. (2003; DGGE), Itio *et al*. (2006; clone libraries), Paungfoo *et al*., (2006; clone libraries and FISH), Fösel *et al*. (2007; clone libraries and FISH), Michaud *et al*., (2009; clone libraries). The main groups of bacteria found in marine RAS were *Proteobacteria* and members of the CFB cluster (*Cytophaga-Flavobacterium-Bacteroidetes*) (Paungfoo *et al*., 2006; Michaud *et al*., 2009; Schreier *et al*., 2010).

Most bacteria involved in the nitrification process also belong to the *Proteobacteria*. Based on function, bacterial nitrification is divided into ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). These bacteria are typically obligatory lithoautotrophic microorganisms that use

ammonia and nitrite as their sole energy source and carbon dioxide as their main carbon source (Winogradsky, 1890). Nitrifiers are ubiquitous and comprise a very diverse group of bacteria, having representatives among the *Alpha-, Beta-, Gamma-,* and *Deltaproteobacteria* and the phylum *Nitrospirae*. They are important key organisms in the global nitrogen cycle, since they oxidize ammonia to the bioavailable nitrate, which is also assimilated by plants and higher organisms and the more mobile form of inorganic N. Nitrate is reduced to nitrogen oxides and molecular nitrogen (denitrification), closing the N-cycle. Nitrification also exists among heterotrophic bacteria and eukaryotes, such as fungi and algae. The heterotrophic nitrification reactions, however, are unlikely to generate energy (Kilham, 1989).

The autotrophic energy generating nitrification is yet not limited to the bacterial domain, which became apparent with the discovery of ammonia oxidizing *Archaea*, isolated first from a marine aquarium by Könnecke *et al*. (2005). These AOA, belonging to the novel phylum *Thaumarchaeota*, possess a similar ammonia monooxygenase (Amo) like AOB. The Amo is one of the two key enzymes for the two-step oxidation of ammonia over hydroxylamine to nitrite. However, the second key enzyme responsible for the oxidation of hydroxylamine to nitrite in AOB, the hydroxylamine oxidoreductase (Hao) was not found in the genomes of three AOA so far sequenced (Walker *et al.,* 2010; Blainey *et al.,* 2011; Hallam *et al.,* 2006). Neither was hydroxylamine detected and instead nitroxyl is the proposed intermediate of ammonia oxidation (Walker *et al.*, 2010). Only in the past decade it became evident that AOA play an important role in the marine nitrogen cycle, especially in oxygen minimum zones (Wuchter *et al*., 2006; Francis *et al*., 2005).

A recently discovered shortcut process in which ammonium is oxidized with nitrite as electron acceptor to molecular nitrogen under anaerobic conditions (anammox) is a promising alternative to aerobic nitrification, since here only 10 % of the ammonia nitrogen is converted to nitrate, no organic substances have to be added for denitrification and energy costs for aeration can be reduced. Only the ammonia oxidizing step has to be accomplished to provide 1.3 mol nitrite per mol ammonia oxidized (partial nitrification) (Strous *et al*., 1999). Key organisms of this process are members of the phylum *Planctomycetes*. Unique to these anammox planctomycetes are the so called anammoxosomes, cell compartments harbouring the enzyme hydrazine oxidoreductase, which converts hydrazine to dinitrogen. To date five anammox genera have been identified: *Brocardia*, *Kuenenia*, *Anammoxoglobus* and *Jettenia*, enriched from wastewater sludge, and *Scalindua*, a marine representative (Jetten *et al*., 2009). The anammox process as part of complete DIN elimination is successfully applied in wastewater treatment systems, though still great research is focusing e.g. on best practices to foster AOB and oppress NOB (partial nitrification), or on undesirable NO or N_2O production by AOB at low oxygen or high nitrite concentrations (Kampschreuer *et al*., 2008). Tal *et al*.

(2006) and Lahav *et al*. (2009) measured anammox activities in the denitrification reactors of a marine RAS and authors of the latter reference identified the fish´s intestines as a source of anammox bacteria. However, aerobic nitrification is still the most commonly used process in mariculture systems.

Marine recirculation aquaculture systems are a relatively new habitat of nitrifiers as research area, compared e.g. to wastewater treatment facilities or soils, combining high salt concentrations with periodically high ammonia concentrations and relatively low organic load (depending on the system and species cultured). However, researchers like Hirayama (1974) or Carmignagni and Bennet (1977) started in the 70´s to examine the nitrification process during the start-up of biofilters in marine recirculation culture systems based on chemical changes. First electron micrographs of nitrifiers in marine RAS were produced by Johnson and Sieburth (1976). They found colonies of *Nitrosomonas* and few *Nitrosococcus*-like cells. Although being the resulting culture of their enrichment efforts, *Nitrobacter* was not found by electron microscopy. Apart from this publication, to the author´s knowledge, began the integration of molecular population analyses only with the work of Tal *et al*. (2003), being inspired by the findings of Hovanec and DeLong (1996), who identified AOB in marine aquaria, and Burrell *et al*. (1998), Hovanec *et al*., (1998) and Daims *et al*. (2000), who found that *Nitrospira* was the dominant NOB in waste water treatment plant and freshwater aquaria. This finding then was a novelty (although already indicated by the findings of Johnson and Sieburth in 1976, see above), since this role was to that date generally allocated to *Nitrobacter,* which grew best in cultivation-based population analyses. Since then, a couple of studies dealt with the identification of the bacteria responsible for the elimination of ammonia and nitrite in marine RAS, but also other cultivation systems:

Nitrification in aquacultural systems or aquaria was reported to be associated in the majority of the publications with *Nitrosomonas* (freshwater: Hovanec and DeLong 1996; marine: Itoi *et al*., 2006; Tal *et al*., 2003; brackish: Paungfoo *et al*., 2007) and *Nitrospira* (freshwater: Hovanec *et al*, 1998; Itoi *et al*., 2007; Sugita *et al*., 2005; marine: Foesel *et al*., 2007; Tal *et al*., 2003). But also *Nitrosococcus* and *Nitrobacter* were detected in a brackish water hatchery system by FISH four months after inoculation with these bacteria (Kumar *et al*., 2009). However, the dominating nitrifiers in a marine RAS have been determined comprehensively so far only by Foesel *et al*. (2007), who used clone libraries and quantitative FISH analyses (*Nitrosomonas* and *Nitrospira*). The same authors found also AOA in the same RAS, though only in very few numbers. As mentioned before, recent studies about N-cycle bacteria in aquaculture facilities also involve anammox planctomycetes and indeed did Tal and colleagues (2003) identify anammox related bacteria even in the aerobic moving bed nitrification reactor of a marine RAS.

Generally, nitrifiers are known to be slowly growing and fastidious and thus difficult to cultivate, hampering the development of specific probes for *in situ* methods. Therefore, knowledge is still limited and speculations about further genera or phyla are common (Paungfoo *et al.*, 2007). This is not surprising, since only about 1 % of all bacteria could be cultured so far (Amann 2000) and still the majority of the proteins, of which sequences are found in e.g. metagenomic approaches, cannot be allocated to functions. Therefore, function specific cultivation is important. So far unknown genera cannot be sought for in environmental samples and fortunately, just before the work for this study started, the nitrite oxidizing *Nitrotoga*, a *Betaproteobacterium*, was enriched and described by Alawi *et al*. (2009) from samples of permafrost affected soil in Siberia. This member of the nitrifying guild thus could be integrated into the PCR based community analyses. As a matter of fact, as this study is being written, the news about a newly discovered NOB came out. Sorokin (not published yet) could isolate the so called *Nitrolancetus hollandicus* from a reactor in the Netherlands. *N. hollandicus* is allocated to the phylum *Chloroflexi* and even grows at temperatures around 60 °C.

AIM OF THE STUDY

The overall aim of this study was to gain new insights into the process of nitrification in marine RAS and the bacteria involved, with special emphasis on the possible causes of malfunction of the crucial conversion of ammonia to nitrate. It was deemed important that the study be strongly applicable to current aquaculture systems, thus determining specific aspects investigated.

The utilization of aerobic nitrification in moving bed filters of marine RAS was described first in the late 1980´s (Rusten *et al*., 2006). Still, such biofilters are regarded as black boxes (Zohar *et al*., 2004), which is unfavorable regarding the importance of the biofilter in a system as well as its costs. Eshchar *et al*. (2006) estimated that a nitrifying biofilter amounts for 20 % of the investment costs of a RAS, or $ 0.72 per kg fish produced per year. Especially start-up phases were subject to research in the past (Carmignani and Bennett, 1977; Perfettine and Bianchi, 1990; Manthe and Malone, 1987; Grommen *et al*., 2001, Gross *et al.*, 2003), but even failure of established systems occurs frequently (Graham *et al.*, 2007 and therein; G. Quantz, personal communication). Such situation is connected with economic and ecological costs, since water has to be immensely replaced, feed input has to be stopped and, in the worst case, cultivated animals have been already harmed or killed.

In this study, a range of aspects of aerobic bacterial nitrification in moving bed filters of marine RAS were examined with the means of physiological experiments in the laboratory, *in situ* measurements

of water quality parameters, cultivation methods and bacterial population analyses mainly based on 16S rRNA gene sequences.

The complicated nature of investigating nitrification in biofilters is mainly due to the biofilm, in which nitrifying bacteria, together with other microorganisms, proliferate. Biofilms are ever regenerating aggregations of cells, kept together by EPS (extracellular polymeric substances) matrices generated by the bacteria themselves. Compositions and quantities of biofilms in moving bed biofilters are influenced e.g. by substrates concentrations, C:N ratios, oxygen levels, shear forces, grazing e.g. by protozoans or nematodes (Lee and Welander, 1992). Biesterfeld and Figueroa (2002) addressed the limitations of work with nitrifying biofilms, whether on bench-scale with synthetic communities or on full-scale with uncontrollable parameters. Van Loosdrecht and colleagues (2002) likewise stressed the disadvantages of results from either microscopic (cannot be linked to overall system dynamics) or macroscopic (difficult to interpret) approaches. The nitrification performance experiments conducted in this study are based on measurements in the bulk water, thus neglecting deviations of substrate concentrations, pH values and dissolved oxygen contents between bulk water and biofilm and within the biofilm (Chen *et al.,* 2006). Emphasis is given on the changes and differences of ammonia and nitrite oxidation under certain circumstances and conditions. Ammonia and nitrite oxidation rates were always evaluated separately, an approach so far not common in biofilter studies and which gave interesting new insights. The step of nitrite oxidation is favored in this study, since on the one hand it was often neglected in the past (due to the rate limiting nature of ammonia oxidation) and on the other hand, it is reported to be the weaker and less stable step in the process (Graham *et al.,* 2007).

DBU PROJECT

The study is part of the DBU project “Operational and microbiological optimization of biofilters in marine recirculation aquaculture systems”, which was launched in 2006. The project consortium consists of various research institutes as well as industry partners. Further research topics of the project are, e.g., the acceleration of attachment by nitrifying bacteria by preconditioning of the biocarriers, the influence of ozone on nitrification, the endocrine effects of leaching substances from biocarrier material, structure and composition of EPS generated by nitrifiers. All results will be summarized for the final report in 2012; interim reports of phase I and II were prepared in 2007 (http://www.dbu.de/OPAC/ab/DBU-Abschlussbericht-AZ-23821_01.pdf) and 2010 (AZ 23821/02).

The project partner of the current phase III are:

LimnoMar, Hamburg (consultancy and recipient of grant)

GEA 2H Water Technologies, Wettringen (provision with plastic material and technical information about polymers and its processing)

Working group "Aquatic Biotechnology" of Prof. Wolfgang Sand at the **Biofilm Centre of the University of Duisburg-Essen** (attachment experiments, surface properties, EPS)

Gesellschaft für marine Aquakultur mbH (GMA), Büsum (marine aquaculture research facility; nitrate and ozone experiments, endocrine effects of plastics, aeration experiments)

The GMA replaced the aquaculture company **Ecomares** as partner, which was a founder member of the project. Ecomares provided their facilities (brackish warm water and marine moderate water) for on-site experiments in phase I and II.

Spranger Kunststoffe, Plauen (provision of material and system components, and system related consultation)

OVERVIEW CHAPTERS AND WORK CONTRIBUTIONS

According to the mentioned emphasis on NOB, **chapter 2** deals alone with the role of the most active nitrite oxidizer in a commercial marine RAS cultivating turbot and sea bass at 17 °C (Ecomares). The role of *Nitrospira* as dominant NOB was identified by a row of methods, and a novel strain of *Nitrospira marina* could be isolated and characterized. This study was in cooperation with the University of Osnabrück; Myriam Kruse and Prof. André Lipski analyzed the fatty acid profiles of the biofilter bacteria.

With the inauguration of a complete new aquaculture research facility of our project partner GMA in Büsum, comprising of three almost identical marine RAS modules of 35 m^3 each, we had the possibility to monitor the development of the nitrifying potentials of AOB and NOB on frequently sampled biocarriers. Parallel DNA extractions and subsequent PCR with a broad set of specific primers were used to identify the bacteria involved during the start-phases of the filter systems, which had been inoculated with inorganic nitrogen compounds or fish feed. The results are documented and discussed in **chapter 3.** The planning and realization for this long-term experiment

were accompanied by several coworkers at the GMA, especially by Stefanie Beth and Markus Griese, who took care for the record keeping of water quality parameters as well as frequent sampling of biocarriers for molecular analyses.

The **chapters 4 and 5** deal with the potential sources of inhibition of nitrification. Here, the impact of elevated nitrate concentrations and low pH values (chapter V) as well as of leaching substances from plastic material, the substrate on which the nitrifiers are immobilized (chapter IV), was examined. Experiments dealing with the attachment of nitrifying bacteria to different plastic foils were performed by Jürgen Schrötz from the working group “Aquatic Biotechnology” of Prof. Wolfgang Sand at the Biofilm Centre in Duisburg-Essen.

The storage of colonized biocarriers from established systems and their subsequent reactivation after was subject of the experiments documented in **chapter 6.** The carriers were taken from the running biofilter of module 1 at the GMA and stored either dry or in water at 4 and 17 °C. After 3, 6 and 9 months, AOB and NOB specific activity tests were conducted with these carriers. The trial after 12 months storage could not accomplished during this study but will be included in the project report.

CHAPTER II

Relevance of *Nitrospira* for nitrite oxidation in a marine recirculation aquaculture system and physiological features of a *Nitrospira marina*-like isolate

S. Keuter, M. Kruse*, A. Lipski*, E. Spieck

*Rheinische Friedrich-Wilhelms-Universität Bonn, Institut für Ernährungs- und Lebensmittelwissenschaften, Abteilung Lebensmittelmikrobiologie und -hygiene, Meckenheimer Allee 168, D-53115 Bonn, Germany

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ABSTRACT

In biofilters of recirculation aquaculture systems (RAS), nitrification by lithoautotrophic microorganisms is essential to prevent the cultivated organisms from intoxication with ammonium and nitrite. In moving-bed biofilters nitrifying microorganisms are immobilized together with heterotrophic bacteria in dense biofilms on carrier elements like plastic beads. Analyses of fatty acid profiles of these biofilms from a marine biofilter revealed a high abundance of *Nitrospira*-related lipid markers (8-12 % of total fatty acids). Further results of a labeling experiment with ^{13}C-bicarbonate in mineral salts medium with 3 mM nitrite confirmed that *Nitrospira* is the major autotrophic nitrite oxidizer in the biofilter system. According to 16S rRNA gene sequence analyses the nitrite oxidizing community in the biofilter consisted of at least two different representatives of *Nitrospira*, one of which could be successfully isolated. The marine isolate "Ecomares 2.1" belongs to cluster IVa and showed 98.8 % 16S rRNA gene sequence similarity to *Nitrospira marina,* whereas the enrichment "M1 marine" is only distantly related (94.0 % 16S rRNA gene sequence similarity *to N. marina*). In laboratory experiments, the isolate exhibited remarkable tolerances against high substrate and product concentrations (30 mM nitrite and 80 mM nitrate) as well as ammonium (50 mM). During the isolation process a strong tendency of this strain to develop biofilms became apparent. Thus, Ecomares 2.1 seems to be well adapted to the attached lifestyle in biofilters and the nitrogenous load prevailing in the effluent waters of RAS. Both members of *Nitrospira* could be detected by PCR-based methods in environmental samples of marine and brackish RAS biofilters and are therefore considered to be characteristic for these engineered ecosystems.

INTRODUCTION

In the fast growing aquaculture industry, the utilization of the recirculation aquaculture system (RAS) technology is increasing worldwide (Losordo and Hobbs, 2000), yet the process of bacterial nitrification in biofilters, especially of marine RAS, can still be regarded as a black box. During this crucial process, lithoautotrophic ammonia oxidizing bacteria (AOB) convert ammonia to nitrite, which is further oxidized to nitrate by lithoautotrophic nitrite oxidizing bacteria (NOB) (Watson *et al.*, 1989). In comparison to ammonia and nitrite, nitrate is far less toxic to the cultivated organisms (Camargo *et al.*, 2005). Ammonia oxidizing *Archaea*, although abundant in marine environments (Francis *et al.*, 2005), seem not to contribute significantly to the purification of marine RAS water (Foesel *et al.*, 2007).

Apart from aerobic nitrification, the process of anammox, in which members of the order *Planctomycetales* oxidize ammonia with nitrite under anoxic conditions to molecular nitrogen (Strous *et al.*, 1999), is a promising alternative for nitrification in biofilters of RAS (Tal *et al.*, 2006). Here, no nitrate is generated and the system runs under oxygen limited conditions, which is economically preferable. However, the use of autotrophic aerobic nitrification is still the prevalent application for the elimination of toxic nitrogen species in RAS as well as in aquariums.

Nitrifying bacteria are generally slow growing due to the relatively low energy yields of ammonia oxidation ($\Delta G^{\circ\prime}$ = -275 kJ mol N) and nitrite oxidation ($\Delta G^{\circ\prime}$ = -74 kJ mol N) (Thauer *et al.*, 1977). Additionally, nitrification rates in marine RAS in general seem to be lower compared to freshwater RAS (Nijhof and Bovendeur, 1990; Rusten *et al.*, 2006). In well running biofilters, though, nitrite concentrations usually remain low, since nitrite is the intermediate of nitrification. However, during start-up phases and due to changes of operational conditions, nitrite peaks may develop, which are often hazardous or even mortal for the cultivated organisms in the rearing tanks (Lewis and Morris, 1986). Graham *et al.* (2007) dealt with the disturbance of the mutualism between ammonia and nitrite oxidizing bacteria in wastewater treatment systems (WWTP) and suggested that NOB are the weaker link in the nitrification process. Reported reasons for the failure of nitrite oxidation in nitrifying reactors are e.g. changes in pH-values or temperature, accumulation of heavy metals or organic compounds and, above all, elevated concentrations of free ammonia (Villaverde *et al.,* 1997; Villaverde *et al.*, 2000).

Only since recently, the microorganisms responsible for nitrification in aquatic habitats with high nitrogen load were explored applying cultivation-independent molecular approaches. Several authors identified representatives of the genus *Nitrospira* as dominant NOB in biofilters of aquaria

and RAS (freshwater and marine) (Hovanec *et al.*, 1998; Tal *et al.*, 2003; Sugita *et al.*, 2005; Foesel *et al.*, 2007; Itoi *et al.*, 2007) as well as WWTPs (Juretschko *et al.*, 1998). *Nitrospira*-like bacteria are members of the deep-branching phylum *Nitrospirae* (Ehrich *et al.*, 1995). At least some species of this genus are not obligate lithoautotroph (e.g. *N. marina*) (Watson *et al.*, 1986) and grow better in the presence of simple organic carbon sources (e.g. pyruvate) or nitrogen sources (e.g. peptone) than in mineral salts medium for autotrophs.

In this study, the relevance of *Nitrospira* as active nitrite oxidizing organism in the moving-bed biofilter of a marine RAS was investigated by fatty acid profiling and ^{13}C-labeling experiments. This method was established by Boschker *et al.* (1998) in order to link microbial processes in natural environments with the organisms involved. Since Lipski *et al.* (2001) found the three different major fatty acids (cis 7 and cis 11 isomers of hexadecenoic acid and 11-methyl-hexadecanoic acid) in different combinations to be characteristic for *Nitrospira* species, they serve as chemotaxonomic markers for the detection and identification of *Nitrospira* species in natural and engineered ecosystems. Image-based methods like electron microscopy and FISH as well as immunoblotting supplemented the results. Further, a *Nitrospira*-strain was isolated from the biofilter and characterized with emphasis on its tolerances against dissolved inorganic nitrogen compounds (DIN).

EXPERIMENTAL PROCEDURES

Cultivation and physiological experiments

The marine mineral medium by Watson *et al.* (1986) in 70 % seawater (North Sea, off the island of Heligoland, Germany) was the basic medium for cultivation and isolation procedures. The pH was adjusted to be 7.0-7.5 after autoclaving. Larger volumes of media were stirred for sufficient oxygen supply. Mixotrophic (with nitrite) and heterotrophic (without nitrite) medium contained additionally 0.15 ‰ w/v yeast extract and peptone and 0.055 ‰ w/v sodium-pyruvate. Limnic (non-marine) mineral medium was prepared according to Ehrich *et al.* (1995).

All physiological tests were carried out with defined volumes (mostly 100 ml) of medium (see above) in 300 ml Erlenmeyer flasks in duplicates at 28 °C (unless noted otherwise). To test tolerance limits, NH_4Cl, $NaNO_3$, $NaNO_2$ were added in different concentrations keeping the same pH values. The media were inoculated with 1 ml of active cultures and incubated in the dark without agitation. Samples (1 ml) were taken regularly and nitrite and nitrate were analyzed by HPLC to control nitrification performances. For temperature optimum tests 10, 17, 22, 25, 28 and 37 °C were chosen.

The denitrifying media were mineral salts media with either 10 mM tri-sodium citrate dihydrate and 10 mM $NaNO_3$ or with 0.15 and 0.75‰ w/v peptone, 0.15 and 0.75 ‰ w/v yeast extract, 0.055 and 0.275 ‰ w/v pyruvate and 5 or 10 mM $NaNO_3$ or 1 mM $NaNO_2$. The media were filled into test tubes containing Durham tubes and closed with rubber stoppers after inoculation. For comparison *Nitrospira marina* strain 295 was used. Cell counts were performed with a Thoma cell chamber (0.02 mm depth).

Electron microscopy

For transmission electron microscopic observations (Zeiss model Leo 906E, Carl Zeiss, Jena, Germany), cells were fixed in 2.5 % (v/v) glutaraldehyde for 1.5 hours and 2 % (w/v) osmium tetroxide over night, and embedded in Spurr (Spurr, 1969). Ultrathin sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). For morphological visualization of whole cells, cell pellets were stained with uranyl acetate (Spieck *et al.*, 1996).

For scanning electron microscopy (Zeiss model LEO 1525) pelletized cells were fixed in paraformaldehyde (1 %) and glutaraldehyde (0.25 %), dehydrated by critical point drying with a Balzers CPD 030 and sputter-coated with gold with a SCD 050 (both Bal-Tec, Schalksmuehle, Germany). SEM micrographs were taken with a LEO 1525 (Zeiss, Jena, Germany).

Chemical analyses

Nitrite and nitrate concentrations were determined by high pressure ion-pair chromatography with a Hypersil ODS C18 column (125 mm, 4.6 mm) (Meincke *et al.*, 1992) followed by UV detection in an automated system (Kontron, Eching, Germany).

Molecular and phylogenetic analyses

The DNA of the cultures as well as environmental samples was extracted using the Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Near complete 16S rRNA genes were cloned and sequenced according to Alawi *et al.* (2007). Clones of the enrichment culture M1 marine were screened with the semi specific primer pair 27f/1158r (Lane, 1991; Maixner *et al.*, 2006) before sequencing. Sequences published within this study were checked for chimera using the Pintail program (Ashelford *et al.*, 2005).

For denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), 16S rRNA gene fragments were amplified by PCR with the specific primer pair GC-60f/662r (Daims *et al.*, 2001; Alawi, 2007) and

the universal primer pair GC-341f/907r (Lane, 1991) which targets most bacteria. The products were loaded into a gel of 60-90 % and 40-70 % denaturants, respectively, and run at 110 V and 59 °C for 17 hours. Bands of interest were excised, re-amplified and sequenced.

The primer pair M1Nsp (forward: 5´GGC AAC CTG GTA GTA AAG TG 3´; reverse: 5´AGG CAG TAA CCT GCC TTA TC 3´; *E. coli* positions 78 to 470; annealing temperature 66 °C) was deduced using the program Clone Manager (Sci-Ed Software, Cary, USA) and evaluated with the Basic Local Alignment Search Tool (BLAST, NCBI) (Altschul *et al.*, 1990) and Probe Match (Cole *et al.*, 2007). Published and new 16S rRNA gene sequences (> 1400 bp) were aligned and a neighbor-joining tree was calculated using the MEGA software (Tamura *et al.*, 2007). The 16S rRNA gene sequences from this study have been deposited in GenBank with the accession numbers HQ686082– HQ686084.

Imunoblot analyses were performed as described in Bartosch *et al.* (1999) with the antibodies Hyb 153-3. The crude extract was obtained from biofilm that was scratched off the biocarriers.

Fatty acid analyses

Cells for the extraction and preparation of complete fatty acid methyl esters (FAMEs) were harvested by centrifugation after cultivation of 3-Liter enrichments. The preparation of FAMEs was performed as described by Sasser (1990) and the extracts were analyzed by GC-MS (gas chromatography mass spectrometry). The chromatographic conditions and the analyses of the fatty acids had been done as described previously (Lipski and Altendorf, 1997; Lipski *et al.*, 2001).

For labeling experiments carriers with biofilm were incubated with stable isotope $NaH^{13}CO_3$ (20 mM) and $NaNO_2$ (3 mM) in gastight flasks for several weeks at 17 °C. The oxygen content was measured regularly by GC-MS and samples were extracted when the oxygen content dropped below 5 %. After incubation, the sample material was concentrated by centrifugation. Lipids were extracted and phospholipid fatty acid methyl esters were prepared and analyzed as described by Knief *et al.* (2003).

Fluorescence in situ hybridization

Aliquots of the isolate were prepared by paraformaldehyde fixation as described by Amann *et al.* (1995). FISH was performed according to the protocol detailed by Manz *et al.* (1992) with the Cy-3 labeled probe S-*-Ntspa-0712-a-A-21 (Daims *et al.*, 2001), targeting the phylum *Nitrospirae*. Cells were stained with DAPI (4',6-Diamidin-2'-phenylindol-dihydrochlorid) afterwards and observations were performed with an Axio ImagerM2 (Carl Zeiss, Jena, Germany).

