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"Ammonia-Oxidizing Archaea in Marine Cold-Water Sponges"

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“Only by walking ahead you get to the end of the journey”

Ovambo, Namibia

1 INTRODUCTION

1.1 Marine Sponges – the Origin of Metazoans

The phylum Porifera is the most ancient branch of the animal kingdom. The fossil record documents that all classes of sponges were present at least since the earliest Cambrian (Reitner and Mehl, 1995; Reitner and Mehl, 1996) possibly starting the cambrian "explosion" of metazoans (Hadzi, 1963). Their simple body plan and the lack of organisation or tissue led to a taxonomic assignment to the animal kingdom only in 1766 (Pallas, 1766). It is assumed that porifera evolved from a colonization of choanoflagellates (Haeckel, 1868) based on the astonishing resemblance of those protozoans to the 'feeding cells' (choanocytes) of a sponge (James-Clark, 1868) and supported by molecular analyses (Lang *et al.*, 2002). The sponge body is composed of functionally independent cells (Pechenik, 2000) and resembles an aggregation of different protozoans. Choanocytes, flagellated cells residing in internal chambers of the mesohyl (Figure 1.1), pick up food particles, such as bacteria and phytoplankton with their sticky collars, which are in turn taken up by amoebocytes via phygocytosis and are transported to other cells within the sponge for digestion (Bergquist, 1978; Simpson, 1984; Osinga *et al.*, 1999). Choanocytes are also responsible for pumping oxygen-rich water to the internal cavity of the sponge by movement of their flagella. However, sponges do not pump constantly and within 15 minutes of inactivity, the sponge mesohyl can g*et al*most anoxic (Hoffmann *et al.*, 2008).

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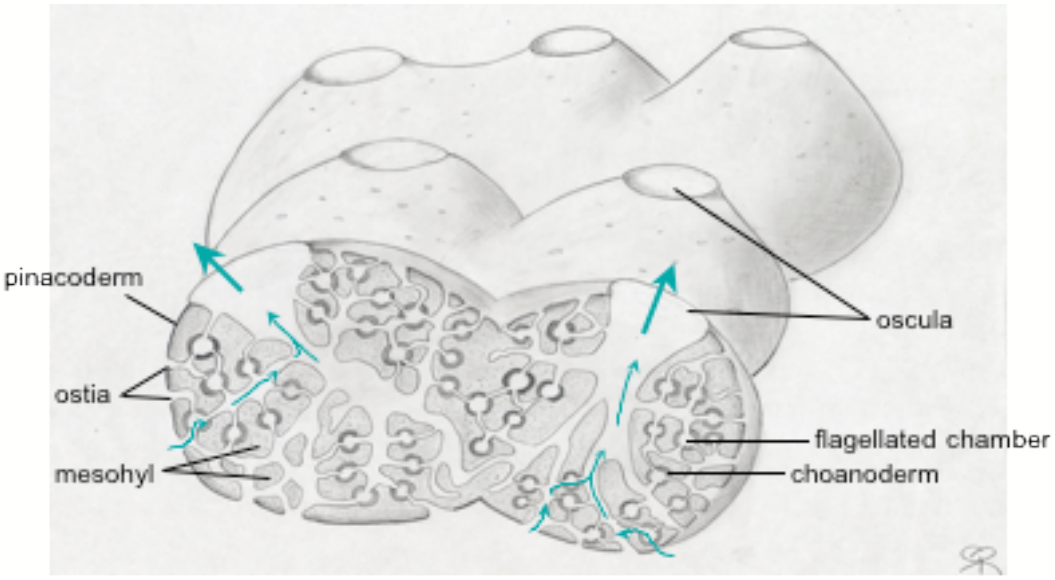

Figure 1.1. Schematic representation of the bodyplan of a marine sponge. Arrows indicate waterflow through channels and choanoderm. Drawing by RR (in Taylor *et al.*, 2007).

The taxonomic classification of the estimated 15 000 species of sponges is still challenging. Classically it was based on the analysis of spicules and nowadays it is refined by molecular genetics. The phylum Porifera encompasses three classes according to their skeletal structure: Hexactinellida with siliceous spicules and no pinacoderm, Calcarea with calcareous spicules and Demospongiae with siliceous spicules and often a mineral skeleton. While Hexactinellida have a very simple structure, Demospongiae have more complexly folded structure and are the most diverse class, comprising 95% of all extant sponge species, with morphologies ranging from encrusting layers to massive tubular sponges up to 1 m in size (Ruppert and Barnes, 1994). Although most sponges described stem from tropical reefs, they also inhabit temperate, polar and deep-sea regions as well as freshwater lakes and streams. Their ecological importance is constituted by their lifestyle, as they are sessile filter-feeding animals, with hourly filtering capacities of up to 1 000 litres per kilogram biomass (Vogel, 1977), leaving the expelled seawater essentially sterile (Reiswig, 1974; Turon *et al.*, 1997). Besides their close association with microorganisms, they are refuge habitats also for other invertebrates, such as bivalvia and crustaceans. Due to their sessile lifestyle, their physical defence is restricted to repelling spicules

and more elaborate mechanisms, like the production of bioactive secondary metabolites preventing predation and biofouling.

Despite their simple structure, sponges have been successful in colonising nearly all contemporary, aquatic environments and are essential components in reef ecosystems. The symbiotic interaction of marine sponges and microorganisms is likely the basis for their sustained evolutionary and ecological success.

1.2 Symbioses of Microbes and Eukaryotes

Microorganisms have evolved about 3.5 billion years ago and more than two billion years ago they helped to shape the chemical environment in which plants and animals have evolved (Schopf, 1978). With the possession of unique metabolisms they carry out many steps of biogeochemical cycles necessary for life to continue on Earth.

A number of marine invertebrates including species of corals, sponges, oligochaete worms, squids, shipworms (Muscatine, 1980; Waterbury *et al.*, 1983; Boettcher and Ruby, 1990; Dubilier *et al.*, 1995; Dubilier *et al.*, 2001) and others are associated with unique bacterial and/or archaeal symbionts. Symbiosis is defined as "two dissimilar organisms living together" (deBary, 1879) and probably represents the most abundant life form on Earth. Nevertheless, the diversity of marine symbionts and the nature of many symbiotic interactions involving microorganisms remains to a large extent an open field. Microorganisms can probably benefit from the physically, nutritionally and chemically different environment when living in association with marine eukaryotes as compared to seawater. The specific symbiotic lifestyle may result in the formation of unique microbial communities. Symbionts can enable their hosts to take advantage of a new environment, for instance by providing nutrients or essential compounds through nitrogen fixation, sulphate reduction, cellulose degradation, or by serving other needs, such as bioluminescence or production of bioactive compounds (Muscatine,

1980; Waterbury *et al.*, 1983; Boettcher and Ruby, 1990; Kobayashi *et al.*, 1993; Dubilier *et al.*, 1995; Dubilier *et al.*, 2001).

Only few symbioses between eukaryotes and specifically adapted microorganisms are well studied. They include the interaction between *Olavius algarvensis* and Proteobacteria (Dubilier *et al.*, 2001), *Euprymna scolopes* and *Vibrio fisheri* (Boettcher and Ruby, 1990), Aphids and *Buchnera* (Zientz *et al.*, 2001), numerous insects and *Wolbachia* (O'Neill *et al.*, 1992) and the interaction between *Elysia chorotica* and Proteobacteria (Rumpho *et al.*, 2008). The association of complex microbial consortia and eukaryotes, like in sponges, is also a common form of symbiosis including the gut microbiome of mammals and insects (Buchner, 1965; Nogge, 1976; Savage, 1977) as well as the microbiota on epithelia including the skin. There is even an association with Gammaproteobacteria living endosymbiotically in Betaproteobacteria inside mealybugs (von Dohlen *et al.*, 2001). Generally, bacteria seem to be more common symbionts of eukaryotes than archaea, although this might be at least in part due to the lack of knowledge regarding the widespread distribution of archaea, which were thought to be restricted to extreme environments until 20 years ago (DeLong, 1992). Thus common approaches, based on polymerase chain reaction (PCR), targeting symbiotic micr-organisms were focused on the detection of bacteria.

An ancient symbiosis was likely the origin of today's eukaryotic cell (Sagan, 1967). The exact timing and mechanisms of this merging event giving rise to Eukarya is not clear and is undergoing intense investigations (chapter 1.5.2). Generally, symbioses facilitate the exchange of genes during evolution. An example for a recent transfer is a ±11kb fragment that moved from the gammaproteobacterial *Wolbachia* genome to the genome of the bean beetle *Callosobruchus* (Kondo *et al.*, 2002).

Genes of microbial origin are commonly found in eukaryotic host genomes, for example with 18% cyanobacterial genes in the nucleus of *Arabidopsis* (Martin *et al.*, 2002) and more than half of the genes in the yeast genome having bacterial homologues (Rivera *et al.*, 1998). The nature of those observations is issue of discussion amongst microbiologists with manifold hypotheses on the

origin of eukaryotic cells and their evolution (reviewed in Hoffmeister and Martin, 2003).

1.3 Sponge-Microbe Associations

The first detection of symbiotic microorganisms in marine sponges dates back to the 1970s (Vacelet, 1970; Vacelet and Donadey, 1977; Wilkinson, 1978a, b, c) followed by the finding that sponges can distinguish food bacteria from symbionts in feeding experiments (Wilkinson, 1984; Wehrl *et al.*, 2007). Since then, our knowledge regarding identity of sponge associated bacteria and archaea increased immensely, while the functions of most sponge symbionts are still unknown. In "Bacteriosponges" (Reiswig, 1981) or high microbial abundance (HMA) sponges (Hentschel *et al.*, 2006) the mesohyl is densely packed with microbes (10^8-10^{10} cells per cm^3) and can comprise up to 30% of the sponge biomass (Vacelet, 1970; Vacelet and Donadey, 1977). The majority of marine sponges harbour a range of microbes, whereby Acidobacteria, Chloroflexi, Proteobacteria, Nitrospira, Firmicutes, Bacteroidetes and Spirochaetes are consistently found (Althoff *et al.*, 1998; Friedrich *et al.*, 1999; Webster *et al.*, 2001; Hentschel *et al.*, 2002; Taylor *et al.*, 2007; Webster and Taylor, 2011). Furthermore, archaea and the candidate phylum Poribacteria, which are not captured by general primer-based PCR searches (Fieseler *et al.*, 2004; DeLong, 1992), are detected commonly in targeted approaches.

The diversity of symbionts in some marine sponges exceeds that of typical symbiotic interactions by far. Furthermore, the microbiota of sponges is distinctively different from the communities of typical seawater (Vacelet, 1970, 1975; Vacelet and Donadey, 1977; Wilkinson, 1978c, b, a; Santavy *et al.*, 1990; Hentschel *et al.*, 2002; Taylor *et al.*, 2007; Webster and Taylor, 2011) and appears highly stable in space and time (Friedrich *et al.*, 2001; Webster *et al.*, 2004; Taylor *et al.*, 2005). Notably, the microbial consortia from various sponges in different oceans share sponge-specific bacterial and archaeal taxa (see section 1.4), giving an indication of specific symbiotic adaptations, as it has been shown, for example, for Cyanobacteria, which provide the host with photosynthates.

Specific associations between microorganisms and their sponge host were also supported by the observation of vertical transmission of some of the symbionts via the gametes (reviewed in (Taylor *et al.*, 2007). In particular, microbes potentially involved in nitrogen cycling, such as potential ammonia-oxidizing archaea and distinct nitrite-oxidizing bacteria from the phylum Nitrospira are transmitted vertically over sponge larvae (Sharp *et al.*, 2007; Schmitt *et al.*, 2008; Steger *et al.*, 2008; Lee *et al.*, 2009) suggesting an evolutionary adaptation.

Amongst others, Actinobacteria, Proteobacteria, Firmicutes and Cyano-bacteria can produce bioactive compounds, which are beneficial for the hosts chemical defence system against biofouling and predators (Bakus *et al.*, 1986; Paul, 1994; Proksch, 1994; Unson *et al.*, 1994; Bewley *et al.*, 1996). The identification of bacteria producing these metabolites leads to the discovery of a high number of substances with biotechnological potential each year (Blunt *et al.*, 2008). Marine sponges are the most promising candidates to find metabolites even against cancer and HIV (reviewed in Thomas *et al.*, 2010) Culture collections of marine microorganisms from sponges thus also provide the foundation for the sustainable production of biotechnological products.

1.3.1 Sponge-Specific Sequence Clusters

In an encompassing survey in 2007, all publicly available sponge-derived 16S rRNA sequences were included in phylogenetic analysis followed by the identification of sponge-specific clusters (Taylor *et al.*, 2007). In overall 16 bacterial phyla and the archaeal lineages Euryarchaeota and Thaumarchaeota group I.1A were identified in sponges from all oceans, from which 12 phyla contained monophyletic, sponge-specific clusters (Taylor *et al.*, 2007). These clusters were originally defined by harboring three or more 16S rRNA sequences from different sponge species, or from the same sponge species but from different geographical locations, and were stable with different treeing algorithms (Hentschel *et al.*, 2002).

Two-thirds of the ≈1500 sponge-derived 16S rRNA sequences available in 2007 were included in one of 33 clusters, with the biggest encompassing 52 sequences retrieved from 21 different sponges. This cluster was affiliated to the bacterial phylum Cyanobacteria, to which the well-studied phototrophic, nitrogen fixing mutualistic symbiont "*Candidatus* Synechococcus spongiarum" belongs to (Usher *et al.*, 2004).

The results of this survey suggested the presence of a mixture of evolutionarily ancient, permanently associated bacteria (transmitted vertically), and those that are acquired horizontally from the water column. In 2011, almost 10-fold the number of 16S rRNA sequences as in 2007 were deposited in public databases, dispersed over more than 25 phyla (Webster and Taylor, 2011). An update of treeing calculations to track sponge-specific sequence clusters is in process and a reduction of sequences falling into those clusters is expected (M. Taylor, pers. comm.), also due to the detection of rare microbial sequences in seawater with deep sequencing methods. High-throughput sequencing techniques allow the detection of low abundant microbes, but often do not allow the high phylogenetic resolution needed to infer the presence of sponge-specific sequence clusters. A study from 2010 applied V6-tagging (analysis of a small variable region of the 16S rRNA gene) on three marine sponge species and matched this region to sequences from previously described sponge-specific sequence clusters (Webster *et al.*, 2009). In two of the adult sponges analysed, approximately a third of all tag-sequences were affiliated to formerly known sponge-specific clusters.

Overall, the formation of phylogenetic sequence clusters found specifically in sponges (and other invertebrates) might indicate an evolutionary adaptation of the symbiotic microorganisms to their hosts and thus can help to distinguish microbial symbionts from other commensalic or food bacteria in these complex consortia.

1.4 Tackling the Functions of Sponge-Associated Prokaryotes

Only in the past few years, investigations on marine sponges have started to focus on the function of sponge symbionts. The challenge and beauty hereby is the need of methods, allowing to link function to community structure to characterise a highly complex microbiome. Many marine sponges and the majority of microbial symbionts resist cultivation in the laboratory. The phylogenetic placement of lineages can, however, only rarely give an indication for the specific physiology of microorganisms. Furthermore, DNA-sequence information of ribosomal RNA (rRNA) genes does neither allow for a prediction of the viability of the respective microbe at the time of sampling, nor is a reliable indication for activity. Studies targeting RNA of sponge symbionts (Kamke *et al.*, 2010) are certainly a step towards capturing metabolically active symbionts (DeLong *et al.*, 1989; Poulsen *et al.*, 1993), although the concentration of rRNA in the cell does not always correlate with growth and activity (Morgenroth *et al.*, 2000). In contrast, messenger RNA (mRNA) is degraded much faster and hence a good indicator for activity on the RNA level.

The characterisation of processes in nutrient cycles can also be adressed by amplification of functional marker genes, encoding enzymes involved in specific reactions. In addition, metagenomics can unravel properties of uncultured organisms, like it was shown for the archaeal symbiont of the marine sponge *Axinella mexicana* (Hallam *et al.*, 2006) and for the gutless marine oligochaete (Dubilier, 2007). While metagenomics describes the genomic inventory of a microbial community, metatranscriptomics targets the subset of genes that are transcribed, which gives a better indication for activity of the respective microorganisms. The first metatranscriptome study involving high throughput sequencing was done on soil in 2006, where Leininger and colleagues were able to show the vast abundance of archaea in different soil communities and the activity of archaeal ammonia oxidizers (Urich *et al.*, 2008). The approach, as also used in this study, involves the simultaneous analyses of rRNA and mRNA sequences, thus providing

taxonomic information of the microbial community side-by-side with functional information from protein encoding genes.

High-throughput sequencing technology was also applied to mRNA analyses of marine surface waters (Frias-Lopez *et al.*, 2008; Gilbert *et al.*, 2008; Hollibaugh *et al.*, 2010) including a step for rRNA depletion for a higher output of mRNA sequence reads (Stewart *et al.*, 2010). The use of deep sequencing technologies in metagenomics and metatranscriptomics has opened a new era in microbial ecology, as it allows to study complex natural microbial communities with a more adequate resolution than it was possible before.

However, major challenges remain. One of the major limitations of shotgun sequencing approaches of DNA and RNA from environmental samples lies in the assignment of genes or transcripts to specific microbial lineages. These assignments are not only dependent on excellent bioinformatic tools that are constantly refined and developed in parallel to novel sequencing technologies, but are also dependent on the availability of fully sequenced reference genomes.

In order to provide such full genomic information from specific taxa that can not be cultured in the laboratory, single-cell genomics was applied, e.g. on a Poribacterium and a Chloroflexi living in symbiosis with the mediterranean sponge *Aplysina aerophoba* (Siegl and Hentschel, 2010; Siegl *et al.*, 2010). A mixotrophic pathway was reconstructed from the poribacterial genome and the potential involvement of Poribacteria in chemical defence and denitrification was suggested due to the identification of genes encoding for PKS (polyketide synthase) and nitrite reductase (NIR) as well as nitric oxide reductase (NOR) (Siegl *et al.*, 2010). However, methods based on sequence homology are dependent on the quality and content of sequence databases and are only of limited use for organisms containing a large set of genes whereof the function is not yet defined. Furthermore, the presence of functional genes in the genome does not necessarily correlate with metabolic activity. Therefore physiological studies used in combination with the detection of functional and/or 16S rRNA genes were employed to describe the

activity of sponge-associated microbes in specific processes, like nitrification (Bayer *et al*., 2008) and sulfate reduction (Hoffmann *et al*., 2005). Also, the combination of modern techniques like FISH (fluorescence *in situ* hybridization) in combination with microautoradiography (MAR), Raman spectrometry or Nano-SIMS (secondary ion mass spectrometry) now offer the possibility of a more direct characterisation of structure-function relationships of uncultured microbes whose activities may have been predicted by metagenomics or metatranscriptomics.

1.5 Conversion of nitrogen compounds in marine sponges

Nitrification, the two-step conversion of ammonia to nitrate via nitrite, is a crucial component of the natural nitrogen cycle (Figure 1.2). In sponges, this process was observed already more than 20 years ago with the detection of ammonia uptake and release of nitrite and nitrate in tropical sponges (Corredor *et al*., 1988; Diaz and Ward, 1997). More recently, the nitrification potential of mediterranean sponges began to be explored, with considerable lower nitrification rates measured in comparison to tropical sponges and the observation of seasonal fluctuation in the uptake and release of ammonia in the sponge *Aplysina aerophoba* (Jimenez and Ribes, 2007; Bayer *et al*., 2008; Schläppy *et al*., 2010). Fluctuations in temperature and nutrient availability are more pronounced in tropical and other shallow water habitats, than they are in deeper marine habitats, as represented by the cold-water sponges investigated in this study.

Microorganisms exclusively belonging to two physiologically distinct groups, the ammonia oxidizers, which convert ammonia into nitrite and the nitrite oxidisers, which further oxidize nitrite to nitrate perform nitrification.

The symbiosis between those nitrifying microorganisms and sponges might be mutualistic if the ammonia, produced as a waste product by the invertebrate, is taken up by microbial symbionts as they can gain energy from inorganic nitrogen compounds. Nitrifyers known from sponges include archaea (phylum

Thaumarchaeota) conducting the oxidation of ammonia (ammonia oxidizing archaea, AOA), and marine sponge-specific bacteria (phylum Nitrospira), that can oxidize nitrite to nitrate (nitrite oxidizing bacteria. NOB) (reviewed in (Taylor *et al.*, 2007; Bayer *et al.*, 2008). Furthermore, putative ammonia-oxidizing bacteria (AOB) related to the subdivisions of Gamma- (*Nitrosococcus*) and Betaproteobacteria (*Nitrosomonas, Nitrosopira*) are sometimes found in 16S rDNA libraries of sponges (Hentschel *et al.*, 2002; Diaz *et al.*, 2004; Bayer *et al.*, 2008; Meyer and Kuever, 2008; Mohamed *et al.*, 2009). The ecological niche partitioning between AOA and AOB is an open question remaining to be solved, therefore the abundance and activity of those groups in various nitrifying sponges offers valuable clues about their requirements to thrive in certain ecological conditions.

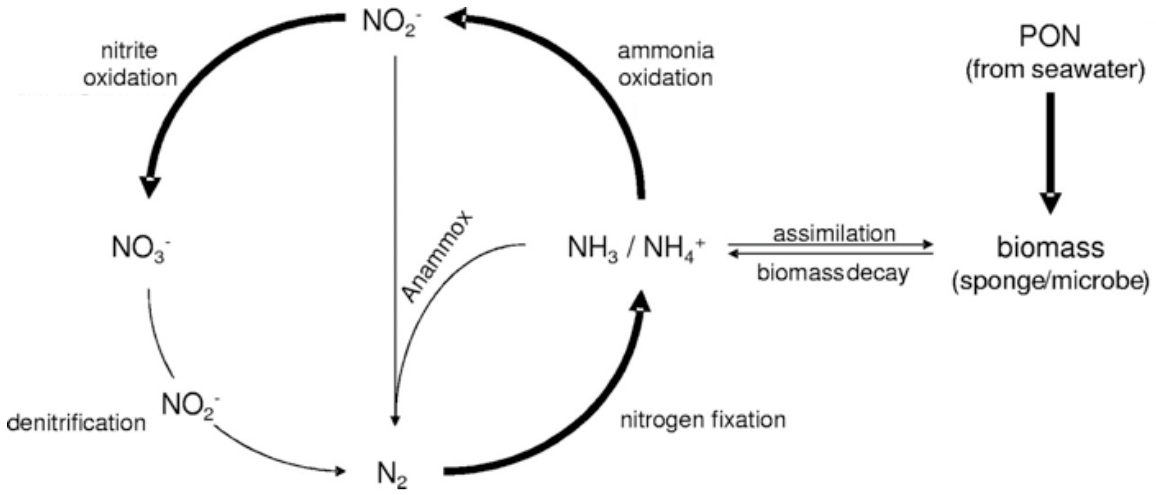

Figure 1.2 Microbial nitrogen cycle in marine sponges: Ammonia-oxidizing bacteria convert ammonia (NH_3) to nitrite (NO_2-), which is further processed to nitrate (NO_3-) by nitrite oxidisers. Nitrate is then metabolized to atmospheric nitrogen (N_2) by denitrifying bacteria. PON = particulate organic matter. Figure adapted from Taylor *et al.* 2007.

Denitrification was suggested to occur in sponge mesohyl because a sponge-derived Alphaproteobacterium, closely related to the denitrifier *Pseudovibrio denitrificans* was isolated (Enticknap *et al.*, 2006). Additionally, it was further proposed that Planctomycetes might be involved in anaerobic ammonia oxidation in marine sponges (this work and (Mohamed *et al.*, 2009). Considering the significant areal coverage and biomass of sponges in certain oceanic regions (Tendal *et al.*, 1993), as well as their high filtration rates,

marine sponges might constitute important sites for regeneration of nitrogenous compounds in marine habitats.

1.6 Archaea – Phylum Thaumarchaeota

After their discovery as a separate domain of life in 1977 (Woese, 1977), archaea were considered for a long time to be specialized in thriving in extreme environments, such as hot springs and hypersaline lakes (Barns *et al.*, 1994; Barns *et al.*, 1996; Jones *et al.*, 1998; Grant *et al.*, 1999; Takai and Sako, 1999) or anaerobic habitats. The first report of mesophilic, aerobic archaea came 15 years later with the discovery of 16S rRNA sequences in the picoplankton of the ocean (DeLong, 1992; Fuhrman *et al.*, 1992) and related species in the sponge *Axinella mexicana* (Preston *et al.*, 1996). Metagenome sequences of "*Candidatus* Cenarchaeum symbiosum" (the *Axinella* symbiont) and other marine and terrestrial archaea unraveled a greater evolutionary and physiological diversity than previously assumed (Könneke *et al.*, 2005; Treusch *et al.*, 2005; Hallam *et al.*, 2006; Hatzenpichler *et al.*, 2008; Blainey *et al.*), see chapter 1.5.2.1 for more detail). Besides their widespread distribution, these archaea are also highly abundant in marine and terrestrial habitats (Francis *et al.*, 2005; Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Jiang *et al.*, 2008), hence possibly play an important role in global biogeochemical cycles. Furthermore, some (if not all) of those archaea harbour genes for a potential ammonia monoxygenase (Venter *et al.*, 2004; Könneke *et al.*, 2005; Treusch *et al.*, 2005; Hallam *et al.*, 2006; Hatzenpichler *et al.*, 2008; Blainey *et al.*, 2011) (Figure 1.3) and might therefore be capable of gaining their energy from ammonia oxidation.