RESULTS

Description of the RAS in Büsum, Germany

The biofilters of the RAS (600 m^3 total volume, commercial production of turbot and European seabass) were started in January 2005 with biocarriers from a previous filter as inoculum. The reactors contained biocarriers of recycled HDPE (high density polyethylene) (type HX09, Stöhr, Marktrodach, Germany) with a surface of 884 m^2/ m^3 (Fig. 2.1). About 3-8 % of the system water was replaced daily by filtered seawater (North Sea) from the adjacent port. The temperature of the process water was 15-20 °C and the salinity was 24-30 ‰. Oxygen concentration was kept at 9-10 mg/l and pH at 7.0. The maximum nitrification potential of NOB on the biocarriers under optimal laboratory conditions was about 1050 nmol nitrite per hour per biocarrier (8.1 cm^2) in September 2006 (data not shown). Between 2007 and 2009 about 6000 µM nitrate were measured in the biofilters, as well as ammonium and nitrite concentrations between 5-60 µM and 10-40 µM, respectively. DIN concentrations in the water from the port of Büsum analyzed by Sudarno *et al.* (2009) in January 2008 were 2.4 µM nitrite, and 37 µM nitrate. Ammonium was not detectable.

Enrichment and isolation of **Nitrospira** *from biocarriers*

Biocarriers were taken in October 2005 from one of the biofilters, incubated in marine medium with 70 % North Sea water at 22 °C and supplemented repeatedly with 0.3 mM of nitrite. A dilution series of this culture resulted in growth of NOB in the 10^{-4} dilution, which was incubated at 17 °C. After ten months a new inoculation was started at 22 °C and occasionally supplied with nitrite, when the substrate had been converted to nitrate. Six months later a bottle containing 3 litres of medium was inoculated (50 ml) and gently stirred at 22 °C.

After several weeks, a thick brownish biofilm developed at the water/air interface at the wall of the bottle (Fig. 2.2). A sample of this film was removed, whirled with Tween 80 (1 % v/v) in medium and served as inoculum for a dilution series. Cells of the highest dilution of this series, in which nitrite oxidation could be detected (10^{-6}), were enriched and served as inoculum for a second dilution series. Again, cells of the 10^{-6} dilution level of this series converted nitrite to nitrate and were enriched and transferred to fresh medium several times until the strain “Ecomares 2.1” was considered pure. In total, the isolation process took about four years. Purity was checked visually by electron microscopy, light microscopy and fluorescence *in situ* hybridization (FISH). Further purity controls were performed under heterotrophic and mixotrophic conditions (Spieck and Lipski, 2011) and DGGE analyses with *Nitrospira*-specific (60f-GC/662r) as well as universal bacterial primer sets

(341f-GC/907r), resulting in identical sequences. Unless otherwise noted, results reported here are based on this final isolate.

The enrichment "M1 marine" was started with biocarriers from the same biofilter, taken in June 2007. Here cells were removed from the HDPE carriers by shaking with glass beads (1.7-2 mm) over night and incubated in marine mineral salts medium containing 1 mM nitrite for half a year. After only one transfer with 1 % inoculum DNA was extracted and 16S rRNA gene sequences (1502 bp) were retrieved by a cloning approach.

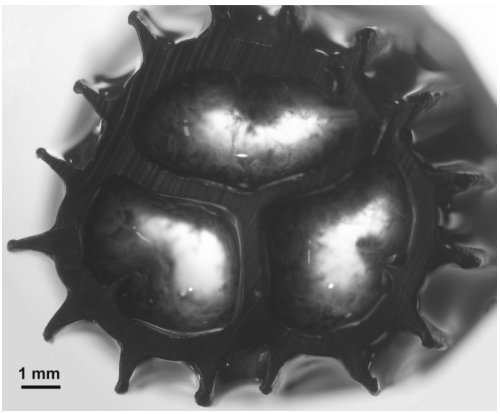

Figure 2.1 (left): Biocarrier (recycled HDPE, type HX09) overgrown by a thick biofilm taken from the biofilter of the RAS in Büsum after approximately one and a half year of operation**.**

Figure 2.2 (right): Biofilm developed at the water/air interface of the culture vessel of the enrichment culture Ecomares 2.1. This biofilm was scratched off the glass wall, whirled with Tween 80 and used as inoculum for further dilution series.

Analysis of the nitrite oxidizing community

A polyphasic approach was applied to identify the dominating and active population of nitrite oxidizing bacteria in the biofilm of the carriers. For fatty acid labeling experiments carriers covered with biofilm were taken from the moving-bed filter system of the marine aquaculture plant in Büsum in April 2008 (at temporarily low feeding rate) and incubated with ^{13}C-bicarbonate and 3 mM nitrite in marine as well as in limnic medium. The *Nitrospira marina*-specific fatty acid compounds 16:1 cis 7 and 16:1 cis 11 were present with a percentage of total fatty acids from 8 % to 12 %. In mineral salts medium with nitrite as sole energy source both acids were labeled with percentages of 20 to 21 (16:1 cis 7) and 13 to 14 (16:1 cis 11) (Fig. 2.3), which indicated for the major role of *Nitrospira* as autotrophic nitrite oxidizer in this habitat. As depicted in Fig. 2.3, also 6-7 % of palmitic acid (16:0) were labeled. Total fatty acids of *Nitrospira marina* consist to 30-37 % of this compound, yet it is not genus specific (Lipski *et al.*, 2001). The fatty acids 18:1 cis 11, 16:1 cis 9 and 14:0, major, but non-

specific compounds of the genera *Nitrobacter, Nitrococcus, Nitrospina* and *Nitrotoga* (Lipski *et al.*, 2001; Alawi *et al.*, 2007), respectively, were not labeled significantly.

Additionally, genera-specific western blot analyses with the monoclonal antibody Hyb 153-3, which recognizes the ß-subunit of the nitrite oxidoreductase, were performed with one year old biofilm from the biocarriers (Fig. 2.4). The antibody did not recognize proteins with the molecular masses of *Nitrobacter* and *Nitrococcus* (65 kDa), but that of *Nitrospira* and *Nitrospina* (46-48 kDa) (Bartosch *et al.*, 1999).

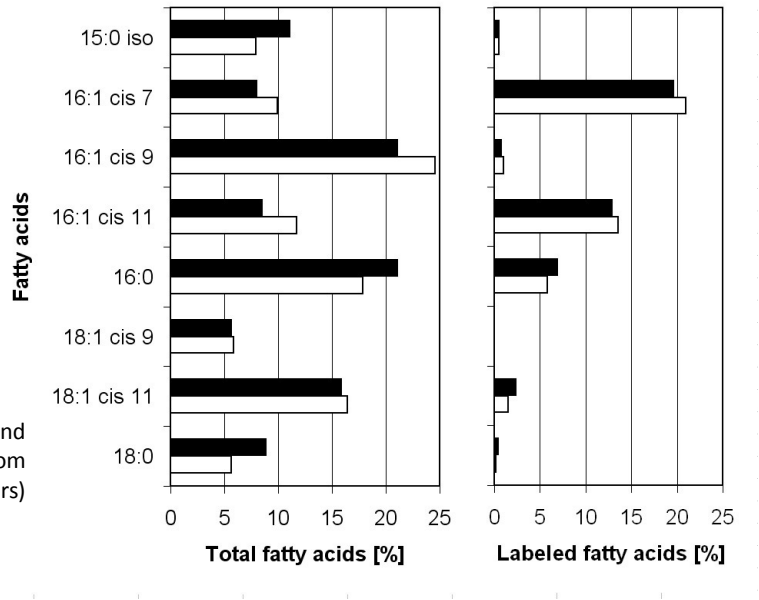

Figure 2.3: Total fatty acids (left) and percentages of labeled fatty acids (right) from carrier biofilms incubated in marine (black bars) or limnic (white bars) medium.

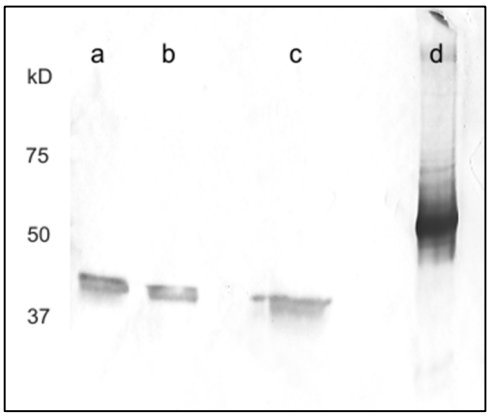

Figure 2.4: Results of western blot analysis with the Hyb 153-3 antibodies. Lanes: *Nitrospira defluvii* A17 (a), a NOB enrichment culture (b), biofilm from carrier elements of the Ecomares biofilter (c), *Nitrobacter hamburgensis* X14 (d). The molecular weight of the ß-subunit of the nitrite oxidizing system of *Nitrospira* is 46 kDa.

Electron micrographs of ultrathin sections, prepared from embedded pieces of the biocarriers from Büsum after one and two years of operation, depict *Nitrospira* colonies, which are very abundant and distributed throughout the biofilm. They are surrounded by less dense colonies of *Nitrosomonas* (AOB) and other bacteria (Fig. 2.5). *Nitrobacter*-like cells were found very rarely, while no cells of other NOB could be identified. Fluorescence microscopy after hybridization with the Ntspa-712

probe, targeting the phylum *Nitrospirae,* likewise revealed numerous hybridized colonies within the biofilm of fixed samples from 2006 (Fig. 2.6).

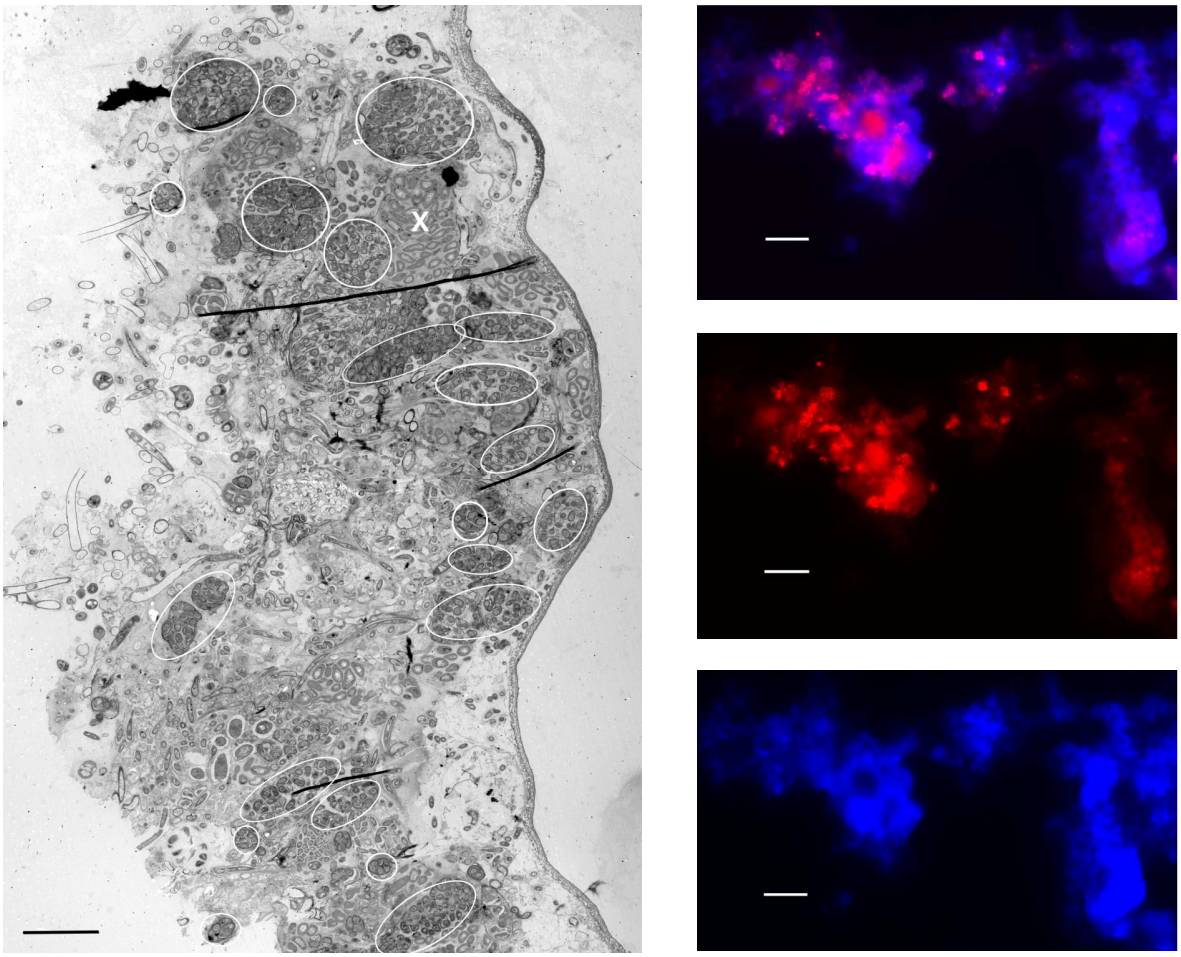

Figure 2.5 (left): Electron micrograph of an ultrathin section trough one year old biofilm, which was embedded directly on the plastic material. Typically, *Nitrospira*-like colonies (circles) dominated the sections throughout the biofilm, whose basis here is on the right side. An example of a *Nitrosomonas*- like colony is marked with an X. Scale bar = 5 µm.

Figure 2.6 (right): Epifluorescence micrographs depicting aggregations of cells hybridized with the *Nitrospirae*- specific probe Ntspa-712 (middle) and DAPI-stained (below) within disrupted biofilm of the filter media from 2006 (above = merged pictures). Scale bars = 10 µm.

Phylogeny and chemotaxonomy of enrichment culture M1 marine and strain Ecomares 2.1

Both cultures comprised at least two different *Nitrospira*-associated 16S rRNA gene sequences at the beginning of the enrichment processes, according to findings of DGGE analyses with the primer pair GC-60f/662r. One of these, henceforth referred to as M1 marine, is rather distantly related to *Nitrospira marina* (94.0 %), but also a member of the cluster IVa (Fig. 2.7). The closest cultured relative (94.3 %) is a marine sponge associated *Nitrospira* recently published by Off *et al.* (2010). The most similar environmental sequences (98.6 %) are those of two clones from a RAS of a shrimp farm

(accession number HM345625 and HM345623) (Fig. 2.7). With specific primers (M1Ntsp) targeting the sequence of M1 marine, we obtained PCR amplicons from further enrichments and environmental samples from the biofilter in Büsum, as well as of samples from a brackish RAS biofilter at the Baltic Sea near Kiel, Germany (data not shown), but not from DNA samples of the final Ecomares 2.1 cultures. Here, the *Nitrospira*-like bacterium M1 marine must have become lost due to repeated transfers and only one 16S rRNA gene sequence (1502 bp) could be found after finishing the isolation process. The remaining strain Ecomares 2.1 is closely related to *Nitrospira marina* (X82559) with a 16S rRNA gene sequence similarity of 98.8 % (Fig. 2.7). The most similar published environmental sequences originated from a marine recirculation plant in Rehovot, Israel (99.1 % and 98.9 % similarity, AM295541 and AM295539), and from chronically oil-polluted retention basin sediment from the Berre lagoon in France (99.0 % similarity, FM242344) (Fig. 2.7). A highly similar sequence was also present in the enrichment culture M1 marine (99.0 %).

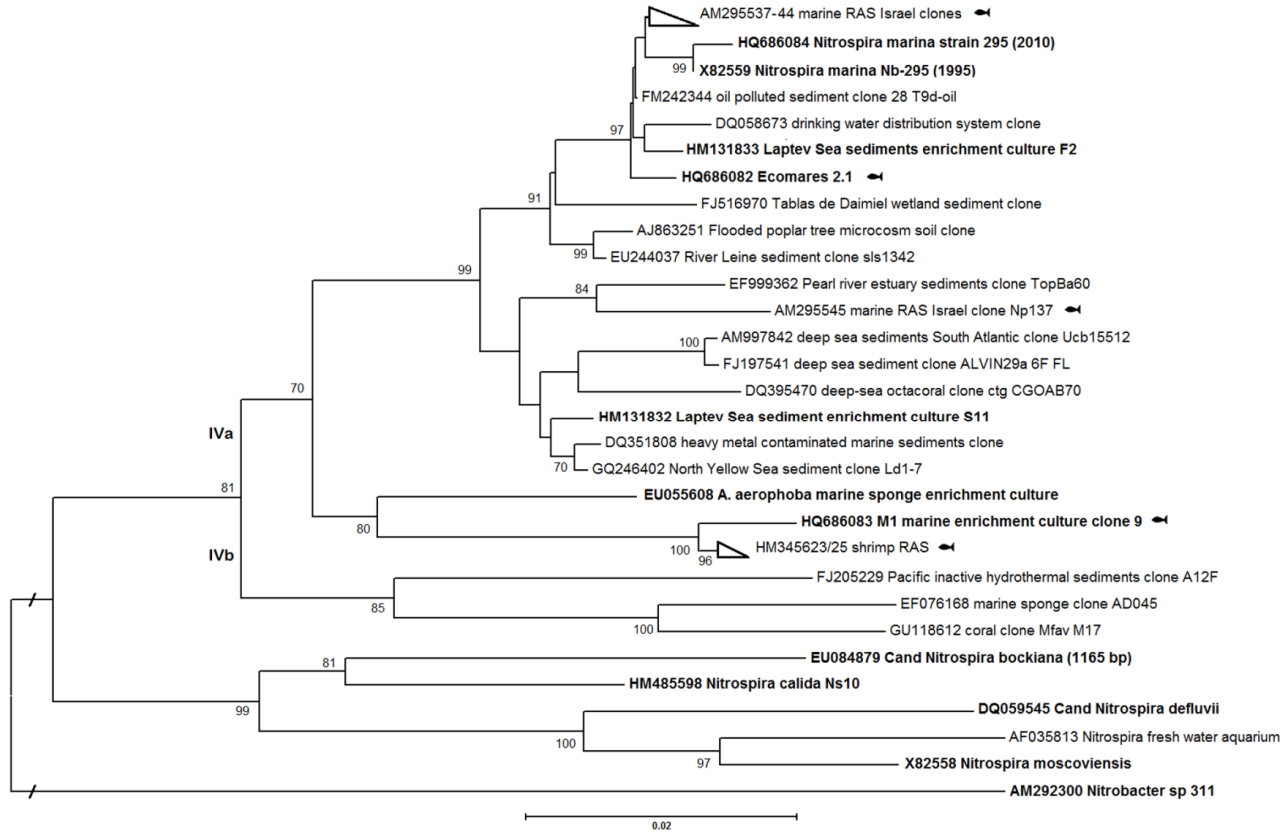

Figure 2.7: Neighbour-joining tree depicting the phylogenetic relationships between the 16S rRNA gene sequences of strain Ecomares 2.1 and other *Nitrospira*-like environmental sequences of cluster IV as well as cultivated representatives (in bold). Sequences originating from aquaculture plants are indicated by a fish symbol. The tree was constructed using sequences of >1400 bp (except *N. bockiana*). Nodes supported by bootstrap values are indicated. Scale bar = 2 % sequence divergence.

The total fatty acids of the enrichment culture and the isolate Ecomares 2.1 grown in limnic and marine mineral salts medium (17, 22 and 28 °C) and mixotrophic medium (28 °C) showed the *Nitrospira*-characteristic fatty acids 16:1 cis 7 with percentages from 37 to 41 % and 16:1 cis 11 with 20 to 32 % (Table 2.1). These compounds were found in *Nitrospira marina* in a similar range (30 to 34 % of the 16:1 cis 7 acid and 15 to 22 % of the 16:1 cis 11 acid) (Lipski *et al.*, 2001).

Table 2.1: Fatty acids profile (in % of the respective acid) of strain Ecomares 2.1 and *Nitrospira marina* 295 at different times and cultivation conditions (auto=autotroph, mixo= mixotroph).

	enrichment culture Ecomares 2.1		**pure culture Ecomares 2.1**			***Nitrospira marina 295* (Lipski *et al.*, 2001)**	
	April 2008		February 2010				
trophic condition	auto	auto	mixo	auto	auto	auto	mixo
temperature	22 °C	22 °C	28 °C	17 °C	28 °C	28 °C	28 °C
medium	limnic	marine	marine	marine	marine	marine	marine
12:0						0.6	0.2
14:0	0.8	0.7		1.0		1.4	0.8
15:0 iso	0.8					0.8	0.4
15:0 anteiso	0.6						
14:0 3OH	0.4						
16:1 cis 7	41.4	41.4	40.4	40.7	37.1	30.4	33.5
16:1 cis 9	1.3	1.2					
16:1 cis 10	0.8	1.9		1.9	2.8		
16:1 cis 11	31.7	31.9	27.6	30.8	19.8	15.5	22.2
16:0	20.5	22.0	29.4	24.4	35.3	36.5	30.7
16:0 11 methyl					2.3	0.8	
17:0 iso	0.2					0.8	0.6
16:0 2OH	0.3						
18:1 cis 7	0.6						
18:1 cis 11	0.7	0.6				0.6	
18:0			2.6	2.2	2.8	8.7	8.4
18:1 cis 9						1.8	0.7
19:0 cyclo 9-10		0.3					

Morphology of the strain Ecomares 2.1

As mentioned above, *Nitrospira* occurred in dense colonies within the biofilm (Fig. 2.8 A). During the ongoing isolation, such microcolonies disappeared and planktonic cells occurred. These cells of the *Nitrospira* strain Ecomares 2.1 were 0.2-0.4 µm and 0.8-1.2 µm in size and formed compact curved rods (Fig. 2.8 B-D). The same shape became visible with the FISH probe Ntspa-712 (Fig. 2.9). Long spiral cells with 2 to 3 turns were found rarely by EM. The periplasmic space was wider than that of other gram-negative cells, which is characteristic for the genus *Nitrospira* (Watson *et al.*, 1986). Although the pronounced tendency of the culture to develop biofilms could not be observed any more in the final culture, electron microscopic pictures of ultrathin sections (not shown) and negative stained cells (Fig. 2.8 D) showed considerable EPS (extracellular polymeric substances) around single cells. Accordingly, cells examined by scanning electron microscopy were coated with a thick and rough layer (Fig. 2.8 C). In comparison, cells of *Nitrospira moscoviensis*, subjected to the same drying procedure (alcohol series, critical point drying), had a very smooth surface and were visually more distinct (not shown).

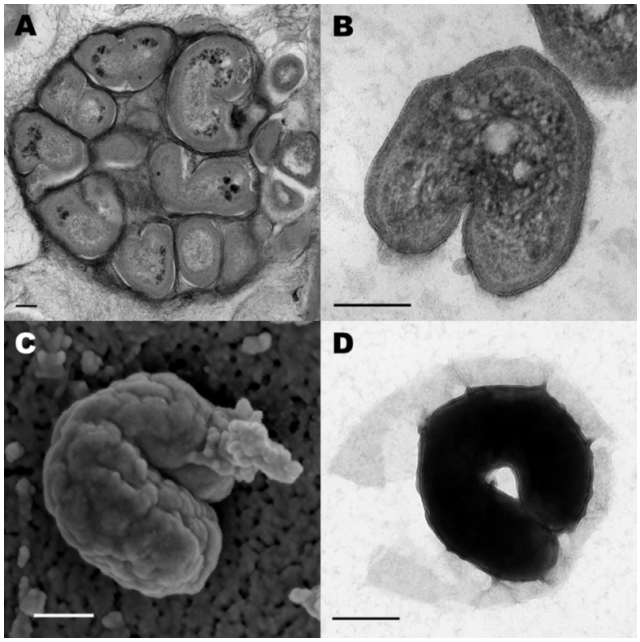

Figure 2.8: Electron micrographs: (A) dense colony of *Nitrospira*-like cells (ultrathin section of the biofilm grown on a biocarrier sampled October 2005); (B) ultrathin section of a typical curved rod of strain Ecomars 2.1 revealing the wide periplasmic space; (C) scanning electron microscopic picture of a cell of strain Ecomares 2.1 showing the thick coat around the cell; (D) whole cell of Ecomares 2.1 stained with uranyl acetate. Scale bars = 200 nm.

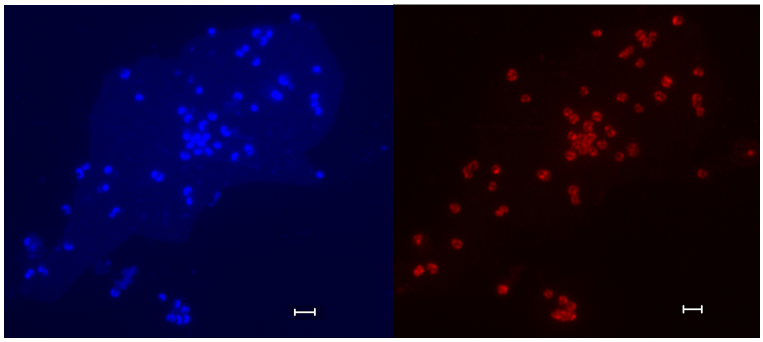

Figure 2.9: Characteristic curved rod-shaped cells of strain Ecomares 2.1 stained with DAPI (left) and oligonucleotide probe Ntspa-712, recognizing the phylum *Nitrospirae* (right). Scale bar = 2 µm.

Physiological experiments with Ecomares 2.1

The growth optimum of the new *Nitrospira* culture was 28 to 30 °C, although the temperature of the rearing water of the fish farm was 17 °C (data not shown). At 4 °C the culture grew extremely slowly (1 mM nitrite was oxidized in 10 weeks), whereas at 37 °C no growth could be observed. Incubated in mineral salts medium, the cell yield of Ecomares 2.1 ranged between 4 x 10^5 and 2 x 10^6 cells per µmol nitrite, and between 5 x 10^6 and 3 x 10^7 cells per µmol nitrite in mixotrophic medium. In the same experiments the generation time of Ecomares 2.1 under autotrophic conditions averaged 2.5 ± 1.3 d (n = 3) and 1.7 ± 0.4 d (n = 6) under mixotrophic conditions (data not shown). Heterotrophic growth could be observed neither on plates nor in liquid media of different compositions after six weeks of incubation at 28 °C.

Ecomares 2.1 did grow in limnic medium, but slowly, and ceased with new inoculations to fresh medium. Neither production of gas or of nitrite nor elimination of nitrite or nitrate could be observed in mineral salts media with citrate plus nitrate or media with peptone, yeast extract, pyruvate plus nitrate or nitrite under oxygen limited conditions.

Increasing substrate concentrations decreased growth of the isolate Ecomares 2.1 gradually; 1 mM nitrite was oxidized within 10 days, while the same amount of substrate was converted within 25 and 53 days, when the nitrite concentration was raised to 20 and 30 mM, respectively. The latter was also the highest tolerated substrate concentration. Similar to nitrite, elevated nitrate concentrations slowed down nitrite oxidation. Whereas in the control (0 mM $NaNO_3$) 1 mM substrate was converted in 15 days, cultures supplemented with 40, 60 and 80 mM $NaNO_3$ oxidized the same amount of substrate in 24, 35 and 40 days, respectively. In contrast, ammonium concentrations up to 50 mM did not inhibit growth significantly and, although not measured quantitatively, growth of Ecomares 2.1 could be observed in the presence of ammonium up to 80 mM.

DISCUSSION

Relevance of* Nitrospira *as nitrite oxidizer in the moving-bed biofilter

Since molecular techniques found their way into microbiological ecosystem studies, the dominant nitrite oxidizers detected by different authors in RAS and aquaria, both marine and freshwater, were members of the genus *Nitrospira*. In this study these outcomes could be expanded to a further marine RAS with the use of chemotaxonomic markers correlating metabolic activities with taxonomic groups. The high degree of labeling of the *Nitrospira*-specific compounds using nitrite concentrations

of 3 mM reflects considerable activity of *Nitrospira* cells in the biofilter, while other nitrite oxidizing autotrophic organisms were not relevant. Especially with regard to the growth characteristics of *Nitrobacter*, this dominance of *Nitrospira* in a habitat of high nitrogen load remains unexplained. In several studies *Nitrobacter* was characterized as r- strategists (high maximum specific growth rate at high nitrite and oxygen levels) (Schramm *et al.*, 1998; Blackburne *et al.*, 2007), whereas *Nitrospira* was described as K-strategist (low maximum specific growth rate, but well adapted to low nitrite and oxygen concentrations). The genus *Nitrobacter* is allocated to the class *Alphaproteobacteria* and differs physiologically and biochemically from the genus *Nitrospira* (Luecker *et al.*, 2010). Due to its high tolerance towards nitrite and fast growth, *Nitrobacter* was historically used as primary model organism to study nitrite oxidation in the laboratory (Starkenburg *et al.*, 2006). The r/K- hypothesis of *Nitrospira* and *Nitrobacter* was approved elsewhere in ecosystem studies (Kim and Kim, 2006; Nogueira and Melo, 2006) but is not consistent with the above mentioned dominant role of *Nitrospira*-like bacteria in habitats with high nitrogenic load like RAS integrated biofilters. Accordingly, Maixner *et al.* (2006) suggested a broader differentiation of the ecology of the genus *Nitrospira* on an imaginary scale reaching from K- to r-strategies.

Analyses of nitrifying biofilms by *in situ* hybridization and the use of microelectrodes revealed a heterogenous distribution of *Nitrospira* microcolonies due to its microaerophilic behaviour mentioned above (Okabe *et al.*, 1999; Schramm *et al.*, 2000). It was found that these NOB were absent at the oxic part of the biofilm, but most abundant at the oxic/anoxic interface. In our investigations of a 10 months old marine biofilm, *Nitrospira* occurred throughout the layer without any preferences, but the *in situ* spatial organization of nitrifying bacteria was not examined in detail. However, as already found by Schramm *et al.* (2000), *Nitrospira* was not restricted to a certain zone during biofilm development and it should be noted that the nitrifying biofilm in the biofilter of the Ecomares plant is exposed to permanent shearing forces.

Apart from metabolic activities, also the amount of specific fatty acids affirmed the dominance of the *Nitrospira* population within the biofilms of the carriers analyzed in this study. Of the total fatty acids, between 8 and 12 % consisted of the *Nitrospira* characteristic compounds 16:1 cis 7 and cis 11. Cell counts after *in situ* hybridization in freshwater aquarium filters (Hovanec *et al.*, 1998) and marine RAS (Foesel *et al.*, 2007) led to percentages of 5 and 15.7 % *Nitrospira* of total cells, respectively. Further, electron microscopy and fluorescence *in situ* hybridization of cells in the biofilm confirmed high abundance of *Nitrospira* as well as a negligible presence of other NOB, which in turn was corroborated by western blot analyses. The fatty acids 16:1 cis 9 and 18:1 cis 11, indeed found in the genera *Nitrobacter, Nitrococcus, Nitrospina* and *Nitrotoga*, yet are major compounds of most *Proteobacteria*. Michaud *et al.* (2009) quantified heterotrophic bacteria in marine RAS biofilters by ARDRA and found that the majority of all bacteria are *Alpha*- and *Gammaproteobacteria* (33.7 and

22.6 %, respectively). Also the clone library of Itoi and colleagues (2006) revealed that half of the bacterial population on filter material of a pufferfish RAS were *Proteobacteria*. These data may explain the high percentages of the mentioned fatty acids in the biofilm on the carriers.

Since the RAS investigated in this study was started and refreshed with water from the North Sea, it is probable that the nitrifying bacteria originate from there. The natural distribution of NOB in oceans, however, is poorly investigated so far. There are, for instance, findings of *Nitrobacter* and *Nitrococcus* (Ward and Carlucci, 1985) or *Nitrospina* (Labrenz *et al.*, 2007) in marine waters. More comprehensive studies were conducted by Watson *et al.* (1986), who assumed the genus *Nitrospira* to be ubiquitous in oceanic environments, and *N. marina* to be the most prevalent in sediments and organic rich marine waters.

Coexistence of different* Nitrospira *in the marine RAS

The phylogenetic and morphological investigations showed that the strain Ecomares 2.1 resembles *N. marina*, which is in accordance with the results of the chemotaxonomic analyses. Variations in medium (marine autotrophic and mixotrophic) or temperatures (17, 22 and 28 °C) did not affect the characteristic fatty acid profile significantly. The genotypic information, here based on the sequence of the 16S rRNA gene, revealed a 98.8 % similarity to *N. marina* strain 295, therefore we consider the isolate Ecomares 2.1 as a new strain of the same species, which is consistent with the recommendations of Stackebrandt and Ebers (2006).

Ecomares 2.1 is the first isolate of sublineage IVa from a technical system with high nitrogenous load. It is, indeed, possible that cultivation procedures may lead to the enrichment of less dominant species which adapt better to laboratory conditions. The similarity of the 16S rRNA gene sequence of Ecomares 2.1 to the sequences retrieved from other marine RAS, however, let us assume, that this strain is characteristic for this habitat. The sequence of M1 marine was likewise found in further samples of marine and brackish RAS and might thus be equally characteristic for marine aquaculture biofilters. The coexistence of different *Nitrospira* (e.g. of cluster I and II) in technical systems was already reported. The authors found out that for instance varying prevailing nitrite concentrations (Maixner *et al.*, 2006) or dissolved oxygen concentrations (Park and Noguera, 2008) influenced the composition and dominance of various *Nitrospira*-species. At the moment, increased effort is put into the isolation of the second representative of *Nitrospira* in the enrichment culture M1 marine for physiological characterization in comparison with Ecomares 2.1.

A robust* Nitrospira *in the marine cluster IV

For cultivation matters, the tendency of the enrichment culture Ecomares 2.1 to form biofilms was utilized, which probably led to a relatively short time for the isolation of a NOB. Although the biofilm formation was far less pronounced at the end of the isolation process, SEM and EM images showed isolated cells of Ecomares 2.1 still coated in putatively extracellular polymeric substances that made it even difficult to cut embedded cells with a diamond knife. Biocarriers in moving-bed biofilters are continuously exhibited to strong shear forces. These influence the formation, structure and stability of the biofilm community (Tay *et al.*, 2001) and thus might select for nitrifying bacteria with strong adherence capability.

In the beginning of the isolation process, electron micrographs of ultrathin sections revealed micro-colonies of *Nitrospira*-like bacteria, as it is known for nitrifying bacteria in general (Watson *et al.,* 1989). These colonies changed during the course of isolation to a single-cell lifestyle and appeared as short curved rods in the pure culture. This morphological transformation during cultivation was reported elsewhere (Lebedeva *et al.*, 2008) and might be correlated to the fact that the bacteria detach from a multi-species biofilm and proceed to an isolated and pelagic lifestyle in complementary medium. For counting purposes, the single cells were advantageous, and generation times as well as cell yields could be generated for Ecomares 2.1. Like *N. marina* (Watson *et al.,* 1986), Ecomares 2.1 doubles more often under mixotrophic than under autotrophic conditions (*N. marina*: 1 d and 3.8 d, respectively). Both marine strains are outpaced in this respect by *N. moscoviensis* (0.5 days) (Ehrich *et al.*, 1995). Autotrophic cell yield per µmol nitrite of Ecomares 2.1 is slightly less than that of *N. moscoviensis* (7.5×10^6; Ehrich *et al.*), but comparable with that of *N. winogradskyi* (2.5×10^6; Laudelout *et al.*, 1974).

A further aspect of the cultivation of NOB is an extreme accumulation of nitrate in the culture medium, especially when high cell densities are required. The inhibition of *Nitrobacter* by nitrate was already studied in the 1960´s, when Schön (1965) found an impaired carbon dioxide uptake of *N. winogradskyi* at 80 mM nitrate, while nitrite oxidation slowed down only slightly after adding 128 mM nitrate. Boon and Laudelout (1962) specified the mechanism of inhibition of nitrite oxidation by nitrate as non-competitive. Apart from *Nitrobacter*, growth of *Desulfovibrio vulgaris* was inhibited significantly at a nitrate concentration of 70 mM (He, 2010), which could not be contributed to osmotic stress alone. So far, the influence of nitrate on *Nitrospira* species was only tested on *N. moscoviensis*, which was inhibited by 75 mM nitrate (Ehrich *et al.*, 1995). Therefore, it cannot be excluded that other *Nitrospira* species exhibit similar tolerances towards the nitrification end product. Interestingly, in some activity tests with biocarriers from similar RAS biofilters, the nitrifying performance of NOB was already impaired at a nitrate concentration of 1.5 mM (data not shown),

which is easily accumulated in RAS. This discrepancy cannot be explained by now, but at least demonstrates once again that inferences from isolates to ecosystems have to be made with caution.

The conditions prevailing in aquaculture plants are characterized by periodically high ammonium loads after feeding events, and the percentage of free ammonia (FA) increases with the temperature and the pH of the rearing water. A particular sensitivity of NOB towards free ammonia was stated elsewhere (Villaverde *et al.*, 2000; Kim *et al.*, 2006). However, this issue seems to be controversial, since authors recently reported relatively high FA tolerances for *Nitrospira* (mixed and pure cultures) and *Nitrobacter* (Simm *et al.*, 2006; Hawkins *et al.*, 2010). These are in accordance with the results in this study, where approximate 0.7 mM NH_3-N did not inhibit growth of Ecomares 2.1.

Nitrite tolerance limits, on the other hand, were determined for most of the cultivated *Nitrospira* strains (Off *et al.,* 2010). *Cand*. N. defluvii, highly enriched from wastewater, was until now the most resistant species in that respect, defying 20-25 mM nitrite. Although tolerance and inhibition experiments with fastidious NOB enrichment cultures or isolated strains are always dependent on the momentary fitness of the respective bacteria, a difference between *Nitrospira* species in terms of tolerances and sensitivity clearly does exist. The isolation and the subsequent physiological experiments of the strain Ecomares 2.1 showed high substrate tolerances in contrast to the otherwise sensitive marine members of cluster IV. Moreover, Ecomares 2.1 seems to be one of the most substrate tolerant *Nitrospira*-strains so far described. These high nitrite concentrations are rather not found in technical systems even of very high nitrogen load, not to mention in aquaculture systems. But still, this feature of the culture Ecomares 2.1 together with its biofilm developing capacity is ecophysiologically as well as technically of high interest.

CONCLUSIONS

In summary, our results show that (i) *Nitrospira* was metabolically active and of importance for the nitrite oxidation in the biofilter of the marine RAS in Büsum (ii) at least two members of *Nitrospira* were present in the filter system and (iii) the strain Ecomares 2.1 seems to be characteristic for and well adapted to its habitat. We thus obtained a new *Nitrospira marina*-like strain which is less sensitive and starts to grow more easily than *N. marina* strain 295 after 20 years of isolation. The cultivation and isolation of relevant NOB enable insights on their physiology. Further studies, e.g. about inhibitory effects of substances occurring in RAS or attachment experiments, can be conducted

specifically with NOB from the habitat of interest, which may help to optimize the critical step of nitrite oxidation in biofilters.

Future work could deal with the question, whether the different *Nitrospira* in RAS also respond to different conditions and what these conditions are. Additionally, with the design of adequate probes for both strains, their coexistence can be validated in further engineered marine ecosystems and their distribution examined *in situ*.

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CHAPTER III

Monitoring nitrification potentials and nitrifying populations during the biofilter activation phases of three marine RAS

ABSTRACT

The start-up phases of three marine moving bed nitrification biofilters identical in construction were monitored for the period of over one year. The first biofilter (17 °C) was started with the mere addition of NH_4Cl and $NaNO_2$, the second (23 °C) with fish feed plus NH_4Cl and the third (17 °C) was inoculated with colonized biocarriers (100 days old) from the first one plus NH_4Cl. The increase of the nitrification potentials of the plastic biocarriers was monitored using activity tests for AOB and NOB respectively in the laboratory and correlated to *in situ* dissolved inorganic nitrogen concentrations, nitrogen input and further parameters (pH, salinity, temperature, oxygen) as well as operational activities (e.g. introduction of fishes, ozone addition, interruption of feeding).

In general, considerable nitrifying potentials developed only slowly in all biofilters. Early activities in the biofilter inoculated with fish feed decreased again after approx. 60 days, probably because the high initial N concentration was not kept at a sufficient level. The activities in the biofilter inoculated with colonized biocarriers from the first one showed the fastest and most consistent increase. In all biofilters NOB activities lagged behind but eventually exceeded AOB activities after 218 to 286 days.

The nitrifying populations, as identified by 16S rRNA gene sequences derived by PCR, differed only slightly between the biofilters. However, the single dominant AOB in the second biofilter differed from the dominant AOB of the others, as indicated by DGGE. These *Nitrosomonas*-like strains changed simultaneously at almost the same time in all biofilters, resulting again in only one dominant AOB per biofilter. Additionally, *Nitrosospira*-like sequences could be retrieved from two biofilters.

The nitrite oxidizers were more divers on the genus level (*Nitrospira*, *Nitrobacter*, *Nitrotoga),* but *Nitrospira* was assumed to be the most dominant NOB; it was detected by PCR for the whole sampling period and observed in huge colonies by electron microscopic inspection of ultrathin sections of biofilm from the carriers. Some 16S rRNA gene sequences of *Nitrospira* were up to 100 % similar to sequences found in other marine RAS, as was also the case for one of the *Nitrosomonas* strains.

INTRODUCTION

In recirculation aquaculture systems (RAS) between 90 to 98 % of the system water is contained (even 100 % is theoretically possible), hence toxic ammonia, the major catabolic end product in intensive fish and shrimps production, has to be eliminated within the system to prevent accumulation. The most common way to remove ammonia from the recirculating water is the utilization of aerobic autotrophic nitrification, the conversion of ammonia to nitrate via nitrite performed by three phylogenetically distinct groups of lithoautotrophic microorganisms, the ammonia and nitrite oxidizing bacteria (AOB and NOB) and the ammonia oxidizing thaumarchaeota (AOA).

The guild of the AOB consists almost only of bacteria belonging to a monophyletic group within the *Betaproteobacteria* (*Nitrosomonas, Nitrosospira*), whereas the exclusively marine representatives of *Nitrosococcus* belong to the *Gammaproteobacteria* (Head *et al*., 1993). The guild of the NOB is phylogenetically heterogenous and so far comprises bacteria of five different genera: *Nitrobacter* (*Alphaproteobacteria*), *Nitrospina* (preliminary affiliated to the *Deltaproteobacteria*), *Nitrococcus* (*Gammaproteobacteria*), *Nitrospira* (*Nitrospirae*) and the recently discovered *Nitrotoga* (*Betaproeobacteria*). Both, AOB and NOB, are known to be fastidious and grow slowly. Therefore, and especially in marine applications, the establishment of the nitrifying biofilm is tedious and hence involves economic losses and elevated discharge of nitrogen-rich wastewater to the environment (Rusten *et al*., 2006; Gross *et al*., 2003). Nitrite is also toxic for the shrimp or fish reared, and build-ups are commonly observed during activation of new biofilters. Accumulations of nitrite may persist as long as 3-4 months (Manthe and Malone, 1987). Several authors (Bower and Turner, 1981; Grommen *et al*., 2002; Gross *et al*., 2002; Manthe and Malone, 1987; Pefettine and Bianchi, 1990; Paungfoo *et al*., 2007) evaluated the application of start-up enhancer, some of which are commercially available, with differing results. Although ammonia or nitrite peaks develop more often during activation, the failure of one or both nitrification steps can also occur in established biofiltration systems and is often due to unknown causes (Muossa *et al*., 2006; Graham and Knapp, 2007). Therefore, Martins *et al*. (2010) or Guttierez-Wing and Malone (2006) stressed the importance of further research to foster a development towards more efficient waste managements which leads to more sustainable fish production.

Autotrophic nitrification is a mutualistic process between AOB and NOB, and Graham and Knapp (2007) considered the NOB to be the weaker link, which has been confirmed by reports of nitrite accumulations (e.g. Svoboda *et al*., 2005). This is also congruent with the general assumption that NOB are more sensitive towards deviating values of pH, oxygen, free ammonia etc. (Villaverde *et al*.,

1997 and therein). Dreaded nitrite peaks are the result of NOB failure in RAS or aquariums and threaten the welfare of the cultivated organisms. Thus, data discriminating between AOB and NOB conversion rates during critical start-up are of special interest.

For this study, we examined nitrification performances of three marine biofilters identical in construction. For the evaluation of different common start-up strategies, the first biofilter (M1) was started with the mere addition of ammonium chloride and sodium nitrite, the second (M5) with fish feed plus ammonium chloride and the third (M3) was inoculated with colonized biocarriers from the first one plus ammonium chloride. No material from other biofilters was used to avoid transfer of pathogens. To monitor the increase of the nitrification potential of the biocarriers, we applied activity tests in the laboratory for AOB and NOB respectively. These data were compared with water sample data to clarify the validity of activity tests *in vitro*. The sampling period spans from start-up over the introduction of fishes to more than a year of operation.

In order to investigate possible connections between activities and community composition, the species of nitrifying organisms that had settled on the biocarriers during the time of the start-up phases were characterized. Therefore, 16S rRNA gene sequence analyses with specific primer sets were applied and the microbial communities of the three biofilters were compared. The knowledge about the bacteria involved as well as their origin may help to understand the course of the start-up phase or vice versa. Besides bacterial nitrifiers, also anaerobic ammonia-oxidizing planctomycetes (anammox) as well as ammonia oxidizing archaea were sought after.

In short, the questions addressed in this study were:

Which start-up strategy (mineral substrate or fish feed) shows better results, i.e. less activation time?

Which nitrifying bacteria/archaea settle on the biocarriers and

Do the nitrifying communities differ between the modules?

Do in vitro activity tests correspond to in situ activities of the biofilters?

EXPERIMENTAL PROCEDURES

Description of the biofilters

The total volume of each of the three identical modules in Büsum is 35 m^3 (25 m^3 in fish tanks), the biofilters have a volume of 5.65 m^3 each and were filled with 1 m^3 HDPE (high density polyethylene)

biocarriers of the type 2H-BCN 012 KLL (GEA 2H Water Technologies, Wettringen, Germany) with a surface of 859 m^2/ m^3 and a protected surface of 704 m^2/m^3. The biocarriers were moved continuously by aerators arranged circularly at the bottom of the biofilter tank. Before use the biocarriers had been washed in freshwater (10 days), disinfected with a chloride-solution for a week and washed 2 to 3 times with freshwater.

Further compartments of the RAS modules are drum filters (60 µm mesh size) and protein skimmers using ozone. The latter were started only shortly before fishes were stocked. The protein skimmer and the biofilter are individually connected to the pump sump. From here, partly oxygenized water is pumped into the rearing tanks. Before returning to the pump sump, the water is filtered by the drum filters. About 3 m^3 system water is replaced daily with water from the adjacent port of Büsum, which is filtered and ozonated.

Calculated feeding rates and feeding regime

10,000 turbots of 10 g each were planned to be introduced in module 1, equalling 100 kg fish. Based on that, the calculated feeding rate in the first days after stocking would have been about 80 g TAN/ day (TAN = total ammonia nitrogen). For orientation, a nitrification rate of about 93 mg/ m^2 TAN and TNN of the 1 m^3 biocarriers in the biofilters would be necessary to remove this amount of TAN in a day (TNN = total nitrite nitrogen). The fishes reared at the GMA are subject of research studies, thus no fast increase of biomass or high densities are necessary. The respective initial feeding regime for the start-up in the three biofilters was set as follows, and mineral feed was added to the pump sump when ammonia or nitrite concentration were low again after the previous spiking.

Module 1:

This module was started first on 02.07.2009. 15 % of the calculated 80 g TAN in form of NH_4Cl (30 g) and $NaNO_2$ (20 g) were added on the first and second day after start to the pump sump, then 120 g on day 41 and 46. 4,000 turbots (*Psetta maximus*) were stocked on day 88 and additionally 3,000 fishes on day 118 (43.86 kg in total). The feed contained 54 % protein.

Module 3:

Module 3 was started on 20.10.2009 as the last one. 50 liters of biocarriers from module 1 (day 109) were added to the biofilter plus 30 g NH_4Cl on day 1, 2, 29, 31 and 60 g on day 34, 36, 38, 40 and 42. Additionally, 1.7 g of KH_2PO_4 were added to module 1 and 3 at each of the feeding days, since phosphate might be a limiting factor for bacterial growth in seawater. 3110 turbots (*Psetta maximus*) were stocked on day 60 (54 kg) and fed with feed containing 54 % protein.

Module 5:

Module 5 was started on 29.09.2010 and spiked with 1.3 kg of fish feed containing 45 % protein (equaling 101 g N) on day 1 and 6. Then 30 g NH_4Cl were fed on day 50 and 52, 60 g NH_4Cl on day 55, 57, 59, 63, 65, 69, 71, 77, 83, 85, 99 and 104 and 40 g NH_4Cl on day 106, 112 and 113 (900 g in total). 5,000 seabass (*Dicentrarchus labrax*) were stocked on day 126 (15 kg) and given feed with 48 % protein.

Water samples

Water samples were taken directly from the biofilters, first every 24h (until February 2010; except weekends), later every 48h. Sampling period was from July 2009 until May 2010 (approx. 220 days for each module). Ammonia was measured with the powder-pillow tests and the DR 2800 spectrophotometer (both Hach Lange, Düsseldorf, Germany) nitrite and nitrate by ion pair chromatography (Elite LaChrom System, Hitachi, Krefeld, Germany). Oxygen and temperature were measured in the biofilters with a handheld Oxyguard Polaris (Birkerød, Denmark), the redox potential with an Aquamedic mV Controller (Bissendorf, Germany), pH with a 1200-S Sensor (Hach Lange). Measurements of these parameters until October/November 2010 were included in this study.

Nitrogen input

The nitrogen input by fish feed as daily average value for each month was calculated using the equation of Timmons *et al*. (2002): P_{TAN} = F * PC * 0.092 with P_{TAN} = total ammonia nitrogen production rate (kg per day), F = feed rate (kg/day) and PC = protein content in feed (decimal value).

Activity tests

Sampling for activity tests and DNA extraction started around day 20 of each biofilter. In the first half year, samples were taken almost fortnightly, later every 4^{th} to 8^{th} week until October 2010 (activity test results shown here until July 2010). In parallels, 5 biocarriers were shaken (at about 170 rpm) in 25 ml marine mineral salts medium (see below) spiked with 0.5 or 1 mM ammonia (AOB) or nitrite (NOB) as substrates. Nitrite and nitrate concentrations were measured with HPLC (chapter 2) and the sum of nitrite and nitrate served as substrate conversion of AOB, since the stoichiometrically conversion of ammonia over nitrite to nitrate is 1:1:1. The maximum nitrification potentials were calculated for AOB and NOB respectively, taking the average of two parallels.

Marine mineral salts media (1 L)

AOB: 5.4 mg KH_2PO_4; 1 mg $FeSO_4$ x 7 H_2O; 1 ml trace elements solution; 5 g $CaCO_3$; 700 ml seawater; 300 ml aqua dest.

NOB: 300 ml aqua dest.; 600 ml seawater; 100 ml stock solution NOB marine; 1 ml trace elements; adjusted to pH 6.5-7.0 before autoclaving

Stock solution NOB marine: 50 mg $CaCl_2$ x 2 H_2O; 1 g $MgSO_4$ x 7 H_2O; 10 mg $FeSO_4$ x 7 H_2O; 17 mg KH_2PO_4; 700 ml seawater; 300 ml aqua dest.

Trace elements solution: 25 mg Na_2MoO_4 x 2 H_2O; 50 mg $MnCl_2$ x 4 H_2O; 0.5 mg $CoCl_4$ x 6 H_2O; 25 mg $ZnSO_4$ x 7 H_2O; 6 mg $CuSO_4$ x 5 H_2O; aqua dest.

DNA extraction

Of each sample, taken on different days from the three biofilters, 10 biocarriers were counted in 50 ml plastic tubes with 10 ml 2 % NaCl, 0.1 % Triton X 100 and 2.5 g of each 1.7-2 mm and 0.45-0.5 mm glass beads. After sonication (1 h) the tubes were shaken vigorously for 1 h before the liquid phase was centrifuged. DNA was extracted from the pellets using the MoBio soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA). For some samples the MoBio Biofilm DNA extraction kit and the QIAmp DNA stool Mini kit (Quiagen, Hilden, Germany) were used additionally to check whether these kits might extract DNA of more or different nitrifying bacteria. Between 10 and 12 DNA samples of each biofilter were prepared and processed. Sample names compose of the module number (M1, M3 or M5) and the time of the sample taken, i.e., M3-99 is a sample of module 3 taken on day 99 after start.

PCR, DGGE, Cloning

The population analyses were based mainly on the analysis of 16S rRNA gene sequences. Therefore, the samples were screened using group specific primer pairs targeting 16S rRNA gene sequences of known bacteria and archaea involved in nitrification (Table 3.1). PCR were conducted with purified 16S rRNA amplificons from previous PCR (nested PCR) except for PCR targeting gene sequences of key enzymes. Where possible, PCR products were directly sequenced after purification. For sample M5-344 amplificons of *Nitrospira*-specific PCR (27f/1158r) were cloned and 40 clones were screened using RFLP with the restriction enzyme HpaII and specific primers M1 mar.

For denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993), 16S rRNA gene fragments were amplified by PCR with the GC-clamped primer pairs CTO189f/CTO654r (ß-AOB; Kowalchuk *et*

al., 1997), 60f/662r (*Nitrospira*; Daims *et al*., 2001; Alawi, 2007) and the universal primer pair 341f/907r (Lane, 1991). The products were loaded into a gel of 40–70% (CTO) and 30-60 % (other primers) denaturants, and run at 110 V and 59 °C for 17 h. Bands of interest were excised, re-amplified and sequenced. The sequences were analyzed using BLASTN (Altschul *et al*., 1990) and the MEGA software (Tamura *et al.*, 2007).

The primer pair Nspina190/934 (forward: 5´-CTCAAAGATGAGTTCGCGGACC-3´; reverse: 5´-CCCAAGCAAGTCAAATCCAG -3´; *E. coli* positions 78–470; annealing temperature 68 °C) was deduced using the program Clone Manager (Sci-Ed Software, Cary, USA) and evaluated with the Basic Local Alignment Search Tool (BLAST, NCBI) and Probe Match (Cole *et al*., 2007).

Electron microscopy

Pellets of cells (see DNA extraction) of M1-477 and M5-388 were fixed in 2.5% (v/v) glutaraldehyde for 1.5 hours and 2 % (w/v) osmium tetroxide overnight, and embedded in Spurr (Spurr, 1969). Ultrathin sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and inspected by transmission electron microscopic (Zeiss model Leo 906E, Carl Zeiss, Jena, Germany).