Recently, they were assigned their own phylum, the Thaumarchaeota (previously considered a subgroup of the phylum Crenarchaeota) (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010), with subgroup I.1a, mainly consisting of 16S rRNA sequences derived from marine habitats (including sponges) and group I.1b, with mainly soil and sediment-derived sequences. Sequence analyses on based on *amo*A, encoding subunit A of the key enzyme

ammonia monooxygenase the marker gene for ammonia oxidation, show a similar phylogenetic pattern (Pester *et al.*, 2011).

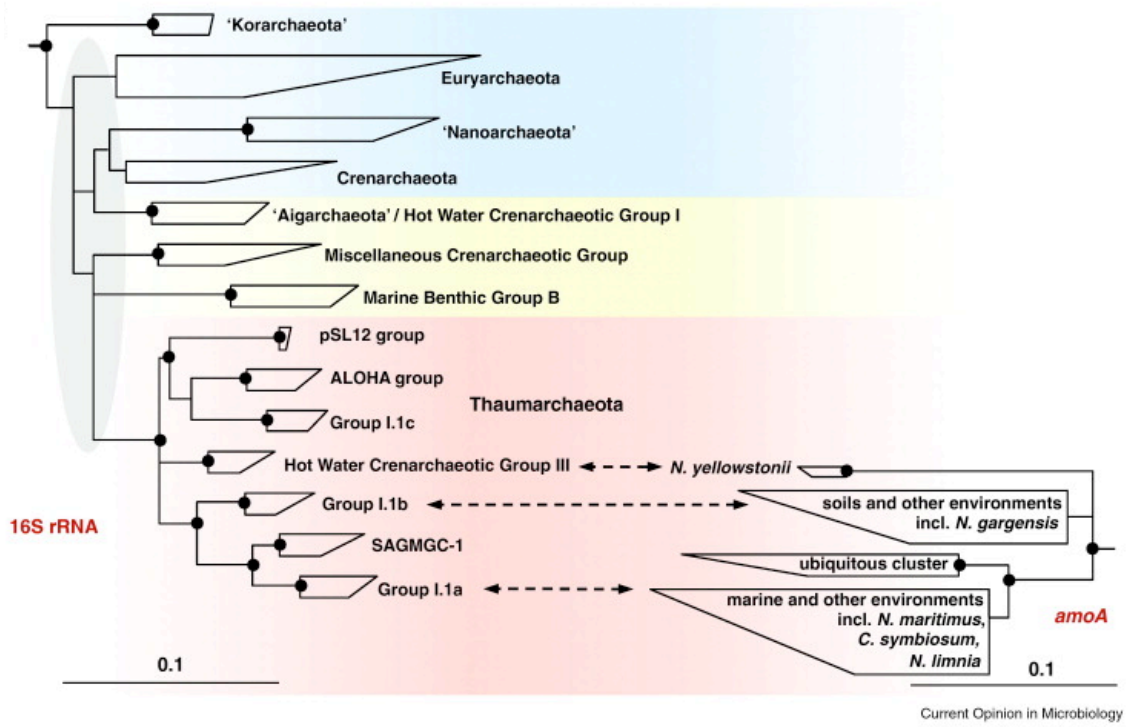

Figure 1.3. 16S rRNA consensus tree of ammonia-oxidizing Thaumarchaeota. Taken from Pester *et al.*, 2011.

There is evidence that Thaumarchaea are a deep-branching lineage in the evolutionary archaeal tree (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). Therefore, the association between these archaea and sponges might constitute a symbiosis between the most basal organisms within the eukaryal and archaeal domains of life. The quest for the last eukaryotic common ancestor (LECA) is intertwined with the archaeal phylogeny, as archaea and eukarya are evolutionary related in a way that is not fully understood yet (Brown and Doolittle, 1997). In particular, most factors involved in cellular information processing in archaea, i.e. in replication, transcription, translation, cell division are homologues of eukaryotic counterparts (Klenk and Garrett, 2006). One proposed scenario for the phylogenetic relationship between archaea and eukaryotes show Eukaryota as a secondary domain, that emerged by phagocytosis of an Alphaproteobacterium by an archaeon (Rivera and Lake, 1992; Lake and Rivera, 1994; Martin and Muller, 1998; Cavalier-Smith, 2002; Embley and Martin, 2006; Poole and Penny, 2007a;

Poole and Penny, 2007b; Cox *et al.*, 2008), equating the most recent ancestor of archaea with LECA. The eocyte hypothesis (Lake *et al.*, 1984; Rivera and Lake, 1992) suggests, that an association between a bacterium and a crenarchaeote (nowadays including a subgroup of the thaumarchaeotes, Forterre 2010; Foster *et al.*, 2009) lead to the origin of eukaryotes. The second scenario for the origin of the eukaryal domain places it as a monophyletic sister group to the archaea, congruent to the 16S/18S rRNA tree of life (Gogarten *et al.*, 1989; Woese, 1990). In this case, the Alphaproteobacterium was an endosymbiont of a protoeukaryote leading to a gene transfer. However, there are arguments for both scenarios (Gribaldo *et al.* 2010) and with the lack of evolutionary intermediate stages it seems difficult to solve this issue (Poole and Penny, 2007b). Advancements in taxonomic sampling, genome sequence analysis and new models for phylogenetic analysis might eventually help to find the missing link(s).

1.6.1 Physiology of Ammonia oxidizing archaea

The mechanism of ammonia-oxidation in archaea is not fully understood. The first isolate exhibiting this metabolism was *Nitrosopumilus maritimus*, which grows chemoautotrophically on low concentrations of ammonia (Martens-Habbena *et al.*, 2009), although there are indications of a heterotrophic mode of carbon assimilation in other thaumarchaea (Ouverney and Fuhrman, 2000; Herndl *et al.*, 2005; Ingalls *et al.*, 2006; Teira *et al.*, 2006; Tourna *et al.*, 2011). The complete genome sequence of the sponge symbiont "*Candidatus* Cenarchaeum symbiosum" (Hallam *et al.*, 2006) exhibits high similarity to the genome of *N. maritimus* (Walker *et al.*, 2010), derived from seawater (marine aquarium). Both archaea are phylogentically related to Group I.1a Thaumarchaea, together with the recently described "*Candidatus* Nitrosoarchaeum limnia" (Blainey *et al.*, 2011). Putative metabolic functions of those archaea coincide in terms of ammonia oxidation and carbon assimilation inferred from genome annotation. Genes encoding the ammonia monooxygenase protein (AMO) and ammonia transporter are consistently

found, together with multicopper oxidases and blue copper domain-containing proteins that are supposed to be involved in electron transfer. Noteworthy is the incessant finding of unannotated proteins, partly in close genomic proximity to genes for subunits of the AMO. The genomic repertoire of group I.1a Thaumarchaota suggests a differing metabolic pathway for the oxidation of ammonia from that of bacteria. The energy-gaining step of AOB involves an enzyme called hydroxylamine oxidoreductase (HAO), which has no homologue in archaeal genomes so far. A homologue of nitrite reductase (*nir*K), reducing nitrite to nitric oxide in bacteria under denitrifying conditions is present in the genome of *N. maritimus*, but not of "*Cand.* C. symbiosum". In this context it will be interesting to explore the genetic setup of other archaeal sponge symbionts and correlate it to nitrification activity.
One hypothesis for the biochemistry of archaeal ammonia oxidation proposes the existence of a – not yet annotated – nitroxyl oxidoreductase (HNO) instead of the bacterial HAO (Walker *et al.*, 2010) and an involvement of the archeal *nir*K in providing nitric oxide (NO), activating AMO together with 0.5 O_2 per NH_3 oxidized (model by M. Klotz in Schleper and Nicol, 2010). This would widen the range of environmental niches of AOA to even microaerophilic environments, congruent with their occurence in suboxic marine waters and sediments (Erguder *et al.*, 2009; Schleper and Nicol, 2010 and references therein, Labrenz *et al.*, 2010;). Possibly there is also an evolutionary adaptation to different oxygen concentrations, as distinct lineages of AOA are found in stratified marine sediments and the Black Sea (Coolen *et al.*, 2007; Durbin and Teske, 2010). This adaptation could certainly be of advantage for symbionts of massive marine sponges, as oxygen concentrations in the mesohyl decrease in zones of no active pumping activity (Hoffmann *et al.*, 2005).

1.6.2 Thaumarchaeota and sponges

Starting with the discovery of a psychrophilic crenarchaeon (now thaumarchaeon) living in association with a sponge (Preston *et al.*, 1996), 16S

rDNA of thaumarchaea were detected in many marine sponges all over the world (Taylor *et al.*, 2007 and referencess therein, Bayer *et al.*, 2008). Archaeal AMO genes were found both in adult sponges and in their free-swimming larvae (Sharp *et al.*, 2007; Schmitt *et al.*, 2008; Steger *et al.*, 2008), suggesting a vertical transmission mode of these symbionts. As mentioned before, different sponge species from various geographic locations share similar thaumarchaeal 16S rRNA sequences, distinct from those derived from seawater samples. This clustering also occurs for archaeal *amo*A sequences, with a potential sponge-specific cluster in phylogenetic proximity to the *amo*A sequence of "*Cand.* C. symbiosum" (Steger *et al.*, 2008; Turque *et al.*, 2011). Further investigations on sponge-derived archaea will tell more about possible adaptations of those organisms to the ecological conditions in different sponges.

1.7 Aims of this study

The overall goal of this study was to investigate the diversity, activity and genomic potential of ammonia oxidizing archaea in cold-water sponges of the North Atlantic and to compare it to their bacterial counterparts, the ammonia oxidizing bacteria (AOB). Nitrification in cold-water sponges has not been investigated before and thus these data should allow for comparison with temperate and tropical environments. The four sponges from the mesopelagic zone of the Norwegian Sea exhibit different morphologies which allows to relate differences in abundance, phylogeny and activity of nitrifyers to chemoclines interrelated with the diffusion boundary layer.
The analyses included physiological tests and molecular and phylogenetic investigations as well as the use of in depth metatranscriptomics to study archaeal and bacterial activity in the frame of the complex microbiota of the high microbial abundance (HMA) sponge *Geodia barretti.*

The complete analysis and results are presented in the following three chapters, which represent 3 manuscripts, one of which has been published

(Hoffmann *et al*., 2009) and two have been submitted for publication on Environmental Microbiology and ISME Journal, respectively.

The first manuscript, entitled "Ammonia-oxidizing archaea as main drivers of nitrification in cold-water sponges" is under review in Environmental Microbiology and describes the occurrence and abundance of potential ammonia oxidizing archaea (AOA) in four morphologically different cold-water sponges from the mesopelagic zone of the Norwegian coast. Nitrification rates in these sponges were determined in laboratory incubations. Phylogenetic analyses confirmed the presence of specific populations of nitrifying microorganisms in the sponge mesohyl, which were either affiliated to groups previously detected in marine sponges or consisted of typical inhabitants of cold and deep water environments. AOA outnumbered their bacterial counterparts as measured by quantitative PCR analyses on DNA and cDNA of the functional gene *amo*A. Fluorescence *in situ* hybridizations (FISH) confirmed their high abundance in the sponge mesohyl and showed spacial distribution of archaeal cells in the sponge *Phakellia ventilabrum*. Our measurements allowed the calculation of cell-specific nitrification rates, which were in the range of planktonic archaea.
(Own contribution: RR sampled sponges, performed incubation experiments with *Phakellia ventilabrum*, *Antho dichotoma* and *Tentorium semisuberites*, quantitative PCR, FISH on *Geodia barretti*, gene library construction, phylogenetic analyses and wrote most of the manuscript)

The second manuscript, published in Environmental Microbiology, is entitled "Complex nitrogen cycling in the marine sponge *Geodia barretti"* describes the quantification of both aerobic (nitrification) and anaerobic (denitrification, anammox) microbial processes of the nitrogen cycle in the sponge *G. barretti* by stable isotope techniques and the identification of microbial taxa possibly involved in those processes by molecular techniques. Thaumarchaeota and nitrite-oxidizing bacteria of the genus Nitrospira were detected based on functional gene and 16S rRNA gene analyses as well as

potentially denitrifying Betaproteobacteria and Planctomycetes, which might be responsible for the anaerobic processes we measured. This was the first proof of anammox and denitrification in marine sponges.
(Own contribution: RR did PCR amplification, gene library preparation and phylogenetic analyses of archaeal *amo*A and bacterial *nir*S genes and wrote parts of the paper)

The third manuscript is entitled "Metatranscriptomics of the marine sponge *Geodia barretti*: Tackling phylogeny and function of an uncharacterized microbial community" and describes a metatranscriptomic approach of the microbiota in *G. barretti*, based on the simultaneous analysis of rRNA and mRNA. Analyses of sequence tags assigned to small subunit rRNA resulted in a detailed qualitative and quantitative community profile, dominated by Chloroflexi, Poribacteria and Acidobacteria, which was different from that obtained in the bacterial clone library produced from the same nucleic acid preparation. Optimized assembly strategies of the metatranscriptome data allowed the reconstruction of full-length rRNA genes and consequently, detailed phylogenetic studies of the dominant phylotypes. Genes encoding key metabolic enzymes of the nitrification process were among the most abundant mRNAs, in particular those from ammonia oxidizing archaea. A number of concomitantly transcribed archaeal genes with unknown function pointed to their putative role in the energy metabolism of these archaea.
(Own contribution: RR sampled sponges, prepared cDNA for sequencing, analyzed data, constructed cDNA 16S gene libraries and wrote the manuscript draft)

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2 AMMONIA-OXIDIZING ARCHAEA AS MAIN DRIVERS OF NITRIFICATION IN COLD-WATER SPONGES

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2.1 Abstract

The association of archaea with marine sponges was first described 15 years ago and their role in the nitrification process in mediterranean and tropical sponges has been suggested. Here we explore the occurrence and abundance of potential ammonia oxidizing archaea (AOA) in four morphologically different cold water sponges from the mesopelagic zone of the Norwegian coast, and relate them to nitrification rates determined in laboratory incubations. Nitrification rates up to 1880 nmol N cm^{-3} day^{-1} were observed; i.e. comparable to those measured in mediterranean sponges. Furthermore, a high abundance of archaeal cells was determined by fluorescent *in situ* hybridizations and quantitative PCR, targeting archaeal *amo*A genes (encoding the alpha subunit of ammonia monooxygenase). *Amo*A genes as well as *amo*A transcripts were either only detectable from archaea or were orders of magnitudes higher in abundance than their bacterial counterparts. Phylogenetic analyses of AOA and bacterial nitrite oxidizers (genus *Nitrospira*) confirmed the presence of specific populations of nitrifying microorganisms in the sponge mesohyl, that were either affiliated to groups detected earlier in marine sponges or were typical inhabitants of cold and deep water environments. AOA cells in sponge mesohyl occur in very high density while exhibiting cell-specific nitrification rates comparable to planktonic organisms (estimated 0.6 to 6 fmol archaeal $cell^{-1}$ day^{-1}). Our results identify AOA as the major drivers of nitrification in cold-water sponges, and indicate an important role of these archaea for nitrogen cycling in ocean regions with high sponge biomass.

2.2 Introduction

Sponges have existed for more than 600 million years and are considered to represent the earliest branching lineage of metazoans (Müller, 1998). They are found in diverse morphologies and live in many environments and varying depths of the oceans. Their evolutionary success is likely correlated with their close association with microorganisms, which can make up over half of the sponge body weight (Wang, 2006; Kennedy *et al.*, 2009), and can amount to up to 10^{10} microbial cells per g of sponge (Hentschel *et al.*, 2006). The sponge-associated microbes are phylogenetically highly diverse (reviewed in Taylor *et al.*, 2007) and many of them are presumably involved in metabolic processes that are directly or indirectly beneficial to the sponge. Some bacteria, as e.g. actinobacteria and proteobacteria (reviewed in (Thomas *et al.*, 2010), produce bioactive compounds that serve as defense against predation and biofouling (Thompson *et al.*, 1983; Teeyapant *et al.*, 1993; Ebel *et al.*, 1997) and are of special interest for pharmaceutical industry. Another well-known example of beneficial symbiotic partners is photosynthetic Cyanobacteria that reside in various sponge species and provide fixed organic carbon and nitrogen to their host through their autotrophic life style (Wilkinson and Fay, 1979; Wilkinson, 1983a; Wilkinson *et al.*, 1999; Thacker, 2005). Correspondingly, cyanobacterial symbionts from sponges all over the world are phylogenetically closely related (Taylor *et al,* 2007) and transmitted vertically via sponge larvae (Usher, 2001; Oren, 2005). The symbiosis between nitrogen transforming microorganisms and marine animals is common in corals, sponges, sea urchins and tunicates and has great influence on their host's ecology and nutrient biochemistry (see Fiore *et al.,* 2010 for a review). The release of nitrite and nitrate, indicating microbial nitrification, was discovered in tropical sponges more than 20 years ago (Corredor *et al.*, 1988; Diaz and Ward, 1997). *Aplysina aerophoba*, a Mediterranean sponge showed considerable seasonal fluctuation with ammonia uptake in spring and release in autumn, paralleled by high and low nitrate excretion, respectively (Bayer *et al.*, 2008). More recently, aerobic

nitrification, but also the anaerobic microbial processes denitrification and anaerobic ammonium oxidation (anammox) were detected in *Geodia barretti* a cold-water sponge from the Norwegian Sea (North Atlantic) (Hoffmann *et al.*, 2009). This demonstrated complex nitrogen cycling within the animal. By quantification of nitrification, denitrification and anammox rates, we concluded that sponge-mediated nitrogen mineralization processes might be more important than sediment processes in certain marine environments with high sponge coverage (Hoffmann *et al.*, 2009), e.g. sponge grounds on continental margins (Klitgaard and Tendal, 2004).

The first step of nitrification, aerobic ammonia oxidation - in which ammonia is converted into nitrite - is known from Proteobacteria (Beta-, Gamma- and Deltaproteobacteria), and from Group I archaea, recently renamed Thaumarchaeota (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). Both lineages, AOB (ammonia oxidizing bacteria) and AOA (ammonia oxidizing archaea) have been detected in sponges (Bayer *et al.*, 2007; Meyer and Kuever, 2008; Mohamed *et al.*, 2009; Taylor *et al.*, 2007; Steger *et al.*, 2008), however, their relative abundance has never been assessed. Archaeal ammonia oxidizers have only recently been discovered by metagenomics (Treusch *et al.*, 2005) and cultivation (Könneke *et al.*, 2005). The first genome sequence from this group was obtained from "*Candidatus* Cenarchaeum symbiosum", a symbiont of *Axinella mexicana*, a Californian sponge (Preston *et al.*, 1996). Together with the genome of the cultivated planktonic isolate *Nitrosopumilus maritimus* it demonstrated the genetic potential of these archaea and their fundamental differences to bacteria (Hallam *et al.*, 2006; Walker *et al.*, 2010)

AOA have been shown to be transmitted to the next generation via sponge larvae (Steger *et al.*, 2008), together with potential bacterial nitrite oxidizers (Sharp *et al.*, 2007; Webster *et al.*, 2010). These bacteria, members of the phylum Nitrospira, perform the second step in the nitrification process converting nitrite into nitrate and are commonly found in bacterial gene libraries of marine sponges, forming a sponge-specific subcluster (named Nitrospira IVb) (Taylor *et al.*, 2007; Bayer *et al.*, 2008; Lopez *et al.*, 2008;

Meyer and Kuever, 2008; Webster *et al.*, 2008; Mohamed *et al.*, 2009; Radwan *et al.*, 2009; Kamke *et al.*, 2010). Together, these findings confirm that nitrification might be a common and important process in sponges. The roles and relative contributions of bacterial and archaeal nitrifiers, however, remain unclear.

We focus here on the role of Archaea and Bacteria in nitrification in cold-water sponges by combining nitrification rate measurements and molecular analysis of potential microbial key players. Quantification of archaeal and bacterial ammonia monooxygenase subunit A, the key enzyme for ammonia oxidation on genomic and transcriptional level allowed an assessment of resident and active potential ammonia oxidizers in sponges for the first time. Direct quantification of archaeal cells by CARD-FISH allowed calculation of cell-specific nitrification rates of sponge-associated archaea in one of the investigated species.

Four morphologically different demosponges from the Norwegian coast were included in this study: *Phakellia ventilabrum* (Linnaeus, 1767), *Geodia barretti* Bowerbank, 1858, *Tentorium semisuberites* (Schmidt, 1870) and *Antho dichotoma* (Linnaeus, 1767). *P. ventilabrum* (Halichondrida, Axinellidae) is a thin, fan-shaped sponge (up to 50cm) that lives attached by a narrow stalk on rocks or other solid substrate in current-exposed regions. Former studies revealed the presence of bioactive compounds (Amade *et al.*, 1982) and quorum sensing activity (Krick *et al.*, 2007), but classified this sponge in low-bacterial abundance sponges based on their amount of mid-chain branched fatty acids (Thiel *et al.*, 2002).

Geodia barretti (Astrophorida, Geodiidae) is a large (up to meter-scale), massive sponge, which harbors high numbers of microbes in both aerobic and anaerobic zones (Hoffmann *et al.*, 2005b). Besides considerable rates of nitrification of 566 nmol N cm^{-3} sponge day^{-1} in laboratory incubations (Hoffmann *et al.*, 2009), microbial sulfate reduction (Hoffmann, 2003; Hoffmann *et al.*, 2005b) as well as denitrification and anammox (Hoffmann *et al.*, 2009) were detected in this sponge.

Antho dichotoma, (Poecilosclerida, Microcionidae) is a thin, dichotomously branching sponge (10-30 cm) in which no microbiological investigations have been undertaken so far. *Tentorium semisuberites* (Hadromerida, Polymastiidae) is a small (1-2 cm), cone-shaped sponge, living partly buried in the (deep-sea) sediment (Barthel and Tendal, 1993) or attached to rocks and corals. A distinctive microbial community consisting of bacteria and archaea has earlier been demonstrated in this species (Pape *et al.*, 2006; Queric *et al.*, 2008).

2.3 Material and Methods

2.3.1 Sampling procedure

The four species of sponges were collected in two fjord localities on the Norwegian west coast by the Norwegian research vessel Hans Brattstrøm. Samples were taken at Landrøypynten in Langenuen (59.58.7N 05.22.89E) on June 26th 2007, and from the nearby locality Skorpeodden in Korsfjord (60.10N 05.10.5E) in October 2006, March 2007 and end of October 2007. All samples were collected using a triangular dredge on rocky bottoms at 200-300m depth. The sponge samples were transported to the aquarium facility at the University of Bergen where they were maintained in natural running seawater from 200 m depth. For nucleic acid extraction, sponge samples were immediately cut, rinsed with artificial seawater and plunged in liquid nitrogen on board of the research vessel. Samples were stored at -80°C until nucleic acid extraction. For CARD-FISH (catalyzed reporter deposition - fluorescence in situ hybridisation), sponges were fixed in 2% formalin and *G. barretti* samples for FISH were fixed in 4% paraformaldehyde after collection for 2-12 hours. Sponges were dehydrated in a 30, 50 and 70% ethanol series and stored in 70% ethanol at -20°C.

2.3.2 Nitrification rates

Sponges were maintained for 1 to 5 days in running seawater before experiments started. For the nitrification experiments with *P. ventilabrum* and *A. dichotoma*, 2-5 sponge fragments of 1-2 cm^3 were placed in 900 ml of natural, unfiltered seawater from 200 m depth amended with 10 µM NH_4^+. For incubations with *T. semisuberites*, individual sponge specimen of 1-3 cm^3 each were placed in 600 ml of seawater. *G. barretti* samples were processed and incubated aerobically according to Hoffmann *et al.* (2009).

Three parallel incubations and two controls (sea water without sponge and sponge without ammonia) were used. Beakers with air bubblers and magnetic stirrer were placed in a temperature-controlled room at 15°C in the dark. Water samples were taken every 4 hours over a time course of 24 hours and immediately frozen at -80°C until analyses. Ammonium concentrations were determined with a Scalar Continuous-Flow-Autoanalyzer using the chemistry described by (Grasshoff, 1983). Nitrite and nitrate were analyzed with a chemoluminescence NOx analyzer (Thermo Environmental Instruments Inc, USA).

Experiments were performed in different seasons: October 2006 and March 2007 with *P. ventilabrum* and *T. semisuberites*; July 2007 with *P. ventilabrum*; and November 2007 with *P. ventilabrum, T. semisuberites* and *A. dichotoma*. Production or consumption rates of ammonium, nitrite and nitrate were calculated from the average concentrations of all sponge replicates minus the concentration in the control without sponge sample.

2.3.3 Fluorescence *in situ* hybridisation and quantification of Archaea

Sponge cubes of *P. ventilabrum* and *G. barretti* and whole specimen of *T. semisuberites* were embedded in cryomedium (Jung Tissue Freeze medium®, Leica Microsystems, Nussloch, Germany). After saturation with liquid cryomedium for 12 h at 4°C, the blocks were embedded in base molds with fresh cryomedium and left to solidify for 12h at -80°C. 5-8µm longitudinal

sections were made using a cryostat microtome (HM 505E, Microm, Walldorf, Germany and Leica CM 3050 S for *G. barretti*) at -35°C. Sponge sections were mounted on gelatinized glass slides and stored at -20° C.