Table 3.1: Overview of primer pairs and annealing temperatures used in this study.

primer forward/ primer reverse	target	sequence (5´-3´)	annealing [°C]	reference
27f 1492r	bacterial 16S rRNA	AGAGTTTGATCMTGGCTC GGTTACCTTGTTACGACTT	55	Lane, 1991
341f* 907r	bacterial 16S rRNA	CCTACGGGAGGCAGCAG GTCAATTCMTTTGAGTTT	55	Muyzer *et al*., 1998 Muyzer *et al*., 1998 (modified)
Arch21f Arch958r	archaeal 16S rRNA	TTCCGGTTGATCCYGCCGGA YCCGGCGTTGAMTCCAAT	58	DeLong, 1992
27f Nsv443r	*Nitrosospira*	CCGTGACCGTTTCGTTCCG	59	Mobarry *et al*., 1996
Nb1000g f Deg2r	*Nitrobacter*	CCATGACCGGTCGCAG GGTTTTTTGAGATTTGCTAGGGG	58	Mobarry *et al*., 1996 modified Alawi, 2007 Degrange and Bardin, 1995, modified Alawi, 2007
341f Nspn347 2r	*Nitrospina* 347	GACCCTATTTCTAGGGCT	57	Muyzer *et al.* 1998 Alawi, 2007
27f NscmaR	*Nitrosococcus oceani*	CCTTCGCCTCTCAGCAAAG	57	Alawi, 2007
Ns60fkurz* 662r	*Nitrospira*	CGGGTGAGGAATACATGG GGAATTCCGCGCTCCTCT	64.2	Alawi, 2007 Daims *et al.* 2000
ArchAmoAf ArchAmoAr	archaeal *amo*A	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTATATGT	53	Francis, 2005
CTO 189f A* CTO 189f C* CTO 654r	ß-AOB	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	58	Kowalchuk *et al*., 1997
M1 mar Nsp f M1 mar Nsp r	M1 mar *Nitrospira*	GGCAACCTGGTAGTAAAGTG AGGCAGTAACCTGCCTTATC	66	this study (chapter 2)
M13f M13r	pGEM-T multiple cloning Site	TGTAAAACGACGGCCAGT CAGGAAACAGCTATGACC	53	Messing, 1983
27f 1158r	*Nitrospira*	CCCGTTMTCCTGGGCAGT	55	Maixner *et al*., 2006
NTG200 NTG840	*Nitrotoga*	CTCGCGTTTTCGGAGCGG CTAAGGAAGTCTCCTCCC	58	Alawi *et al.,* 2007
Nspina190 for Nspina934 rev	*Nitrospina*	CTCAAAGATGAGTTCGCGGACC CCCAAGCAAGTCAAATCCAG	68	this study
Pla46 Amx820	anammox-planctomycetes	GGATTAGGCATCCAAGTC AAAACCCCTCTACTTAGTGCCC	50	Neef *et al.*, 1998 Schmidt *et al.*, 2001
Nso190f NITBr	*Nitrosomonas*	GGAGAAAAGCAGGGGATCG TTACGTGTGAAGCCCTACCCA	59	Mobarry *et al.,* 1996 Voytek and Ward, 1995
F1norA R1norA	*nxr*A of *Nitrobacter*	CAGACCGACGTGTGCGAAAG TCYACAAGGAACGGAAGGTC	55	Poly *et al*., 2008

*with CG clamp: 5´ CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3`

RESULTS

Establishment of nitrification in the biofilters

The development of maximum nitrifying potentials is illustrated here in two ways: in Figure 3.1 and 3.2 the nitrification rates of AOB and NOB on the biocarriers of all three biofilters, respectively, are compared over time given in TAN and TNN per square meter per day (total area of biocarriers, not the so called protected area). These are thus biocarrier-specific rates. As the two parallels in these tests were always almost identical, standard deviations are not given. In all tests the concentration of the endproducts equalled the concentration of the substrates given; no great fractions of other nitrogen species by different reactions than nitrification therefore were produced. Figures 3.3 - 3.5 include biofilter-specific nitrification rates, calculated by multiplying biocarrier-specific rates with the amount of biocarriers in the biofilters.

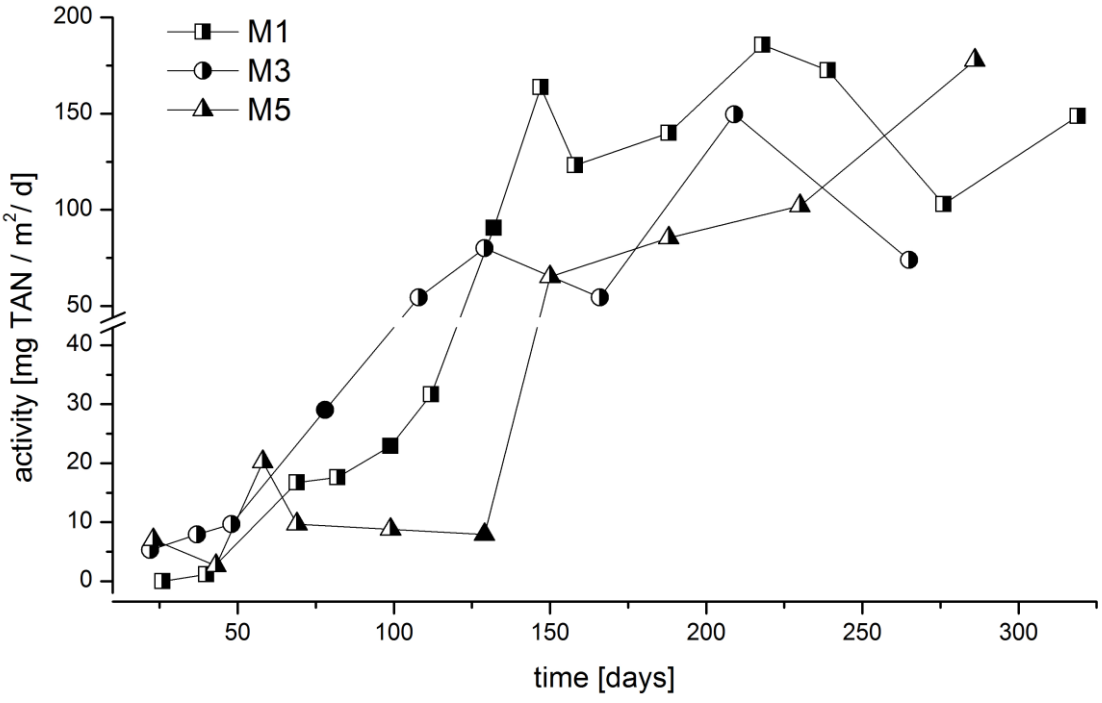

Fig. 3.1: Development of potential nitrifying activities of ammonia oxidizing microorganisms on the biocarriers over time given in TAN removal per square meter per day. Filled symbols denote first sampling after the introduction of fishes.

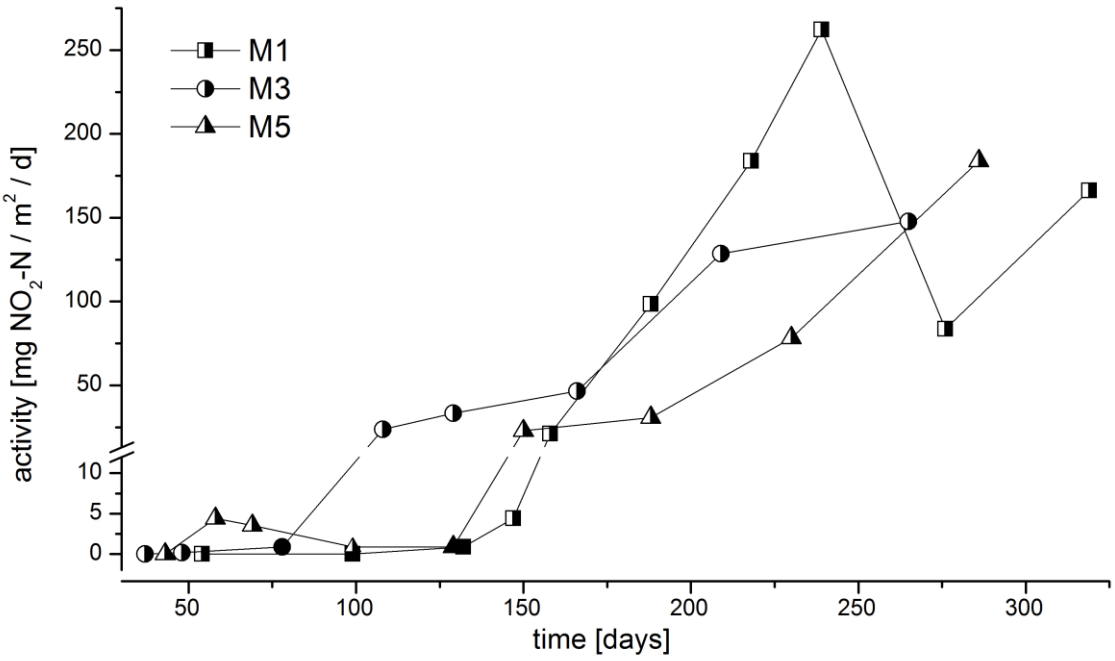

Fig. 3.2: Development of potential nitrifying activities of nitrite oxidizing microorganisms on the biocarriers over time given in TNN removal per square meter per day. Filled symbols denote first sampling after the introduction of fishes.

MODULE 1 (inoculated with NH_4Cl and $NaNO_2$):

Changes in DIN-concentrations in module 1 during start-up followed the known start-up scheme (Fig. 3.3); an elevated ammonia concentration (around 1 mg TAN/l) was followed by a nitrite built-up (up to 4 mg TNN/l), which was then reduced due to the formation of nitrate. Until fishes were added on day 88, the nitrite peak remained relatively constant (between 3 and 4 mg/l TNN), since only on day 41 and 46 additional portions of 120 g NH_4Cl were fed. One day before the introduction of the fishes, the foam fractioner as well as ozone addition were started, the nitrite concentration decreased and the nitrate concentration started to increase. After day 118, when further 3,000 turbots were stocked, another nitrite peak developed which was less pronounced and decreased after fewer days. In contrast to the other modules, no ammonia peak evolved after stocking. The average temperatures before and after the fishes had arrived were 22.3 °C and 17.2 °C respectively (Table 3.2), an increase to 18.4 °C average temperature and 29.8 ppm salinity could be observed during summer time (between 04.06.2010 and 22.09.2010). On day 226, 0.5 m^3 of new biocarriers were added to the biofilter.

The potential nitrifying activities of AOB in module M1 started only after 40 days, while biocarriers of modules M3 and M5 showed AOB activities more or less right after start (Fig. 3.1). The ammonia oxidizing potentials of the biocarriers from M1 increased very fast after the introduction of turbot on day 88 and 118, until they reached 164 mg TAN per m^2 *d^{-1} around day 147. The rise of NOB activities started slightly delayed after the stocking but then increased from 21 to 99 mg TNN per m^2 *d^{-1} between day 158 and 188 and overtook the AOB in terms of activity on day 218 with 184 mg TNN per m^2 *d^{-1} (Fig. 3.2). A drastic decrease of both AOB (40 %) and NOB (68 %) activities was observed between day 239 and day 276. A lot of water was exchanged and the fish were temporarily not fed during this time.

Until almost day 200, the amount of N input as feed exceeded NOB removal rates considerably. The correlation coefficients between the nitrogen input into the system and the development of AOB and NOB potential activities until day 218 (February 2010) were 0.92 and 0.89, respectively.

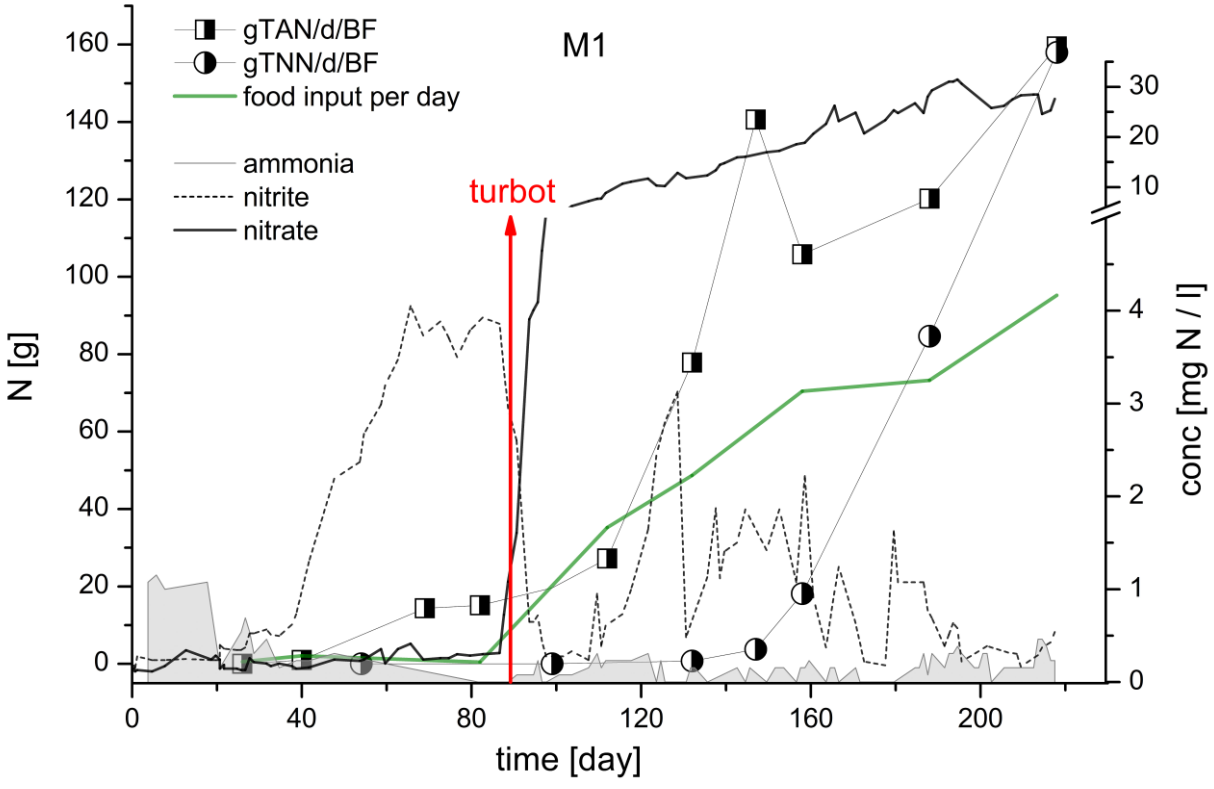

Fig. 3.3: Overview of ammonia and nitrite removal rates (in g N per day per biofilter), food input (in monthly average g N per day), and DIN concentrations in mg N per litre in biofilter M1. The arrow indicates the time of introduction of fishes.

MODULE 3 (inoculated with biocarriers from M1):

The AOB on the biocarriers from M1, which were used to inoculate M3, were already slightly active (32 mg TAN per m^2 *day^{-1} on day 112) and comparing the potential nitrifying activities (in mg TAN or TNN per m^2 *day^{-1}, Fig. 3.4), M3 had the earliest onset and the most steady increase of activities, which could be measured a bit earlier for AOB than for NOB (day 22 and 48, respectively). Until the stocking with turbot on day 60, additional NH_4Cl was fed intermittently (420 g in total). The early oxidation of the given ammonia resulted in a nitrite peak of 2.8 mg/l TNN, which decreased shortly after the activation of the foam fractioner and ozonation on day 54 with the increase of the nitrate concentration. In this time also the temperature was lowered from approx. 24 °C around day 34 to 16 °C for the acclimatization of the juvenile turbot (54 kg). On day 67, TAN concentrations reached 2.3 mg/l directly after excessive ozonation caused by a defect. As abruptly the TAN peak then disappeared within a few days, a second considerable TNN peak evolved up to 3.5 mg/l and persisted until day 95. On day 117, 0.5 m^3 new biocarriers were added and according to that, the activity data in g TAN and g TNN per biofilter per day have been adjusted in Figure 3.4. A further remarkable TNN peak occurred around day 164 (2.6 mg/l), two weeks after a stoppage of feeding due to tagging and grading of fish. Average temperature and salinity values before and after the introduction of fish were 21.5 °C to 17.3 °C and 32.5 ppm to 27.9 ppm (Table 3.2). In the summer months (June to August 2010) the average temperature increased to 18.9 °C, and salinity to 29.8 ppm.

As shown by activity tests in the laboratory, AOB and NOB activities developed relatively steadily until day 129, but with the samples of day 166 a decrease (AOB) and a slightly less steep increase (NOB) of activities could be observed, the same occurred on sampling day 265 (Fig 3.1 & 3.2). The advance of the nitrifying potentials of the AOB inverted after day 265, when NOB activities increased almost 3-fold in 60 days to 412 mg TNN per m^2 *day^{-1} (not shown). The level of N input (feed) was more or less congruent with the development of the TAN removal rates until day 110, though it exceeded TNN removal rates considerably until day 169. Between the AOB and NOB activities measured in the laboratory and the increase of monthly average nitrogen input until day 209, the correlation coefficient was 0.99 for both nitrifying guilds.

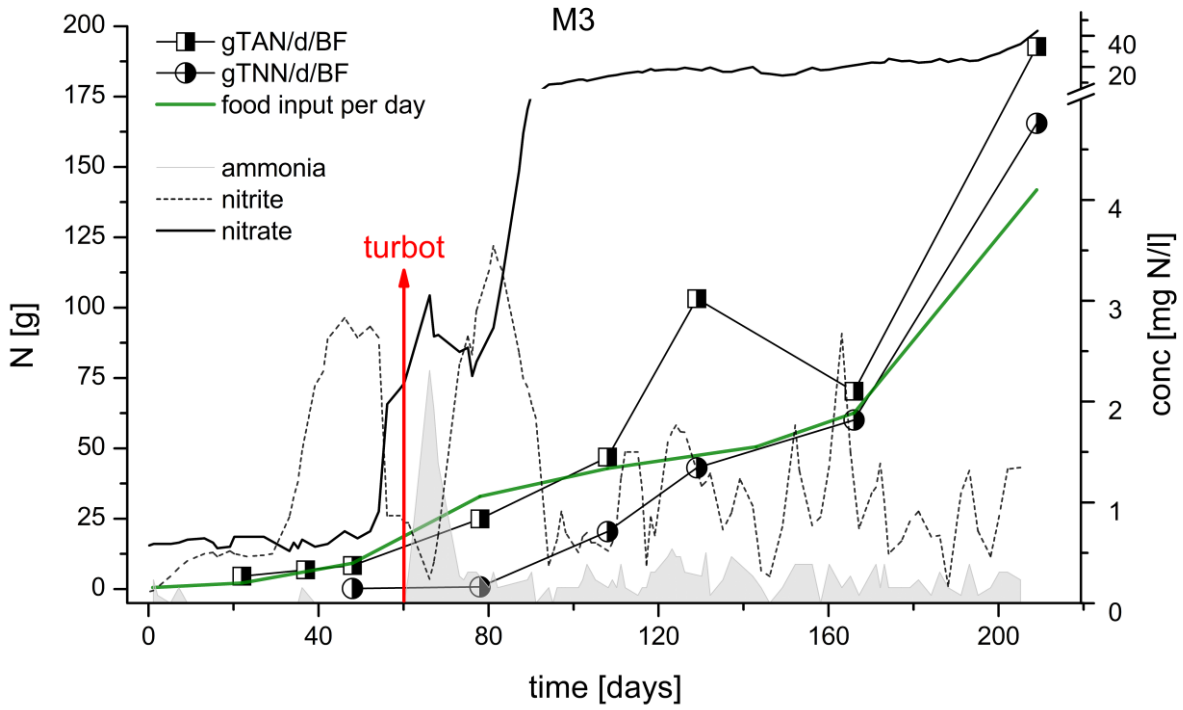

Fig. 3.4: Overview of ammonia and nitrite removal rates (in g N per day per biofilter), food input (in monthly average g N per day), and DIN concentrations in mg N per litre in biofilter M3. The arrow indicates the time of introduction of fishes.

Table 3.2: Average values before and after the introduction of fish for main parameters measured in the system (with standard deviations). Oxygen and temperature were measured directly in the biofilters.

	Oxygen [mg/l]				**Temperature [°C]**				**pH**			
	before	+/-	after	+/-	before	+/-	after	+/-	before	+/-	after	+/-
M1	7.54	0.43	8.33	0.66	22.30	2.63	17.21	1.10	8.23	0.10	7.57	0.32
M3	7.44	0.62	8.29	0.57	21.53	2.97	17.28	1.45	7.74	0.65	7.48	0.21
M5	7.54	0.39	7.57	0.74	22.68	2.49	22.26	2.86	8.31	0.18	7.53	0.33
	Salinity [ppm]				**Redox [mV]**							
	before	+/-	after	+/-	before	+/-	after	+/-				
M1	29.23	1.39	28.77	1.92	163.79	44.14	141.45	44.04				
M3	32.50	2.22	27.85	2.02	170.77	34.57	122.15	31.46				
M5	33.86	2.17	29.25	2.00	189.45	42.12	145.22	29.54				

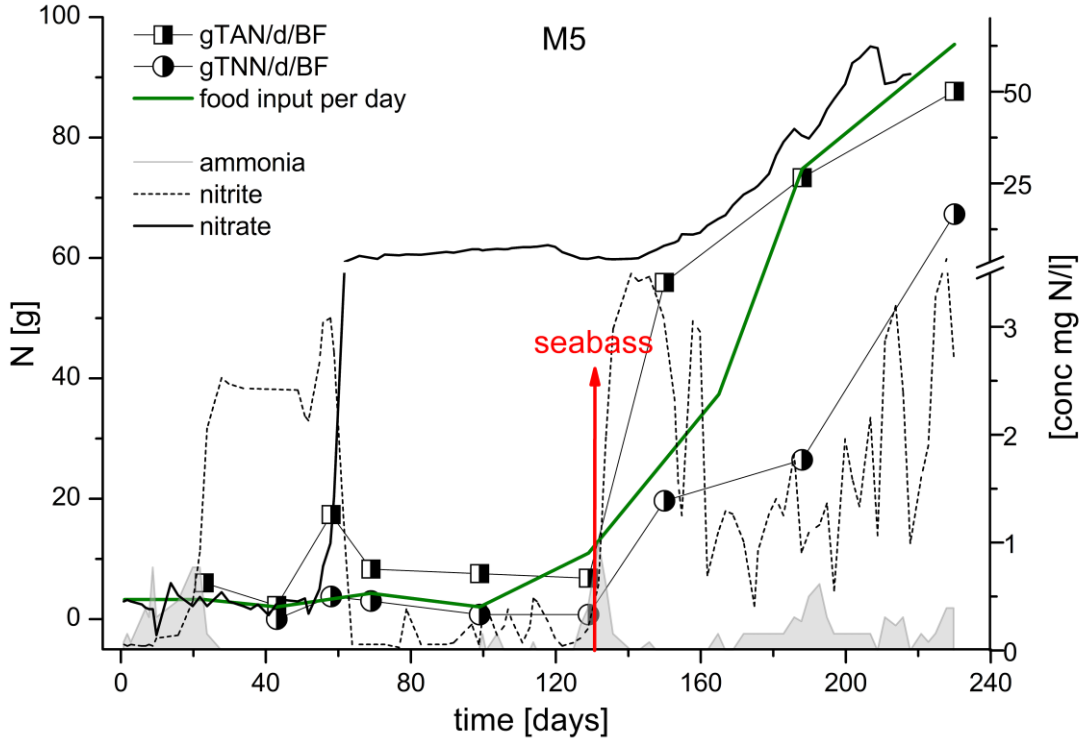

Fig. 3.5: Overview of ammonia and nitrite removal rates (in g N per day per biofilter), food input (in monthly average g N per day), and DIN concentrations in mg N per litre in biofilter M5. The arrow indicates the time of introduction of fishes.

MODULE 5 (inoculated with fish feed and NH_4Cl):

Module 5 was stocked with European seabass (5000 fishes of 3 g each) not before day 126. An initial nitrite peak (2-3 mg/l TNN) developed after two weeks and persisted for almost 40 days, when it decreased quite rapidly, simultaneously with the onset of some NOB activity (4.4 mg TNN per m^2 * d^{-1} on day 58) and the increase of nitrate concentrations (Fig. 3.5). Between day 50 and day 113, 900 g TAN were fed in total (in portions between 40 and 80 g NH_4Cl), while ammonia and nitrite concentrations remained relatively low after the nitrite peak had disappeared. For the acclimation of the fishes the temperature was reduced from 24.4 to 12.7 °C and the foam fractioner and ozone addition were started on day 105. After the fishes were introduced, an ammonia peak (1 mg/l TAN) developed, followed by an extreme nitrite peak (3.5 mg/l TNN). The average temperature in module 5 did not change considerably after the introduction of the sea bass, the salinity, though, was averagely 33.9 ppm before and 29.3 ppm after. In the week before the sea bass arrived, the salinity even dropped from 37 to 28 ppm. In the summer months the average temperature climbed up to 25.1 °C and salinity to 30.8 ppm (Table 3.2).

AOB activity already started around day 20 (Fig. 3.1), which apparently caused the nitrite built-up, but decreased again after day 58 (20 mg TAN per $m^2 * d^{-1}$) and remained at around 8 mg TAN per $m^2 * d^{-1}$ until the fish arrived. The NOB activities followed the same pattern, but on a lower level, so that values dropped from 4.4 to 0.97 mg TNN per $m^2 * d^{-1}$ on day 129 (Fig. 3.2). The following rapid increase of the AOB and NOB activities was somehow decelerated after day 150, and also increased much more slowly than the activities of M1 and M3 in the further course.

Water had to be exchanged often to maintain acceptable nitrite concentrations after stocking. For module 5, the correlation coefficient between activities and nitrogen load until day 230 was 0.99 for AOB and 0.93 for NOB. Average daily N input was less than the potential activities of AOB until day 100, but then feed input was increased and exceeded TAN removal levels at least until day 286 (data not shown).

In general, the establishment of efficient nitrification in all three biofilters took relatively long time of a year and more and significant development of activities occurred only after the introduction of the fishes. It is to be kept in mind, that the potential activities between the sampling days might have changed unnoticed. Two main events (in February and summer) were observed especially for M1 and M3, where activity rates of AOB dropped dramatically and those of NOB increased less.