For CARD-FISH, the slides were dehydrated in an ascending ethanol series (1 min in 50%, 70%, 2x 99% ethanol each). They were then incubated for 10 min in 0.01M HCl for inactivating of endogenous peroxidases, and washed twice in MilliQ water. Slides were dipped for 30 seconds in 0.1M HCl and washed in MilliQ water and ethanol. CARD-FISH targeting Marine Group I.1.A Crenarchaeota (now Thaumarchaeota) was performed with the oligonucleotide probe CREN554 (Massana *et al.*, 1997): Sequence 5'→3' TTA GGC CCA ATA ATC MTC CT; *E. coli* position number 554-573; 20% Formamid concentration in hybridisation buffer). Hybridization conditions were optimized for CARD-FISH applications by conducting a formamide series at 35°C hybridization temperature. Carboxyfluorescein-labeled tyramide (Invitrogen, Karlsruhe, Germany) was used. For CARD-FISH hybridisation, we followed the standard protocol by (Pernthaler *et al.*, 2002).

After CARD-FISH, sections were treated with 4,6-diamidino-2-phenylindole (DAPI). Color micrographs were taken using a Zeiss AxioImager M1 microscope with an AxiCam MRc camera system. Digital image processing was performed using AxioVision 4.4 software. An ocular with 122 µm x 122 µm-counting grid and scale bar was used for counting the DAPI- and CARD-FISH-stained microbial cells at 1000 times magnification.

For Dope-FISH (Stoecker *et al.*, 2010) the sections were exposed to UV light for 15min to bleach parts of autofluorescence before dehydration with a graded series of ethanol (3 min in 50, 80 and 96% ethanol each). 30 ng of Cy5-labelled EUB-I-III probe (Amann *et al.*, 1995; Daims *et al.*, 1999), and double-Cy3-labelled probes Cren569 (Jurgens *et al.*, 2000) and Arch915 (Stahl and Amann, 1991) each were hybridized and incubated in an isotonically equilibrated chamber at 46°C in hybridization buffer, and 20% formamide concentrations for 3-4h. After hybridization, slides were incubated 10 min in preheated washing buffer (composition corresponds to hybridization stringency) at 48°C, subsequently dipped in ice-cold distilled water and air-

dried. For visualization slides were mounted with the anti-fadent Citifluor (Citifluor Ltd., London, UK) and placed on a LSM 510 scanning confocal microscope (Zeiss).

To distinguish signals resulting from unspecific binding of the probes and autofluorescence, 60ng of double-labelled Non338-probe (Wallner *et al.*, 1993) was applied on a separate well and visualised with the exact same adjustments as for samples with archaeal probes. NonEUB-probes as negative controls were also applied on separate sections following the CARD-FISH protocol.

2.3.4 Nucleic acid extraction

Frozen sponge pieces were quickly cut into approx. 1 mm^3 pieces and parallel extraction of RNA and DNA was performed using a modified version of the Griffiths protocol (Griffiths *et al.*, 2000). Cells of approximately 0.5 g sponge (fresh weight) were solubilized with 0.5 ml of CTAB buffer and PCI by bead beating in a lysing matrix E tube (Q-Biogene) for 45 seconds with at speed of 4.5.

2.3.5 cDNA synthesis for real-time PCR

Diluted nucleic acid extracts were treated with RQ1 DNase (Promega) before reverse transcription of RNA with random hexamer primers (Invitrogen, Superscript III) was performed according to the manufacturer's protocol for first strand cDNA synthesis.

2.3.6 Real-time quantitative PCR

Archaeal *amoA* genes of the four different sponge species (*G. barretti*, *P. ventilabrum, A. dichotoma* and *T. semisuberites*) were quantified on DNA and cDNA level with 1x QuantiTect SybrGreen PCR Kit solution (Quiagen), 1 µM forward primer 19F (5' - ATG GTC TGG CTW AGA CG – 3') (Leininger *et al.*, 2006) and 1 µM reverse primer thaum-amoA-628R (5' - TGG ACA TAC MGR

TGG ATG G - 3') (this study), 0.2 mg ml^{-1} BSA and H_2O up to a final volume of 20 µl for each reaction. Dilution series of the linearized fosmid 14b11 from a metagenomic library of the sponge *P. ventilabrum*, containing the archaeal *amoA* gene, generated a standard curve spanning over six orders of magnitude from 460 to $4.6*10^8$ copies. Cycling conditions were 95°C for 15min and 45 cycles of 95°C for 1min, 50°C for 1min and 72°C for 1min. Reading of fluorescence intensities were measured after each cycle, before and after incubation for 1s at 78°C to avoid measuring potential primer dimerization. A final elongation on 72°C was followed by a melting curve analysis form 30-95°C.

Bacterial *amo*A genes were quantified using 500nM of primers amoA-1F* (Stephen *et al.*, 1999) and amoA-2R (Rotthauwe *et al.*, 1997) and known amounts of linearized plasmids (pCR4-TOPO, Invitrogen) containing the *amo*A gene of *Nitrosospira multiformis* ATCC25196 ranging from 110 to $1.1*10^8$ copies per reaction. Cycling conditions were as described above, but annealing temperature was 55°C for bacterial *amo*A amplification.

All reactions (20µl) were performed in low-profile thermostrips and ultra clear cap-strips (ABgene) in a DNA Engine Opticon2 real-time thermocycler (MJResearch) and analysed with Opticon Monitor software version 3.01 (Biorad). Reactions were performed in triplicate using of 5ng of DNA or 2µl of cDNA per sponge individual respectively. For *G. barretti*, *A. dichotoma, P. ventilabrum* three specimen were analysed, while for *T. semisuberites* only two specimen were available.

2.3.7 Clone library constructions

16S rRNA genes of nitrite-oxidizing bacteria of the phylum Nitrospira were amplified with the universal primer 616V (Juretschko *et al.*, 1998) and the specific primer 1158R (Maixner *et al.*, 2006). Universal 16S rRNA genes of archaea were amplified with primer set 21F and 958R (DeLong, 1992) and archaeal *amoA* genes with primer set 19F (Leininger *et al.*, 2006) and 643R (Treusch *et al.*, 2005). The respective amplicons of three different sponge

specimen were cut out from a low-melting agarose gel and pooled for cloning using the TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Clones with inserts of correct size were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

2.3.8 Phylogenetic analyses

16S rRNA sequences related to Archaea and Nitrospira were edited and analysed using the ARB program package (Ludwig *et al.*, 2004) after proofreading and combined with an updated version of the sponge symbiont database (Taylor *et al.*, 2007). Three phylogenetic treeing methods were employed and compared. Maximum likelihood trees are presented for Thaumarchaeota group I.1.A and the bacterial phylum Nitrospira, which were constructed including relevant reference sequences and closest BLAST 16S rRNA sequences from NCBI. For the archaeal 16S rRNA tree an individual filter was used, including 875 base pairs, for Nitrospira a 50% conservation filter for this phylum was used over the full length of the 16S rRNA. Bootstraps were calculated from 100 maximum parsimony trees. DNA sequences of ammonia monooxygenase subunit A (*amo*A) were also edited and analyzed in ARB. The tree includes closest BLAST hits to sponge derived sequences from NCBI. Maximum likelihood tree was calculated in ARB from 486 nucleotide positions.

Phylogeny and distance matrix for statistical analysis were calculated using the distance matrix algorithm in ARB based on the same alignment as for treeing calculations. The calculation of UniFrac significance and clustering based on phylogenetic distance was done via the FastUniFrac homepage (Hamady *et al.*, 2010), with 1000 permutations, weighted and normalized option.

For operational taxonomic unit (OTU) analysis and downstream calculations all *amo*A and archaeal 16S rRNA sequences over 800 base pairs were used. Analysis was performed via the program mothur (Schloss *et al.*, 2009).

16S rRNA gene sequences, from Archaea and Nitrospira and archaeal *amo*A sequences are available in GenBank under accession numbers JF802616-JF802783.

2.4 Results

2.4.1 Nitrification rates

Nitrite and nitrate production was followed over a time course of 24 hours in laboratory incubation experiments with sponge fragments. Nitrification activity was observed independent of the addition of ammonia (10 µM), indicating that nitrification was not limited by ammonia supply under these conditions.

Table 2.1 shows the production/consumption rates of ammonium, nitrite and nitrate from three sponge species analyzed, as calculated from the average of all sponge replicates during the first 24 hours minus the concentrations of the control incubation without sponge.

Table 2.1. Ammonia, nitrite, nitrate production and nitrification rates[#] in four cold-water sponges

Species	Time of incubation	NH_4^+	NO_2^-	NO_3^-	total nitrification
P. ventilabrum	October 2006	3240	130	380	510
	March 2007	3590	10	110	120
	July 2007	4510	160	1720	1880
	November 2007	4100	-20	360	340
A. dichotoma	November 2007	3810	270	0	270
*G. barretti**	March 2007	0 - 8000	150	410	560

[#] in nmol N cm^{-3} sponge day^{-1}, rates were calculated from incubations of triplicate experiments
* Hoffmann *et al.* 2009

P. ventilabrum showed a constant release of ammonium in all experiments, with highest ammonium excretion in July (Fig. 2.1). In that month, the highest nitrate and nitrite excretion rates were also observed. Nitrite

production was generally lower than nitrate production, and in November 20 nmol nitrite cm^{-3} sponge day^{-1} was consumed.

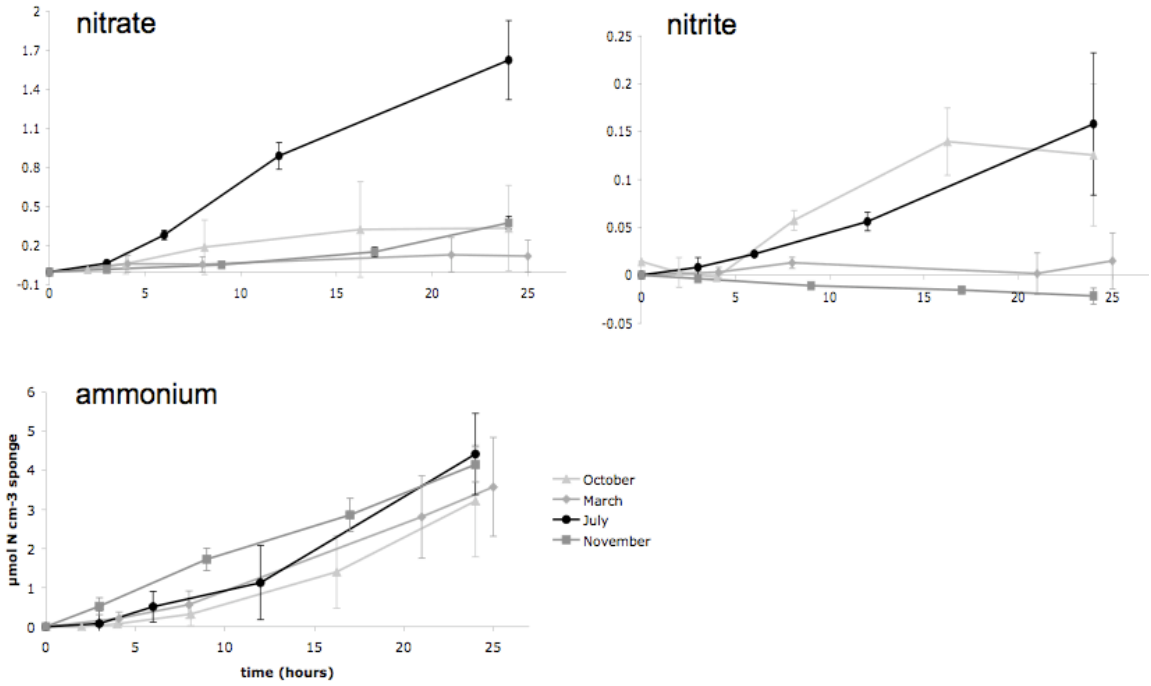

Figure 2.1. Incubation of *P. ventilabrum* fragments with 12 mM NH_4^+: production of nitrate and nitrite and ammonium per cm^3 sponge (as average water concentrations in three replicate incubations minus concentration in control incubation without sponge) at a given time point. Axis labels and months of the different data series as shown for the graph for ammonium production.

Total nitrification, the sum of nitrate and nitrite release, varied considerably and was highest in July with 1880 nmol N cm^{-3} sponge day^{-1} (Table 2.1) and lowest in March, releasing 120 nmol N cm^{-3} sponge day^{-1}.

Incubation experiments with *Antho dichotoma* in November 2007 showed production of ammonia and nitrite within the range of *Phakellia ventilabrum*, but production of nitrate was not observed (Table 2.1).

In earlier experiments nitrate and nitrite production of *G. barretti* was demonstrated to be 410 and 150 nmol N cm^{-3} sponge day^{-1}, respectively, i.e. similar to that of *P. ventilabrum* in July and October. Only ammonium release was different, as consumption exceeded production in the first hours, while after that, net production of ammonium was observed (Hoffmann *et al.*, 2009).

Incubation experiments with *T. semisuberites* did not reveal reproducible results. In October 2006, high nitrate production (4530 nmol N cm^{-3} sponge

day^{-1}) was observed, while during following experiments in March and November 2007, no difference between incubation experiments and seawater control was detectable.

2.4.2 Quantification of archaeal and bacterial *amo*A genes and transcripts

Specific real-time PCR assays were employed to test and compare the abundance and activity of ammonia monooxygenase-encoding archaea and bacteria in the sponges.

Highest copy numbers of *amo*A genes were found in *G. barretti* with 6*10^{8} copies per µg of nucleic acids and approx. 4*10^{11} copies g^{-1} sponge fresh weight, while bacterial *amo*A genes were below the detection limit (10 copies per reaction).

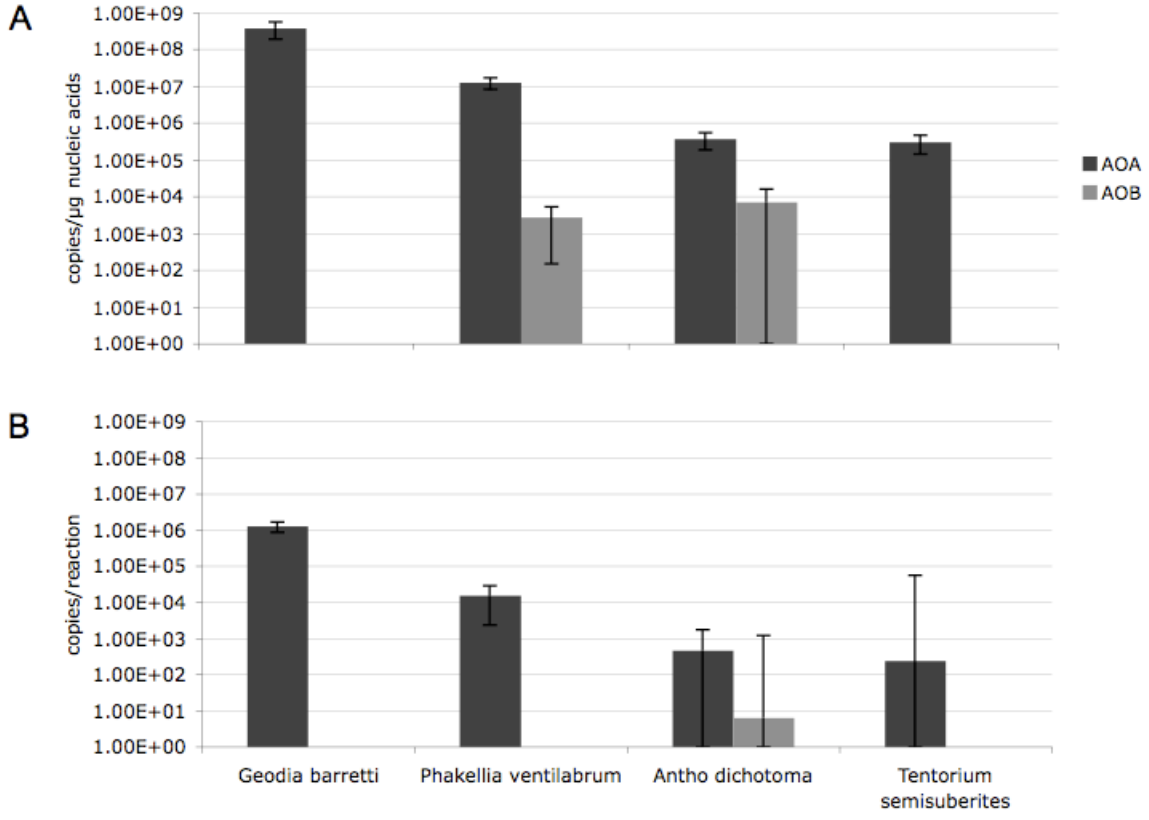

Figure 2.2. Abundance of *amo*A genes (a) and transcripts (b) as estimated by qPCR on four cold-water sponges. Biological replicates were: n=3 for *G. barretti, P. ventilabrum* and *A. dichotoma* and n=2 for *T. semisuberites*. Three technical replicates per sponge individual were performed.

P. ventilabrum exhibited $1.5*10^7$ copies per µg of nucleic acids (approx. $5*10^9$ copies g^{-1} sponge fresh weight) but only $5*10^3$ bacterial *amo*A gene copies per µg of nucleic acids ($9.7*10^4$ copies g^{-1} sponge fresh weight). In *A. dichotoma*, *amo*A genes of archaea were considerably lower, but still about 150x more abundant than *amo*A of bacteria. Only archaeal *amoA* genes were detected in *T. semisuberites* (Fig. 2.2a). In line with the high numbers of archaeal *amo*A genes found in the mesohyl of all four sponges, highest mRNA levels were also detected for archaea, while no AOB transcripts were detected in *G. barretti, P. ventilabrum* and *T. semisuberites* (Fig. 2.2b). The relative amount of archaeal *amo*A transcripts between the different sponges correlated well with the relative abundance of *amo*A genes, indicating that the majority of archaea detected are indeed actively transcribing this marker gene.

2.4.3 Phylogenetic analyses of archaea from cold water sponges

16S rRNA genes of archaea from the four sponges were amplified by PCR, cloned and sequenced for phylogenetic analyses. In total, 85 sequences (between 14 and 38 sequences for each sponge) were obtained, of which 79 were included in operational taxonomic unit (OTU) calculations because of their length and quality. The sequences were grouped based on 97 and 99% sequence similarity respectively, leading to 2 and 5 OTUs respectively (see Fig. 2.3 with OTUs on 99% threshold). Phylogenetic analysis shows a low diversity of archaeal 16S rRNA sequences, restricted to the Thaumarchaeota group I.1A, all of which supposedly represent ammonia oxidizers. *G. barretti* and *P. ventilabrum* exhibited highest diversity on 16S rDNA level, with a fraction of clones (OTU4) included in the open sponge specific cluster defined by "*Candidatus* Cenarchaeum symbiosum". All 16 archaeal 16S rDNA sequences of *A. dichotoma* were identical and clustered in a phylogenetic group similar to most of the sequences of *G. barretti* and *P. ventilabrum*. Those sequences clustered in OTU1 with 99% sequence identity and showed highest sequence similarity to marine clones from cold- and deep-sea regions.

16S rDNA sequences amplified from *T. semisuberites* (TS13 and OTU5) and one sequence derived from *P. ventilabrum* (PV8) showed closest affiliation to Antarctic sponge-derived and (hydrothermal) deep-sea clones, whereas OTU3 - including sequences from *G. barretti* and *P. ventilabrum* - was affiliated to marine sediment and hydrothermal vent clones.

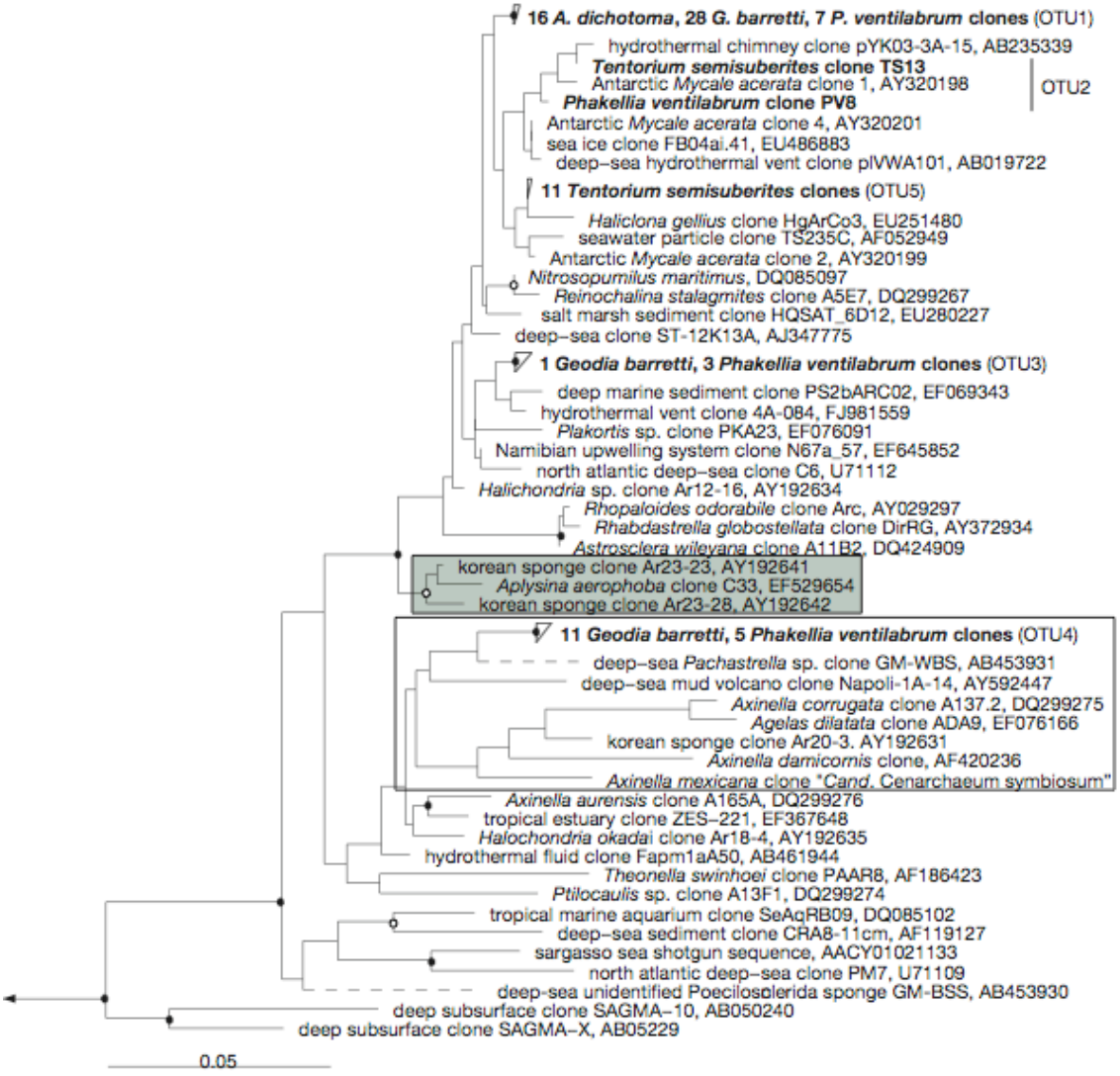

Figure 2.3. Maximum likelihood tree of archaeal 16S rRNA sequences derived from cold-water sponges (shown in bold), belonging to the phylum Thaumarchaeota, group I.1A. Short sequences (under 1000 base pairs) were added using the parsimony insertion tool in ARB after tree calculation and are indicated with a dashed line. The sponge-specific sequence clusters (see Taylor *et al.*, 2007) include only sponges or sponges and one non-sponge derived sequence and were reproduced with three different treeing methods. Bootstrap values, derived from maximum parsimony algorithm, are shown by closed (>97%) and open circles (>75%). The scale bar indicates 10% 16S rRNA sequence divergence. The outgroup (not shown) consisted of 16S rRNA sequences of group I.1B Thaumarchaeota.

The highest similarity to the 16S rRNA gene of the only cultivated marine ammonia-oxidizer *Nitrosopumilus maritimus* was 98.3-98.6% (OTU1 and

OTU5) and sequences of all four sponges were 93.5-94.4% similar to the 16S rDNA sequence from the Californian sponge *Axinella mexicana*, "*Cand.* C. symbiosum".

Coverage calculations (Good, 1953) for OTUs with 99% threshold ranged from 90 to 100%. Clustering on 3% sequence difference led to coverage of 100% for all four archaeal 16S rRNA gene libraries (suppl. material Figure S2.1).

Sequences from *A. dichotoma* and *T. semisuberites*, and from *G. barretti* and *P. ventilabrum* respectively, showed clustering with 95.5 and 71% jackknife confidence in clustering analyses based on phylogenetic distances (see suppl. material Figure S2.2).

The presence and diversity of archaea potentially involved in ammonium oxidation was also investigated by targeting the *amo*A gene. Out of a total of 49 cloned genes 48 (22 from *T. semisuberites*, 17 from *A. dichotoma*, 9 of *P. ventilabrum*) were included in statistical analysis. Clustering nucleotide sequences on 5% distance threshold resulted in 5 operational taxonomic units (OTUs) and clustering on 1.5% amino acid level led to 4 comparable OTUs. The coverage estimation for *amo*A gene sequences from all sponges investigated was between 88.9 and 100% on 5% threshold on nucleotide level.

Figure 2.4 displays a phylogenetic tree based on *amo*A gene sequences (nucleotides) of the investigated cold-water sponges, and other related sponge-derived *amo*A gene sequences and seawater sequences from different locations. The sequences of archaeal *amo*A genes showed around 83% similarity to the respective sequence of *N. maritimus* with one clone of *P. ventilabrum* (PVamo19) showing 92% nucleotide sequence identity (98% on protein level) to this ammonia-oxidizer. Sequence identities to "*Candidatus* C. symbiosum" were highest for *P. ventilabrum* and *G. barretti* with 78.4-80% on DNA and 90-94.4% on protein level while the other sponges had an *amo*A gene sequence similarity of around 76%.

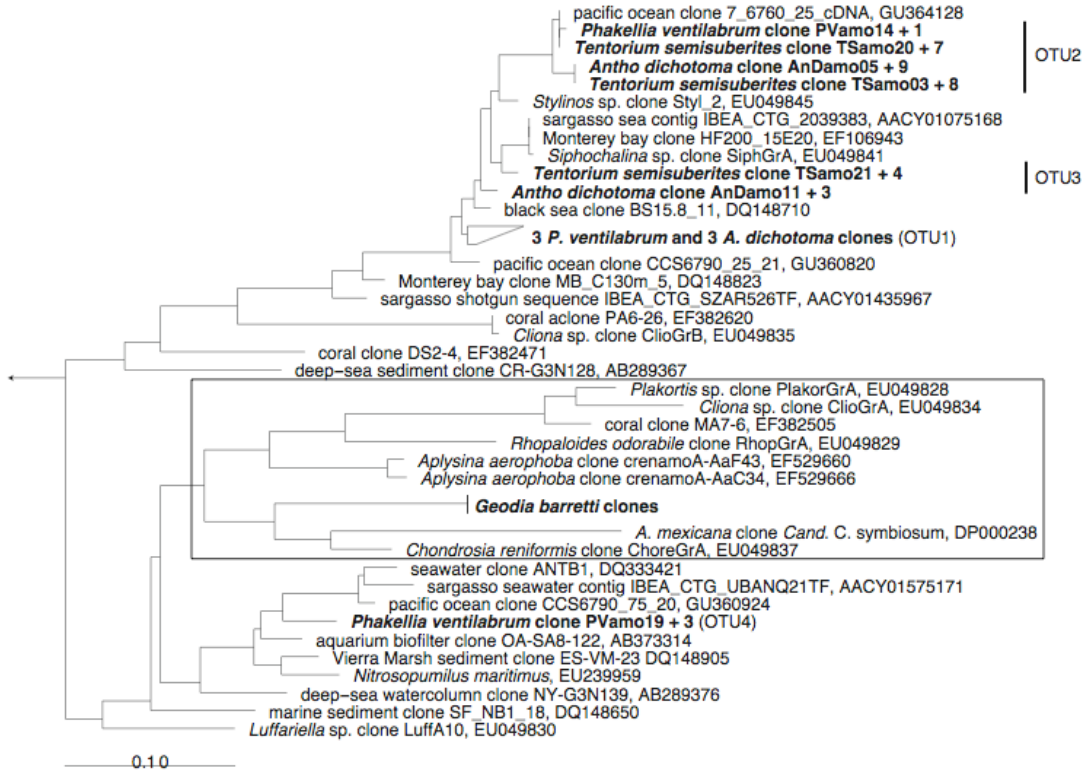

Figure 2.4. Maximum likelihood tree showing the phylogenetic diversity of archaeal *amo*A sequences from cold-water sponges (bold). 486 nucleotide positions were included in phylogenetic treeing calculation. The open invertebrate-specific sequence cluster (see Taylor *et al*., 2007) includes sponges and one coral and was reproduced with three different treeing methods. The scale bar indicates 10% sequence divergence. The outgroup (not shown) consisted of *amo*A sequences of soil organisms.

2.4.4 16S rRNA genes of nitrite oxidizing bacteria (NOB)

To assess the presence and diversity of NOB in cold-water sponges, 16S rRNA gene libraries with primers specific for the phylum Nitrospira were constructed. In total, 34 clone sequences (13 from *P. ventilabrum*, 11 from *A. dichotoma* and 10 *T. semisuberites* clones) were sequenced and phylogenetic analysis showed the affiliation of sequences to the sponge-specific cluster in the phylum Nitrospira (Fig. 2.5) in proximity to the marine isolate *Nitrospira marina*. NOB received from *G. barretti* had 87.3-91.4% similarity to *N. marina* (Hoffmann *et al*., 2009), whereas sequences of *A. dichotoma* were 88.7-93.2%, *P. ventilabrum* were 90.8-92.8% and *T. semisuberites* were 87.3-97.6% identical to this nitrite oxidizing isolate.

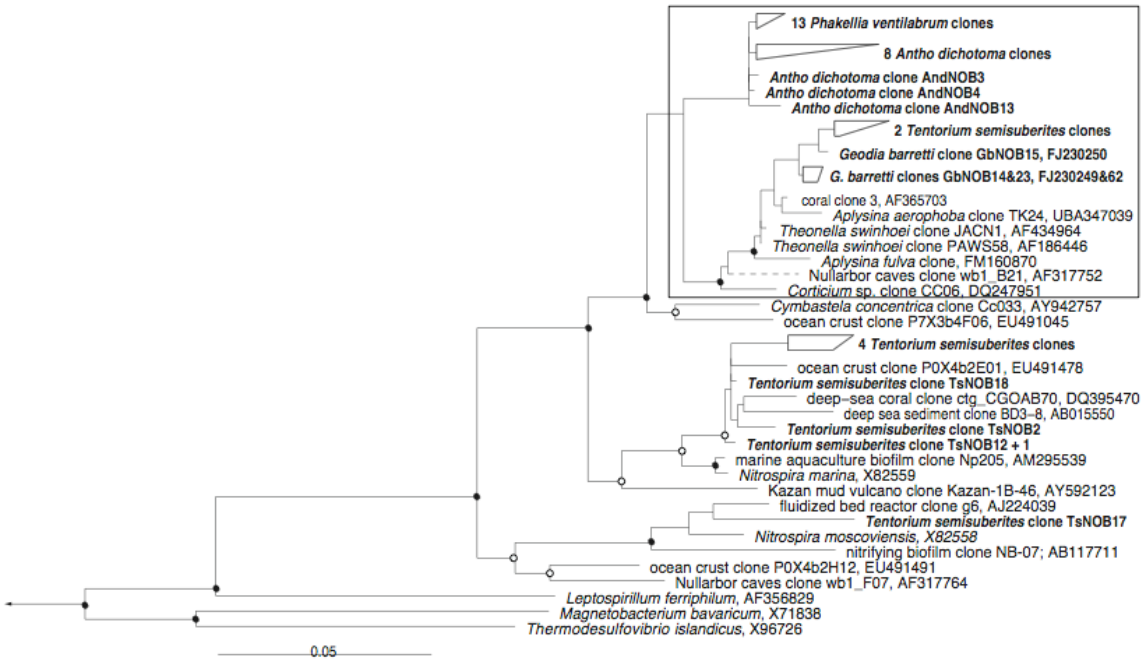

Figure 2.5. Maximum likelihood tree showing the distribution of 16S rRNA gene sequences of the phylum Nitrospira from cold water sponges. Sponge-derived sequences from this study and Hoffmann *et al.* (2009) are highlighted in bold. Short sequences (under 800 base pairs) were added using the parsimony insertion tool in ARB after tree calculation and are indicated with a dashed line. The open invertebrate-specific sequence cluster (see Taylor *et al.*, 2007) includes sponges and one coral and was reproduced with three different treeing methods. Filled circles indicate at least 90% bootstrap support and open circles indicate 75-89% parsimony-bootstrap support. The scale bar indicates 5% 16S rRNA sequence divergence. The outgroup (not shown) consisted of 16S rRNA sequences of several other bacterial phyla.

In the clone library of *T. semisuberites* some clones showed up to 94.2% similarity to *Nitrospira moscoviensis* whereas the other cold-water sponges had max. 89.5% similarity to this isolate. *N. moscoviensis* stems from a corroded iron pipe in Moscow (Ehrich *et al.*, 1995) but is also found in waste water treatment plants (nitrifying fluidized bed reactor).

2.4.5 Fluorescence *in situ* hybridisation of archaea in cold-water sponges

The distribution and abundance of archaea were investigated on the cellular level in three sponges using FISH (*G. barretti*) and CARD-FISH (*P. ventilabrum*, *T. semisuberites*). *A. dichotoma* was not included in the study because of the dense occurrence of autofluorescent spicules. *G. barretti* showed archaeal signals evenly distributed in the sponge mesohyl of all

specimen investigated. The dense background of bacterial signals (blue in Fig. 2.6a) reflects the high microbial biomass in the mesohyl of *G. barretti* making total cell counts impossible. The archaeal cells were relatively small (approximately 1-2 µm long) and their morphology resembled *N. maritimus* (Könneke *et al.*, 2005) and the sponge symbiont "*Cand.* C. symbiosum" (Preston *et al.*, 1996). No spatial distribution of archaeal cells was observed in *G. barretti*. However, the sponge surface is enriched in highly auto-fluorescent spicules and was therefore not specifically analysed.

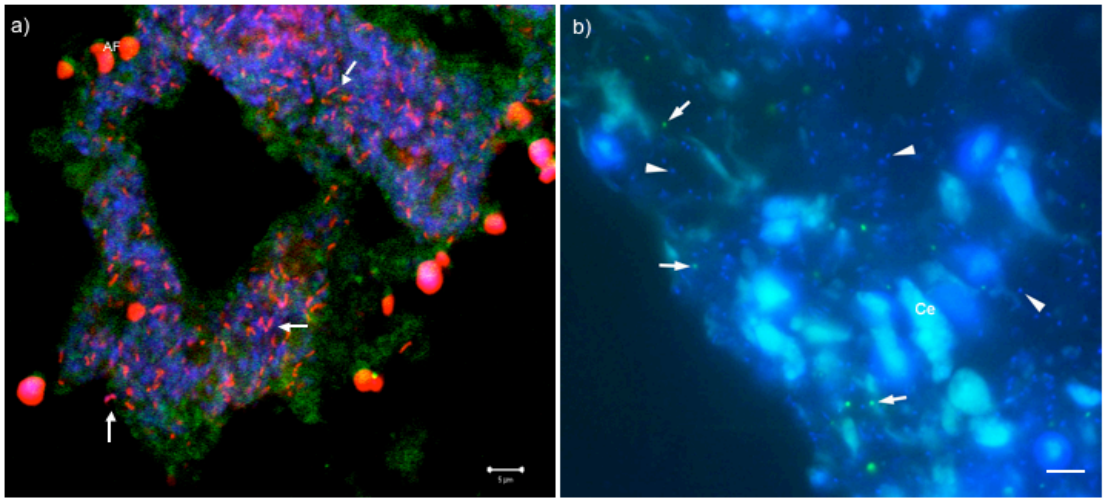

Figure 2.6. Fluorescence *in situ* hybridisations of *Geodia barretti* (a) Group I thaumarchaea in the mesohyl of the sponge (small red dope-FISH signals, arrow), the mesohyl is green fluorescent and bacterial signals (EUB) are shown in blue. Bigger red/yellow signals are autofluorescence (AF), i.e. also detected with a nonsense probe (not shown) and (b) *Phakellia ventilabrum*, Group I thaumarchaeota (green CARD-FISH signals, arrows) represent approx. 10% of total microbial cells (blue DAPI signals, arrowheads) in the uppermost layer beneath the sponge surface, but are more sparse further away from the sponge surface (towards the upper right corner of the image). Sponge cell = Ce. Note that *G. barretti* is classified as sponge with high-microbial abundance (HMA) and therefore harbors more bacteria and archaea than *P. ventilabrum* (low microbial abundance sponge, LMA). The bars represent a distance of 5 µm.

While no clear archaeal CARD-FISH signals were obtained in tissues of *T. semisuberites*, archaea were detected in all 5 investigated specimen of *P. ventilabrum*. They were found unevenly distributed in the sponge mesohyl (Fig. 2.6b), with different abundances in the different layers: In the uppermost 50 µm beneath the sponge surface, archaeal numbers were up to 3 x 10^9 cells cm^{-3} sponge, which represents approx. 10% of the total microbial cells stained by DAPI (3 x 10^{10} cells cm^{-3} sponge) (Fig. 2.6b). At greater

distance from the sponge surface, archaeal signals were less frequent with approximately 2 x 10^7 cells cm^{-3} sponge, which represented only 0.1% of DAPI counts. This means that the archaea were approximately 100x more abundant in the outer 50 µm of the sponge compared to the interior.

2.5 Discussion

2.5.1 High abundance and activity of archaeal ammonia oxidizers in cold-water sponges

A prevalence of archaeal versus bacterial *amoA* genes and transcripts (Fig. 2.2) indicated that ammonia-oxidation in cold-water sponges should be mostly due to the activity of archaea. On the DNA level, archaeal *amo*A genes were either the only *amo*A genes detectable or were orders of magnitude more abundant than bacterial *amo*A. Archaeal *amo*A was actively transcribed in all sponges, while bacterial *amo*A transcripts were only found in the detectable range in *Antho dichotoma*. Only in this latter sponge both groups of ammonia oxidizers might indeed be active.

Highest gene copy numbers (2-4*10^{11} g^{-1} sponge wet weight) were found for *Geodia barretti*, a sponge with active ammonia oxidizing archaeal communities as confirmed in a metatranscriptomic study (Radax *et al.*, submitted). This number is 5 to 7 orders of magnitude higher than in Atlantic ocean waters (Karner *et al.*, 2001; Wuchter *et al.*, 2006; Mincer *et al.*, 2007), and even 10^8 times higher than in the water column of the Eastern Mediterranean Sea (De Corte *et al.*, 2009), highlighting the high density of microbes in sponges, and thus their potential influence on the marine ecosystem in areas with high abundance of these animals.

The relative number of AOA was determined to be about 10% of the total microbial cell count in the outer cortex of *Phakellia ventilabrum,* indicating that also in this (low microbial abundance) species the ammonia oxidizers represent a considerable fraction of the sponge microbiota.

2.5.2 Nitrification rates of cold-water sponges

Nitrification rates in *P. ventilabrum* varied greatly between the different experiments from 120 nmol cm^{-3} sponge day^{-1} in March to 1880 nmol cm^{-3} sponge day^{-1} in July, while ammonium production appeared to be relatively stable (around 4000 nmol cm^{-3} sponge day^{-1}). In all experiments with *P. ventilabrum*, nitrate production exceeded nitrite production; in November, nitrite was even consumed. The higher rates of both nitrification and ammonium production in summer in *P. ventilabrum* could be explained by a higher metabolic activity caused by increased food availability in deep waters, due to export production from the photic zone. Temperature is stable throughout the year at the sponge collection sites and also for all incubation experiments, and can therefore not explain the observed differences. However, more seasonal studies on cold-water sponges would be needed to confirm the observed trends.

In all our incubation experiments throughout the year, sponges produced excess ammonium and only a small proportion of it was oxidized by nitrification. We therefore conclude that nitrification in cold-water sponges is never limited by the amount of available ammonium. In contrast to our study, Bayer and colleagues observed clear seasonal trends in the ammonium metabolism of *Aplysina aerophoba*, a Mediterranean shallow-water sponge (Bayer *et al.*, 2008). While nitrification rates were mostly stable in all seasons, net ammonium consumption was observed in April. In ammonia stimulation experiments an up to 4-fold increase in nitrate production was observed indicating that nitrification in this Mediterranean sponge is limited by the availability of ammonium in spring, while it seems available in excess later during the year.

Table 2.2 shows that nitrification rates of cold-water sponges are comparable to those of other sponge species investigated so far. While the highest rates of nitrification of tropical sponges exceeded rates of cold-water sponges, their lower rates are also within the range of those observed in our study. Notably, most of the studies – except Hoffmann *et al.* (2009) and Schläppy *et al.*, (2010) – did not take into account possible nitrate loss by sponge denitrification. The

rates reported here are net nitrification rates; the unconsidered factor of denitrification may explain some of the observed variability of nitrification rates in the sponges, but in any case might rather lead to an underestimation. Interestingly, comparable nitrification rates were observed in another marine invertebrate, an ascidian colony from the Mediterranean Sea, in which archaea were also found. (Martinez-Garcia *et al.,* 2008)

Table 2.2. Overview of nitrification rates from different sponge species investigated and compared to seawater, sediment and another invertebrate

Sponge species	Marine area, depth	nmol N cm^{-3} day^{-1}	nmol N g^{-1} dry wt h^{-1}	Reference
P. ventilabrum, Antho dichotoma, Geodia barretti	Norwegian coast, 200-300m	120-1880	24 - 376	this study, (Hoffmann *et al.*, 2009)
Chondrosia reniformis, Dysidea avara	Mediterranean Sea, shallow	211 - 360		(Schläppy *et al.*, 2010)
Aplysina aerophoba	Mediterranean Sea, 2-15m		89 - 344	(Bayer *et al.*, 2008)
Agelas oroides, Chondrosia reniformis, Ircina oros, Aplysina aerophoba, Axinella polypoides	Mediterranean Sea, 10-20m		180 - 780	(Jimenez and Ribes, 2007)
Pseudoaxinella zeai, Chondrilla nucula, Plakortis halichondroides	Carribean sea, shallow		30 - 2640	(Diaz and Ward, 1997)
Chondrilla nucula, Anthosigmella varians	Carribean sea, shallow		19 - 600	(Corredor *et al.*, 1988)
Seawater	Southern Californian coast	0.04 (*AO)		(Ward *et al.*, 1982)
	Monterrey bay, 13°C	0.021-0.074		(O'Mullan and Ward, 2005)
	North pacific	0.001-0.137 (*AO)		Dore and Karl, 1996
	North pacific	0.028-0.103 (*AO)		Beman, 2011
Ascidian colony	tunic layer actively nitrifying	240-2160		(Martinez-Garcia *et al.*, 2008)
Estuarine sediment			0.02-7.7	(Bernhard *et al.*, 2007)

*AO = ammonium oxidation rates

In line with lower observed cell counts, reported nitrification rates in seawater and sediments are orders of magnitudes lower than rates measured from sponges (see Table 2.2). They range from about 0.001 to maximally 0.14 nmol N ml^{-1} day^{-1} (Ward *et al.*, 1982; Dore and Karl, 1996; O'Mullan and Ward, 2005; Beman *et al.*, 2010).

2.5.3 Phylogeny of microbes involved in nitrogen cycling indicates the presence of distinct ecotypes in sponges

Microorganisms most probably responsible for nitrification processes in the investigated sponges were thaumarchaeota and members of the bacterial phylum Nitrospira.

Archaea were affiliated to group I.1A, forming at least one sponge-specific sequence cluster based on 16S rRNA phylogeny (boxed in Fig. 2.3). Notably, in particular those sponges that exhibited highest nitrification rates and transcriptional activity, i.e. *P. ventilabrum* and *G. barretti* contained phylotypes of this sponge cluster. These organisms might be particularly adapted to life in the sponge habitat, e.g. to higher ammonia concentrations. Highest transcriptional activity of AOA of this cluster was observed in a metatranscriptomic study (Radax *et al.*, in review). Additionally, all sponges contained archaeal phylotypes that were related to sequences from deeper and colder waters (upper part of tree in Fig. 2.3) indicating cold-adapted ecotypes. Nitrite-oxidizing bacteria of *G. barretti, P. ventilabrum*, *A. dichotoma* and some clones of *Tentorium semisuberites* were related to the earlier identified sponge-specific sequence cluster with other sponge-derived NOBs (reviewed in Taylor *et al.*, 2007). Different from all other sponges *T. semisuberites* showed additional 16S rRNA sequences more closely related to *N. moscoviensis* and other sludge or sediment-derived sequences. In this sponge, there might be a variable microbial community due to microbe exchange with the surrounding sediments (Queric *et al.*, 2008) as the sponge exhibits a partly buried lifestyle. Unlike earlier studies of this sponge from the Arctic in which the presence of high amounts of archaea were suggested

(Pape *et al.*, 2006), we found little archaeal presence in *T. semisuberites* and also un-reproducible nitrification rates. The microbial community of this species might be unstable and might change in composition and activity between different individuals, between specimen from different sampling sites or even within the same specimen over time.

The quantitative dominance of *amo*A-encoding archaea over AOB in marine sponges with high nitrification activity has not been shown before. This successful symbiosis might be due to the adaptation of archaea to environmental constraints that their bacterial counterparts can possibly not cope with, like fluctuating oxygen and nutrient (ammonia) concentrations. Ammonium concentration in seawater may vary, dependent on the region and anthropogenic input in coastal areas, but are generally low. While we measured low ammonium concentrations (<500 nM) in the water column of the sampling site, high ammonium production rates (4000 nmol cm^{-3} sponge day^{-1}, see Table 2.1.) might cause high ammonium accumulation within the sponge, especially in phases of non-pumping (Hoffmann *et al.*, 2008). *N. maritimus*, an ammonia-oxidizing isolate of the Thaumarchaeota group I.1A, shows inhibited growth above 2 mM (=2000 nmol cm^{-3}) (Martens-Habbena *et al.*, 2009) and "*Candidatus* Nitrososphaera gargaensis", a thermophilic ammonia-oxidizer is partially inhibited from 3080 nmol cm^{-3} on (Hatzenpichler *et al.*, 2008). This might hint towards the presence of a specialized group of Thaumarchaeota in marine sponges, which are able to tolerate fluctuating ammonium concentrations.

2.5.4 Spatial distribution of archaea in *P. ventilabrum*

The abundance of Thaumarchaeota in *P. ventilabrum* was up to 100x higher in the uppermost 50 µm beneath the sponge surface than further into the interior (Fig. 2.6.b). The observed pattern might indicate that the balance between ammonium and oxygen availability is most favorable for archaea in the outermost 50 µm of this sponge. This may be especially important in

situations when the animal is not pumping water: in non-pumping sponges, oxygen is only available in the uppermost layer of the sponge due to diffusive flux over the sponge surface (Hoffmann *et al.*, 2005a; Hoffmann *et al.*, 2008). Due to the lack of ventilation, ammonium concentration may become too high inside the body of non-pumping sponges due to extensive ammonium excretion by the sponge cells (see above).

2.5.5 Quantification of archaea and cell-specific nitrification rates in *P. ventilabrum*

Assuming an average thickness of 1 mm of *P. ventilabrum*, the uppermost 50 µm of a given sponge section would represent 10% of the sponge volume. An average archaeal number in *P. ventilabrum* can therefore be calculated from the CARD-FISH counts:

$2*10^7$ cells cm^{-3} *0.90 + $3*10^9$ cells cm^{-3} * 0.1 = $3.2*10^8$ cells cm^{-3} sponge.

An average archaeal number can also be calculated from qPCR results of AOA *amo*A g^{-1} wet weight (Table S2.1.), assuming a single *amo*A gene copy per genome and cell (Hallam *et al.*, 2006; Walker *et al.*, 2010) and that 1.2 g wet weight corresponds to 1 cm^3 sponge (see above).

This reveals an average archaeal number of $4 * 10^9$ cells cm^{-3} sponge, i.e. roughly 10-fold higher than the results obtained by FISH counts. However, it should be noted that very small portions of sponge were used for the DNA preparation and these were preferably taken from the outermost regions of the sponge body that contains more archaeal cells. Another explanation could be the different time of sampling for the two analyses: samples for CARD-FISH were taken in October, while samples for qPCR were taken at the end of July – in a season where nitrification rates were 10x higher than in the rest of the year. It is therefore also possible that archaeal numbers are 10x higher in summer.

For calculating cell-specific nitrification rates, we used the average numbers

obtained by CARD-FISH counts of 3.2 *10^8 cells cm^{-3} sponge. The lowest nitrification rate measured in March (0.12 µmol N cm^{-3} day^{-1}) thus corresponds to a specific nitrification rate of 0.38 fmol N archaeal $cell^{-1}$ day^{-1}, while the highest nitrification rate obtained in July (1.88 µmol N cm^{-3} day-1) obtains a specific nitrification rate of 6 fmol N archaeal $cell^{-1}$ day^{-1}. Assuming a 10x higher number of archaea in summer, as *amo*A gene quantification might indicate, specific nitrification rates in July would be around 0.6 fmol N archaeal $cell^{-1}$ day^{-1}.

Cell-specific nitrification rates in enrichment cultures of planktonic AOA were reported to be 2 and 4 fmol NH_3 per archaeal cell day^{-1} at 22 and 25°C respectively (Wuchter *et al.*, 2006) and in pure cultures 12.7 fmol N per archaeal cell day^{-1} (0.53 fmol N per *N. maritimus* cell $hour^{-1}$, Martens-Habbena *et al.*, 2009). An *in situ* estimation of archaeal nitrification rates of in the North Sea was maximally 7 fmol of NH_3 $cell^{-1}$ day^{-1} (Wuchter, 2006), but might include sedimentary nitrification. It can therefore be concluded that the estimated activity of AOA in cold-water sponges at 13°C is similar to or slightly below AOA in seawater at higher temperatures.

2.6 Conclusions

Our study indicates that archaea are the major drivers of ammonia oxidation and thus of nitrification in marine cold-water sponges and show high specific nitrification rates. Since sponges have been identified as important contributors to marine nitrogen cycling (Wilkinson, 1983b; Corredor *et al.*, 1988; Diaz and Ward, 1997; Hoffmann *et al.*, 2009; Mohamed *et al.*, 2009), sponge AOA may play an important and so far unrecognized role for nitrogen cycling in ocean regions with high abundance and biomass of sponges in both warm and cold waters. This contribution should be considered in current ocean biogeochemistry models.