Nitrifying microbial community analysis

The 16S rRNA analyses, mainly based on direct sequencing after specific PCR, revealed that representatives of almost all known groups of autotrophic ammonia and nitrite oxidizing bacteria were present in the biofilters examined. Details are given below, and the sequences can be found in the appendix. Only for the ɣ-proteobacteria *Nitrococcus* and *Nitrosococcus* all PCR results were negative or unclear (positive PCR run but poor sequences). *Nitrospina*-specific PCR was positive for several samples, however, sequences retrieved from M1-477 (445 bp) and M5-388 (265 bp) revealed a low similarity to *Nitrospina* sp 3005 with 83 to 86 %, a longer sequence from M3-169 (704bp) was 92.8 % similar to this δ- proteobacterium isolated from deep sea sediment. PCR with primers targeting *Archaea* as well as archaeal *amo*A (encoding subunit A of the ammonia monooxygenase) were negative for all samples taken (from day 20 up to one and a half year). Primers targeting planctomycetes led to amplificons in a range of samples of all modules. Two of them (M1-477 and M5-99) were sequenced, but only M1-477 was 85.6 % (265 bp) similar to *Cand.* Brocadia fulgida, a bacterium known to perform anammox. By visual inspection of ultrathin sections of cells from biocarriers of M1 and M5 (samples of October 2010) by electron microscopy, colonies of

Nitrosomonas-typic cells as well as *Nitrospira*-like cells could be identified (Fig 3.6). Further nitrite oxidizers like *Nitrobacter* or *Nitrotoga*, which are morphologically quite noticeable, were not found here.

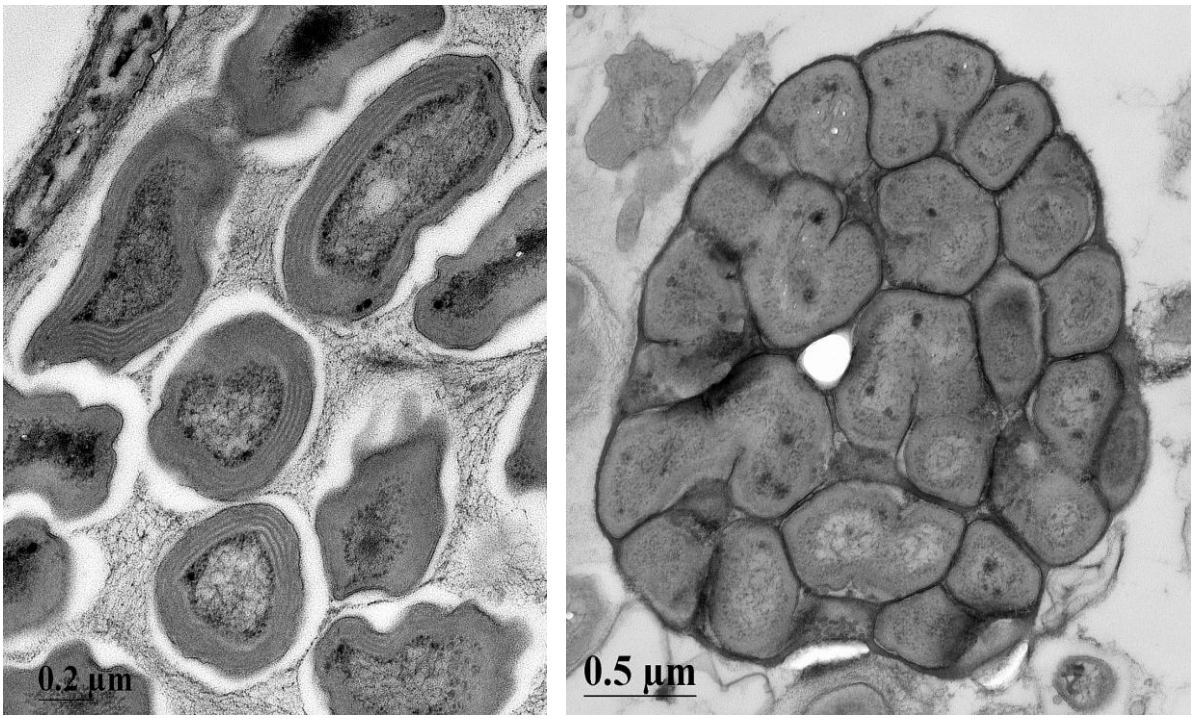

Fig. 3.6: Electron micrographs of ultrathin sections through biofilm shaken off biocarriers from biofilter M5 in October 2010. Left: close-up of colony of *Nitrosomonas*-like bacteria with intracytoplasmic membranes, right: small colony of *Nitrospira*-like cells.

Ammonia-oxidizing bacteria

The sequences of ammonia oxidizers belonging to the subclass *Betaproteobacteria* (ß-AOB) were obtained by DGGE (Fig. 3.7) from sampling days 54 (M1) and 23 (M3 and M5) on. In total, three dominant bands could be detected during the study (referred to as lower, middle and upper band). Mostly only one main representative of ß-AOB could be detected for each biofilter at any one time. The dominant band of modules 1 and 3 (lower band) disappeared between March and May 2010 with the appearance of the middle band. Already in February, the dominant band of module 5 had altered from the middle band into the upper band, though both bands could be detected together until May, when the lower band eventually disappeared. Further faint bands that could be observed occasionally were heterodupleces and of the same sequences as the adjacent dominant bands respectively.

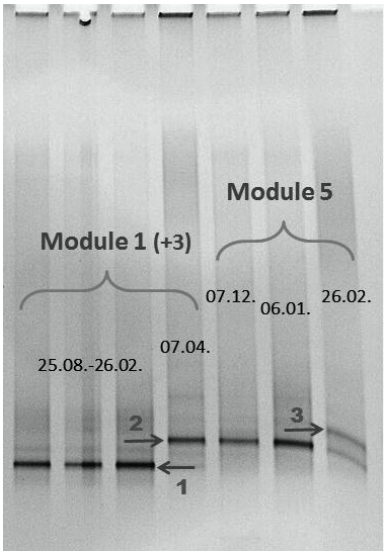

Fig. 3.7: DGGE band pattern of ß-AOB sequences of module 1 and 5 during change of dominant AOB in the biofilters as indicated by sampling dates. Patterns of module 3 were identical with that of M1 (not shown). 1 = lower band; 2 = middle band; 3 = upper band.

The sequences recovered from the dominant bands of several DGGE-gels (approx. 400 bp) were identical, respectively, corresponding to their position. PCR products amplified with the primer pair Nso190/NitB were sequenced directly; these sequences were identical with the corresponding bands from the DGGE approach, thus longer sequences of approx. 1000 bp could be phylogenetically analysed: all three sequences can be allocated to the *Nitrosomonas marina*-cluster with similarities of 97.1 % (lower band), 95.7 % (middle band) and 97.8 % (upper band) to *N. marina.* More detailed, the sequences of the lower band (1005 bp) are most similar (100 %) with a sequence derived from a marine RAS in Israel (AM295532), and 99.8 % similar to the strain Is343 (AJ621032), isolated from the Schelde estuary (Netherlands); the sequences of the middle bands are most similar (96.6 %) to a clone derived from prawn farm sediments (EU155069), the closest described relative is the *Nitrosomonas* strain Is79A3 (96.1 %), which stems from the lake Drontemeer (Netherlands); the sequences of the upper bands are similar to the isolate BF16c57 from a freshwater aquarium (AF386746) and to *N. aestuarii* (AF272420) with 99.4 % each (Fig. 3.8.).

ß-AOB specific DGGE with DNA extracted with the biofilm kit and the stool kit did not result in further or different bands. No bands could be allocated to *Nitrosospira*. However, with the *Nitrosospira*-specific primer pair 27f/Nsv443r, respectively two selected samples between day 22 and 188 from each biofilter showed a positive signal; of module 1 and 3 *Nitrosospira*-like sequences could be retrieved. The 405 and 372 bp long sequences of M1-188 and M3-108 were 99.75 % and 99.73 % similar to *Nitrosospira* sp. Nsp17 (AY123804), which was isolated from Icelandic soil.

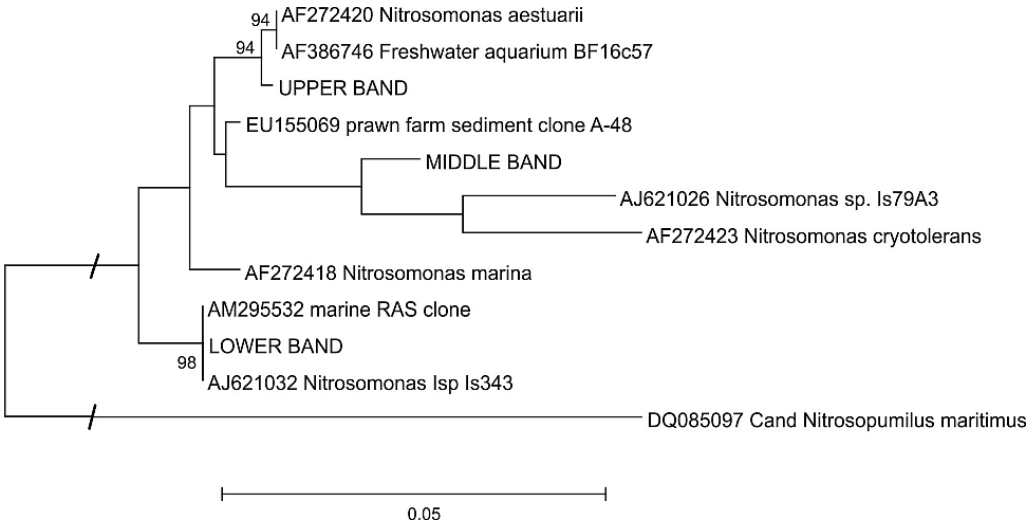

Fig. 3.8: Maximum likelihood tree depicting the phylogenetic relationships between the 16S rRNA gene sequences of the ß-AOB sequences obtained by DGGE with the CTO primer pair and the primer pair Nso190/NitB. The tree was constructed using sequences of approx. 1025 bp. Nodes supported by bootstrap values are indicated. Scale bar = 5 % sequence divergence.

Nitrite oxidizing bacteria

Nitrobacter-specific PCR was positive with samples from all three biofilters from day 20 on, though between the sampling days 17.05.2010 and 08.09.2010 (3 samples) PCR runs were negative for all samples of M1 and two samples of M3 and M5. Amplificons from all three biofilters at different stages were sequenced resulting in three slightly different 245 bp long rRNA gene sequences (98-99.5 % similarity to each other). The sequence retrieved from the samples M1-26, M1-279, M3-22 and M5-23 was 99.1 % similar to the marine *Nitrobacter* strain 311 (AM292300) and 99.6 % to the *Nitrobacter* sp strain PBAB17 (AY508476); the sequence of M1-188, M5-99 and M3-78 was identical with a strain enriched from a wastewater treatment plant (FJ26062); and the sequence of the samples M1-478, M5-389 and M3-368 was identical with *N. vulgaris* (AM292301) and *N. hamburgensis* (CP000319). PCR with primers targeting the subunit A of the Nor (nitrite oxidoreductase) of *Nitrobacter* did not lead to amplifications.

The specific PCR targeting *Nitrotoga*-like bacteria led to positive results from day 26, 48 and 58 (M1, M3, M5) on with few exceptions in M1 (day 112, 147, 188) and M5 (day 99). A total of 7 sequences (around 585 bp) were recovered from samples between day 58 and 389 and all were identical to each other and, with the exception of 2 nucleotides, identical to *Cand*. Nitrotoga arctica (99.7 %; DQ839562).

Nitrospira-specific PCR results were positive with DNA from all biofilters from the first sampling day on. PCR products of early samples could not be sequenced directly (mixed sequences) except for M1-40 (533 bp; 97.0 % similar to *N. moscoviensis*, isolated from a heating system). *Nitrospira*-specific DGGE with the primer pair 60fGC/662r resulted in mixed sequences that were not analysed further.

Sequences (between 900 and 1160 bp) from later samples of the biocarriers from all biofilters (M1-478, M3-323, M5-344) were 99.3 to 100 % identical to the strain Ecomares 2.1 (HQ686082) (chapter 2), isolated from another marine RAS in Büsum and a close relative of *Nitrospira marina* (98.8 % 16S rRNA gene sequence similarity).

The very specific primer M1 mar targeting the sequence of a clone likewise retrieved from the Ecomares RAS in Büsum, matched only with the DNA of samples of M5 on day 344 and 399, the sequence (250 bp) of day 344 being 99.6 % similar to the sequence of the clone M1 mar. Of the same sample, clones were produced using the semi-specific *Nitrospira*-primer pair 27f/1158r. 40 of these clones were screened by RFLP and all of the same sequence, similar to the Ecomares 2.1 strain (99.3 %; 1150 bp).

DISCUSSION

Development of nitrifying potentials: general observations

The ammonia and the nitrite oxidation potentials of biocarriers were obtained in laboratory experiments for each of the sampling days over the period of almost a year and could be correlated to the development of the nitrifying performances of the respective biofilters, calculated with the average daily feed input value during each month. Changing activities of AOB as well as NOB could be putatively related to events like temporarily feeding stoppages as observed for module 1 around day 276 and for module 3 around day 166 (prior to that the fishes were not fed for almost two weeks). No greater effect, on the other hand, seemed to have the addition of virgin biocarriers to M1 (day 226) and M3 (day 117), since on the directly following sampling days activities only decreased little (M1) or still had increased (M3) (assuming that the probability to grab exclusively “old” carriers for the tests is rather small).

It seems not to be possible, however, to calculate the exact *in situ* nitrifying capacity of a biofilter with the data of the activity tests; although the curves of the N input in the graphs 3-5 were about

congruent with or even lower than the nitrification rates at most of the time until about day 200, still water had to be exchanged often and feed input could not be increased as planned, to keep ammonia and especially nitrite concentrations within limits. Control activity tests conducted with process water from the biofilters as medium or at a lower temperature (22 °C) led to the same calculated nitrifying potentials (data not shown); the discrepancies were thus not caused by these factors of the experimental setup. We suppose therefore that the extrapolation from 5 to almost half a million biocarriers in the biofilters is not realistic due to the optimal aeration and substrate provision during activity tests, which is not given for an unknown fraction of biocarriers in the filters. Additionally, organisms other than AOB and NOB (heterotrophs, protozoa, planctomycetes) possibly involved in nitrifying biofilm ecology were not taken into account using activity tests tailored for aerobic nitrification, and the so called passive nitrification, taking place elsewhere in the system but not in the biofilter, was neglected. According to Losordo and Hobbs (2000), it can account for as much as 30 % of the total nitrification in a RAS.

Thanks to the decoupling of the two nitrification steps, the ammonia oxidation is not the rate limiting step in the activity tests and both guilds can be compared independently (Fig. 3.1 and 3.2); the nitrification potentials of the AOB increased earlier and faster, yet were more fluctuating and reacted more quickly and intense to the above mentioned suspensions of TAN input. Similar observations could be made during the monitoring of nitrifying potential in the RAS of the Marifarm in Strande at the Baltic Sea (data not shown). Also Holl *et al.* (2011) reported an earlier onset of ammonia oxidation and designated the nitrite oxidation as the rate limiting step for full nitrification due to the slower growth rates of NOB.

A further hindrance for the development of NOB activities might have been the use of ozone; nitrite reacts with ozone to nitrate especially when further competitive substrates for the reaction with ozone (e.g. yellow substances) are absent (Summerfelt *et al*., 2003), leading to a deprivation of substrate for bacterial nitrification (Schröder *et al.*, 2011). In M5 nitrate concentrations replaced nitrite concentrations of around 3 mg/l before ozonation started, and at the same time NOB activities had increased to 4.4 mg TNN/m^2 *d^{-1} on day 58 (Fig. 3.5). In M1 and M3, however, equally high nitrite peaks were replaced by nitrate just after ozone addition had started (days 87 and 54 respectively; Fig. 3.3 and 3.4). From these points of time on it still took some 40 days until NOB activities in M1 and M3 reached 1 mg TNN/m^2 *d^{-1}. These circumstances indicate nitrite elimination by ozone in M1 and M3, possibly hampering NOB activities development. Ammonia, in contrast, can only be oxidized primarily to nitrogen gases in reactions catalyzed by brominated ozone-produced oxidants, which in turn are produced in the presence of sufficient dissolved organic material (Rosenthal and Krüner, 1985). The ammonia peaks detected in the modules in this study, however,

appeared either before ozone addition had started, or AOB activities were already high enough to convert these peaks into nitrite.

However, the potential nitrite oxidation activities in the biofilters reached the ammonia oxidation activities around day 218 (M1), 265 (M3) and 286 (M5) and with the sampling days 433, 223 and 344, NOB activities exceeded AOB activities by far with 230, 200 and 157 %, respectively (data not shown). Since the nitrification process consists of two subsequent steps of the same stoichiometry regarding substrate and product, one would expect similar nitrifying activities. The authors suspect therefore a coherence with the before mentioned consistency of NOB activities in comparison with AOB activities. Bollmann and her coworkers (2002), e.g., observed an immediate reactivation of *N. europea* after 10 weeks starvation. However, Spieck and Bock (2005) reported an ability of NOB to survive long periods of starvation and dryness up to 24 months (*Nitrobacter vulgaris*). Luecker and colleagues (2010) could detect the NxrB protein, subunit of the nitrite oxidizing enzyme of *Nitrospira*, still after 110 days of starvation. It might therefore be possible that AOB are more liable to starvation and NOB, once grown in the biofilm, may persist longer through times of substrate deprivation and reactivate quicker when sufficient substrate is provided, as it is during activity tests (see also chapter 6).

Development of nitrifying potentials until arrival of fishes

Module 5 was spiked two times at start with 101 g N in form of organic compounds (fish feed), which is about 10 times more than the initial N inputs of module 1 and 3, here in form of mineral salts. This might explain the fast increase of AOB and even NOB activities in M5 at the beginning, following the Monod equation model (zero-order expression; Chen *et al*., 2006), since after the initial spiking of the biofilters, ammonia chloride was added only when ammonia concentrations were low again, indicating nitrifying activity. Thus, module 1 and 3 were never deprived of ammonia for long, but due to times near or below minimum substrate concentrations, nitrification rates probably could not develop equally quickly as in module 5. In fact, in module 1, nitrite oxidation activity was not or only barely measured before the fishes were added. In contrast, activities in module 3 developed right after start, too, like in M5, yet not as fast. The effect of inoculation of new biofilters with nitrifying bacteria was subject of a range of studies, and results are divers (Bower and Turner, 1981; Grommen *et al*., 2002, Manthe and Malone, 1987; Perfettine and Bianchi, 1990). However, it may sound reasonable that activation can be accelerated in any case, when the inoculation derives from an established system with identical operational conditions (Carmignani and Benett, 1977; Gross *et al*., 2003).

The decrease of activities in module 5 after day 60 was probably caused by exceeding rate limiting substrate concentrations and could possibly have been adverted by feeding more TAN. Kaiser and Wheaton (1983) report this problem of activated biofilters; the nitrifying efficiency drops when feeding rates are too low to maintain bacterial biomass, which has built up.

Development of nitrifying potential after fish arrival

After the fish had been added to the systems on day 88 (M1), 60 (M3) and 126 (M5), nitrification rates lagged behind for quite a long period, and in all modules the fish feed input could not be increased as planned. Especially in module 5, water had to be exchanged very often to avoid hazardous nitrite peaks. In contrast to the other modules, where turbot was cultivated, the feed for the sea bass in module 5 would have had to be increased much more rapidly. It was observed elsewhere, that nitrification potentials of biofilters break down for unknown reasons, when fishes are introduced (Gutierez-Wing *et al.,* 2006; G. Quantz, personal communication).

Activation times of brand-new media in marine applications are reported to take much longer time, and in general nitrification rates are up to 60 % lower than in comparable freshwater systems (Rusten *et al*., 2006; Chen *et al*., 2006). However, the extremely long activation time until satisfactorily high nitrification rate as required in commercial RAS, even long after the introduction of the fishes, cannot be fully explained. Suggestions include a possible inhibition of bacteria by the migration of substances from the new plastic material the system is made of (see chapter 4). Such effects of migrating substances from polymers on nitrification were reported for EPDM (ethylene propylene diene monomer) Horowitz *et al*. (2001). Further potential events to impair nitrification could have been the decrease of water temperature in module 5 from 24.4 to 12.7 °C, for the acclimatization of the fish as well as the simultaneous decrease of salinity from 37 to 28 ppm (Kir, 2009).

Nitrifying community

The microbial nitrifying community attached on the filter media were analysed using PCR based approaches supported by electron micrographs of ultrathin sections. Generally, DNA extraction methods, as well as PCR and subsequent DGGE involve certain biases (von Wintzigerode *et al.*, 1997). Additionally, nitrifying bacteria are known to form dense clusters embedded in EPS (Watson *et al.,* 1989), which has been suspected to prevent complete DNA extraction (Schramm *et al*., 1998; Foesel *et al*., 2007). The results of the DGGE analyses - the presence of mainly only one band for ß-AOB in all biofilters - has let us speculate, that the DNA extraction methods applied was not sufficient.

Therefore, further isolation kits were tested, but still, no other than the before obtained *Nitrosomonas marina* -like AOB (cluster 6a) could be detected by DGGE with the ß-AOB specific primer set CTO189/CTO654 (Kowalchuk *et al.*, 1997). The results and sequences derived from the CTO-DGGE were confirmed by further amplification with *Nitrosomonas*-specific primers and direct sequencing. Diversity of AOB populations in WWTP varies among studies between single populations to the coexistence of several different AOB (Wittebolle *et al*., 2009 and therein). Still incongruent with the results of the ß-AOB primer pairs is the occurrence of *Nitrosospira* sp Nsp17 sequences in module 1 and 3, which could indicate that the size of the *Nitrosospira* population is around the detection limit of DGGE (less than 1 % of total cells) (Muyzer *et al*., 1993). *Nitrosospira*-like bacteria also happen to be found even as dominant AOB in biofilters (Sakano *et al*., 1998; Schramm *et al.,* 1998; Terada *et al*., 2010), although they are characterized as dominant AOB in habitats with low substrate concentrations (Prosser, 1989) or other adverse conditions (Li *et al*., 2007).

In early 2010, the *Nitrosomonas* populations in the biofilters changed collectively, which gives rise for speculations about the cause(s). Interestingly, the sequences of the new presumably dominant AOB in M1 and M3 after the transition were identical to the sequences of former dominant AOB in M5. However, no corresponding pattern could be observed for any of the parameter measured (pH, temperature, redox potential, salinity). Temperature is an elementary parameter differing between M1/M3 and M5 and was described as key factor influencing AOB population structures (Urukawa *et al.*, 2008), while Egli *et al*. (2003) set the importance of changes of pH values over temperature variations. Further key factors in this respect are e.g. ammonia concentrations (Lydmark *et al.,* 2007), salinity (Bernhard *et al.,* 2007; de Bie *et al*. 2001) and oxygen (Rowan *et al*., 2003).

Neglecting the species pattern of the change of AOB in the biofilters, there are, indeed, other events that could have triggered the change in AOB communities: in M5 the fishes arrived shortly before the change of AOB populations and might have transferred bacteria from the hatchery system, or could have changed parameters which were not measured. Due to the acclimatization of the fishes the temperature was lowered and salinity dropped, possibly caused by decreased evaporation. Further, in all biofilters feeding was stopped to different extends in March due to tagging and/or grading of the fishes, and potential activities of NOB and AOB increased more slowly (M3 NOB and M5) or even decreased (M1 and M3 AOB). Also the addition of 500 L virgin biocarriers to the biofilters of module 1 and 3 fell in that time (February); however, these had no direct effect on activities, as mentioned before, but might have had an influence on the bacterial population. All these will remain co-events that occurred around the change of AOB communities within the biofilters, which cannot be explained here determinately.

Most PCR amplificons could be sequenced directly after group-specific PCR, indicating rather dominant representative of the respective genera or species. Three of the five known genera of NOB, namely *Nitrobacter*, *Nitrotoga* and *Nitrospira*, were found almost continuously by specific or semi-specific PCR in all biofilters with sequences being very similar to sequences of known species. Some sequences gave reason for speculations concerning the presence of *Nitrospina-like* bacteria with a similarity of 92.8 % to *Nitrospina* strain 3005. This richness in genera of a nitrite oxidizing community was reported most infrequently so far (Alawi *et al.,* 2007; Xiao *et al*., 2009). *Nitrospira* and *Nitrobacter*, however, were detected together in a range of habitats (Schramm *et al*., 2000; Cébron and Garnier, 2005; Attard *et al*., 2010; Spieck *et al*., 2006; Kumar *et al.,* 2010). The existence of the nitrite oxidizing ß-proteobacterium *Nitrotoga* was only discovered in 2007 (Alawi *et al.,* 2007) and the present study is the first report of *Nitrotoga* in a marine habitat. Hence, studies conducted before could not include this NOB to be sought for. Therefore, *Nitrotoga*-specific primer pairs and probes should be included in any future research dealing with nitrifying communities. Several studies published to date identified *Nitrospira* as the dominant NOB in marine RAS (chapter 2; Foesel *et al.,* 2007; Tal *et al*., 2003) and this also is indicated by the results of this chapter. Samples were not quantified in detail in this respect, however, by electron microscopic inspection of late samples of M1 and M5 only *Nitrosomonas*-like and *Nitrospira*-like cells were found in colonies throughout the ultrathin sections, although *Nitrobacter* and *Nitrotoga* would have been easy to identify. Furthermore, all *Nitrospira*-specific PCR were positive from the first to the last sampling days, which was not the case for *Nitrobacter* and *Nitrotoga*. Except for one *N. moscoviensis*-like sequence found in an early sample of M1, all other sequences retrieved were similar to the sequences found in other marine RAS in Büsum and Rehovot, Israel. In the latter, interestingly, the non-marine *N. moscoviensis* was likewise detected by FISH in low cell numbers (Foesel *et al*., 2007). However, the amount of sequences that were obviously mixed and therefore not evaluable, although indicating relatedness to *Nitrospira* (data not shown), let us assume that mostly more than one *Nitrospira* were present in the biofilters at the GMA. The occurrence of several *Nitrospira*-like bacteria in one habitat was commonly reported (Maixner *et al*., 2006; Daims *et al*., 2006).

Concerning the putative diversity within the genus *Nitrobacter*, it is problematic to determine the strains by comparing 16S rRNA gene sequences. *Nitrobacter* strains are phylogenetically young and do not differ much with respect to their 16S rRNA gene sequences (Orso *et al*., 1994) and the differences described before comprise of about 4 nucleotides within the relatively short sequences. PCR with primers targeting the nitrite oxidoreductase gene sequence, however, were not successful, which remains unexplainable to the authors.

Neither archaeal 16S rRNA nor amoA gene sequences could be amplified in any sample. As supported by several authors, AOA activity is of importance in low nitrogen load systems like seawater aquaria

(Urukawa *et al*., 2008; Könnecke *et al.*, 2005), yet not of high ammonia concentrations (Foesel *et al*., 2007). Still, other archaea than AOA would have been expected within the biofilm. Again, it is possible that the applied DNA extraction methods were not sufficient, since the archaeal cell wall and membrane structures are distinct from those of bacteria (Urukawa *et al*., 2010).