2.7 Acknowledgements

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2.9 Supplemetary Figures

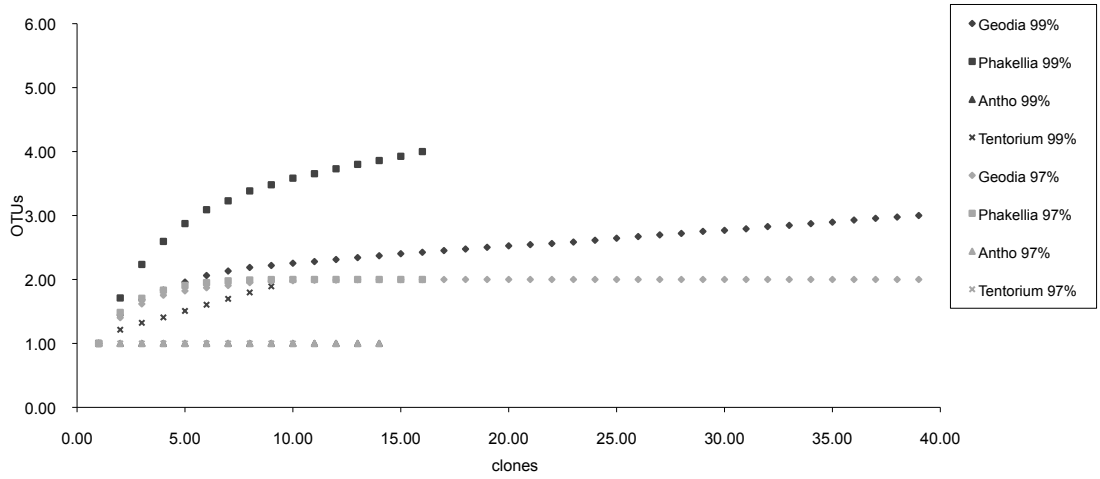

Figure S2.1. Rarefaction curves of 16S rDNA clone libraries of four cold-water sponges with OTU calculation thresholds of 99% (dark grey) and 97% (light grey).

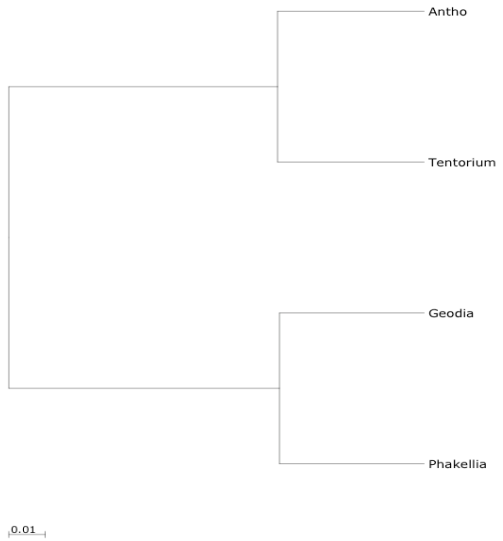

Figure S2.2. Clustering analysis of archaeal 16S rRNA, normalized abundance weights, 1000 permutations. The scale shows the distance between clusters in UniFrac units: a distance of 0 means that two samples are identical, and the distances of 1 means that two samples contain mutually exclusive lineages.

2.10 Supplementary Tables

Table S2.1. Number of archaeal and bacterial *amo*A gene copies in investigated sponges

		AOA amoA copies μg^{-1} NA (S.D.)	AOB amoA copies μg^{-1} NA (S.D.)	AOA amoA copies g^{-1} fresh weight (S.D.)	AOB amoA copies g^{-1} fresh weight (S.D.)	AOA amoA copies rxn^{-1} (S.D.)	AOB amoA copies rxn^{-1} (S.D.)
Geodia barretti	GB1	$2*10^8$ ($2*10^7$)	< 10	$2*10^{11}$ ($2*10^{10}$)	< 10	$1.5*10^6$ ($2*10^5$)	< 10
	GB2	$3*10^8$ ($1.6*10^7$)	< 10	$2*10^{11}$ ($1*10^{10}$)	< 10	$1.4*10^6$ ($4*10^5$)	< 10
	GB3	$6*10^8$ ($8*10^7$)	< 10	$4*10^{11}$ ($5*10^{10}$)	< 10	$8*10^5$ ($3*10^5$)	< 10
Phakellia ventilabrum	PV1	$7*10^6$ ($4*10^5$)	< 10	$2*10^9$ ($1*10^8$)	< 10	n.d.	<10
	PV2	$1.5*10^7$ ($2*10^6$)	$2.8*10^3$ ($6*10^2$)	$5*10^9$ ($6*10^8$)	$7*10^4$ ($2*10^4$)	$1.8*10^4$ ($1.8*10^3$)	<10
	PV3	$1.5*10^7$ ($3.5*10^6$)	$5.2*10^3$ ($2*10^3$)	$8*10^9$ ($2*10^9$)	$9.7*10^4$ ($4*10^4$)	$2.8*10^4$ ($8.8*10^3$)	<10
Antho dichotoma	AD1	$2.3*10^5$ ($2*10^4$)	n.d.	$1*10^8$ ($9*10^6$)	< 10	$1.1*10^2$ ($1.8*10^2$)	n.d
	AD2	$4.5*10^5$) ($6*10^4$)	$1.8*10^4$ ($1.8*10^3$)	$1.4*10^8$ ($2*10^7$)	$4.8*10^6$ ($4.7*10^5$)	n.d.	11.7 (1.3).
	AD3	$4.1*10^5$ ($3*10^5$)	$2.7*10^3$ ($4.7*10^3$)	$1.1*10^8$ ($7.6*10^7$)	$2.3*10^6$ ($2*10^6$)	$1.3*10^3$ ($2.2*10^3$)	7.6 (13).
Tentorium semi suberites	TS1	$1.9*10^5$ ($6.6*10^4$)	< 10	$6.5*10^7$ ($2.3*10^7$)	< 10	< 10	n.d
	TS2	$9*10^5$ ($1.3*10^5$)	not determined	$2.5*10^8$ ($3.7*10^7$)	not determined	$4.7*10^2$ ($1.6*10^{2)}$	not determined

Abbreviations: <10, under 10 copies per 5ng NA or 2µl cDNA; n.d., not detected, unspecific amplification

Table S2.2. qPCR data quality

	Geodia barretti	*P. ventilabrum*	*Antho dichotoma*	*T. semisuberites*
AOA	6x (100-10^7), e=92% y=-3.524x+41.14, r^2=0.995	6x (100-10^7), e=88% y=-3.659x+40.74; r^2=0.994	6x (100-10^7), e=92% y=-3.528x+34.51; r^2=0.996	4x (100-10^5), e=82%, *92% y=-3.862x+29.8; r^2=0.997 *y=-3.519x+42.54; r^2=0.962
AOB	6x (10-10^6), e=80,25% y=-3.908x+37.08; r^2=0.991	6x (10^3-10^8), e= 91% y=-3.573x+35.6; r^2=0.993	4x (10-10^4), e=92.5%, y=-3.516x+37.78; r^2=0.985	4x (10-10^4), e=94% y=-3.487x+25.83; r^2=0.981

3 COMPLEX NITROGEN CYCLING IN THE MARINE SPONGE *GEODIA BARRETTI*

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3.1 Abstract

Marine sponges constitute major parts of coral reefs and deep-water communities. They often harbour high amounts of phylogenetically and physiologically diverse microbes, which are so far poorly characterized. Many of these sponges regulate their internal oxygen concentration by modulating their ventilation behaviour providing a suitable habitat for both aerobic and anaerobic microbes. In the present study, both aerobic (nitrification) and anaerobic (denitrification, anammox) microbial processes of the nitrogen cycle were quantified in the sponge *Geodia barretti* and possible involved microbes were identified by molecular techniques. Nitrification rates of 566 nmol N cm^{-3}sponge day^{-1} were obtained when monitoring the production of nitrite and nitrate. In support of this finding, ammonia-oxidizing Archaea (thaumarchaeotes) were found by amplification of the *amo*A gene, and nitrite-oxidizing bacteria of the genus *Nitrospira* were detected based on rRNA gene analyses. Incubation experiments with stable isotopes ($^{15}NO_3^-$ and $^{15}NH_4^+$) revealed denitrification and anaerobic ammonium oxidation (anammox) rates of 92 nmol N cm^{-3} sponge day^{-1} and 3 nmol N cm^{-3} sponge day^{-1}, respectively. Accordingly, sequences closely related to "*Candidatus* Scalindua sorokinii" and "*Candidatus* Scalindua brodae" were detected in 16S rRNA gene libraries. The amplification of the *nir*S gene revealed the presence of denitrifiers, likely belonging to the Betaproteobacteria. This is the first proof of anammox and denitrification in the same animal host, and the first proof of anammox and denitrification in sponges. The close and complex interactions of aerobic, anaerobic, autotrophic and heterotrophic microbial processes are fueled by metabolic waste products of the sponge host, and enable efficient utilization and recirculation of nutrients within the sponge-microbe system. Since denitrification and anammox remove inorganic nitrogen from the environment, sponges may function as so far unrecognized nitrogen sinks in the ocean. In certain marine environments with high sponge cover, sponge-mediated nitrogen mineralization processes might even be more important than sediment processes.

3.2 Introduction

Phylogenetically complex, yet highly sponge-specific microbial communities live in close association with numerous marine sponge species, sometimes in such high densities that sponges can be viewed as "microbial fermenters" (Hentschel *et al.,* 2006). While our knowledge about the phylogeny of sponge microbes is increasing rapidly (Hentschel *et al.,* 2002, 2003; Taylor *et al.,* 2007), many open questions remain concerning their metabolic functions and their possible interactions with the host.

The role of associated microbes in nitrogen cycling in sponges has received particular attention. Symbiotic cyanobacteria may contribute to the sponge nitrogen budget via fixation of atmospheric nitrogen (Wilkinson and Fay, 1979). This was observed for shallow-water sponges in oligotrophic waters, for example in coral reefs. Usually, sponges ingest nitrogen with their food and excrete NH_4^+ as a metabolic end product (Brusca and Brusca, 1990), which can fuel microbial ammonia and nitrite oxidation. Nitrification rates based on the release of nitrite and nitrate have been reported from numerous tropical and temperate sponges (Diaz and Ward, 1997; Bayer *et al.,* 2007; Jimenez and Ribes, 2007; Southwell *et al.,* 2008). Additionally, 16S rRNA gene sequences of several clades of ammonia-oxidizing Gamma- and Betaproteobacteria and nitrite-oxidizing Nitrospira were recovered from sponges (Hentschel *et al.,* 2002; Diaz *et al.,* 2004; Bayer *et al.,* 2007). Recently, the involvement of Marine Group I Thaumarchaea (Archaea) in ammonia oxidation has received particular attention, and their stable affiliation with numerous sponge species has been demonstrated (Preston *et al.,* 1996; Margot *et al.,* 2002; Lee *et al.,* 2003; Schleper *et al.,* 2005; Hallam *et al.,* 2006b; Pape *et al.,* 2006; Holmes and Blanch, 2007; Steger *et al.,* 2008; Bayer *et al.,* 2008). Archaeal symbionts can be transmitted vertically through the larvae (Sharp *et al.,* 2007; Steger *et al.,* 2008), implying an important role of this host-microbe-interaction. A fully sequenced genome of a potential archaeal ammonia oxidizer from the sponge *Axinella mexicana.* is available, and demonstrates the genetic potential for ammonia oxidation (Hallam *et al.,*

2006a,b), as predicted earlier through metagenomics (Venter *et al.,* 2004; Treusch *et al.,* 2005). The isolation of an autotrophic, free living ammonia-oxidizing marine crenarchaeote gave the final proof for the predicted metabolism (Könneke *et al.,* 2005).

Anaerobic processes of the nitrogen cycle have not been investigated in sponges so far. Until recently, sponge metabolism was viewed as being based on aerobic respiration, similar to metazoan respiration in general. Oxygen is usually supplied in excess to the sponge body through the water current created by the choanocytes (flagellated cells) (Reiswig, 1974). The remarkable ability of sponges to pump large amounts of water through their body has led to the assumption that permanent oxygen saturation exists within the sponge body. The application of oxygen-sensitive microelectrodes on different sponge species, however, showed remarkable oxygen deficiencies in the sponge matrix, as a consequence of reduced pumping activity (Gatti *et al.,* 2002; Schönberg *et al.,* 2004; Hoffmann *et al.,* 2005a,b; Hoffmann *et al.,* 2007; Schläppy *et al.,* 2007). Sponges with a massive growth form that stop pumping become anoxic within 15 minutes; oxygen is only present in the first millimeter of the sponge surface, due to molecular diffusion (Hoffmann *et al.,* 2008). Fluctuating ventilation behaviour, as frequently observed for sponges both in the field and in cultivation (Reiswig, 1971; Vogel, 1977; Gerodette and Flechsig, 1979; Pile *et al.,* 1997; Schläppy *et al.,* 2007; Schläppy *et al* submitted), thus leads to fluctuating oxygen concentrations in sponges.

Consequently, both aerobic and anaerobic microbial processes can be expected in sponges. However the only proof so far of an anaerobic microbial process in a sponge was the detection of microbial sulfate reduction in the cold-water sponge *Geodia barretti,* in line with anoxia in this species as observed with oxygen-sensitive microelectrodes (Hoffmann *et al.,* 2005b). Bacterial denitrification, the anaerobic reduction of nitrate (NO_3^-) to nitrogen (N_2), coupled to the oxidation of organic matter or reduced sulfur species, is a major sink for nitrogen in global nitrogen budgets, and most denitrification takes place in the seafloor (Middelburg *et al.,* 1996). Recently, anaerobic ammonium oxidation (anammox), which combines NO_2^- and NH_4^+ to produce

N_2, was discovered as an alternative pathway for the loss of inorganic nitrogen (Van de Graaf *et al.,* 1995), and has so far been identified in a broad range of natural environments such as marine sediments (Thamdrup and Dalsgaard, 2002), oxygen minimum zones (Kuypers *et al.,* 2005), anoxic fjords/basins (Dalsgaard *et al.,* 2003; Kuypers *et al.,* 2003) and in arctic sea ice (Rysgaard and Glud, 2004). Under certain environmental conditions, anammox can even exceed denitrification as the main N-loss process (Kuypers *et al.,* 2005; Hannig *et al.,* 2007). Molecular methods and stable isotope approaches allow exploration of both the presence and the activity of denitrifying and anammox bacteria in the environment.

The presence of microbial denitrification and anammox in sponges has been hypothesized (Taylor *et al.,* 2007) but neither the processes nor the microbes involved were detected until now.

G. barretti, which is common in the North Atlantic shelf and slope area, is a sponge with high microbial abundance, hosting > 10^{10} microbes cm^{-3} (Hoffmann *et al.,* 2006). The detection of both anaerobic zones (Hoffmann *et al.,* 2005a,b) and sulfate reduction (Hoffmann *et al.,* 2005b), as well as the possibility to grow explants of this species in the lab (Hoffmann *et al.,* 2003), make it a suitable candidate to explore the anaerobic nitrogen cycle in sponges. The aim of the present study is to quantify the aerobic (nitrification) and anaerobic (denitrification, anammox) processes of the microbial nitrogen cycle in *G. barretti*, and to identify the microbes that are potentially involved.

3.3 Materials and methods

3.3.1 Sponge sampling and explant culture

Sponges were sampled near the city of Bergen on the west coast of Norway, between 100 and 200 m depth on a hard bottom slope in Korsfjord (60°09'12"N; 05°08'52"E) using a triangular dredge operated from the research vessel R/V Hans Brattstrøm. Since it is not possible to maintain whole specimens of *G. barretti* in the lab, explant cultures of sponge tissue were established: Cube- or cuboid-shaped fragments of 0.3 – 0.4 cm^3 were cut

from the choanosomal part of freshly retrieved *G. barretti* and kept in half-open cultivation systems with unfiltered seawater as previously described (Hoffmann *et al.,* 2003): Sponge explants were placed on fine mesh plastic grids (3 mm mesh) with no artificial connection, allowing the entire surface to be in contact with the ambient water. Explants can be maintained up to one year (Hoffmann *et al.,* 2003); tissue and skeleton regeneration, growth and even the production of egg cells was observed during cultivation. Within the first months of cultivation, explants of *G. barretti* have no canal system and can not pump water through their body. Most of the animal is anoxic, except a surface layer of 1 mm where oxygen enters the sponge by molecular diffusion (Hoffmann *et al.,* 2005a). Thus, explants of *G. barretti* reflect conditions of both pumping (oxic) and non-pumping (anoxic) sponges and are useful model organisms to investigate aerobic and anaerobic processes in sponges.

3.3.2 Incubation experiments

3.3.2.1. NH_4^+ incubation – nitrification

Explants, which had been cultivated for 5 months and showed a visually healthy appearance, were used for the experiments. For the nitrification experiment, 2 explants of 0.3 – 0.4 cm^3 (total sponge volume: 0.7 – 0.8 cm^3) were placed in 500 ml natural, unfiltered sea water from 200 m depth amended with 12µM NH_4^+. Three parallel incubations and one control (sea water without sponge) were used. Beakers with air bubblers and magnetic stirrers were placed in a temperature-controlled room at 15°C (same as used for explant cultures) in the dark. Water samples were taken over a time course of 48 hours and immediately frozen at -80°C until nutrient analyses were carried out. Ammonium and phosphate concentrations were determined with a Scalar Continuous-Flow-Autoanalyzer using the chemistry described by (Grasshoff, 1983). Nitrite and nitrate were analysed with a chemoluminescence NOx analyzer (Thermo Environmental Instruments Inc, USA).

3.3.2.2. ^{15}N incubations - denitrification and anammox

The denitrification process combines two NO_3^- ions to form one molecule of N_2. When $^{14}NO_3^-$ is present in addition to the added $^{15}NO_3^-$, denitrification produces $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ through random isotope pairing. The anammox process, in contrast, combines one NO_2^- and one NH_4^+ ion, which result in the production of $^{14}N^{14}N$ or $^{14}N^{15}N$ in incubation experiments with $^{15}NO_3^-$, but no production of $^{15}N^{15}N$ (Thamdrup and Dalsgaard, 2002).

For ^{15}N incubations we slightly modified a previously published method (Dalsgaard *et al.,* 2003; Hannig *et al.,* 2007). Sponge explants of 0.3 -0.4 cm^3 (average 0.35 cm^3) were incubated in gas-tight 12.5 ml glass vials (Labco exetainer, Labco Limited, UK) completely (bubble-free) filled with natural seawater. Seawater was amended with 10µM $^{15}NO_3^-$ ($^{14}NO_3^-$ background: 8.2 µM) for the denitrification experiment and 10µM $^{15}NH_4^+$ + 2µM $^{14}NO_2^-$ (background: 0.4 µM NH_4^+, 0.3 µM NO_2^-) for the anammox experiment. Respiration rates of sponge explants (9 µmol O_2 cm^{-3} sponge day^{-1} (Hoffmann *et al.,* 2005a) depleted 90% of the initial oxygen concentration (280µM) in the exetainers during the 24 h of the experiment. Biological activity was stopped at 0, 3, 6, 12 and 24 hours by adding 150µl of saturated $HgCl_2$ solution. A headspace of 2 ml of He gas was added to trap the produced N_2. 1-2 replicates and 2 controls (sea water without sponge) were sampled per time point. To avoid any leakage of gas, samples were stored upside-down at room temperature until analyses. The isotope ratio ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) of the headspace nitrogen was determined by gas chromatography-isotopic ratio mass spectrometry by direct injections from the headspace according to (Kuypers *et al.,* 2005). The concentrations of the produced $^{14}N^{15}N$ and $^{15}N^{15}N$ were assessed as excess relative to air and the N_2 production rates were calculated from the slope of increase (Nielsen, 1992; Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al.,* 2003).

3.3.3 Molecular and phylogenetic analyses of sponge microbes

3.3.3.1. DNA extraction

For anammox bacterial 16S rRNA gene libraries, as well as for microbial community patterns, samples of freshly collected *G. barretti* were immediately fixed in 99% ethanol, washed once in 99% ethanol, and frozen at -20°C. Prior to DNA extraction, the sponge material was ground with mortar and pestle, and left at room temperature for several minutes to evaporate the ethanol. DNA was extracted using the Fast DNA spin kit for soil (BIO 101) according to the manufacturer's instructions. For construction of gene libraries containing 16S rRNA genes of *Nitrospira*, *amoA* genes of archaea and *nirS* genes of bacteria, samples of freshly harvested *G. barretti* were fixed in liquid nitrogen directly on the boat and frozen at -80°C until DNA extraction with phenol-chloroform was performed as described before (Leininger *et al.,* 2006).

3.3.3.2. Microbial community patterns obtained by ARISA

Since DNA for the molecular studies was obtained from freshly sampled *G. barretti*, while cultivated sponge explants were used for the physiological experiments, it was necessary to check if explants and wild sponges contain similar microbial communities. The high-resolution molecular fingerprinting technique ARISA (Automated rRNA intergenic spacer analysis) was used for this comparison. DNA of 3 freshly sampled *Geodia barretti*, of two explants which had been used for the nitrification experiments (2 independent DNA extractions per explant), and of 5 explants that died during cultivation and showed obvious signs of decay (black colour, bad smell) was extracted as described above. Comparison with the microbial community of dead explants was done to provide an additional proof that experimental explants were viable and healthy; this is not always obvious in explant cultures which lack an aquiferous system and thus can not pump (Hoffmann *et al.* 2005a). Universal bacterial ARISA was performed in triplicate using the primers ITSF and the HEX-labelled ITSReub (Cardinale *et al.,* 2004). ARISA-PCR,

fragment analysis and processing of ARISA profiles was performed as described elsewhere (Böer *et al.,* 2009). Non-metric multidimensional scaling (nmds, Chord distance) was performed with the PAST data analysis package. Testing for significant differences between distinct sample groups (field sponges, experimental explants, dead explants) was done by non-parametric analysis of similarities (ANOSIM) in PAST.

3.3.3.3. Construction of clone libraries

In order to obtain 16S rRNA gene sequences from putative anammox bacteria, we constructed four clone libraries using primers with different specificities. The first clone library was constructed with the planctomycete specific primer PLA46F (Neef *et al.,* 1998) and with BS820R targeting the anammox bacteria "*Cand.* Scalindua wagneri" and "*Cand.* Scalindua sorokinii" (Kuypers *et al.,* 2003). For the second clone library we used PLA46F in combination with Amx820R (Schmid *et al.,* 2000), which targets the anammox bacterial genera "*Cand.* Brocadia" and "*Cand.* Kuenenia". The third clone library was constructed with the primer Amx368F that should targ*et al*l anammox bacteria (Schmid *et al.,* 2003) and the universal primer 1392R (Stahl *et al.,* 1988). For the fourth clone library we used the planctomycete-specific primer PLA46F and the universal primer 1392R in order to targ*et al*l planctomycetes. 34 PCR cycles were used for amplification with the first and the third primer set, 33 cycles for the second primer set and 26 cycles for the fourth primer set. All PCR reactions were conducted in 12 replicates, the replicates were pooled and the pooled PCR products were cloned. Preparative gels ensured cloning of PCR products of the correct size.

PCR reactions were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), prior to cloning with TOPO TA Cloning kits for sequencing with vector pCR4 (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Clones were screened by PCR for inserts of correct size and these were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

16S rRNA genes of Nitrospira were amplified with the universal primer 616V (Juretschko *et al.,* 1998) and the Nitrospira-specific primer 1158R (Maixner *et al.,* 2006). Amplification of archaeal *amoA* genes was performed with primer set 19F (Leininger *et al.,* 2006) and 643R (Treusch *et al.,* 2005) and amplification of *nirS* genes was achieved with primers nirScd3AF (Michotey *et al.,* 2000) and nirSR3cd (Throbäck *et al.,* 2004), using touchdown PCR conditions (Braker *et al.,* 1998) in combination with GoTaq Polymerase (Promega Corporation, Madison, WI). The respective amplicons of three sponge individuals were cut out from a low-melting agarose gel and were pooled for cloning with the TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Clones were screened for inserts of correct size and these were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

3.3.3.4. Phylogenetic analysis

16S rRNA sequences of anammox bacterial clone libraries were edited with the Sequencing Analysis Software (Applied Biosystems) and analysed for their closest relatives using the ARB 16S rRNA gene database (Ludwig *et al.,* 2004) and BLAST (Altschul *et al.,* 1997). Sequences of about 800 base pair length were assembled from individual reads with the Sequencher v4.6 software (Gene Codes Corporation, Ann Arbor, USA).

The 16S rRNA gene sequences were checked for the presence of chimeric sequences by using the CHIMERA_CHECK program from RDP II (Cole *et al.,* 2003) and imported into the ARB 16S rRNA gene database. Phylogenetic analyses of the 16S rRNA sequences were made using distance matrix, maximum parsimony, and maximum likelihood algorithms in ARB with and without 50% variability filters. Consensus trees were constructed thereafter.

16S rRNA sequences related to Nitrospira were edited and analysed using the ARB program package (Ludwig *et al.,* 2004) after proofreading and were combined with the sponge symbiont database (Taylor *et al.,* 2007). Sequences

with high similarity to *G. barretti*-derived sequences were identified by BLAST searches and were also imported into ARB. A consensus tree, based on three different treeing methods (neighbor joining, maximum likelihood and maximum parsimony) was constructed including relevant reference sequences and other available sponge-derived 16S rRNA sequences.

Amino acid sequences of ammonia-monooxygenase (AmoA) and dissimilatory nitrite reductase (NirS) were also edited and analyzed in ARB. Databases for both genes were generated by importing sequences from NCBI, after BLAST searches using *G. barretti*-derived sequences were performed. Fitch trees (Fitch and Margoliash, 1967) were calculated in PHYLIP and imported into ARB for further formatting and the addition of shorter NirS sequences.

Sequence identities were calculated using the similarity function of the distance matrix algorithm in ARB. Anammox bacteria-related 16S rRNA gene sequences, 16S rRNA gene sequences for *Nitrospira*, AmoA and NirS sequences are available in GenBank under accession numbers FJ230205-FJ230287.

3.4 Results

3.4.1 Nitrification, denitrification and anammox rates

For nitrification measurements, small sponge explants were incubated in ammonium-amended natural seawater under aerobic conditions and ammonium, nitrite and nitrate concentrations were monitored over a time course of 48 hour. Figure 3.1a shows the production of NO_3^- and NO_2^- per cm^{-3} sponge as an average of 3 replicate experiments. The amount of NO_3^- and NO_2^- production in a control incubation (without sponge) has been deducted from these values. Ammonium consumption exceeded production in the first 24 hours, while after that, net production of ammonium was observed (Fig. 3.1b). Ammonium depletion was also observed in the control incubation without sponge, which indicates algal growth during the experiment. Though we do not know to which extend the bottle effect of unwanted algal growth

may have masked some of the turnover of inorganic nitrogen, it is obvious that (1) the sponge-microbe system was always supplied with NH_4^+ in excess and (2) both nitrite and nitrate production was observed in all sponge treatments. A total net nitrification rate of 566 nmol N cm^{-3} sponge day^{-1} was calculated from the linear slopes of increase of NO_3^- and NO_2^- production as presented in Fig. 3.1a.

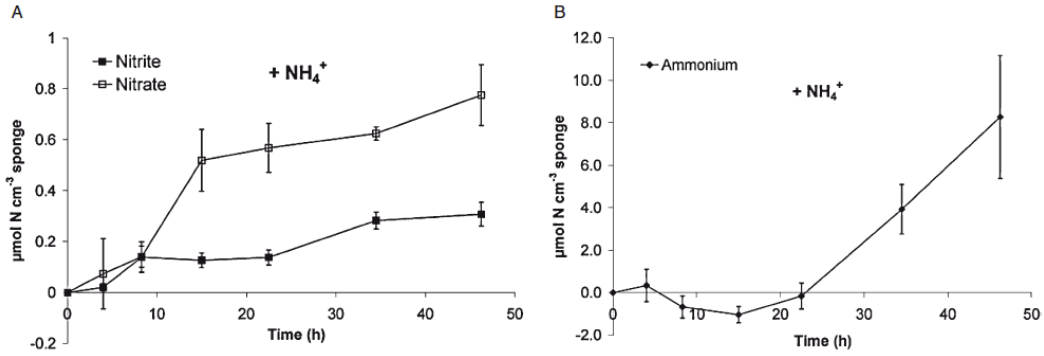

Figure 3.1. Incubation of *G. barretti* explants with 12 µM NH_4^+: production/ consumption of ammonium, nitrite and nitrate per cm^{-3} sponge as average water concentrations in 3 replicate incubations minus concentration in control incubation at a given time point. Nitrite and nitrate were produced during the experiment. A net nitrification rate of 566 nmol N cm^{-3} sponge day^{-1} was revealed by the linear slope of increase (a). Ammonium consumption exceeded production in the first 24h of the experiments, thereafter, a net production of ammonium was observed (b).

Production of N_2 as an end product of denitrification and anmmox was examined by incubating sponge pieces in gas-tight glass vials filled with natural seawater. Seawater was amended with 10µM $^{15}NO_3^-$ ($^{14}NO_3^-$ background: 8.2 µM) for the denitrification experiment and 10µM $^{15}NH_4^+$ + 2µM $^{14}NO_2^-$ (background: 0.4 µM NH_4^+, 0.3 µM NO_2^-) for the anammox experiment. Linear production of both $^{14}N^{15}N$ and $^{15}N^{15}N$ was observed in the $^{15}NO_3^-$ incubation (Fig. 3.2a). We assume that denitrifiers were not nitrate limited: The small diameter of the sponge explant and the high natural $^{14}NO_3^-$ concentration of 8 µmol/l allowed a fast diffusive transport that exceeds the denitrification rate. Therefore, the rates of $^{28}N_2$, $^{29}N_2$ (corrected for the $^{29}N_2$ rate of the anammox experiment, see below) and $^{30}N_2$ production were used to calculate a total denitrification rate of 92 nmol N cm^{-3} sponge day^{-1}.

The source of $^{14}NO_3^-$ used for denitrification is either the ambient water (8 µmol/l of $^{14}NO_3^-$) or $^{14}NO_3^-$ produced via nitrification in the sponge. The

sources can be distinguished by comparing the ratio of labelled and unlabelled NO_3^- in the ambient water with the ratio calculated from the $^{29}N_2$ and $^{30}N_2$ production (Nielsen, 1992).

Of 49 nmol $^{14}NO_3^-$ cm^{-3} sponge day^{-1}used for denitrification only 26% can be attributed to coupled nitrification-denitrification whereas 74% derived from $^{14}NO_3^-$ in the ambient water.

In $^{15}NH_4^+$ + $^{14}NO_2^-$ incubations, a linear production of $^{14}N^{15}N$, but no production of $^{15}N^{15}N$ was observed (Fig. 3.2b). This indicates the process of anammox and an anammox rate of 3.0 nmol N cm^{-3} sponge day^{-1} was calculated. Since no lag phase in the production of $^{14}N^{15}N$ could be observed, coupled nitrification-denitrification of $^{15}NH_4^+$ can be neglected, and the observed $^{14}N^{15}N$ production must be due to anammox.

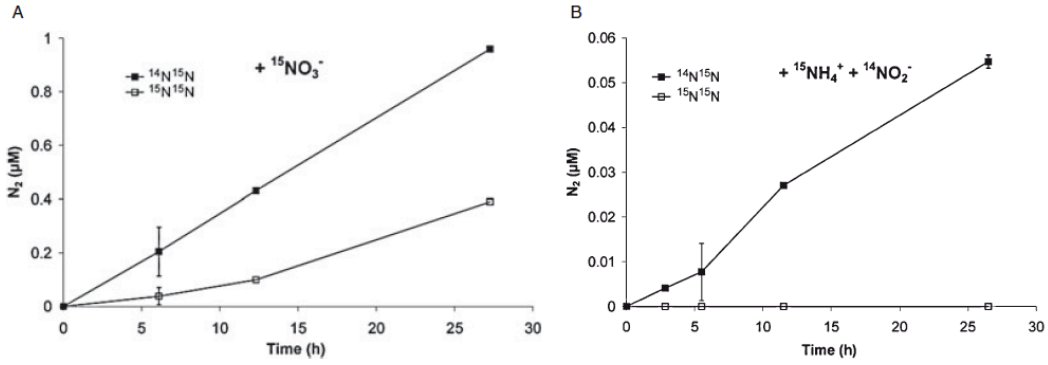

Figure 3.2. Incubation experiments with linear production of $^{14}N^{15}N$ and $^{15}N^{15}N$ from $^{15}NO_3^-$ indicating denitrification (a), and $^{14}N^{15}N$ from $^{15}NH_4^+$ + $^{14}NO_2^-$ indicating anammox (b).

3.4.2 Microbial community patterns in freshly sampled and cultivated sponges

ARISA (Automated rRNA intergenic spacer analysis) and statistical analyses were used to compare microbial communities in samples of sponge explants (used for the incubation experiments) to those of freshly sampled *G. barretti* sponges (used for the molecular studies). Nmds (Non-metric multidimensional scaling) – plots and ANOSIM (non-parametric analysis of similarities) of ARISA results showed that sponge explants and fresh sponges had overlapping community patterns. An R-value of 0.29 ($p<0.001$) was obtained by R test statistic measures. R values > 0.75 are commonly

interpreted as well separated, R>0.5 as separated, but overlapping, and R<0.25 as barely separable (Ramette, 2007). In contrast, microbial communities of explants used for the experiments were well separated from dead explants (R = 0.75, p<0.001). It can thus be concluded that explants that were used for the experiments were alive and healthy, and contained microbial communities that were similar to those in freshly sampled *G. barretti.*

3.4.3 Identification of microbes involved in nitrogen cycling

3.4.3.1. Ammonium monooxygenase of archaea

The presence and diversity of archaea potentially involved in ammonia oxidation was analysed by targeting the *amo*A gene in PCR-based studies. *Amo*A encodes an archaeal homologue of subunit A of the ammonia monooxygenase enzyme. The homologous enzyme in bacteria has been shown to catalyze the transformation from ammonia to hydroxylamine, the first step in ammonia oxidation. The deduced amino acid sequences of 38 cloned PCR products of archaea obtained from *G. barretti* formed a specific, monophyletic cluster, which was stable with four treeing methods (neighbor joining, maximum parsimony, maximum likelihood, fitch). Although the phylogenetic classification of other marine AmoA sequences cannot be unambiguously resolved (see Fig. 3.3) the *G. barretti* cluster was clearly affiliated to the crenarchaeotal marine group and together with these was separated from the second major group of archaeal AmoA sequences mostly derived from soils (100 % bootstrap support). Similarities of *G. barretti* -derived amino acid sequences to those of "*Candidatus* Cenarchaeum symbiosum" ranged from 93.3 to 94.4% and to those of *Nitrosopumilus maritimus* from 92 to 93.2% (both on amino acid level). The closest related sponge-derived sequence was from *Chondrosia reniformis* with a similarity of 95.6%.

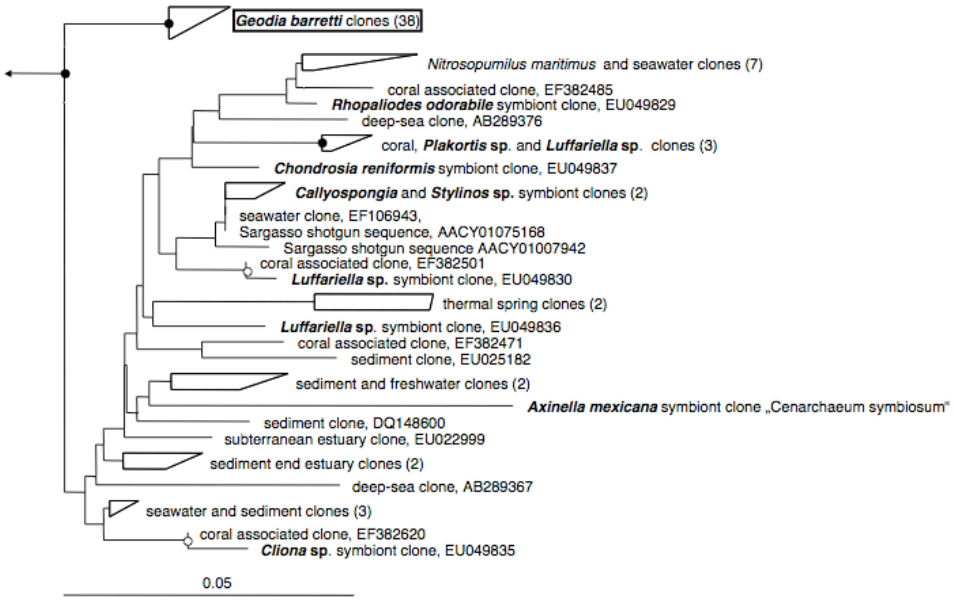

Figure 3.3. Phylogenetic tree based on archaeal AmoA sequences of ammonia-oxidizing archaea showing the placement of AOA associated with the marine sponge *Geodia barretti* (bold and framed) within the thaumarchaeal group of mostly marine sequences. In this group are also numerous other sponge-derived sequences (bold) and the AmoA sequence from "*Cand*. Nitrosopumilus maritimus", a known ammonia-oxidizing archaeon. 200 amino acids were used for tree construction and parsimony-based bootstraps values are given. Filled circles indicate ≥90% bootstrap support and blank circles indicate ≥70% bootstrap support. The scale bar indicates 5% sequence divergence. Tree is rooted with a second group of AmoA sequences of *Crenarchaeota* that mostly stem from soils and sediments. The tree was constructed from a distance matrix using the Fitch-Margoliash algorithm.

Intracluster similarities, i.e. differences between *Geodia barretti*-derived sequences only, were 99.38 -100%. PCR amplifications of bacteria-derived *amo*A genes were often at the detection limit, indicating far lower amounts of bacterial ammonia oxidizers (not shown). These results were confirmed by metatranscriptomic studies and quantitative PCR data in which *amo*A genes of archaea outnumbered by three to six orders of magnitude *amo*A genes of bacteria in different sponge individuals, both on cDNA and on DNA level (Radax *et al*., in review). Therefore, a gene library of bacterial *amo*A genes was not constructed.

3.4.3.2. 16S rRNA genes of nitrite oxidizing bacteria (NOB)

To assess the presence and diversity of potential nitrite oxidizers in *Geodia barretti*, a 16S rRNA gene library with primers specific for the phylum *Nitrospira* was constructed. The produced amplicons were approximately 1100 nucleotides in length. Phylogenetic analysis of 20 genes suggested the

presence of sponge-specific nitrifiers from the phylum Nitrospira (Fig. 3.4) with 87.3-91.4% identity to the *Nitrospira marina* 16S rRNA gene and 84.2 to 87.5% identity to the *N. moscoviensis* 16S rRNA gene. The nearest full length sequence derived from a sponge (AJ347039) had an identity of 98.6-98.9% to the *G. barretti*-derived sequences. *G. barretti*-derived clones formed an internal monophyletic group within this cluster with parsimony-bootstrap support of 95%. The 16S rRNA similarity range within the *G. barretti* group was 95.3 to 99.7 % and within the sponge-cluster it was 92.8 to 99.7 %.

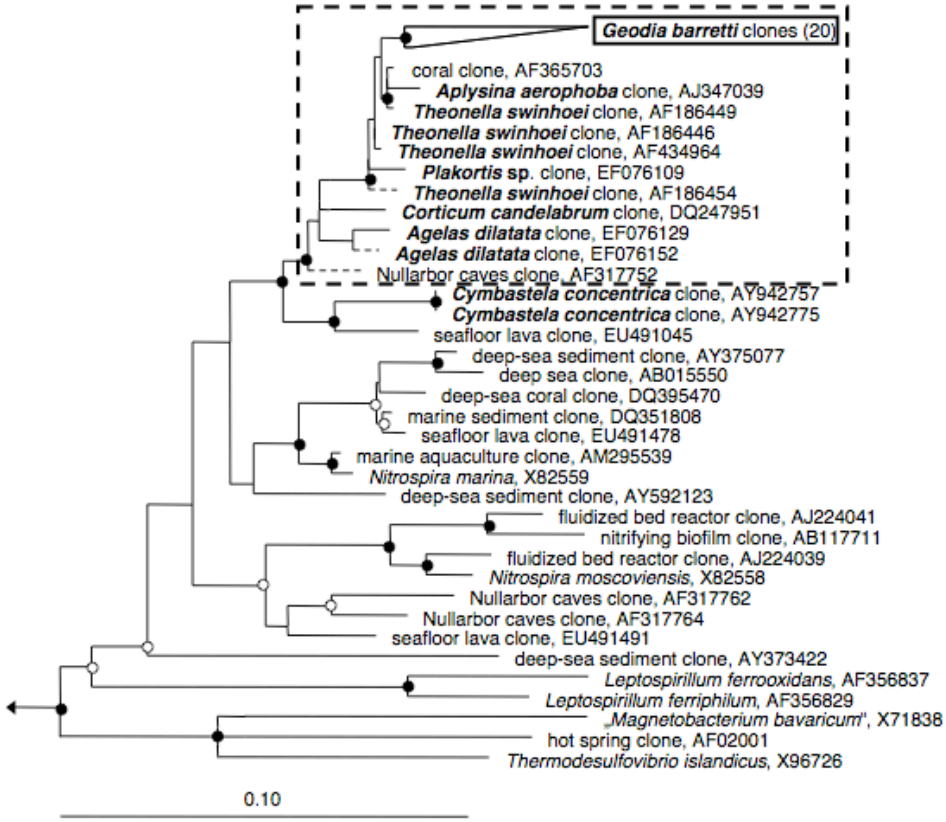

Figure 3.4. Consensus tree based on 16S rRNA gene sequences affiliated to the phylum *Nitrospira*, recovered from the marine sponge *Geodia barretti*. Sponge-derived sequences are highlighted in bold and sequences from this study have an additional frame. The tree shown is based on neighbor joining, maximum likelihood and maximum parsimony treeing methods. Branches not unambiguously resolved by different treeing methods are indicated by polytomies. Short sequences were added using the Parsimony insertion tool from ARB after tree calculation and are indicated with a dashed line. The dashed box depicts a sponge-specific monophyletic sequence cluster including one coral-derived sequence. Filled circles indicate ≥ 90% bootstrap support and open circles indicate ≥ 75% parsimony-bootstrap support. The scale bar indicates 10% 16S rRNA sequence divergence. The outgroup (not shown) consisted of 16S rRNA sequences of several other bacterial phyla.

3.4.3.3. 16S rRNA genes of anaerobic ammonium oxidizing (anammox) bacteria

Based on the process measurements we concluded that another group of microbes involved in the nitrogen cycling in *G. barretti* should be the anaerobic ammonium oxidizing (anammox) bacteria. Four different primer combinations were used to amplify the 16S rRNA genes of putative anammox bacteria: PLA46F/ BS820R, PLA46F/Amx820R, Amx368F/1392R, and PLA46F/1392R. Only the primer combination PLA46F and BS820R was successful in retrieving 6 gene sequences related to anammox bacteria, the rest of the sequences of this clone library were related to the candidate phylum *Poribacteria. Poribacteria* related sequences were also retrieved with the primer pairs PLA46F/Amx820R and PLA46F/1392R. The clone library with primers Amx368F and 1392R produced sequences related to the phylum *Acidobacteria*. Of the six anammox bacterial sequences retrieved with primers PLA46F and BS820R (Fig. 3.5), 5 sequences (clones 2G8_1, 2G8_6, 2G8_7, 2G8_8 and 2G8_12) were almost identical to each other (sequence identity of 99.8 to 100%). Based on these calculations with full length anammox bacterial sequences they were most closely related to a sequence from Barrow Canyon sediment (Alaska, DQ869384, 97.5% sequence identity), whereas the sequence identity with "*Candidatus* Scalindua sorokinii" and "*Candidatus* Scalindua brodae" (Kuypers *et al.,* 2003; Schmid *et al.,* 2003; Schubert *et al.,* 2006) was only ~95%. One sequence (clone 2G8_47) showed only 96% sequence identity to the former cluster of anammox bacterial sequences from *G. barretti* and was more closely related to "*Candidatus* Scalindua sorokinii" and "*Candidatus* Scalindua brodae" (~98% sequence identity). The highest sequence identity was observed with amplified gene fragments from the Peruvian upwelling system (Woebken *et al.,* 2008) (98.3%, e.g. clones Peru_23, Peru_54 and Peru_88 of the Peruvian OMZ sea water cluster I).

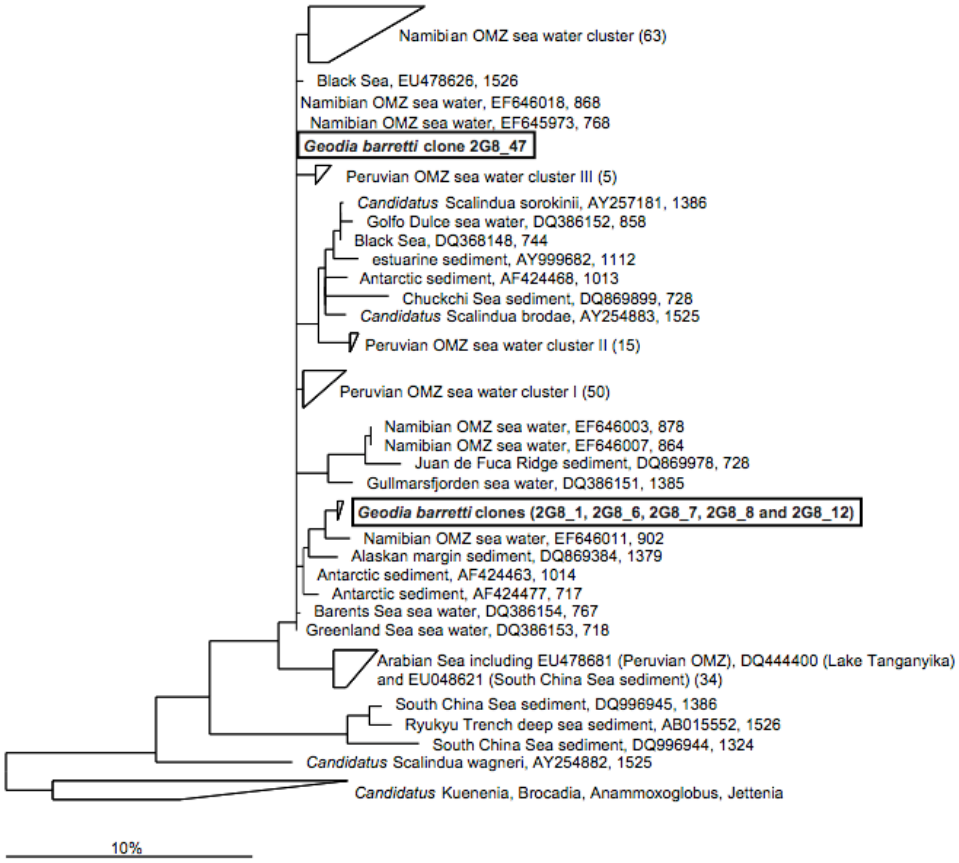

Figure 3.5. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic affiliation of the anammox bacterial sequences retrieved from *G. barretti* (bold, framed). The consensus tree was constructed based on maximum likelihood, neighbor joining and maximum parsimony trees, calculated without and with 50% position variability filters using other cultured and uncultured planctomycete-sequences as outgroup (not shown in tree). The bar represents 10% estimated sequence divergence.

3.4.3.4. Cytochrome cd1 nitrite reductase (*nir*S) of bacterial denitrifiers

The denitrification rates measured in *Geodia barretti* implied the presence of denitrifying organisms. Since this metabolic capacity is spread between species of different bacterial phyla, we attempted to amplify genes encoding the key metabolic enzyme of this process, i.e. the dissimilatory nitrite reductase. Two unrelated forms of this enzyme exist, the copper-dependent enzyme, NirK, and the cytochrome cd1 nitrite reductase, NirS. Several attempts to amplify *nir*K genes from *Geodia barretti* failed, as only PCR products of unexpected size were obtained. Nevertheless, 20 potential candidate genes obtained with nirK1F and nirK5R (Braker *et al.,* 1998) under conditions of low stringency, were sequenced, but as expected were no *nir*K

genes. Similarly, the use of the same reverse primer in combination with the forward primer nirK583F (Santoro *et al.,* 2006) resulted in no products. In contrast, we were able to amplify bacterial *nir*S genes encoding the cytochrome cd1 nitrite reductases of bacterial nitrifiers. Thirteen out of 19 NirS sequences were related to NirS of *Thauera mechernensis* with 99.2 to 100% amino acid sequence identity and the second group with 6 sequences had 89 to 90.6% sequence similarity to NirS of *Pseudomonas stutzeri* and 91.3 to 92.9% sequence identity to NirS of *Alcaligenes faecalis* on the protein level (Fig. 3.6).