Nitrifying communities and nitrifying potentials

The modules, though identical in construction, exhibited a range of differences. While only the time until stocking, feed increase and further minor operational activities varied between module 1 and 3, the conditions in module 5 were of major differences compared to the others, caused mainly by the different fish species (sea bass) requiring different (higher protein content) and more feed as well as higher temperatures than turbot. Next to temperature also slight variances in oxygen contents between M5 (7.57 ± 0.74 mg/l) and M1 or M3 (8.33 ± 0.66 and 8.29 ± 0.57 mg/l) after fish arrival (Table 3.2) could be observed, possibly caused by the higher temperature and stock density. Additionally, stocking occurred in module 5 much later than in the other two modules and another type of the protein skimmer was installed.

The compositions of the bacterial nitrifying populations, with the exception of dominant AOB, were remarkably similar between module 5 and the other two modules and did not change considerable over time. Wittebolle and colleagues (2009) assumed that the inoculum (in this case seawater from the port of Büsum) is more important for the further composition of nitrifying bacteria than is the operational parameters of biofilters.

However, it is generally believed that diversity within functional groups of bacteria correlates with stability of a system (Egli *et al.,* 2003). With respect to the nitrite oxidizing guild, certain diversity would be presented by the representatives of different genera, although one might discuss in the first place, whether the biofilter systems can be called especially stable and resilient towards disturbance. For the AOB, only one dominant band was found at any time. It is possible that by the means of DGGE underrepresented groups of bacteria may have been missed, which on the other hand would have most probably not contributed greatly to the ammonia removal.

It is still to be clarified, by what factors the bacterial composition is influenced and to what extend it is responsible for varying nitrifying performances. The here given information paralleling changes in sequences or activities have to be understood as possibilities, no certain correlations between nitrifying populations and potential activities could be inferred in this study.

With respect to the different start-up regimes using mineral salts and fish feed as substrate for the bacteria, the operational variances mentioned before do not allow definite conclusions here. The

early data of AOB and NOB activities indicated a clear advance in module 5, spiked with fish feed, though one has to be careful, since the initial N input was far larger here compared to module 1. It was shown, however, that continuous feeding is of importance to maintain and increase activities once developed. It is further possible that conducting the start-phase at the same operational parameters like temperature, salinity and pH (as far as possible) might support stability and development of nitrification potentials after the introduction of fishes.

CHAPTER IV

Substances migrating from plastics impair marine nitrifiers

ABSTRACT

In many aquaculture facilities, employing moving bed biofilter systems, biocarriers made of plastics (e.g. HDPE) are used for the immobilization of nitrifying bacteria. A quick colonization of virgin biocarriers is desirable to minimize biofilter activation time and to reduce the risk of accumulation of toxic ammonia or nitrite. Plastics contain a range of additives for their processing, stabilization, preservation, etc., which migrate into the surrounding water. In this study, the effect of such substances on aerobic nitrification was examined by monitoring the nitrifying performances of bacteria from marine biofilters in the presence of granule plastic material and plastic foils. All plastics tested (HDPE, LDPE, PP, with different admixtures) had an inhibitory effect on the nitrification performances and degrees of inhibition differed between the materials. The results of blank tests showed that the inhibiting substances must be volatile.

To standardize the plastic material, seven foils were extruded with polypropylene alone and with different admixtures like talcum, carbon black, or recycled PP material. Again, all foils inhibited both ammonia and nitrite oxidation, though differences between admixtures could not be observed.

INTRODUCTION

Recirculation aquaculture systems are land-based facilities for the larvae production or grow out of aquatic organism for human consumption. The processing and reuse of more than 90 % of the water reduces greatly water consumption and facilitates waste water management by biofiltration and reduction of effluent volumes. On the other hand increases the recirculation of the water the risk of accumulation of unwanted or toxic substances, which are not eliminated by standard mechanical, chemical or biological processes.

The accumulation of ammonia, the main product of fish and crustacean metabolism and highly toxic to these, is avoided by biological filtration. The aerobic nitrification of ammonia via nitrite to nitrate is performed by phylogenetically distinct groups of chemolithoautotrophic organisms, the ammonia oxidizing bacteria (AOB) and archaea (AOA), and the nitrite oxidizing bacteria (NOB). In moving bed filters of marine RAS, the nitrifiers are immobilized on filter media, which are often beads, made of HDPE or other long living plastic material, having a vast area per cubicmeter for the bacterial biofilm to grow on (Hutchinson *et al.*, 2004).

Due to the infamous sensitivity of nitrifying bacteria, they are used for toxicity tests primarily but not exclusively in sewerage systems (Inui *et al*., 2002; Stevens, 1988), where their own nitrifying performance is essential and at risk of being inhibited. Also, they can be used for toxicity assessments in other areas (Tanaka *et al*., 2002; Sverdrup *et al*., 2006). Ren and Frymier (2004) selected *Nitrosomonas* as best candidate among other bacteria for aquatic toxicity test batteries.

Unlike in sensitive toxicity tests, is the tediousness of nitrifying bacteria extremely unfavorable when employed in biofiltration of aquaculture facilities. In this study we used nitrifying bacteria from such biofilters to assess toxic effects of plastics, on which they are immobilized within the biofilter.

Beside the biocarriers, a great part of the components and assemblies in RAS facilities are made of plastics, like tanks and tubing. The huge plastic polymers themselves are mainly inert and long living, easy to process and handle. However, processed plastics contain a range of additives for their preservation and stabilization. Further, substances facilitating the processing of the plastics end up in the final product. These substances or degradation products thereof may migrate into the surrounding media. Several authors, like Marcato *et al*. (2002), Song *et al*. (2003), Skjevrak *et al*. (2003) and Figge and Freytag (1984), analyzed leaching compounds from HDPE, PP and other plastics which are also used for packaging of food and beverages or drinking water system tubing and found a range of toxic substances.

Due to the nature of the plastic processing industry, exact compositions of ingredients remain secret or are simply unknown, especially with respect to recycled material (Zitko, 1994). Horowitz *et al*. (2001) tested the effect of migrating substances from EPDM (ethylene propylene diene monomer), used as tank liners in an intensive shrimp production on shrimps and nitrifying bacteria and reported mortality of shrimp and inhibited nitrification to high extends. The authors suggested therefore a thorough testing of any plastic material before use in aquaculture, since even after washing procedures of brand new material, the toxic effects on both shrimps and bacteria became apparent in their trials.

Apart from chemical additives also different filling materials are added to modify mechanical, electrical or magnetic properties of plastics. Talcum e.g. increases rigidity and scratch-resistance. Carbon black is added to recycled plastics to absorb UV radiation but also to make up the greyish color. These substances can also have an effect on certain surface properties of the plastics. The primary adhesion of bacteria to abiotic surfaces is determined by the net sum of attractive or repulsive physical and chemical forces generated between the two surfaces. These forces are e.g. van der Waals attraction force, electrostatic charge, hydrophobic interactions (Katsikogianni and Missirlis, 2004). In the second phase of adhesion, after the surfaces got close by physicochemical interactions, the formation of adhesives like exopolysaccharids or pili consolidates the binding. Once attached, the bacteria develop dense colonies within extracellular polymeric substances (EPS) and a biofilm matures (Dunne, 2002).

Apart from physicochemical forces, determined by the chemical composition of the material, also irregularities of surfaces (roughness) as well as porosity of materials promote bacterial adhesion (Scheuermann *et al*., 1998). Certain surface properties of the plastic polymers therefore might facilitate the attachment of nitrifying bacteria during start-up phases of new biofilters. One aim of the DBU project “Operational optimization of marine recirculation aquaculture systems” is the identification of suitable plastics as material for biocarriers. Our approach included attachment preferences of nitrifying bacteria to varying plastic foils, as well as surface properties of these. The experiments were performed by Jürgen Schrötz at the Biofilm Centre of the University of Duisburg-Essen. To test different plastic materials for inhibitory effects on nitrifying bacteria, we picked up the approach of Horowitz *et al*. (2001), and the results thereof are discussed in this chapter.

EXPERIMENTAL PROCEDURES

Plastic materials used

The plastic materials were supplied by our project partner, GEA 2H Water Technologies (Wettringen, Germany). The granules (Fig. 4.1) were made of high and low density polyethylene (new HDPE, recycled HDPE [with addition of approx. 8 % carbon black], HDPE certified for potable water and LDPE) and polypropylene (new PP, new PP with 30 % glass fibers, recycled PP with 40 % glass fibers and carbon black and recycled PP with 70 % talcum). Granules are the basic plastic substrates which, mixed with others or further substances, are extruded and pressed into the desired form.

The foils (No. 1-7), made by GEA 2H Water Technologies, were extruded mixing polypropylene with different admixtures of talcum, carbon black, recycled PP material or an antioxidant component. The foils were cut into small pieces suitable for the experimental set ups.

Fig.4.1: Plastic granules tested for inhibition of nitrification. Clockwise from upper left: recycled HDPE, new HDPE, HDPE certified for potable water, LDPE, recycled with 70 % talcum, recycled PP with glass fibers, PP with glass fibers, PP.

Inhibition tests in presence of plastic granules and foils

For inhibition tests nitrifying bacteria from moving bed biofilters from the marine aquaculture plants Ecomares in Büsum, Marifarm in Strande and GMA in Büsum were used, always sampled at most a couple of days beforehand. 100 biocarriers and glass beads (1.7-2 mm) in 100 ml 2 % NaCl were shaken vigorously for 3 h, then 5 ml of the cell suspension were pipetted into 100 ml of marine AOB or NOB medium (see chapter 3) with differing concentrations of substrate (between 2 and 5 mM; depending on the momentary activity of the biofilters) in 300 ml flasks. To all flasks (2 to 3 parallels), except the controls, plastics were added. The substrate oxidation at 28 °C of AOB and NOB in the shaken flasks were monitored: nitrite and nitrate concentrations over time were analyzed by HPLC as

described in chapter 2. The decrease of nitrite and the increase of the sum of nitrite and nitrate represented nitrite oxidation and ammonia oxidation respectively.

For the tests 1-6, plastic granules were used (30 g wet weight per 100 ml medium) and between 3 and 7 different plastics were tested simultaneously. For the inhibition tests with foils (6 with AOB and 8 with NOB) the plastic foils were cut into pieces (21 g, with equals approx. 300 cm^2 surface). Each PP foil was tested in 3 to 4 experiments. All plastics were washed and stored in aqua dest. before each test (between one week and one year; same procedure for plastics tested together).

Two inhibition tests (AOB and NOB) with granules were conducted to test pure and enrichment cultures (*Nitrobacter* strain 311, *Nitrospira marina* strain 295, *Nitrospira* strain Ecomares 2.1, *Nitrosomonas marina* strain 22, *N. cryotolerans* strain 55 and AOB enrichment culture *BUE* 10^{-4} *A*).

To check for concentration dependent inhibition, a test (only AOB) was conducted applying different amounts (18 and 36 g wet weight) of LDPE and recycled HDPE granules, respectively.

Inhibition tests in absence of plastics (blank tests)

For blank tests, 200 to 400 g (depending on density; approx. 6000 cm^2 surface area respectively) of the granules new polypropylene with glass fibers, recycled PP with glass fibers, new PP, recycled HDPE, new HDPE, and LDPE were stirred in 500 ml distilled water for 10 days at 22 °C. Media (AOB and NOB) were prepared containing 70 % of these “granule waters”, 30 % seawater and additional sea salt, and the inhibition test was conducted as described without addition of the plastics themselves.

The foils 1 and 7 were used in a further blank test (only NOB). After stirring in media without bacteria for two months, the foils were removed and bacteria were added. Nitrite oxidation was tested in comparison to a control with 21 g of foil 2 and a control without plastic.

Evaluation of degree of inhibition

As means for the evaluation of the inhibitory effects in tests 1-6, points were given to the different plastic granules based on the time differences (in days) between complete substrate oxidation in the control flasks and in the media with plastics. The control thus was always the basis, and from the different amounts of days, mean averages and standard deviations were calculated for each test for AOB and for NOB. If the substrate oxidation in a flask was completed in less time than the mean average, 1 point was given to the respect plastic. If it was less than mean average minus standard deviation, two points were given. The same was done in the negative way; here the standard

deviation was added to the mean average. The points were then divided by the amount of tests evaluated, so that the maximum amount (positive and negative) was two points. Zero points signify the average strength of inhibition.

RESULTS

Inhibition tests with granules

The presence of any plastic granules tested inhibited ammonia and nitrite oxidation compared to the controls without plastics. With respect to their degree of inhibition, the different plastics could only be evaluated in relation to each other and the controls, due to the changing potential activities of the bacteria from the running biofilters. Figure 4.2 shows an overview of the points the plastics "achieved" in the 6 evaluated tests and the amount of tests in which each plastic was applied. For both test series discriminating between AOB and NOB oxidation performances, recycled PP with glass fiber was the least inhibiting plastic and recycled PP with talcum, LDPE and HDPE certified for potable water were the most inhibiting. The order according to positive and negative points only varies between AOB and NOB in the case of new PP with glass fiber and recycled HDPE, and new PP and recycled PP with talcum. If parallels were not congruent, tests have not been used for evaluation. Completed inhibition tests lasted between 20 and 150 days.

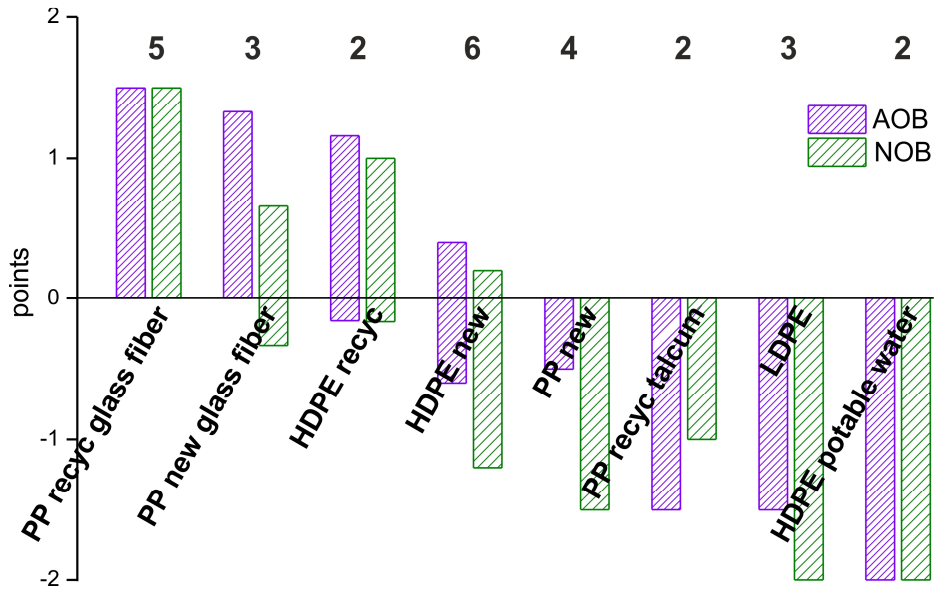

Fig. 4.2: Evaluation of inhibitory effects towards AOB (left, purple) and NOB (right, green) of granules of different plastic material. Numbers above indicate the amount of tests for the respective plastic material. Positive points signify less than the average strength of inhibition, negative points more than the average strength of inhibition.

The ammonia oxidation in the flasks with different amounts (18 and 36 g wet weight) of the same plastics was completed earlier in the flasks with the low amount of plastic (HDPE 9 days; LDPE 15 days) than in the flasks with the double amount (both 19.5 days).

Ammonia and nitrite oxidation of pure and enrichment cultures of AOB and NOB was also affected by the presence of plastic granules compared to the controls. The parallels in these tests differed greatly from each other and were not further evaluated.

Inhibition tests with foils 1-7

The presence of all foils decreased nitrification compared to the controls, extremely so in some NOB tests. Fig. 4.3 (left) depicts the results of one of the NOB tests as example. Here, the oxidation of 5 mM nitrite in the presence of foils 1 and 7 was completed almost 30 days later than in the control.

In 4 of 6 (AOB) and 5 of 7 (NOB) tests with the foils, no considerable difference in the degree of inhibition between the plastics could be observed, and the results of the remaining tests, however, were contradictive in this respect (data not shown).

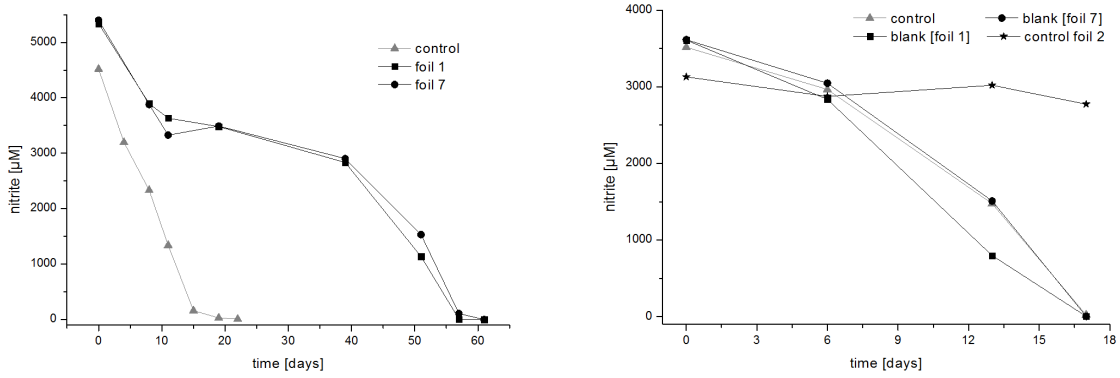

Fig. 4.3: Oxidation of nitrite in presence of foils 1 and 7 and without foil (on the left). The same foils were stirred for 2 months in media and then removed. Bacteria were added to these "blank" media and nitrite oxidation was monitored (on the right): nitrite oxidation here was completed in the same time as the control, whereas the NOB in the presence of foil 2 (control foil 2) were strongly inhibited.

Inhibition tests in absence of plastics

The nitrification in the media prepared with 70 % "granule water" was not inhibited compared to controls (AOB 6 days; NOB 16 days), neither was the nitrite oxidation in the flasks, in which the foils 1 and 7 had been stirred for 2 months (3.5 mM nitrite oxidized in 17 days, Fig. 4.3 on the right).

DISCUSSION

As mentioned before, Horowitz and colleagues (2001) recommended nitrification inhibition tests with synthetic polymer material before use in aquaculture, not only to prevent retardation of nitrification performances but also for the sake of the organisms cultivated and eventually sold for human consumption. This recommendation was corroborated by the results of the inhibition tests in this study. In all conducted inhibition tests during the course of the study (27 in total, data not shown) either with granules or extruded foils and whether with bacteria from the biocarriers or with cultures, the oxidation of ammonia and nitrite in the presence of plastics was completed later, mostly by far, than in the absence of plastics (controls). Even after one and a half year of storage in water, the effect could be observed (recycled and new HDPE granules, data not shown). However, the process of washing-off was not examined systematically in this study. Further research in this respect and thereupon based recommendations for different plastics would be valuable.

The results of the two test set ups to assess inhibition in the absence of the plastics themselves after a certain time of incubation (blank test) indicated that the leaching substances, designated causes of nitrification inhibition in the inhibition tests, are volatile.

The assessment of 6 tests with plastic granules revealed that different plastics cause inhibitions of differently strong degrees. The rank order of the plastics in terms of their inhibitory effects was similar but not identical between AOB and NOB. NOB seem to be more sensitive towards the migrating substances, which was also observed by Horowitz *et al*. (2001). Least inhibiting were the PP materials with glass fiber admixtures. It is possible that here the amount of leaching substances from the plastic is considerably reduced due to the 30 to 40 % glass fibers. The experiment with different amounts of plastics (200 and 400 g) showed that the concentration of plastics, and therefore of the leaching substances, has an influence on the degree of inhibition. This explanation, however, is incongruent with the high degree of inhibition caused by the PP with 70 % talcum. The strong inhibiting effect by the HDPE certified for potable water in our study (Fig. 4.2) is probably caused by antimicrobial agents added to this special HDPE to avoid microbial colonization.

In the experiments of Figge and Freytag (1984), testing the migration of the antioxidant n-octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate from various plastics into test fat, the migration rate decreased in the order LDPE>HDPE>PP. Mercato *et al*. (2003), on the other hand, found the migration rate of the antioxidant additives Irganos 680 and Irganox 1010 from PP into test fats greater than that from HDPE. The order presented in figure 4.2 is more similar to that of the first reference. Direct comparison, however, would be inappropriate due to differing media (aqueous and

fat). Furthermore, it is not clear, whether antioxidants or other substances caused the inhibition of the nitrifiers in the here presented experiments.

The inhibitory effects of the 7 different foils were similar, indicating that the basic material including additives is the determining factor rather than the admixtures. As mentioned earlier, it is nearly impossible to track down the origin of plastic material not to mention the composition of additives and it is to be assumed that migrating substances as well as their migration characteristics differ widely between plastics, their additives and processing state. Therefore, the results of the evaluation are restricted to the batches of plastics used here and should not be generalized.

Despite no differences in inhibition by migrating substances were found between the foils, the AOB *Nitrosomonas marina strain 22* and the NOB *Nitrospira* Ecomares 2.1 preferred to adhere to foil 3. This was the result of computer-based analyses of DAPI stained cells attached to the foils, which were performed by Jürgen Schrötz at the Biofilm Centre at the University of Duisburg-Essen. This preference could have been caused by differences of the plastics´ surface properties. Contact angle (θ) measurements are used to determine the wettability of surfaces, indicating whether a surface is hydrophobic (higher contact angle) or hydrophilic (lower contact angle).

Contact angle measurements in water with nitrifying bacteria led to different results ranging from 15° (Sousa *et al.,* 1997; Teixera *et al*., 1998) over 23° (Kim *et al*., 1997) to 61° (Khan *et al*., 2010). Bacteria with a contact angle below 40° are considered hydrophilic (Rouxhet and Mozes, 1990). Consequently would nitrifiers attach easier to hydrophilic surfaces. Kim *et al*. (1997) could confirm this theory with nitrification experiments in the case of cellulose carriers with a contact angle of zero. Sousa *et al*. (1997), on the other hand, recommended more hydrophobic support material for the use in nitrifying reactors to make use of van der Waals forces of attraction that increase with surface hydrophobicity. Mazumder and colleagues (2010) found that the initial biofilm formation on their CN-coated surfaces resulted in a decrease of hydrophobicity of the surfaces as measured by contact angles in water.

However, contact angles of the 7 polypropylene foils were measured by Jürgen Schrötz but did not differ significantly. With angles between 104° and 110° (in water) and 78° to 85° (in ethylene-glycol) the foils are hence extreme hydrophobic (Bright and Fletcher, 1983; Mazumder *et al*., 2010). The differences in initial attachment of AOB and NOB could therefore not be explained with the results of the contact angle measurements. Other types of interactions therefore must create differences between the foils with the different admixture. Roughness of surfaces, e.g., was identified to influence bacterial adhesion (Tang *et al*., 2009; Pedersen, 1990). The foils are being examined further in this respect by Jürgen Schrötz using AFM (atomic force microscopy), and the overall results will be presented in the final project report.

CHAPTER V

Effects of high nitrate concentrations and low pH on nitrification in marine RAS

ABSTRACT

Nitrate accumulates to high concentrations in RAS and was thus a candidate for us to be examined as potential inhibitor of ammonia and nitrite oxidation. Pure cultures, enrichment cultures as well as colonized biocarriers were exposed to nitrate concentrations in mineral salts media up to 200 mM. Further, the influence of low pH values on nitrifiers on biocarriers was examined using activity tests.

Nitrite oxidation decreased with increasing nitrate concentration, this effect was especially observed in activity tests with biocarriers from systems with low nitrate concentrations. *Nitrobacter* turned out to be less sensitive towards nitrate than *Nitrospira* strains. Inhibition of ammonia oxidation was neither observed for biocarriers nor for cultures.

In activity tests with biocarriers (at pH 5.3 and 7.5) from a RAS system that temporarily run at a pH 6, the difference of 2 pH units reduced the potential activities of NOB by about 36 % and of AOB by about 57 %. After incubation without substrates at pH 7.5 for 6 days, nitrite oxidation rates had increased considerably. Ammonia oxidation rates, in contrast, were even lower. Here, especially substrate deprivation as effect of low pH is discussed.

INTRODUCTION

Nitrifying bacteria are slowly growing due to their source of energy generation and they are generally known to be difficult to cultivate. As a consequence, failure of or troubles to maintain steady nitrification in aquaculture facilities or waste water treatment plants occur frequently. As mentioned earlier, the syntrophic association of AOB and NOB is a critical factor leading easily to instability when disturbed. Failure is rather credited to the NOB, which are considered to be the weaker part in the nitrification process (Graham *et al*., 2007). Wheaton *et al*. (1994) stated that more than 20 physical, chemical and biological factors can have a direct or indirect influence on the performance of a nitrification biofilter. However, the reasons for acute disturbances in the nitrification process in biofilters often enough remain unknown.

Several factors were repeatedly suspected and identified to cause irregularities, among these are e.g. temperature, alkalinity, salinity, dissolved oxygen content, organic compounds, heavy metals and, above all, free ammonia and nitrous acid (Villaverde *et al*., 1997, and therein). Thresholds and degrees of inhibitions are not equal between the genera and species and depend on fitness, growth phases, and life form (culture or biofilm); therefore, habitat specific research is mandatory.

A better knowledge about the influences of different environmental factors could help to understand, why impaired nitrification performances occur and how they can be avoided or remediated. This chapter deals with the influence of high nitrate concentrations on nitrification, since the end product accumulates in recirculation systems without denitrification stages and is therefore of special interest. The drastic down-regulation of pH values in one of the fish farms (to combat pathogenic bacteria) which were subjects of investigations, led us to perform also experiments in this respect. Most other water quality parameters, which can possibly affect nitrification and were mentioned before, can either not be controlled (e.g. heavy metals) or do not or should not vary in running aquaculture systems for the well-being of the animals cultivated (e.g. salinity, DO, temperature, organic compounds).

NITRATE INHIBITION TESTS WITH ENRICHMENT CULTURES AND ISOLATES

Nitrate, as the end product of nitrification, is the most oxidized form of nitrogen (+V). It is assimilated by plants and microorganisms, dissimilated to ammonia (dissimilatory nitrate reduction to ammonia,

DNRA) or serves as electron acceptor in dissimilatory denitrification. The stoichiometry of nitrification:

$NH_3 + O_2 + 2\ H^+ \rightarrow NO_2^- + 5\ H^+ + 4\ e^-$ (ammonia oxidizing bacteria)

$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$ (nitrite oxidizing bacteria)

shows that one mol ammonia is eventually oxidized to one mol nitrate. For the reared organisms in RAS, nitrate is far less toxic than ammonia or nitrite, which is credited to its lower branchial permeability (Camargo *et al*., 2005). Comparable to nitrite, nitrate causes the formation of methemoglobin or methemocyanin, blocking oxygen transport in the blood or hemolymph of fishes and crustaceans, and accumulates in muscle and organ tissues (Cheng *et al.,* 2002). Guilette and Edwards (2005) proposed different mechanisms of how nitrate could even function as endocrine disruptor in vertebrates. The toxicity of nitrate increases with its concentration and the residence time and decreases with the size of the organisms affected and the salt content of the rearing water (competition for transport with chloride ions), which is an advantage in marine applications. DIN toxicities in general strongly depend on species, development stages, and acclimatization. Camargo *et al*. (2005) recommended in their review, not to let nitrate concentrations in marine systems exceed 20 mg NO_3-N/L (= 1.4 mM), while only 2 mg NO_3-N/L are recommended as safe level for sensitive freshwater species. Some specific values for shrimp and marine fish are given in Table 5.1.