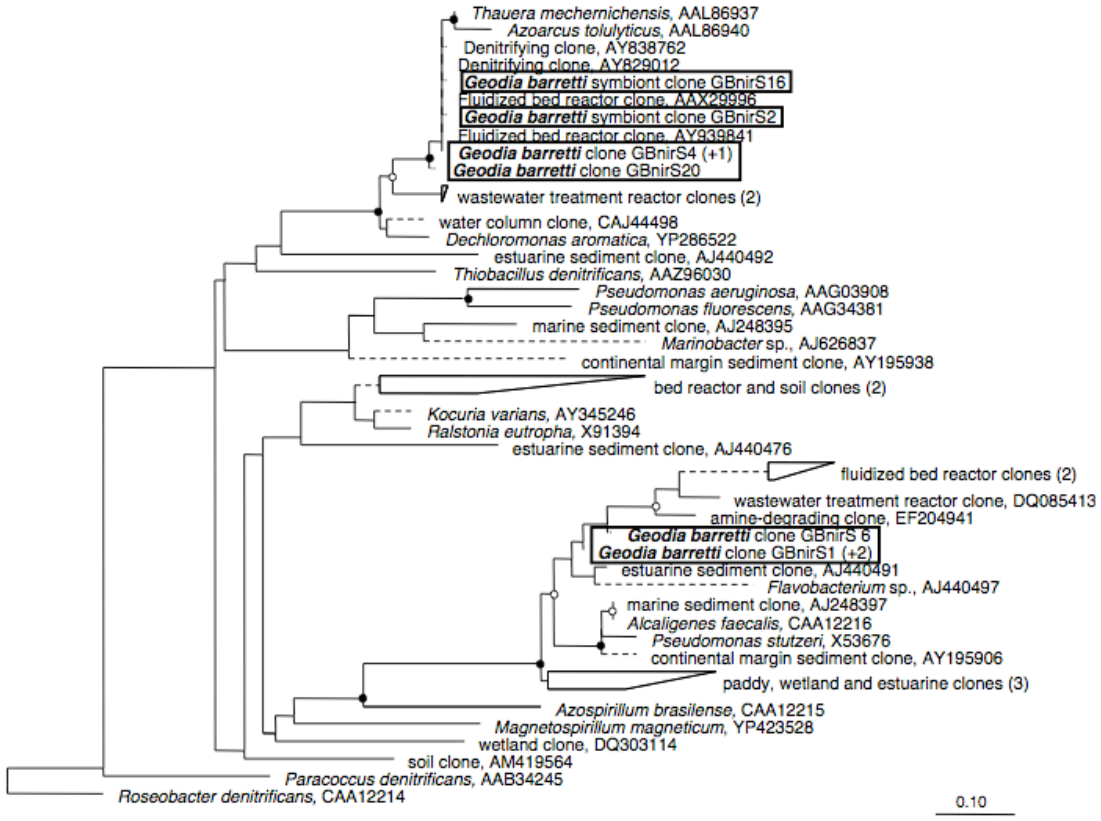

Figure 3.6. Phylogenetic Fitch tree based on bacterial NirS protein sequences showing the distribution of denitrifying organisms associated with *Geodia barretti* (bold and framed). The tree was calculated based on sequences with 192 amino acids, whereas short sequences (139 amino acids) were added via the parsimony interactive tool and are indicated by a dashed line. Filled circles indicate ≥90% and blank circles indicate ≥70% bootstrap support. The scale bar indicates 10% sequence divergence. The tree is rooted with *Roseobacter denitrificans.*

3.5 Discussion

3.5.1 Nitrification, denitrification and anammox rates in sponges

Both, anammox and denitrification are important processes for nitrogen cycling in virtually all habitats on Earth, as they remove nitrogen from the environment and recycle it back to the atmosphere. While denitrification in other invertebrates was described recently (Stief *et al.,* 2009), our study represents the first indication for anammox to occur in an animal and shows that both nitrification, denitrification and anammox can occur simultaneously in one sponge individual. Rates for nitrification, denitrification and anammox were quantified and putative involved microbes identified. Under suboxic and micro-oxic conditions as in *G. barretti* explants, these aerobic and anaerobic processes can apparently happen at the same time, as recently described for the suboxic zone of Black Sea sediments (Lam *et al.,* 2007). Our bulk nitrification rate (566 nmol N cm^{-3} day^{-1}) must thus be considered as a net rate, since part of the products are used to fuel denitrification and anammox. As pointed out in the results section, 26% of the nitrate used for denitrification (denitrification rate: 92 nmol N cm^{-3} day^{-1}) stem directly from nitrification. Adding this amount to the net nitrification rate reveals a total nitrification rate of 582 nmol N cm^{-3} day^{-1}. Similarly, the calculated anammox rate is a conservative estimate, since alternatively to the $^{15}NH_4^+$ added, the microbes could use unlabelled NH_4^+ as a substrate which is continuously excreted by the sponge. This would result in the production of unlabelled N_2, which would not be detected with our analysis method and thus is not attributed to the calculated anammox rate.

We also observed nitrification and denitrification in a parallel study with two Mediterranean sponges (Schläppy *et al.,* 2011). In *Dysidea avara*, net nitrification was 218 nmol N cm^{-3} day^{-1} while denitrification was 242 nmol N cm^{-3} day^{-1}; in *Chondrosia reniformis*, nitrification was 319 and denitrification 360 nmol N cm^{-3} day^{-1}. Compared to the present study, nitrification rates were lower while denitrification rates were higher in the Mediterranean

sponges. Anammox was under the detection limit (Schläppy *et al.,* 2011). These results show that complex nitrogen cycling processes as we observed in *G. barretti* may be common in sponges, but rates may be variable in different sponge species. It will be important to investigate the activity in further species to obtain a broader view on the significance of these processes in these and other animals.

Besides our studies, there are no other reports on denitrification or anammox rates in sponges, whereas nitrification has been reported. The net nitrification rate we observed in *G. barretti* (566 nmol cm-3 day-1; approx. 196 nmol g^{-1} dry wt h^{-1}, assuming a sponge water content of 90%), is within the range of nitrification rates reported for Mediterranean sponges (89 – 1325 nmol g^{-1} dry wt h^{-1} (Bayer *et al.,* 2008); 180 – 780 nmol g^{-1} dry wt h^{-1} (Jimenez and Ribes, 2007) as well as tropical sponges (30 – 2650 nmol g^{-1} dry wt h^{-1}, Diaz and Ward, 1997). It is also within the range of nitrification rates reported from other benthic animals (3 - 1020 nmol g^{-1} dry wt h^{-1}) and it is orders of magnitude above nitrification rates of estuarine sediments (0.02 – 7.7 nmol g^{-1} dry wt h^{-1} Bernhard *et al.,* 2007).

Rates of anammox have not been reported from animal hosts so far. The rate we found in the sponge *G. barretti* (3 nmol N cm^{-3} sponge day^{-1}) is at least one order of magnitude higher than pelagic anammox rates (0.02-0.38 nmol ml^{-1} day^{-1}, Kuypers *et al.,* 2003; Kuypers *et al.,* 2005; Hannig *et al.,* 2007).

Denitrification rates of microbes dwelling in the guts of benthic invertebrates (insects, mollucs and shrimps) range between 7 and 38 nmol N ind^{-1} day^{-1}, which equals 29 – 365 nmol cm^{-3} day-1 (Stief *et al.,* 2009), and are thus comparable to denitrification rates we found in *G. barretti*.

3.5.2 Microbial community patterns in freshly sampled and cultivated sponges

ARISA analysis showed that healthy explants kept in the laboratory contained similar communities of microbes as wild *G. barretti*. This is consistent with a previous FISH (fluorescence *in situ* hybridization) study

targeting the main groups of the microbial community of *G. barretti* during 8 months of cultivation. Hoffmann and co-workers (2006) showed that the respective microbial groups were stable throughout several months of cultivation; while aberrant microbes occurred during the first days.

Microbial processes observed in healthy explants as monitored in this study can thus also be expected in complete sponges; and *vice versa*, the microbial sequences that we recovered from freshly sampled sponges should also be present in the explants. A more extensive study on the microbial community patterns of *G. barretti* investigating specimens from different sampling sites and seasons using ARISA and DGGE (denaturing gradient gel electrophoresis) revealed a species-specific microbial community in *G. barretti* (F. Hoffmann, S. Fortunato, unpublished). Moreover, these studies showed that the microbial community of *G. barretti* was very close to that of other sponge species of the family of Geodiidae; indicating that the complex nitrogen cycling processes described here may be found in many or even all sponges of the Geodiidae family.

3.5.3 Phylogeny of microbes involved in nitrogen cycling

We used PCR-based studies to screen specifically for microorganisms potentially responsible for nitrification, denitrification and anammox processes in the sponge *G. barretti*. The *Nitrospira* found in *G. barretti* and other sponge species formed a sponge-specific sequence cluster within the *Nitrospira marina*-related group (Hentschel *et al.,* 2002; Taylor *et al.* 2007). The ammonia-oxidizing archaea found in *G. barretti* were closely related to other sponge-derived sequences within the group of Thaumarchaeota, mainly consisting of marine sequences. A prevalence of archaeal versus bacterial *amoA* genes as obtained by quantitative PCR as well as whole genome transcription patterns of the microbial communities in *Geodia barretti* (Radax *et al*, submitted) indicate that the ammonia oxidation we observed in *G. barretti* is most likely driven by the activity of archaea. In total, the phylogenetic analyses imply that sequences of *G. barretti* -associated

nitrifiers (AOA and Nitrospira) form monophyletic sequence clusters. This indicates a long-term stable association and/or adaptation of the microbes to their host sponge, which lives in environmental conditions notably different from that of shallow water sponges in tropical and temperate areas, i.e. cold water, darkness and high pressure. Anammox bacteria sequences derived from *G. barretti* were most closely related to the "*Candidatus* Scalindua" branch, like most other marine anammox bacteria sequences. The recovery of two groups of genes potentially encoding cytochrome cd1-type nitrite reductases (NirS) suggests that the denitrification activity, as measured in the sponge, is most probably performed by more than one group of bacteria. Based on sequence distributions the most likely denitrifying population to be specifically associated with *Geodia* could be related to the *Thauera* group (see Fig. 3.6). Clear phylogenetic assignments based on the *nir*S gene are not possible as horizontal gene transfer (HGT) occurs frequently and can lead to wrongly assigned taxa (Braker *et al.,* 1998). From cultivation studies it is known that organisms harboring the *nirS* gene are most prevalent in the Betaproteobacteria (Heylen *et al.,* 2006). In our study we have found two clusters, one related to sequences from Betaproteobacteria, and another cluster related to sequences of Beta- and Gammaproteobacteria as well as a short Bacteroidetes-*related* sequence. Since this study provides the first phylogenetic analysis for denitrifying and anammox bacteria from a marine sponge, we cannot assign any group as being sponge-specific. It is worth noting in this context that a common alphaproteobacterial associate of marine sponges (Hentschel *et al.,* 2001; Webster and Hill, 2001; Enticknap *et al.,* 2006) was found to be very closely related to the marine denitrifier *Pseudovibrio denitrificans*, and that at least some of these sponge-derived strains had tested positive for denitrification (Enticknap *et al.,* 2006).

3.5.4 Function and effects of complex nitrogen cycling in sponges

It seems obvious that autotrophic partners such as nitrifying microbes may provide an additional source of carbon for the sponge. The exact nature of metabolic exchange between the respective partners is not well understood yet; however, direct feeding of sponge cells on associated microbes is well described (Wilkinson and Garrone, 1980; Ilan and Abelson, 1995; Vacelet *et al.,* 1996) and we made similar observations by transmission electron microscope investigation of *G. barretti* (F. Hoffmann, not shown). The ratio of carbon fixation to nitrification by bacterial ammonium and nitrite oxidizers is approximately 1 mol of CO_2 for every 9 mol of NH_4^+ oxidized to NO_3^- (Feliatra and Bianchi, 1993). Thus, nitrification rates of *G. barretti* (582 nmol cm^{-3} day^{-1}, regarding nitrate lost by denitrification) allow the nitrifying community in sponges to fix up to 64 nmol CO_2 cm^{-3} sponge day^{-1}. Differences in carbon fixation mechanisms of archaeal ammonia oxidizers (Hallam *et al.,* 2006a) could alter this ratio. In any case, the possible carbon fixation rates are small compared to respiration rates of *G. barretti* explants (9 µmol O_2 cm^{-3} day^{-1}, Hoffmann *et al.,* 2005a). Assuming that 1 µmol O_2 consumption per cm^3 sponge per day is due to aerobic oxidation of ammonium to nitrate and nitrite, and the remainder is due to carbon oxidation, microbial nitrification would provide less than 1% of the carbon demand of *G. barretti*. Due to the even lower rate, carbon fixation by anammox bacteria can be neglected.

Heterotrophic microbes consume simple compounds (dissolved organic carbon, DOC), while sponge cells prefer small particles (particulate organic carbon, POC) like pelagic bacteria or phytoplankton (Willenz, 1980; Pile *et al.,* 1996; Witte *et al.,* 1997; Ribes *et al.,* 1999). Sponges with high amounts of associated microbes however have been identified as important DOC sinks (Yahel *et al.,* 2003; De Goeij *et al.,* 2008b). This leads to the conclusion that DOC uptake in sponges is mediated by sponge microbes. In fact, a recent investigation by (De Goeij *et al.,* 2008a) showed that assimilation of ^{13}C-labelled DOC was both direct and bacteria mediated, as tracer carbon was recovered both in bacteria-specific and non-bacteria fatty acids within the

sponge. Considering an anoxic situation in the sponge matrix, DOC may also be excreted as a result of fermentation processes in sponge cells, and could thus feed an anaerobic heterotrophic microbial community, such as sulfate reducing prokaryotes (Hoffmann *et al.,* 2005b) and denitrifiers (this study). The importance of these processes for sponge nutrition remains to be proven.

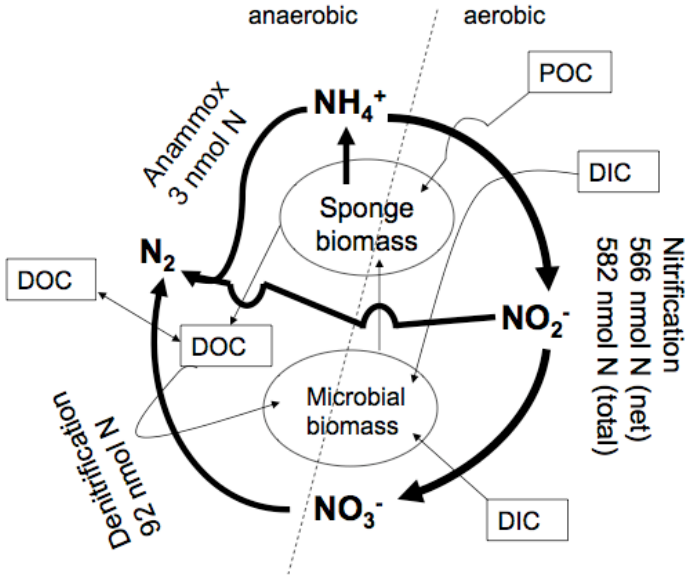

Figure 3.7. Complex nutrient cycling in the sponge *G. barretti* showing the interaction of heterotrophic, autotrophic, aerobic and anaerobic processes within the sponge-microbe system and between sponge and environment. Rates of microbial processes of the sponge nitrogen cycle are given in nmol N cm^{-3} sponge day^{-1}. Proposed processes of carbon transfer between sponge and microbes are indicated with dashed arrows. Thickness of the arrows reflect the quantitative importance of this process. DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, POC = particulate organic carbon, PON = particulate organic nitrogen

In conclusion, the physiologically complex community of microorganisms involved in sponge nitrogen cycling has the potential to make additional carbon sources available to the host sponge, although its impact on sponge nutrition may be rather small.

We assume therefore, that the main benefit of the complex nitrogen cycling in sponges lies within the efficient removal of waste products. Ammonium removal by ammonium oxidizers (aerobic and anaerobic) may even exceed sponge ammonium excretion, as seen in the first 24 hours of the nitrification experiment (Fig. 3.1b). Similar observations were made with the Mediterranean sponge *Aplysina aerophoba*, which functions as an "ammonium sink" during the cold season (Bayer *et al.,* 2008). Accumulation of detrimental nitrite and nitrate is counteracted by nitrite oxidation and

nitrate reduction, respectively. The energy gain and possible carbon transfer from sponge microbes to sponge cells is a positive, but minor side effect of this efficient waste product treatment. Nitrogen fixation by symbiotic cyanobacteria has been described for shallow-water sponges (Wilkinson and Fay, 1979; Mohamed *et al.,* 2008). We do not expect this process in *Geodia barretti*, which lives in deep water where inorganic nitrogen is always available in excess as NO_3^-, and where cyanobacterial symbionts are not present due to the absence of light.

Complex nitrogen cycling within *G. barretti* and nitrogen fluxes between the sponge and the marine environment are summarized in Figure 3.7.

3.5.5 Impact of sponge nitrogen cycling on marine ecosystems

In sponge mass occurrences at the North Atlantic slope at about 200 – 500 m water depth (Klitgaard *et al*, 1997; Klitgaard and Tendal, 2004; Rapp, unpublished data), sponges of the family Geodiidae can be found in densities of up to 30 kg/m^2. Assuming similar rates *in situ* to those we obtained in this study, sponge-mediated nitrification would transform up to 16 mmol N m^{-2} day^{-1}, while sponge-mediated denitrification would remove 2.7 mmol N m^{-2} day^{-1} as N_2. Nitrogen removal by these sponge mass occurrences can thus be 2-10 times higher than by continental shelf sediments at similar water depths, where denitrification rates are 0.1-1 mmol N m^{-2} day^{-1} (Middelburg *et al.,* 1996; Seitzinger and Giblin, 1996).

Sponge mass occurrences, which can cover several km^2 at certain areas in the North Atlantic (Klitgaard *et al.,* 1997; Klitgaard and Tendal, 2004), can therefore be more important for nitrogen mineralization processes than marine sediments at these depths.

The importance of sponge-mediated nitrification and the resulting fluxes of dissolved inorganic nitrogen in areas with high sponge cover, e.g. tropical coral reefs, has already been pointed out (Southwell *et al.,* 2008 and references therein). Denitrification and anammox, in contrast, remove these

nutrients from the environment. If denitrification and anammox rates as we observed them in *G. barretti* turn out to be common processes in sponges all over the world, then marine areas with high sponge cover, e.g. sponge mass occurrences of the North Atlantic as well as tropical and cold-water coral reefs, may function as so far unrecognized sinks for inorganic nitrogen. In order to evalulate the impact of sponges as nitrogen sinks in the ocean it will be important to measure such processes in other marine environments and in more sponge species.

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4 METATRANSCRIPTOMICS OF THE MARINE SPONGE *GEODIA BARRETTI*: TACKLING MICROBIAL DIVERSITY AND FUNCTION OF AN UNCHARACTERIZED MICROBIAL COMMUNITY

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4.1 Abstract

Geodia barretti is a marine cold-water sponge, which harbours high numbers of microorganisms. High levels of nitrification have been observed in this sponge indicating that it might contribute substantially to nitrogen turnover in marine environments with high sponge cover.

In order to get closer insights into the phylogeny and function of the active microbial community and of its interaction with its host, *G. barretti*, a metatranscriptomic approach was employed, using simultaneous analysis of rRNA and mRNA. Of the 262 298 RNA tags obtained by pyrosequencing, 109 325 were assigned to small subunit ribosomal RNA and resulted in a detailed community profile, dominated by Chloroflexi, Poribacteria and Acidobacteria and clearly different from that obtained in the bacterial clone library produced from the same nucleic acid preparation. Optimized assembly strategies allowed reconstructing full-length rRNA genes from the metatranscriptome for detailed phylogenetic studies of the dominant phylotypes. Some of these phylotypes were visualised by FISH analyses. Among the most abundant mRNAs were those encoding key metabolic enzymes of nitrification, in particular from ammonia oxidizing archaea. A number of concomitantly transcribed archaeal genes with unknown function point to their putative role in the energy metabolism of these archaea.

This analysis demonstrates the potential of using a combined rRNA and mRNA analysis approach to explore the microbial community profile, phylogenetic assignments and metabolic activities of a complex, but little explored microbial community.

4.2 Introduction

Marine sponges offer a particularly exciting environment to study microbial community composition and function, because i) they harbour highly complex and specific communities with some lineages and even a whole phylum (Poribacteria, Fieseler *et al.*, 2004) being exclusively found in sponge symbioses, ii) the ecological function of most microbes inside the sponge

system is not understood and iii) sponges and their symbionts are of biotechnological importance as they produce a range of secondary metabolites that are often useful for clinical applications (e.g. treatment against HIV or cancer, (Andavan and Lemmens-Gruber 2010 and references therein).

Although the role of most microbes in the complex sponge community has not been elucidated, several functions have been identified or suggested. Besides the complex chemical defence system of sponge-microbe associations, the role of bacteria in sponges is often coupled with food uptake (Reiswig, 1971, 1975; Pile *et al.*, 1996), supply of photosynthates (Hinde, 1988; Wilkinson, 1992) as well as structural rigidity by mucous production (Wilkinson, 1978) and protection from sunlight (Becerro and Paul, 2004). Microbial processes like sulfur-oxidation and nitrification play a role in eliminating toxic metabolic end products such as hydrogen sulfide and ammonia (Hoffmann *et al.*, 2005; Hoffmann *et al.*, 2009).

In particular, the role of associated microbes in nitrogen cycling has received attention. Usually, sponges ingest nitrogen with their food and excrete ammonia as a metabolic endproduct (Brusca and Brusca, 1990), which can fuel microbial ammonia and nitrite oxidation (Bayer *et al.*, 2008). Using stable isotopes we have recently demonstrated high nitrification rates, but also denitrification and anammox in the Norwegian cold-water sponge *Geodia barretti* (Hoffmann *et al.*, 2009) that harbours high microbial abundance. Beside bacteria, the presence of ammonia oxidizing archaea has been demonstrated in *G. barretti* and other sponges (Preston *et al.*, 1996; Margot *et al.*, 2002; Lee *et al.*, 2003; Pape *et al.*, 2006; Holmes and Blanch, 2007; Taylor *et al.*, 2007; Bayer *et al.*, 2008; Steger *et al.*, 2008). Their high relative abundance (Radax *et al*, in review) indicates a major role in nitrification. Thus sponges offer a great opportunity to characterize these largely uncharacterized and newly detected ammonia oxidizers.

While many studies on marine sponges aim at identifying key players or functions on the DNA level, i.e. by metagenomics (Siegl *et al.*, 2010; Thomas *et al.*) they do not address the question if those microbes are active *in situ*. With the filter-feeding lifestyle of sponges, the detection of a portion of food-

microbes is always a possibility. Therefore, this study aims at providing data on the diversity and function of (likely) metabolically active microbes with a particular focus on characterising the active nitrifying populations in this system.

In the last few years metatranscriptomics has been added to the toolbox in microbial ecology and has been more and more used to characterize the functional response of environmental microbial communities to changing environmental conditions (Poretsky *et al*., 2005; Bailly *et al*., 2007; Frias-Lopez *et al*., 2008; Urich *et al*., 2008; Poretsky *et al*., 2009; Gilbert *et al*. 2008; Shi *et al*. 2009; Stewart *et al*., 2010). This approach in combination with high throughput sequencing technology allows a deeper insight into those genes of the community that are actively transcribed and thus most probably belong to the gene pool that is functional in the given moment of sampling. The information is gathered independent of the knowledge about the taxonomic composition of the microbial community, but often metagenomic analyses or ribosomal rRNA gene studies are performed in parallel to address this. We have recently explored the use of reversely transcribed total RNA for both determination of the structure of the active community and gene expression studies in soil samples, simultaneously (the "double RNA approach", Urich *et al*., 2008). With this approach, the actively transcribing genes of a community can be studied in a single preparatory and sequencing effort on both levels (taxonomy and function) while it allows validation of taxonomic assignments of mRNA. Furthermore, it allows studying the composition of even relatively unexplored microbial communities with respect to all three domains of life simultaneously (Bacteria, Archaea and Eukaryota), and is independent of PCR-based methods that potentially introduce biases. Here we have applied an extended version of this RNA analysis to the characterisation of the microbial community of a sponge-microbe system.

We demonstrate, that this metatranscriptomic approach allows a parallel and quantitative assessment of the active fraction of all three domains of life through analysis of rRNA tags, thus extending beyond the information

obtained by the novel deep sequencing approaches of PCR amplicon libraries (Sogin *et al*., 2006). In addition, it allows characterizing the most abundantly transcribed protein encoding genes concomitant with rRNA-based taxonomic information from the same cDNA preparation. Furthermore we demonstrate, that the approach can also be used to assemble up to full-length rRNA genes from the large dataset of randomly reversely transcribed ribosomal rRNA suitable for phylogenetic studies. Comparing the obtained data to those from 16S rRNA clone libraries and FISH studies, revealed major biases introduced by primer-based studies, which can be circumvented with the approach shown here.

4.3 Material and Methods

4.3.1 Sampling procedure

G. barretti specimen were collected on June 26th 2007 with the Norwegian research vessel "Hans Brattstrøm" using a triangular dredge at Landroeypynten in Langenuen at 59.58.7 N 05.22.89 E in 200-300 m and on October 24th 2007 from Skorpeodden, Korsfjord at 60.10 N 05.10.5 E in 200-300 m. Both sites are located on the Norwegian west coast near the city of Bergen. The samples were cut, rinsed with artificial seawater and immediately plunged in liquid nitrogen on board for storage and further processing.

G. barretti samples for FISH were collected on November 7th, 2008 with the "Hans Brattstrøm" using a triangular dredge from Skorpeodden, Korsfjord and immediately fixed in a solution of 2% formalin, 0.04% glutaraldehyde. After 2-4h the samples were dehydrated in an ethanol series of 15 (min. 1 h), 30 (2 h or over night) and 50% and stored at -20°C in the latter solution of ethanol and artificial seawater.

4.3.2 Nucleic acid extraction

Deep-frozen sponge pieces were cut into small pieces quickly before parallel extraction of RNA and DNA using a modified version of the Griffiths protocol (Griffiths *et al*., 2000), lysing the cells of approximately 0.5 g of sponge (fresh weight) with 0.5 ml of CTAB buffer and PCI in a lysing matrix E tube (Q-Biogene) for 45 seconds with a speed of 4.5 followed by additional phenolisation to eliminate residual RNases and DNases. After precipitation with PEG8000 for 2h and a centrifugation step of 60min, extracts were cleaned and stored at -20°C after addition of RNase inhibitor.

4.3.3 cDNA synthesis for transcriptomics

To increase the amount of RNA transcribed into cDNA per reaction, input for DNase digestion was approx. 5µg and the standard protocol for reverse transcription with superscript III (Invitrogen) was optimised for the amount of input RNA and amount of random primer and enzyme as well as incubation times. In brief, first strand cDNA synthesis was performed with 400 U enzyme and 200 µM random hexamer primers per reaction and transcription at 42°C for 240 min. Two reactions resulted in approximately 500 ng double-stranded cDNA per sample.

For quantitative PCR, only first strand cDNA was prepared according to manufacturer's instructions, whereas 1-2 µg of double stranded cDNA was prepared with an adapted protocol for sequencing. To rule out DNA contamination, respective RNAs were included in qPCR and PCR respectively. Quantity and quality of nucleic acids were measured on a Bioanalyser (Agilent) and Nanodrop.