The nitrate concentrations of the process water of the facilities of this study partly rose up to 11 mM (module 5, GMA), Pierce *et al*. (1993) even reported up to 35.7 mM in RAS. When no denitrification stages are implemented, the nitrate concentration is dependent on water exchange rates. North Sea water in Büsum port, which serves as replacement water for the facilities in Büsum, has a, in this respect, negligible nitrate concentration (37 µM as determined by Sudarno *et al*., 2009).

EXPERIMENTAL PROCEDURES

Nitrate inhibition tests with cultures and enrichments

For these inhibition tests 100 ml mineral salts medium with 1, 2 or 3 mM substrate and 0 to 200 mM nitrate were prepared for the NOB and medium with 2 or 4 mM substrate and 0 to 150 mM nitrate for the AOB (see chapter 3). Mostly two parallels were inoculated with 1 or 1.5 ml of the cultures. In order to test a possible adaption to high nitrate concentrations, 1.5 ml cell suspension of *Nitrospira*

strain Ecomares 2.1 were transferred anew to NOB media with 0 and 100 mM nitrate, after the oxidation of 2 mM nitrite in medium amended with 100 mM nitrate.

Cultures and enrichments with maximum nitrate concentration tested:

AOB: *Nitrosomonas marina* strain 22 (marine); 150 mM
Nitrosomonas cryotolerans (marine); 60 mM
BUE M1 marin (enrichment from marine RAS; *Nitrosomonas*); 60 mM
Percoll U (enrichment from marine RAS; *Nitrosomonas*); 150 mM

NOB: *Nitrobacter strain* 297 (marine); 120 mM
Nitrobacter strain 311 (marine); 200 mM
Nitrospira marina strain 295 (coculture with *Actinobacteria**); 80 mM
Nitrospira strain Ecomares 2.1 (isolate from marine RAS); 150 mM
BUE M1 marin (enrichment from marine RAS: several *Nitrospira*, no *Nitrobacter*); 80 mM

* repeated efforts to activate pure cultures to be used for the inhibition tests were not successful

Activity tests in the presence of elevated nitrate concentrations

For activity tests at high nitrate concentrations, biocarriers were taken from the Marifarm and the GMA RAS. In parallels, 5 or 10 biocarriers were counted into 25 or 50 ml mineral salts medium or water from the biofilters (sterile), respectively, containing either 1 mM NH_4Cl or 1 mM $NaNO_2$, and different concentrations of nitrate. The tests were carried out as soon as possible after sampling (mostly 1-2 days storage at 4 °C).

In both experimental set ups, nitrite and nitrate concentrations were measured by ion pair chromatography as described in chapter 2 and substrate conversions were plotted against time.

RESULTS

Nitrate inhibition tests with cultures and enrichments

Both *Nitrobacter* strains were tested with concentrations up to 120 mM nitrate after trials with 30 mM. No considerable inhibition could be observed: each strain completed the conversion of the substrate in presence of 120 mM nitrate within three days more than without nitrate (strain 311: 13 days; strain 297: 17 days). The respective curves indicated that the lag-phases were prolonged due to

high concentrations of nitrate, and the actual conversion rates of strain 311 during the log-phases were between 667 µM per day (0 mM nitrate) to 583 µM (120 mM nitrate) (Fig. 5.1). With *Nitrobacter* strain 311 a further test was conducted with 150 and 200 mM $NaNO_3$, but even here the 2 mM nitrite was oxidized not much later (8 and 10 days) than in the control (6 days) (Fig. 5.1).

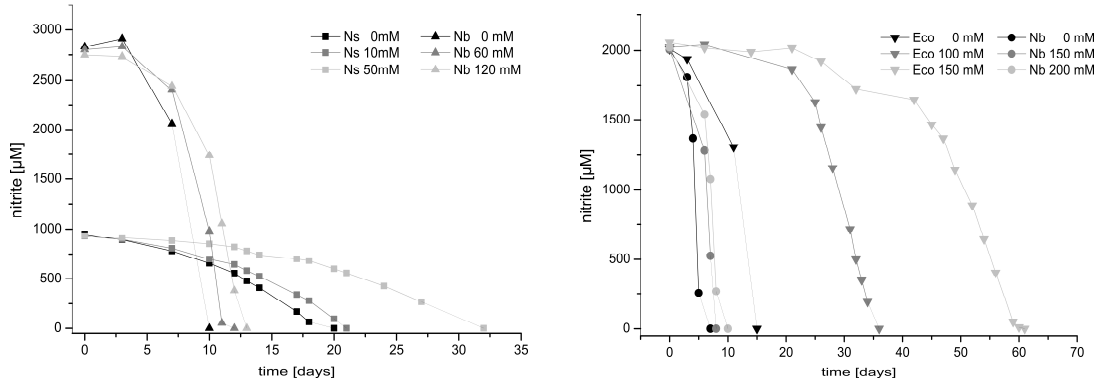

Fig 5.1: Oxidation of nitrite by *Nitrospira marina* (Ns) and *Nitrobacter* strain 311 (Nb; left) and *Nitrospira* strain Ecomares 2.1 (Eco) and *Nitrobacter* strain 311 (Nb; right) at high nitrate concentrations in comparison to controls with initially 0 mM nitrate.

In contrast to *Nitrobacter*, *Nitrospira marina* strain 295 showed a more sensitive behavior against elevated nitrate concentrations: already between 0 and 50 mM nitrate the curves spread from each other from day 7 on (Fig. 5.1). The 1 mM substrate in the control flasks was oxidized after less than 20 days, after 21 days in the media with 10 mM, and only after 32 days no nitrite could be measured anymore in the media with 50 mM nitrate. At this time, not even the half of the substrate in the media with 80 mM nitrate was converted by *N. marina*. The highest conversion rates of nitrite per day were between 108 µM (0 mM nitrate) and 68 µM (50 mM). Almost identical results were measured for the enrichment BUE M1 mar.

The strain Ecomares 2.1 reacted less sensitive towards elevated nitrate concentrations than *N. marina*. Whereas in the control the substrate was converted in 15 days, cultures supplemented with 40, 60 and 80 mM $NaNO_3$ oxidized the same amount of substrate in 24, 35 and 40 days, respectively. Like in the case of *N. marina* and BUE M1 mar, and in contrast to the *Nitrobacter* strains, the activities decreased considerably with increasing concentrations of nitrate (260 µM NO_2 per day in the control and 86 µM NO_2 per day in presence of 60 mM nitrate) in this test. In a further test with Ecomares 2.1, nitrate concentrations of 100 and 150 mM were applied, but even these concentration seem not to be near the maximum tolerable limit for that strain (Fig. 5.1); 2 mM nitrite were oxidized in 15 (control), 36 (100 mM) and 60 (150 mM) days with maximum daily oxidation rates of 326 µM (control), 161 µM (100 mM) and 115 µM (150 mM).

The cells of the *Nitrospira* strain Ecomares 2.1 seem not to have adapted to high nitrate concentrations in such short time of 36 days. The quotients calculated with the amount of days needed to oxidize 2 mM nitrite in the controls and in the test medium was exactly the same as in the first test.

The AOB strains, in contrast, showed no sign of inhibition by the different nitrate concentrations up to 150 mM (*Nitrosomonas marina* 22 and Percoll U); ammonia was oxidized to nitrite within the same time as the respective control. Table 5.1 summarizes data of nitrate inhibition and toxicity levels of different organisms collected from the literature as well as derived by this study.

Table 5.1: Effects of high nitrate concentrations on different organisms.

ORGANISM	NITRATE CONCENTRATION	OBSERVATION	REFERENCE
Nitrobacter strain 311	200 mM	nitrite oxidation slowed down slightly	this study
Nitrobacter winogradskyi	80 mM	impaired carbon dioxide	Schön, 1964
Nitrobacter winogradskyi	128 mM	nitrite oxidation slowed down slightly	Schön, 1964
Nitrobacter agilis (initial/active culture)	81 mM	complete inhibition/ no effect	Aleem and Alexander, 1960
Nitrospira Ecomares 2.1	150 mM	nitrite oxidation slowed down	this study
Nitrospira moscoviensis	75 mM	inhibition	Ehrich *et al.,* 1995
Nitrosomonas marina 22	150 mM	no inhibition of ammonia oxidation	this study
Nitrosomonas enrichment from RAS	150 mM	no inhibition of ammonia oxidation	this study
Desulfovibrio vulgaris	70 mM	significant growth inhibition	He *et al.,* 2010
Pennaeus monodon juveniles	165 mM (35 ‰ seawater)	96 h LC50	Tsai and Chen, 2002
Pennaeus monodon juveniles	10 mM (15 ‰ seawater)	safe level	Tsai and Chen, 2002
Trachinotus carolinus, fingerling	71 mM (32 ‰ seawater)	96 h LC50	Pierce *et al*., 1993
Psetta maximus	9 mM	behaviour change	Jan Schröder, personal communication
Homo sapiens	0.8 mM	safety value for potable water	Trinkwasserverordnung, 2010

Activity tests in the presence of elevated nitrate concentrations

A total of 7 activity tests were conducted using biocarriers from the Marifarm and the GMA biofilters. For NOB a decrease of nitrite oxidation with increasing nitrate concentrations could be observed at

higher nitrate levels (> 1.5 mM). However, thresholds for the onset of inhibition by nitrate cannot be determined by the results:

In tests 1, 2 and 3 (Fig 5.3) an inhibition of the NOB on the carriers could be observed at already 1.5 and 3 mM nitrate. It has to be noted that in test 1 the overall activities were relatively low. The nitrate concentrations in the biofilters, from which the biocarriers were extracted, were 1.4 mM (test 1) and 2.3 mM (test 3) at the times of the tests.

Activity tests with water from the biofilter (Marifarm) led to similar results: For test 2 (Fig. 5.2) mineral salts media were spiked with 1.7 mM nitrate, as was the nitrate concentration of the biofilter water at that time. After 7 hours, the nitrite in the control media without initial nitrate was completely oxidized, whereas only 400 - 500 µM was converted in the biofilter water and the media spiked with nitrate at that time.

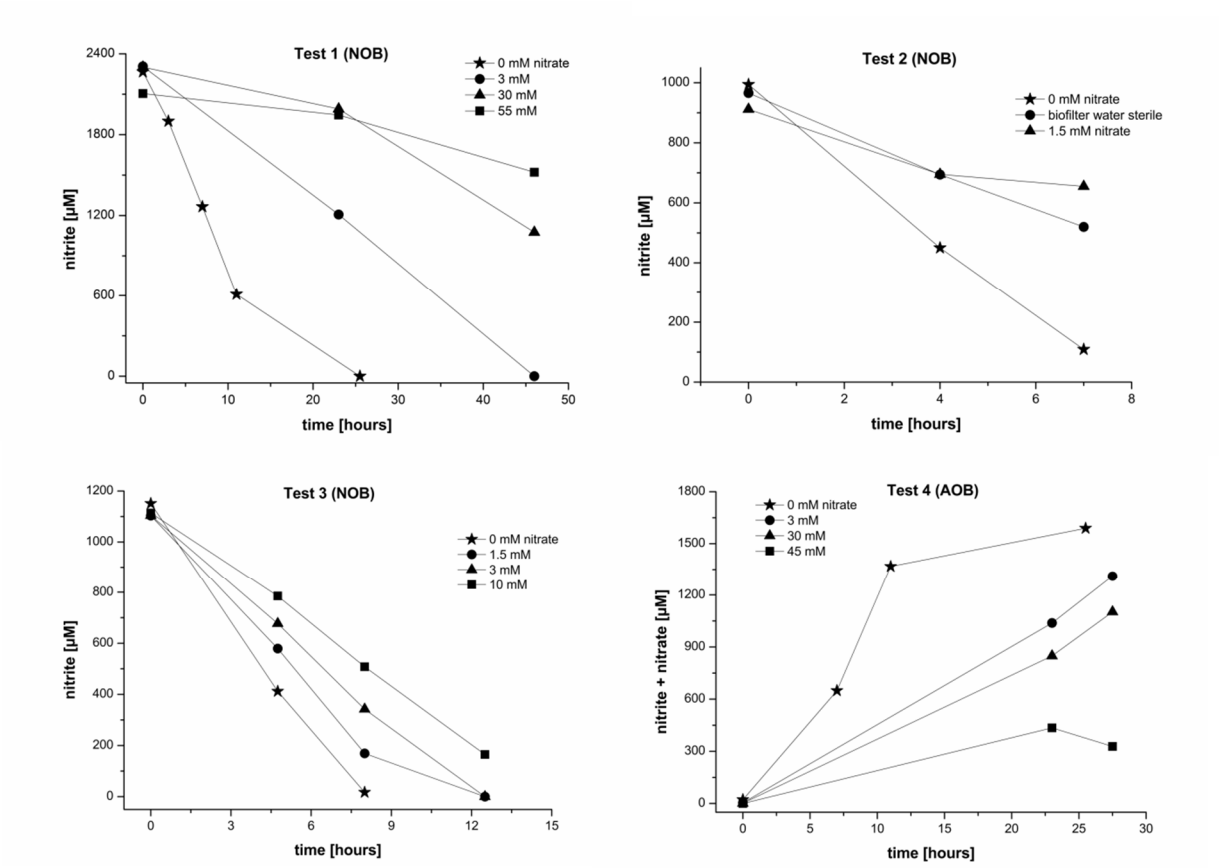

Fig. 5.2: Nitrifying potentials of NOB and AOB at different nitrate concentrations of biocarriers taken from the biofilter of the Marifarm in Strande.

With respect to the AOB on biocarriers, only the results in test 4 (Fig. 5.3) indicated a nitrate inhibition compared to the control. The graphs show the ammonia oxidation over time yet for just one parallel respectively; in this test white and black biocarriers from the same biofilter were used as

parallels, but they turned out to be more different to each other than between increasing nitrate concentrations. None of the other AOB activity tests indicated inhibition by nitrate.

In four tests conducted between April 2010 and February 2011 with biocarriers from two biofilters at GMA in Büsum with biofilter water as well as nitrate concentrations up to 4 mM, inhibition of nitrifying activity could be observed neither for AOB nor for NOB. Nitrate concentrations in the biofilters were between 2.5 mM (April 2010) and 8.5 mM (February 2011).

DISCUSSION

AOB

Nitrification is a syntrophy of two very specialized autotrophic organisms; therefore it appeared to be important to the author to investigate possible effects on AOB by the accumulation of nitrate, the end product of the syntrophy. The ammonia oxidation of the cultures tested was not affected by nitrate concentrations up to 150 mM. Furthermore, only in one of the 7 activity tests with biocarriers did the increasing nitrate concentrations slow down the AOB activities. In the corresponding test with NOB using the same batch of biocarriers from the RAS in Strande, the NOB were inhibited by the nitrate concentrations. It is therefore possible that the AOB in turn were inhibited by accumulating nitrite concentrations in the test flasks (over 1 mM in the flasks with 30 and 45 mM nitrate concentration) (Anthonisen *et al*., 1976). In all other AOB activity tests (6), no inhibition by nitrate could be observed. Dunfield and Knowles (1995) found a non-competitive inhibition of methane oxidizers in soils at >10 mM nitrate, however, they attributed this effect rather to the osmotic stress and pH levels. The key enzyme of methane oxidizers, the methane monooxygenase, is phylogenetically closely related to that of ammonia oxidizers.

NOB

The NOB, in contrast, seem to be more sensitive towards elevated nitrate concentrations. The results of the nitrate inhibition experiments with pure and enrichment cultures indicate that *Nitrobacter* strains react more tolerant in presence of high nitrate concentrations compared to *Nitrospira*; the strains exhibited longer lag-phases, yet no considerable decrease in activities. The *Nitrospira* cultures, on the other hand, slowed down nitrite oxidation already at relatively low nitrate concentrations. These differences of tolerances towards nitrate of different *Nitrobacter* and

Nitrospira species correspond to the findings of other authors (see Table 5.1); Schön (1964) found an impaired carbon dioxide uptake of *Nitrobacter winogradskyi* at 80 mM nitrate and nitrite oxidation slowed down only slightly after adding 128 mM nitrate, while *Nitrospira moscoviensis* (Ehrich *et al*., 1995) was inhibited by 75 mM nitrate. The mechanism of nitrate inhibition of the nitrite oxidation of *Nitrobacter* was specified by Boon and Laudelout (1962) as non-competitive. An end product inhibition would be restricted to NOB, but also growth of the heterotrophic *Desulfovibrio vulgaris* was inhibited significantly at a nitrate concentration of 70 mM (He *et al*., 2010), which could not be contributed to osmotic stress in this case.

Nitrobacter and *Nitrospira* belong to different phyla (*Alphaproteobacteria* and *Nitrospirae*), and according to that their key enzymes, the nitrite oxidoreductases (Nxr), bear a range of differences. *Nitrobacter* species generally have a lower affinity to nitrite and are considered to be r-strategists (Schramm *et al*., 1998), outcompeting *Nitrospira* in nitrite-rich environments. The higher nitrite oxidation activity in experiments with sufficient substrate supplied might be the most obvious explanation for the higher nitrite oxidation performances at high nitrate concentrations of *Nitrobacter*. Further, it is possible that the Nxr of *Nitrospira*, located in the broad periplasmic space (Spieck *et al*., 1996), is more exposed to nitrate ions penetrating the outer membrane. The Nxr of *Nitrobacter*, in contrast, is located in a layer on the inside of the cytoplasmic and intracytoplasmic membranes (Sundermeyer and Bock, 1981).

The recommendation of Camargo *et al*. (2005), not to let nitrate concentration exceed 1.42 mM for the welfare of the organisms cultivated, is rarely realized in marine RAS without denitrification steps. RAS are very dynamic systems, the composition of the process water changes continuously as well as bacterial composition and nitrification potentials. Local nitrate concentrations in the biofilm could be influenced by factors that were not taken into account in the tests performed here. The results of the activity tests, however, have shown that NOB can be inhibited by nitrate concentrations of already 1.5 mM. The activity tests in which such inhibition occurred were conducted with biocarriers from systems with lower nitrate concentrations than in the tests without observed inhibition. It might be possible that tolerances against nitrate are developed by the bacteria with gradually increasing nitrate concentrations of the system water. Cultivation of NOB is not possible without high accumulation of nitrate; the mentioned hypothesis could therefore explain the high tolerances of the isolates and enrichments in contrast to the nitrite oxidizers on the biocarriers. To exclude possible inhibition of NOB, particularly during critical start-up phases of new biofilters in marine RAS, nitrate concentrations should be controlled and, if necessary, lowered.

PH VALUES

In RAS, pH values are permanently reduced by carbon dioxide produced by the fishes or shrimps and by the hydrogen ion released during ammonia oxidation; with every mol ammonia oxidized, 2 mols of acidity are produced (the oxidation of 1 mg TAN results in the elimination of 7.14 mg alkalinity). The optimal pH values published for nitrifiying bacteria in pure cultures and different biofilters range between 7.2 to 8.8 for *Nitrosomonas* and from 7.2 to 9.0 for *Nitrobacter* (Chen *et al*., 2006, and therein).

As an easy and effective method for combating fish pathogens in aquaculture, pH values can be decreased temporarily. In some facilities low pH values at high TAN concentrations are applied to prevent fish toxicity of ammonia, since ammonium is regarded to be much less toxic to the fish (Person-Le Ruyet *et al*., 1995). The pH in module 1 of the Ecomares RAS in Büsum was decreased in June 2007 to combat fish pathogens. Additionally, fish feed input was reduced by 50 %. After two month at low pH values of temporarily below 6, activity tests were conducted in the laboratory to assess the effect of acidity on the nitrifying bacteria on the biocarriers.

EXPERIMENTAL PROCEDURES

Samples for activity tests (see above) under acidic conditions were taken in August 2007. The pH value of the process water was 6.1 by then (normal value around 7.2). Temperature of the water was 22 °C and oxygen content 8.7 mg/l. The pH values of the test media (mineral salts media, see chapter 3) were adjusted to 5.3 and 7.5. After completion of the first test series, the biocarriers were incubated in process water at pH 7.5 for 6 days (22 °C), before another activity test was started in media of pH 7.5. Both series were conducted at 28 °C with two parallels.

RESULTS

In the NOB activity tests the biocarriers from module 1 showed in medium with a pH value of 5.8 a nitrification rate of 270 nmol/BC*h^{-1}, and in medium with a pH of 7.5 420 nmol/BC*h^{-1}. For AOB the

rates were 435 nmol/BC*h^{-1} (pH 5.3) and 1005 nmol/BC*h^{-1} (pH 7.5). Thus, the difference of 2 pH units reduced the potential activities of NOB by about 36 % and of AOB by about 57 %. After incubation in process water without substrates at pH 7.5 for 6 days, an increase of nitrite oxidation rates to 745 nmol/BC*h^{-1} could be observed (Fig. 5.4). This value is even higher than the nitrite oxidation rates of biocarriers im module 1 were before the pH value had been decreased (AOB: 395 nmol/BC*h^{-1}; NOB: 465 nmol/BC*h^{-1} in June 2007). Hence, a temporarily acidification led to an acute decline in nitrification performances, which is yet reversible. AOB activities, though, reached only 220 nmol/BC*h^{-1} after incubation of 6 days (Fig. 5.4).

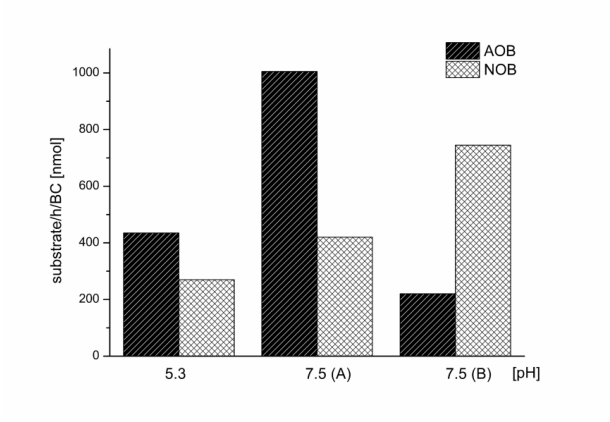

Fig. 5.4: Activities in nmol substrate converted per hour per biocarrier of AOB (black) and NOB (white) on the biocarriers at medium of pH 5.3 and 7.5 before (A) and after (B) one week incubation.

DISCUSSION

The influence of pH, especially on AOB, has been subject to many studies; however, according to Chen *et al*. (2006), still disagreement exists about the causes and the thresholds of nitrification inhibition at low or high pH values. Anthonisen *et al*. (1976) stressed that factors like cell numbers, acclimatization and reaction temperatures prevent strict thresholds, which can certainly be extended to further factors when dealing with multispecies biofilms *in situ*.

According to Villaverde *et al.* (1997), pH values can affect nitrification rates in three different ways: deactivation-activation of enzymes by reversible blockage of active sites by binding of H^+ and OH^-, depletion of carbon source for autotrophs (low pH: stripping, high pH: insoluble carbonate) and inhibition by substrates at low pH (HNO_2) and high pH (NH_3). The opposite occurs with decreasing pH values, when increasing protonation of ammonia and nitrite deprives nitrifiers of their substrates: Suzuki (1974) and Painter (1986) suggested that free ammonia and not ammonium is the substrate taken up by ammonia oxidizing bacteria. Protonated nitrite (nitric acid) on the other hand, disproportionates to nitrate and gaseous nitric oxide at low pH values (Cai *et al*., 2001).

Substrate deprivation is probably the reason for the extreme difference in ammonia oxidation (i.e. nitrite production) at low and neutral pH values in the tests conducted. Since the biocarriers were taken from the RAS that had run already at low pH values since two months and the results for AOB of the first activity tests at pH 7.5 were even higher than NOB activities in the same test, one would have expected a similar recovery of activities after the incubation time. Instead, the rates were as low as in the test at pH 5.3. This might be explained by the prolonged starvation period the AOB had to suffer after the extraction from the system. Within the system at low pH values, the AOB could still have used urea as energy source. Urea is also excreted by the fish cultivated. Since the AOB that were found in the RAS investigated in this study almost exclusively belong to the *Nitrosomonas marina* cluster, they most probably possess the enzyme urease (Koops and Pommerening-Röser, 2001). The presence of urea thus diminishes the effect of low pH values with respect to substrate depletion (Burton and Prosser, 2001).

The activity test medium does not contain urea, and the free ammonia concentrations available for the AOB was very low (1 mM TAN at pH 5.3 and 28 °C results in 0.14 µM NH_3). This might be one reason for the lower oxidation potential at pH 5.3 than at 7.5 in the first tests. During the storage time of 6 days in the laboratory the AOB were not provided with substrates. It might be possible that this time of substrate scarcity was already too long for the AOB to be reactivated at high substrate oxidation rates like the NOB (see chapter VI) in the second tests. Further activity test might have given the chance to the AOB to recuperate.

The NOB, in contrast, have fully recovered, with regard to potential activities, after the incubation period. Also the differences between nitrite oxidation rates at low and neutral pH values were not as pronounced as for the AOB. The inhibition of NOB at low pH was probably caused by high free nitrous acid concentrations (Anthoniesen *et al*., 1976), and this effect was reported to be reversible (Kaiser and Wheaton, 1983). NOB even seem to adapt to long term exposure at low pH values (Willke and Vorlop, 1986; Gieseke *et al*., 2006). The authors of the latter reference checked the hypothesis of conducive microenvironments by microsensor measurements, but found no deviating pH values throughout a nitrifying biofilm.

In summary, results obtained from the low pH experiments were congruent with the reports existing. Obviously, most of these deal with soil bacteria, since soils are acidic to extreme acidic, whereas these conditions do not occur in natural marine habitats (apart from some exceptions). With regard to nitrification performances, lowering of pH values for several weeks in marine biofiltration systems seems not to produce severe effects on the nitrifying microorganisms. However, these assumptions are based on only a few activity tests and as the topic of inhibition of nitrification in marine RAS by pH is of high interest, further research and experiments are strongly recommended.

CHAPTER VI

Residual nitrification potentials after long term storage of biocarriers

ABSTRACT

For the reactivation of biofilters after idle phases or to start new biofilters, the use of already colonized biocarriers (BC) is of advantage. Nitrifiers are known for their capability to resist starvation periods. This chapter deals with the question, how much of the nitrifying potentials become lost after 3, 6 and 9 months of storage at 4 and 17 °C. Biocarriers (1465 nmol ammonia and 1323 nmol nitrite oxidation per BC per day) were stored either in a tap water/ seawater mixture or dry.

The BC stored dry at 17 °C lost their nitrifying potentials after 3 months storage, whereas the ones stored at 4 °C could still be reactivated (1 mM substrate conversion within 7-8 weeks). The BC stored in water still showed instantaneous nitrifying potentials as measured with activity tests. The decrease of activities with the time was greater when the BC were stored at 17 °C and nitrite oxidation potentials were always greater than ammonia oxidation potentials. After 9 months storage, the nitrifiying potentials of AOB and NOB on carriers stored at 4 °C still were 4 and 25 % of the original activity respectively. The results showed that colonized BC can be stored in a simple and inexpensive way and for extended periods for the reuse in marine RAS biofilters after idle phases.

INTRODUCTION

In aquaculture systems, juvenile fish or shrimps are cultivated until they reach market size and are then extracted from the system. As long as the systems run exceptionally balanced and a certain amount of animals of different sizes are present at any time, variations of N input remain low and near steady-state biofilm might develop, which is defined as a biofilm that has neither net growth nor decay over time (Rittman and McCarthy, 1980).
Due to the grow-out cycles, however, systems run on very low or no nitrogen load at all after phases of extreme capacity utilization. Here it is of special advantage to be able to store colonized biocarriers (BC) without major loss of nitrifying capabilities to minimize activation times of afresh started biofilters after idle periods. In the same way, start-ups of new biofilters could be accelerated.

Contrary to heterotrophs, for nitrifying bacteria an exceptional starvation resistance and quick resuscitation was reported by several researchers. In most of the studies AOB were the primary subjects. Due to long lasting periods of substrate deprivation in natural habitats and to their low ability to compete for available substrate, it is important for AOB (and thus for NOB) to maintain cell homeostasis as well as energy generating capacities (Geets *et al*., 2006; Gerards *et al*., 1998). Wilhelm and coworkers (1998) found that even 342 days of starvation did not affect activity of the hydroxylamine oxidoreductase (Hao) or protein patterns of *Nitrosomonas europea* in general. Pinck *et al*. (2001) even measured twice as much ammonia monooxygenase subunit B (AmoB) of *N. eutropha* after 2 months starvation, and also *N. europea* and *N. cryotolerans* showed immediate reactivation after 5 and 10 weeks starvation (Bollmann *et al*., 2002; Johnstone and Jones, 1988). A range of remarkable survival strategies of AOB (e.g. low maintenance energy, quorum sensing, stable housekeeping enzymes or -most astonishing- use of self-produced dinitrogen bubbles to escape zones of low DO concentrations) were summarized in a review by Geets and colleagues (2006). But also NOB showed hardiness in terms of starvation survival. Spieck and Bock (2005) for instance reported an ability of NOB to survive long periods of starvation and dryness up to 24 months *(Nitrobacter vulgaris*) and Luecker *et al.* (2010) could detect the NxrB protein, subunit of the nitrite oxidizing system of *Nitrospira*, after 110 days of starvation. Morgenroth and colleagues (2000) observed that *Nitrospira* as well as *Nitrosospira* maintain high ribosome content during starvation, which in turn affects FISH analyses.

The here conducted starvation experiments focus simply on residual activities of AOB and NOB in the biofilm on biocarriers taken from an established biofilter and stored either in water or dry at different temperatures (4 and 17 °C). With activity tests, for AOB and NOB respectively, it was

determined, how long the populated carriers can be preserved and how much of their original activity they lose after 3, 6 and 9 months. The simple and inexpensive storage conditions were chosen for possible future application in aquaculture facilities.

According to Laanbroek *et al*. (2002), nitrite can function as a stimulus for AOB reactivation after starvation periods, the role of NO as a product of nitrite reduction was mentioned here, as NO seems to play a role in aerobic ammonia oxidation (Schmidt *et al.*, 2001). Seeing an opportunity to enhance reactivation of biocarriers by the simple addition of nitrite, this approach was integrated into the starvation activity test series after 6 months storage.

EXPERIMENTAL PROCEDURES

Biocarriers (type 2H_BCN 012 KLL; GEA 2H Water Technologies, Wettringen, Gemany) were taken from module 1 of the facility in Büsum (GMA, see chapter 3) in October 2010 (1.5 years after start-up). Maximum nitrifying potentials of AOB and NOB per day at this time were 1465 nmol ammonia and 1323 nmol nitrite per biocarrier respectively.

The biocarriers were stored in triplicates at 4 and 17 °C. The latter is also the temperature of module 1. Respectively about 3 layers of carriers were filled into 1L Erlenmeyer flasks. One half of the flasks were filled with 250 ml seawater plus 250 ml tap water (Fig. 6.1). The biocarriers in the other flasks were dried at 28 °C for a period of one and a half months to prevent development of fungi prior to the storage at the colder temperatures. Flasks were either closed with paper bags (dry) or with metal caps (wet).

The reactivation experiments were qualitative, checking for any conversion of substrate at all, and quantitative, measuring substrate conversion over time. The quantitative activity test set up was described in chapter 3. Shortly after the respective substrates were oxidized, the biocarriers were transferred to new medium and activities were measured again. These two consecutive tests are further referred to as 1st and 2nd round. For qualitative observations of substrate oxidation the presence of ammonia and nitrite was checked using test sticks (QuantoMerck, Merck, Darmstadt, Germany) and the modified Griess-Ilosvay spot test (Belser and Schmidt, 1994) respectively. In case initial substrate was converted, again 1 mM substrate was added to the media to check for consistent nitrification.

For the first tests series after 3 months the reactivation test were conducted at 28 °C, after 6 and 9 months at 22 °C.

RESULTS

The results of the qualitative (dry stored biocarriers) are listed in table 6.1. At 17 °C storage, nitrite oxidation could not be recovered, and the AOB were not able to convert a second dosage of ammonia until the test was terminated after 1.5 months. Accordingly, no further nitrification was achieved with these BC after 6 or 9 months storage. The nitrifying guilds on the BC stored at 4 °C both were able to oxidize their substrates still after 9 months storage, while the duration until complete conversion increased over time. Following dosages of ammonia and nitrite were oxidized after two, then one day (data not shown).
Regular checks for substrates during the tests allowed us to assume extensive lag-phases rather than very slow substrate conversion rates: the concentrations started to decrease only shortly before the substrate were not detectable any more, as observed with the ammonia test sticks and the spot test for nitrite.

Fig.6.1: Flasks with biocarriers, stored dry (left) and in water (right).

The biocarriers that were stored in water at both temperatures showed still high residual activities of both AOB and NOB. Table 6.2 summarizes these potential nitrifying activities in relation to the original activity measured before storage. Given activities are the average maximum potential nitrification rates of the three parallels measured during the course of the experiment. Standard deviations were acceptable, mostly between 10 and 40 % of the nitrite (NOB) and nitrite plus nitrate (AOB) concentrations measured by HPLC. The shift from 28 to 22 °C as test temperature resulted

Table 6.1: Times to complete substrate conversion of nitrifiers on dry stored biocarriers.

DRY				
storage duration		**3 months**	**6 months**	**9 months**
reactivation temperature		28 °C	22 °C	22 °C
substrate concentration		1mM (AOB); 0.5 mM (NOB)	1 mM (AOB and NOB)	1 mM (AOB and NOB)
			complete substrate oxidation after:	
AOB	**4°C**	10-15 days	24 days	4 weeks[2]
	17°C	3 days[1]	x	x
NOB	**4°C**	10-15 days	24-27 days	3 weeks[3]
	17°C	x	x	x
duration of test		1.5 months	2 months	2 months

x no nitrification detectable
[1] no further substrate conversion
[2] one of three parallels
[3] two of three parallels

Table 6.2: Residual activities of nitrifiers on biocarriers stored in tap water/seawater:

WET								
storage duration			**3 months**		**6 months**		**9 months**	
temperature of activity test			28 °C		22 °C		22 °C	
			activity (nmol/BC/h)	% of initial activity	activity (nmol/BC/h)	% of initial activity	activity (nmol/BC/h)	% of initial activity
AOB	**4°C**	1st round	270	18	85	6	75	4
		2nd round	235	16	170	12	125	9
	17°C	1st round	185	13	35	2	35	2
		2nd round	115	8	105	7	135	9
NOB	**4°C**	1st round	570	43	340	26	295	22
		2nd round	1085	82	585	44	195	35
	17°C	1st round	380	29	40	3	30	2
		2nd round	483	36	50	4	65	5

from high evaporation rates observed during the activity tests after 3 months. It is therefore possible, that results of this test would have been slightly different at the lower temperature. Extensive lag-phases were not observed, especially nitrification of biocarriers stored wet at 4 °C started immediately even after 9 months (AOB and NOB).

For better overview, figure 6.2 illustrates the percentages of the original activities of the biocarriers for AOB (left) and NOB (right) after 3, 6 and 9 months and in the first and second activity test. Generally, the expected pattern, i.e. that activities decreased with time and increased in the second test, can be observed here. The only exceptions are the AOB activities in the second test after 3 months, which for both temperatures are lower than in the first tests, and the AOB activities of the biocarriers stored at 17 °C in the second tests after 6 and 9 months, which are as high as after 3 months (7, 9 and 8 nmol TAN/BC*d^{-1}, respectively). Highest residual activities were measured with the biocarriers stored at 4 °C, and NOB activities were always higher than AOB activities, although the original nitrification potentials before storage were similar (1465 and 1325 nmol per BC per day for AOB and NOB). The greatest decreases of residual nitrifying potentials were observed for the time between 3 and 6 months. This is especially true for the biocarriers stored at 17 °C (AOB and NOB).

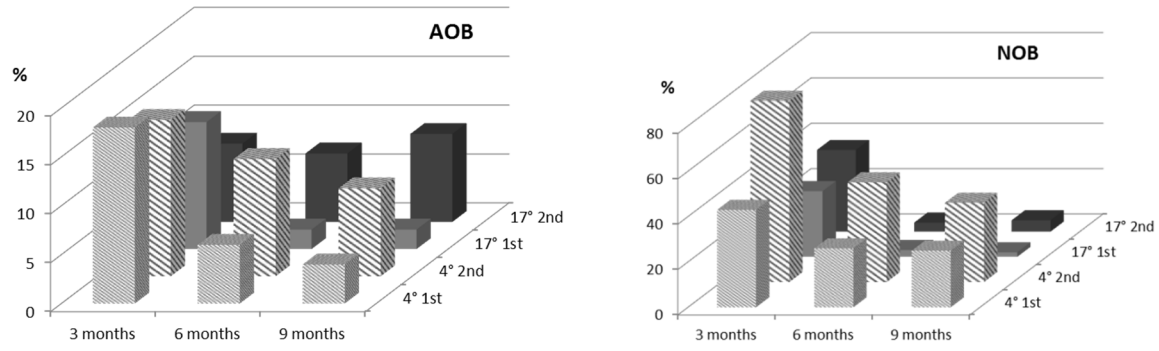

Figure 6.2: Residual activities of AOB (left) and NOB (right) on biocarriers stored in tap water/seawater in percentages of original activities in October 2010.

To check possible stimulation of reactivation of AOB activity by the addition of nitrite, biocarriers stored wet at 4 °C were used after 6 months storage. The AOB medium of three parallels was amended with 0.7 mM $NaNO_2$. No acceleration of ammonia oxidizing activity could be observed compared to controls without nitrite; within 23 hours 273 µM (with nitrite) and 372 µM (control) substrate were converted per 25 ml test medium. The initially added nitrite was oxidized to nitrate within 8 hours.

DISCUSSION

As slow-growing autotrophs that rely on fluctuating provision of ammonia, it is not surprising these bacteria are extra resourceful to survive periods without substrates compared to heterotrophs. Examples of this capability especially for AOB were given in the introduction, and the periods of starvation investigated ranged between a few days to several months to even years. The results of this chapter likewise showed a remarkable resistance of nitrifying bacteria towards substrate deprivation up to 9 months.

In general both bacterial groups showed the same ability to reactivate after 3, 6 and 9 months when stored in tap water/seawater at 4 and 17 °C and dry at 4 °C, and both guilds were unable to convert the given substrate when stored dry after 3 (and more) months at 17 °C within 1.5 months. In all activity tests with wet stored biocarriers, and especially at 4 °C, however, the NOB exhibited by far higher activity rates than the AOB, although the activities before storage were similar. This finding also correlates with the activity tests results during biofilter start-phases in chapter III, where ammonia oxidation rates declined faster after pauses of feeding. Tappe *et al*. (1999), in contrast, observed nitrite accumulations in recovery experiments with *Nitrosomonas europea* and *Nitrobacter winogradskyi* immobilized together on nylon after 3 and 6 months storage at 4 °C and attested *N. winogradskyi* to need a longer period for reviving. Similarly, Morgenroth and colleagues (2000) reported more sensitive NOB to be responsible for nitrite peaks after long idle phases in sequencing batch reactors. They also measured a higher decay rate of the NOB than of the AOB by FISH analyses. Further comparisons of oxidation performances of AOB and NOB after starvation treatments are scarce, and most studies so far concentrated on non-marine AOB. One exception is the study of Bower and Turner, who conducted starvation experiments of nitrifiers in closed seawater systems in 1983. They found similar oxidation rates for AOB and NOB up to 16 weeks after the removal of fishes and arrest of feed input. Since they amended their test aquaria only with ammonia, they could only state that the decline of the nitrite oxidation rate over time did not exceed the decline of the ammonia oxidation rate.

The question rises, why the NOB in this study were by far more active than the AOB after any period of starvation? One difference to other studies would be the bacteria species involved. The direct comparison is of importance, since all other conditions vary considerably in comparison with other studies (e.g. multispecies biofilm on biocarriers, storage medium, temperature, mode of reactivation). The dominant NOB on the biocarrier from the GMA is considered to be *Nitrospira* and the dominant AOB was *Nitrosomonas* (chapter 3). This combination, not to mention of marine

species, could not be found by the author as subject to starvation experiments in other studies and instead mostly *Nitrobacter* and *Nitrosomonas* were used. Specific PCR (for details see table 3.1) with DNA from the biofilm of reactivated biocarriers stored dry for 9 months resulted in sequences similar to *Nitrosomonas aestuarii* (99.4 %; identical to the dominant strain in late samples of module 5 in chapter III) and the strain Ecomares 2.1 (99.8 %). *Nitrobacter*- and *Nitrotoga*- specific PCR were negative. These results, however, give no hints about different decay rates between genera of NOB, since *Nitrobacter* and *Nitrotoga* were less abundant within the biofilm than *Nitrospira*, as observed by EM (chapter 3).

Back to the differences between AOB and NOB: both marine *Nitrospira* strains so far isolated, *N. marina* (Watson *et al.*, 1986) and the strain Ecomares 2.1 (chapter 2) grow better in the presence of pyruvate and peptone as C- and N-source. Some *Nitrobacter* strains are likewise capable of using organic compounds (Steinmüller and Bock, 1976), although the efficiency of the use is different among strains.

The use of organic carbon as C source is also known from AOB strains, *Nitrosomonas europea* e.g. is even able to use fructose (Hommes *et al*., 2003). The ammonia oxidation of *Nitrosococcus mobilis* (belongs to the *Nitrosomonas europea/ Nitrosococcus mobilis* cluster; Purkhold *et al*., 2000), on the other hand, was rather inhibited by organic substances and another *Nitrosomonas* strain isolated from marine water was only slightly affected, as shown in experiments conducted by Krümmel and Harms (1982). Therefore it might be possible that the NOB on the biocarriers make better use of organic compounds than the AOB and are not as early affected by substrate deprivation.

A further strategy to survive substrate-poor times is the use of endogenous substrates (endogenous respiration). The presence of storage compounds in NOB cells were reported for *Nitrobacter* (poly-ß-hydroxybutyrate (PHB), glycogen, polyphosphates), *Nitrospira* (glycogen), *Nitrospina* and *Nitrococcus* as well as *Nitrosolobus* (glycogen and polyphosphates) based on observations by electron microscopy of ultrathin sections by Watson and Waterbury (1971) and Watson *et al.* (1989). Arp *et al*. (2007) disrupted genes encoding glycogen synthase of *N. europea*, which led to less resistance of the bacterium towards ammonia deprivation. Further, Arp and colleagues (2007) found indicators for the use of polyphosphates in the genomes of four AOB (*Nitrosomonas europea, N. eutropha, Nitrosospira multiformis, Nitrosococcus oceani*).

The genome analyses of Arp and his colleagues even led to the finding of two genes for sucrose production. Since no hint of the use of sucrose within the metabolism of AOB is known so far, the authors suspected that the sucrose rather serves as cell protection against osmotic stress. Osmotic stress occurs also during desiccation, which the bacteria on the dry stored biocarriers had to suffer

during storage and especially during the pre-drying at 28 °C; despite of that, nitrification could be observed even after 6 (9) months (4 °C) within 24 days (3-4 weeks) incubation. Lin (1994) conducted a comprehensive study about the use of sucrose and other compatible solutes as protective agents against desiccation in nitrifying bacteria. Among the AOB tested under osmotic stress, only bacteria of the genus *Nitrosovibrio* were able to produce sucrose. In contrast, all *Nitrobacter* strains tested were able to produce trehalose, and were able to incorporate sucrose as well as glycinbetaine from the media. Betaines and sugars are known classes of compatible solutes (Galinski, 1995). However, the use of compatible solutes is known for the majority of microorganisms (deCosta *et al.*, 1998), thus cannot satisfactorily explain the exceptional survival of the nitrifiers colonizing the dry stored biocarriers.

The studies about the physiological response of nitrifiers towards starvation and desiccation are only possible using pure cultures or mixed cultures. Further protection against adverse conditions, however, is guaranteed by a multispecies biofilm, in which the nitrifiers on the biocarriers thrive. Here, the microorganisms are protected in the highly hydrated extracellular polymeric matrix against osmotic stress (storage in only 50 % seawater) and dryness. Furthermore, starvation does not necessarily begin with the interruption of external ammonia supply. Mineralization of organic material continues within multispecies biofilms as long as heterotrophs survive storage conditions; these in turn can feed on the nitrifiers´ soluble microbial products (SMP) (Kindaichi *et al*., 2004). An indicator for ongoing ammonification and nitrification are the nitrate concentrations in the storage water after 9 months (2789 µM [± 175 µM] at 17 °C and 1202 µM [± 126 µM] at 4 °C). The processes seemed to have happened more rapidly at 17 °C than at 4 °C. The prolonged sustenance could explain the extreme decline of oxidation rates between 3 and 6 months of storage in this study, while the difference between 6 and 9 months storage was much less pronounced.

The presence of quorum sensing molecules like AHL (*N*-acyl-homoserine-lactones) seem to be of importance for the reactivation after starvation of AOB and NOB in biofilms, as was shown in studies with *Nitrosomonas europea* by Batchelor and colleagues (1997) with N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), a known AHL produced by Gram-negative bacteria. It is not yet clear, what molecules are analogues of AHL in *Nitrospira*; autoinducer synthase or receptor genes were not found by genome analyses so far (Luecker *et al*., 2010).

The fact that no pronounced or considerable lag-phases were observed before ammonia or nitrite oxidation started is in congruence with findings of several yet not all authors. Proteins, in terms of energy most costly products of the cells, are kept intact for long periods of starvation, while other compounds like mRNA transcripts are degraded after short periods (Sayavedra-Soto *et al*., 1996). That way the cells can react immediately to substrate impulses and gain energy for any further

syntheses. Protein patterns of AOB were found almost unchanged after 342 days by Wilhelm *et al*. (1998), and Luecker *et al*. (2010) could detect the energy-generating enzyme of *Nitrospira* still after 110 days of starvation. The results of the 2nd round activity tests have shown that the immediate oxidation capacities were stable.

The best results with respect to reactivation and residual nitrifying potentials were achieved with biocarriers stored in water at 4 °C. The lower temperature slows down the decay of nitrifiers but also prevents the production of unwanted substances by heterotrophs (e.g. H_2S), especially since the wet storage method did not include active aeration. Vlaeminck *et al*. (2007) stored anammox biomass for 5 months at -20, 4 and 20 °C, and came up with the same recommendation concerning the temperature. The results of the starvation experiment in this study showed that it is possible to store colonized biocarriers in a simple and inexpensive way and for extended periods for the reuse in marine RAS biofilters after idle phases.

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APPENDIX

Supplements Chapter 3:

ANAMMOX PCR

>M1-477

TATGTCCTATCAGCTTGTTGGTGGGGTAACAGCCTACCAAGGNAATGACGGGTAGCCGACCTGAGAGGGTGACCGGCCA
CACTGGGACTGANACACTGCCCAGACTCCTACGGGAGGCTGCAGTCGAGAATCTTCCGCAATGGGCGAAAGCCTGACGG
AGCGACGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTGTCCTAGGTTAANAAACGTTTAGGTGTCAATAGCA
NCTAGACTTGACAAAGGCC

AOB PCR

Nitrosospira-like

>M1-188

ACCATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATGCATCGG
AACGTGTCCTTAAGTGGGGGATAACGCACCGAAAGGTGTGCTAATACCGCATAATCTCTACGGAGAAAAGCA
GGGGATCGCAAGACCTTGCGCTTTTGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTTACCA
AGGCGTCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAAG
GCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAACGAAACGGTCACGG

>M3-108

CGAACGGCAGCACGGGGGCAACCCTGGGTGGCGAGTGGCGAACGGGTGAGTAATGCATCGGAACGTGTCCT
TAAGTGGGGGATAACGCACCGAAAGGTGTGCTAATACCGCATAATCTCTACGGAGAAAAGCAGGGGATCGCA
AGACCTTGCGCTTTTGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTTACCAAGGCGTCGAT
CAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTT
GTAAAGCTCTTT

Nitrosomonas-like

>Lower band

GGAGGAAAGCAGGGGATCGAAAGACCTTGCGCTTTGGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGGGG
TAAAGGCCTACCAAGGCGACGATCAGTAGTTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCG
TGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGTCGAGAAGAAAAAGCTGCAGTGAATAACTGTAGTT
CATGACGGTATCGACAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGC
GTTAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGCTTTGTAAGTCAGATGTGAAATCCCCGGGCTTA
ACCTGGGAATTGCGTTTGAAACTACAAGGCTAGAGTGTGGCAGAGGGAGGTGGAATTCCATGTGTAGCAGTG
AAATGCGTAGAGATATGGAAGAACATCGATGGCGAAGGCAGCCTCCTGGGTTAACACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGG
CCTTATTAGGCTTAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACT
CAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTA
CCTACCCTTGACATGTAGCGAATTTTCTAGAGATAGATTAGTGCCTTCGGGAACGCTAACACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAATTGCCAT
CATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA

>Middle band

GGAAAGYAGGGGATCGCAAGACCTTGCGCTTTTGGAGCGGCCGATGCCTGATTAGCTAGTTGGTRGGGTAAA
GGCCTACCAAGGCGTCGATCAGTAGTTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAG
TGAAGAAGGCYTTCGGGTTGTAAAGCTCTTTCAGTCGAGAAGAAAAGATTGCGACTAATAATCGTAATTCATG
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ATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGCTTTGTAAGTCAGATGTGAAATCCCCGGGCTTAACCT
GGGAACTGCGTTTGAAACTACAAGGCTAGAGTGTAGCAGAGGGGGGTGGAATTCCATGTGTAGCAGTGAAAT
GCGTAGAGATATGGAAGAACATCGATGGCGAAGGCAACCCCCTGGGTTAACACTGACGCTCATGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCGGGTCTTA
CTAGACTTGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAA
GGAATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTAC
CCTTGACATGTCAGGAAGTTTCCAGAGATGGAWTCGTGCCCGAAAGGGAATNTGAACACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAATTGCCATC
ATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCA

>Upper band

GAGRAAAGYAGGGGATCGCAAGACCTTGTGCTTTTGGAGCGGCCGATGCCTGATTAGCTAGTTGGTGGGGTA
AAGGCCTACCAAGGCAACGATCAGTAGTTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTG
AGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGTCGAGAAGAAAAGGTTGTGACAAATAATCACAACTTA
TGACGGTATCGACAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGCTTTGTAAGTCAGATGTGAAATCCCCGGGCTTAACC
TGGGAATTGCGTTTGAAACTACAAAGCTAGAGTGTAGCAGAGGGGGGTGGAATTCCATGTGTAGCAGTGAAA
TGCGTAGAGATATGGAAGAACATCGATGGCGAAGGCAGCCCCCTGGGTTAACACTGACGCTCATGCACGAAA
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ACTAGGCTTGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTA
CCCTTGACATGTAGCGAATATTTTAGAGATAAAATAGTGCCTTCGGGAACGCTAACACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAATTGCCATCAT
TTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGANGACGTC

NOB PCR

Nitrospina-like

>M1-477

CGACGATNGTTAACCGGNCTGANAGGGTGAACGGTCACACTGGAACTGANACACGGTCCANACTCCTACNGGAGGCAGC
ANTGGGGAATTTTGNGCAATGGGNGANNGCNTGACGCNNNNACGCCNCNNGNNGGATGAAGGCTTTAGGGTCGTAAA
CCGCTGTCAAGTGGGAAGAATCCTGACGGTACCACTGAAGGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGGGCAAGCGTTGTTCGGAATCATTGGGCGTAAAGCGTATGTAGGCGGCTAGATAAGTCGGGCGTGCAAGCCCGT
GGCTCAACCACGGAATTGCGTTCGAAACTGTCTAGCTTGAGTGCAGAAGAGGAAAGCGGAATTCCCAGTGTAGCNGTGA
AATGCGTTGATATTGGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTG

>M3-169

GCGACGATGGTTAACCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGACACGGTCCANACTCCTACGGGAGGCAG
CAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGCGGGATGAAGGCTTTAGGGTCGTAAAC
CGCTGTCAAGTGGGAAGAATCCTGACGGTACCACTGAAGGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG
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GCTCAACCACGGAATTGCGTTCGAAACTGTCTAGCTTGAGTGCAGAAGAGGAAAGCGGAATTCCCAGTGTAGCGGTGAAA
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GAGCGAACGGGATTAGATACCCCGGTAGTCCTGGCCGTAAACGATGGGTACTAGGTGTAGGGGGTGTCNATCCCCTCTGT

GCCGAAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGATTTGACTTGCTTGGG

>M5-388

265BPCTNNCCAAGGCGACGATGGTTAACCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGANACACGGTCCANACT
CCTACGGGAGGCAGCANTGGNGAATTTTGCNCAATGGGCGAAAGCCTGACGCANCAACGCCGCGTGCGGGATGAAGGC
TTTAGGGTCGTAAACCGCTGTCAAGTGGGAAGAATCCTGACNGTACCACNGAANGAANCCNNGGCTAACTTCNTGNCAN
CNGCCGCGGTTATACGAAGGGGGNAAGCGTTGTT

Nitrobacter-like

>M1-26

GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTAGTTG
CTACCATTTAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGG
CCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAAGCTAAGGGGTGACCCCTAGCAAATCTCAAA
AAAC

>M1-188

GGTGCTGCATGGCTGTTGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTAGTTG
CTACCATTTAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGG
CCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGAAGCAAAGGGGCAACCCCTAGCAAATCTCAAA
AAAC

>M1-478

GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTAGTTG
CTACCATTTAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGG
CCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGAAGCAAAGGGGCAACCCCTAGCAAATCTCAAA
AAAC

Nitrotoga-like

>M3-108

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Nitrospira-like

>M1-40

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>M1-478

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>M3-323

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>M5-344

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>M5-344

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DANKSAGUNG

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