4.3.4 Clone library construction

Double stranded cDNA applied in metatranscriptomics was also used for 16S rRNA clone library construction. Archaeal and bacterial 16S rRNA, DNA and cDNA clone libraries were constructed individually with primers 21F, 958R

(DeLong, 1992) for archaeal 16S rRNA and 616V, 1492R (Lane, 1991; Juretschko *et al.*, 1998) for bacterial 16S rRNA amplifications, respectively. The gel-purified PCR product was cloned into a pCR4 TOPO vector (TOPO TA cloning kit, Invitrogen) and positive clones were further processed and sequenced at AGOWA (LGC Genomics, Berlin, Germany)

4.3.5 Pyrosequencing

Double stranded cDNA was sequenced on a GF FLX sequencer (Roche Applied Sciences/454 Life Sciences, Barnford, CT) in the Centre for Ecological and Evolutionary Synthesis, Department of Biology, University of Oslo. Samples were independently test-sequenced, resulting in small subsets of RNA-tags (GB1: 200 tags; GB2: 361 tags). The sample GB1 was subsequently sequenced in depth and data in form of FastA files were used for the bioinformatics analyses.

4.3.6 Bioinformatic analyses

4.3.6.1. Separation of rRNA and mRNA tags

Sequences stemming from rRNA and putative mRNAs were identified by comparing all sequences against a combined database of small and large subunits of rRNA (SSU and LSU rRNA) using *blastN* (Altschul *et al.*, 1997) and MEGAN (Huson *et al.*, 2007; Urich *et al.*, 2008). Sequences with a bit score lower than 70 were tentatively assigned as putative mRNA tags. These were further screened for remaining rRNAs by parallel *blastN* and *blastX* analysis against the NCBI nt and nr databases, respectively. Reads with best hits against rRNAs in *blastN* were removed from the putative mRNA fraction, after verification with the *blastX* best hits. If the latter were identified as wrongly annotated hypothetical proteins derived from rRNA genes, the putative mRNA sequences were considered as rRNA and removed from the mRNA fraction.

4.3.6.2. Taxonomic assignment of SSU and LSU rRNA tags

The rRNA sequences were compared against the SSU and LSU reference databases using *blastN*. The LSU rRNA database was beforehand updated with additional Chloroflexi LSU rRNA sequences from the SILVA LSUref database version 94 (www.arb-silva.de, Pruesse *et al.*, 2007). For taxonomic assignment, the 100 best blast hits of each sequence were analysed with MEGAN (Huson *et al.*, 2007) using the following LCA parameters: minimum support 1, minimum bit score 150, top percent 10.

4.3.6.3. Ribo-contig assembly

The ribo-tags had an average length of 223 bases and thus were of limited use for phylogenetic reconstruction using treeing methods. To overcome this problem an assembly approach on the taxonomically binned rRNA reads was applied. Therefore, the parameters of the program CAP3 (Huang and Madan, 1999) were adjusted for it's application on ±250 bp tags as follows. Ribo-tags, binned with MEGAN as phyla Chloroflexi, Poribaceria, Proteobacteria (Alpha-, Gamma- and Deltaproteobacteria), Archaea, Nitrospira, Actinobacteria and Acidobacteria were assembled using the CAP3 program. Parameters were optimized to two rounds of assembly with (1) an minimum overlap of 150 bp with a min. similarity threshold of 99% and mismatch and gap score of -130 and 150 and (2) min. overlap 150 and min. 97% similarity threshold respectively (see suppl. text: Optimisation of parameters for ribo-contig assembly). All assembled ribo-contigs were imported into ARB for inspection and contigs over 800 bp containing a minimum of 100 reads – in addition to all four archaeal contigs were used for phylogenetic analyses.

4.3.6.4. mRNA sequence annotation

The putative mRNA sequence reads were filtered for containing a segment of at least 60 nt length having an average maximum error probability of 2% using LUCY (Chou and Holmes, 2001). The remaining reads were compared to the non-redundant NCBI protein database (Sayers *et al.*, 2010), to a compilation of environmental databases: NCBI env (Sayers *et al.*, 2010),

IMG/M (Markowitz *et al.*, 2008) and HOT02/H179/H186 (courtesy of Y. Shi, E.F. DeLong) as well as an EST database of the marine sponge *Amphimedon queenslandica* (formerly known as *Reneira* sp., JGI-2005, NCBI trace archive) in order to identify homologues and assign a putative function.

The cutoff to assign mRNA tags to a reference gene by *blastX* (using the option –w 15 to correct for sequencing errors causing frame-shifts) was a minimum E-value of 10^{-4} and an alignment covering at least 80% of the shorter sequence length (adapted from Mou *et al.*, 2008). To obtain a list of highest transcribed genes for each kingdom, best *blastX* matches to the UniRef90 (Jain *et al.*, 2009) database were parsed with a custom made perl script, separated according to the taxonomic affiliation of the respective homologue and normalized over the length of the corresponding gene sequence.

4.3.7 Fluorescence *in situ* hybridization (FISH)

Fixed samples of three *G. barretti* individuals were cut in small cubes and embedded in O.C.T. compound (Tissue Tek), followed by cryosectioning to 8-9 µm thin sections. The sections were exposed to UV light for 15min to bleach parts of autofluorescence before dehydration with a graded series of ethanol (3 min each in 50, 80 and 96% ethanol). The hybridization was performed in an isotonically equilibrated chamber at 46°C in hybridization buffer, and 20% to 35% FA concentrations for 3-4 h. Probes and concentrations are given in Table S1. After hybridization, slides were incubated 10 min in preheated washing buffer (composition corresponds to hybridization stringency) at 48°C, subsequently dipped in ice-cold distilled water and air-dried. For visualization slides were mounted with the anti-fadent Citifluor (Citifluor Ltd., London, UK) and placed on a confocal laser-scanning microscope.

Table S4.1: FISH probes used in this study

4.3.8 Data deposition

The pyrosequenced metatranscriptomic data of *G. barretti* have been deposited in the EBI short reads archive (acc. No). Assembled ribo-contigs are available from the authors upon request. The SSU rRNA clones are deposited under accession numbers xxxxx to xxxxx (16S rRNA genes of archaea) and xxxxx to xxxx (16S rRNA genes of bacteria).

4.4 Results and Discussion

4.4.1 Sequencing of the *G. barretti* Metatranscriptome

RNA preparations from two different biological samples of *G. barretti* were randomly reversely transcribed into double stranded cDNA without any intermediate amplification steps. The cDNA was subjected to pyrosequencing on a Roche GS FLX Sequencer, resulting in sequences with a mean read length of 223 bp.

A small-scale pyrosequencing run of the biological replicates was performed to verify reproducibility of the analysis and revealed that approx. 25% of the SSU rRNA tags originated from the eukaryotic host in both samples (see Fig. S4.1). The remaining fraction was assigned to twelve bacterial phyla and one archaeal phylum (as discussed in detail below for sample GB1) with comparable relative distributions in both samples.

Replicate GB1 was sequenced in-depth and yielded 262 298 “RNA-tags”. After comparison to the compiled rRNA reference databases more than 90% (240 973) of all sequences were assigned as ribosomal RNA reads (“ribo-tags”), while 8.1% (21 325) were assigned as non-rRNA sequences. Putative mRNAs were identified by *blastX* searches. While 3 929 tags had a homologue in the NCBI-nr database, additional 5 471 and 327 reads were similar to sequences in the compiled environmental and sponge EST databases, respectively (see Table 4.1). This yielded 10 023 putative mRNA-tags in total.

Table 4.1. Statistics of cDNA sequence analysis

	No. of reads	%
total RNA-tags	262 298	100.0
rRNA	240 973	91.9
non-rRNA	21 325	8.1
putative mRNA*	10 023	3.8
NCBI-nr	3 929	1.5
Env	5 471	2.1
sponge ESTs	327	0.1

*The putative mRNAs were considered significant when a *blastX* hit to a homologue in one of the three databases had an e-value < 10^{-4}

4.4.2 Taxonomic community profile based on ribosomal RNA tags

The analysis of the SSU rRNA community profile revealed Chloroflexi as dominant members of the community in *G. barretti* (36% of all and 50% of the bacterial ribo-tags) (Fig. 4.1). The number of their SSU ribo-tags exceeded even that of the host, *G. barretti*, which only constituted 27% of all ribo-tags, despite the considerably bigger cell size and therefore probably higher ribosomal content of the eukaryotic cells. With 73% of ribo-tags from bacteria and archaea, the sponge holobiont was constituted mostly of microorganisms.

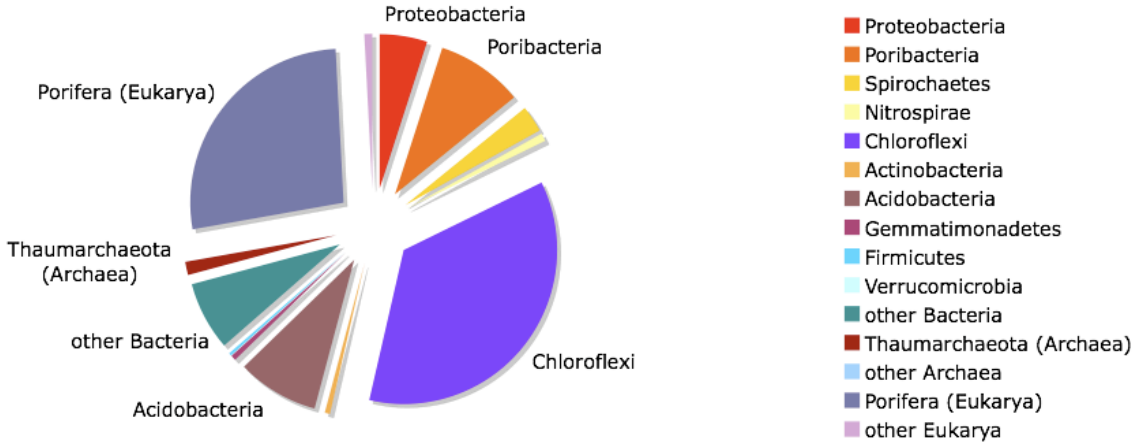

Figure 4.1. Three-domain community profile of SSU ribo-tags (rRNA tags) from the metatranscriptome of *Geodia barretti*. Phyla of Bacteria, Archaea and Eukaryotes with ≥ 0.01% relative abundance are displayed.

Other abundant bacterial phyla were Poribacteria (13.2% of bacterial ribo-tags), Acidobacteria (12.2%), Proteobacteria (6.9%), and Spirochaeta (3.8%) (Fig. 4.1).

According to SSU profiling, archaea accounted for 2% of the total microbial community, and 99.98% thereof were affiliated to group I.1A Thaumarchaeota, i.e. the lineage of ammonia-oxidizing archaea that is found in high abundance in marine environments, including sponges (DeLong, 1992; Preston *et al.*, 1996; Wuchter *et al.*, 2006; Francis *et al.*, 2007; Mincer *et al.*, 2007; Martin-Cuadrado *et al.*, 2008; Mosier and Francis, 2008; De Corte *et al.*, 2009; Beman *et al.*, 2010; Bernhard *et al.*, 2010; Church *et al.*, 2010; Dang *et al.*; Santoro *et al.*, 2010; Urakawa *et al.*, 2010). In total, ten prokaryotic phyla were present in considerable abundance (≥ 0.1%) in *G. barretti*, while rRNA of five phyla and candidate divisions was sequenced in lesser amount (not shown).

4.4.3 Assembly of longer rRNA contigs from the metatranscriptome

The reverse transcription yielded random regions on the 16S rRNA and therefore allows for *in silico* assembly of long, ideally full length, rRNA sequences for certain abundant taxa. This allows for phylogenetic analysis of the most abundant organisms in addition to a mere taxonomic assignment of reads. The alteration of variable and conserved regions on the SSU rRNA molecule poses challenges to a reliable assembly without chimeric rRNA formation from the short ribo-tags. We have therefore performed pilot studies on model "communities" *in silico* to evaluate the assembly of SSU rRNA sequences of closely and more distantly related species from the six most abundant bacterial higher taxa (phyla, classes) found in *G. barretti*. Briefly, each of the test communities consisted of three known rRNA genes with one (the "outlier") being 87- 95% identical to the other two, which were more closely related to each other (97-99% identity). The rRNAs were randomly cut into 250bp fragments, mixed and reassembled using the program CAP3

(Huang and Madan, 1999). After extensive testing of different parameter (see materials and methods and supplementary information for details) a 2-stage assembly process was developed. The first stage involved assembly with highly stringent fragment-overlap (≥150 bases) and sequence similarity parameters (≥ 99%), resulting in contigs with increased read-lengths. These were assembled in the second stage with slightly more relaxed parameters (≥97% sequence identity, ≥150 bp overlap to obtain longer rRNAs, and to account for pyrosequencing errors. The tests showed good separation of the "outlier" rRNA from the more closely related sequences in all test-communities, whereas a clear separation of the more similar sequences was not always achieved.

From the metatranscriptomic dataset of *G. barretti*, 304 SSU rRNA contigs were assembled with sizes up to 1 820 base pairs and a mean length of 729 base pairs (Table 4.2). 76.2 % of the 66 743 used SSU ribo-tags assembled into contigs. For Poribacteria even 85.6 % of the reads were assembled (Table 4.2). Notably, some contigs extented over the 3' end of the 16S rRNA into the intergenic spacer region, giving a good indication for active populations. This was observed for Poribacteria, Chloroflexi, Gemmatimonadetes and Nitrospira.

Table 4.2. Ribo-contig assembly statistics

	no. of reads	**assembled reads**	**% assembled**	**No. contigs**	**Ctgs>800bp (% reads)**	**Mean contig length**
Archaea	1316	1052	80	4	2 (68%)	940
Acidobacteria	8946	6006	67	49	19 (43%)	701
Actinobacteria	640	283	44	10	2 (31%)	605
α - proteobacteria	1284	701	55	10	3 (51%)	618
γ - proteobacteria	2582	1263	49	36)	4 (25%)	523
δ - proteobacteria	598	236	39	7	-	334
Cand. div VC2	343	256	75	2	-	440
Chloroflexi	36978	29428	80	137	46 (65%)	720
Gemmatimonadetes	606	241	40	13	2 (16%)	532
Nitrospirae	805	657	82	3	2 (76%)	1194
Poribacteria	9748	8347	86	22	12 (79%)	1014
Spirochaetes	2897	2424	84	11	6 (74%)	920
overall assembly	**66743**	**50894**	**76.2%**	**304**	**98**	**729**

The quality of assembled ribo-contigs was evaluated by three different methods: (i) Similarity analysis against the NCBI nt database revealed that all contigs exhibited high similarity to sequences deposited in the databases. (ii) Alignments to reference sequences in ARB revealed that a fraction of short contigs (average of 543 bp) with a small number of reads (average of 24 ribo-tags) were potential chimeras by manual inspection in ARB taking into account secondary structure and reference sequences. Those 5.3% of all contigs contained 0.8% of assembled ribo-tags and were excluded from further analysis. (iii) Comparison of assembled contigs with sequences from bacterial and archaeal 16S rRNA clone libraries (obtained from the same cDNA preparations) showed high sequence identity (see also phylogenetic studies below).

4.4.4 Phylogenetic analysis of abundant sponge-derived sequences

Ribo-contigs used for phylogenetic calculations were at least 800 base pairs long and contained 100 or more reads. Shorter contigs with more than 100 reads were also added into phylogenetic trees. Furthermore, sequences from SSU rRNA clones obtained from PCR products with bacterial and archaeal primers, respectively, of the same cDNA preparations were included (224 bacterial and 50 archaeal).

Figure 4.2a and b display the phylogenetic relationships of two groups, which are potential nitrifying organisms in *G. barretti*, the ammonia oxidizing Thaumarchaeota and the nitrite oxidizing Nitrospira.

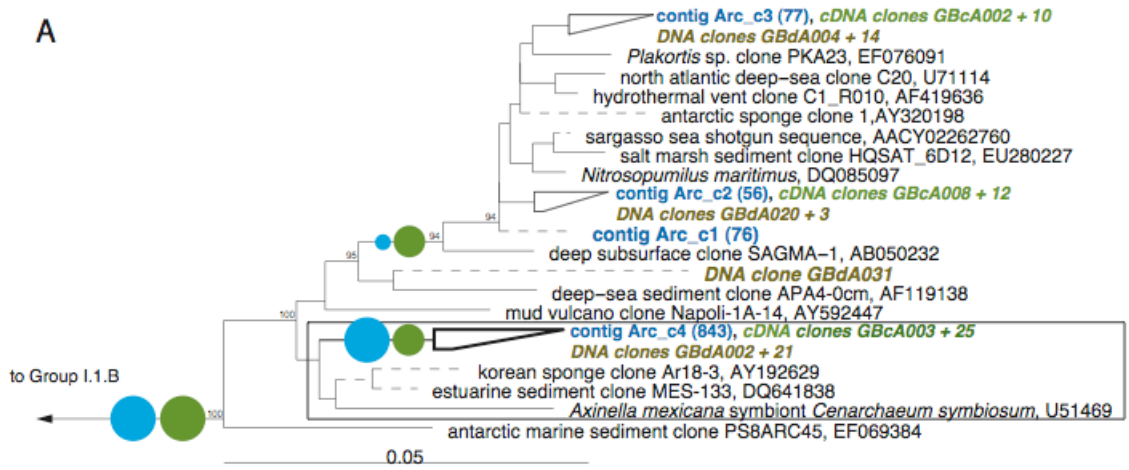

Figure 4.2a. Phylogenetic analysis (maximum likelihood algorithm) of 16S rRNA sequences from microorganisms potentially involved in nitrification, i.e. a) the archaeal phylum Thaumarchaeota group I.1a (potential ammonia oxidizers). Assembled ribo-contigs of the *Geodia barretti* metatranscriptome are depicted in blue, while clones from the DNA and cDNA 16S rRNA clone libraries are shown in green. The correspondingly colored circles at major branches of the tree indicate the relative amount of ribo-contigs or clones in the respective monophyletic group. The number of ribo-tags in each contig is given in brackets. Full-length sequences were used for treeing calculation and shorter sequences were added via the parsimony interactive tool in ARB. Parsimony bootstrap numbers are given if branch support is more than 75%. Size bar indicates 10% sequence divergence. Various sequences from Thaumarchaea group I.1b (in a) or other bacterial phyla (in b) were used as outgroup.

The archaea are restricted to group I.1.a Thaumarchaeota, wherein they form two main phylotypes. The most abundant contig (843 ribo-tags) is affiliated to "*Candidatus* Cenarchaeum symbiosum" (95% similarity) and other sponge symbionts. The other three contigs (209 ribo-tags overall) cluster with seawater-derived clones, such as *Nitrosopumilus maritimus* (98-99% similarity). Both ribo-contigs and clones share the same phylogenetic clusters and are approximately 99% similar.

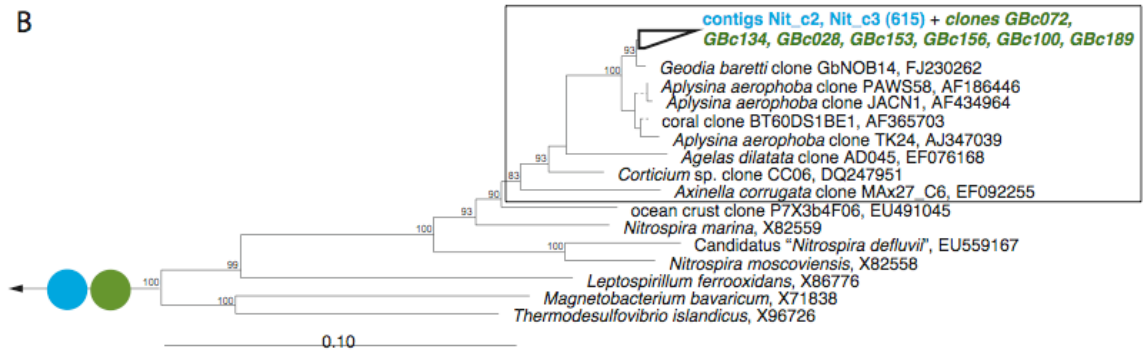

Figure 4.2b. Phylogenetic analysis (maximum likelihood algorithm) of 16S rRNA sequences from microorganisms potentially involved in nitrification, i.e. b) the bacterial phylum Nitrospira (potential nitrite oxidizers). For further details on phylogenetic analysis, see Figure 2a.

Similarly, sequences affiliated with the nitrite-oxidizing genus Nitrospira (Fig. 4.2b), are recovered from ribo-tag assembly as well as in the clone library. They belong to a sponge-specific cluster (Nitrospira sublineage IVb, Maixner 2009) recognized in earlier diversity studies of sponges (reviewed in Taylor *et al.*, 2007).

Table 4.5. Results of contig assembly verification and overview of genome similarity

	Maximum similarity to NCBI	Percentage of contigs with min. 97% similarity to clones	Maximum similarity to genome
Archaea	99%	100	98%
Acidobacteria	99.8%	79.2	85.4%
Actinobacteria	99.6%	100	92%
α - proteobacteria	100%	62.5	92%
γ - proteobacteria	100%	77	91.7%
δ - proteobacteria	99%	83	88.4%
Cand. div. VC2	98%	100	89.2%
Chloroflexi	99.6%	19.5	85.7%
Gemmatimonadetes	98.9%	73	86.9%
Nitrospirae	99.7%	66	92%
Poribacteria	97.4%	0	97%
Spirochaetes	98%	90	87.9%

High congruence between ribo-contig phylogenies and rRNA clones was also obtained for Gamma-proteobacteria (suppl. Fig. S4.3: Phylogenetic analysis of 16S rRNA sequences from gammaproteobacterial sponge symbionts), Delta-proteobacteria, Actinobacteria, Spirochaetes and the candidate division VC2 (see Table 4.5), indicating that the assembly of long, often full-length, rRNA molecules from metatranscriptomic datasets can be performed with high reliability.

The assembly of full-length 16S rRNA contigs also allowed inferring the phylogenetic position of those highly abundant symbionts that were not (or poorly) recovered in the clone libraries. Most ribo-contigs of Chloroflexi belonged to the little characterised, yet uncultured SAR202 clade (Fig. 4.3) (Morris *et al.*, 2004). The monophyletic sequence cluster comprising contig Chl_c24 and Chl_132 with 6 269 ribo-tags together (21.3% of assembled

Chloroflexi ribo-tags), appeared to be a sponge-specific cluster including sequences of at least four other sponges (Fig. 4.3).

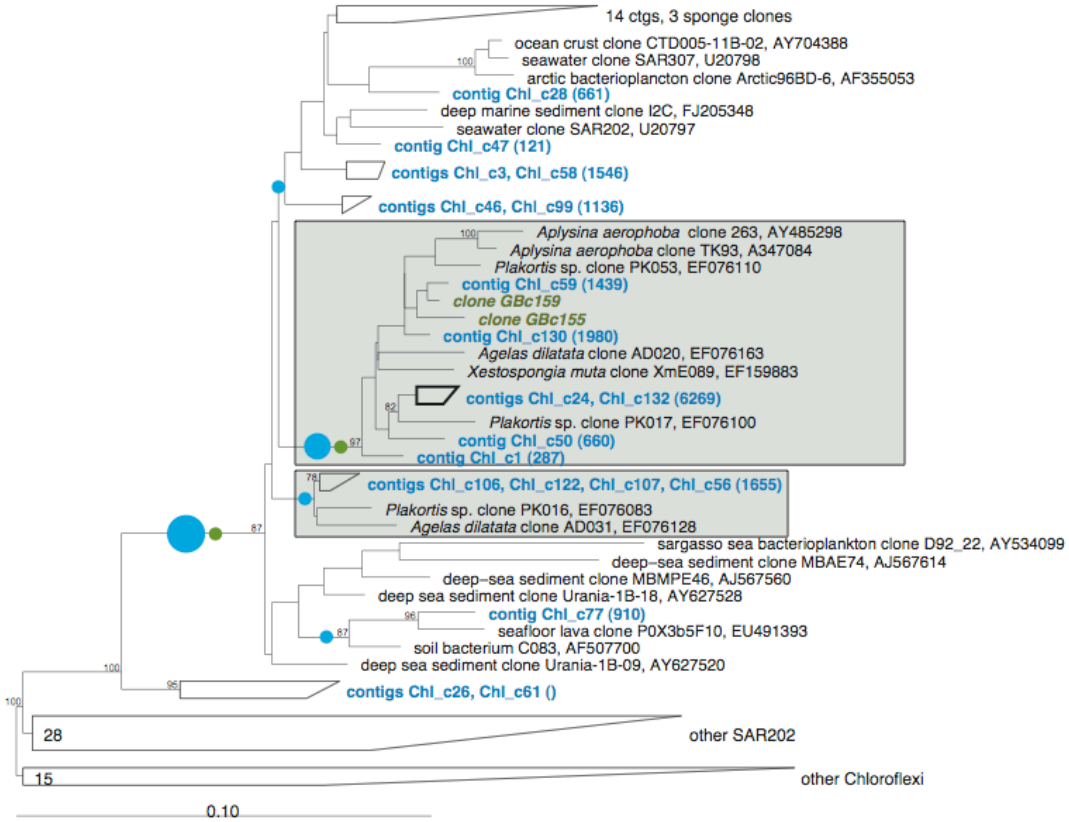

Figure 4.3. Maximum likelihood tree for the subgroup of the SAR202 cluster (phylum Chloroflexi) with the majority of sequences from the metatranscriptome. Assembled ribo-contigs from the *Geodia barretti* metatranscriptome are depicted in blue, while clones from the cDNA 16S rRNA clone library are shown in green. The correspondingly colored circles at major branches of the tree indicate the relative amount of ribo-contigs or clones in the respective monophyletic group. The number of ribo-tags in each contig is given in brackets. Full-length sequences were used for treeing calculation and shorter sequences were added via the parsimony interactive tool in ARB. Parsimony bootstrap numbers are given if branch support is more than 75%. Size bar indicates 10% sequence divergence. Other sequences from SAR202 group were used as outgroup.

Overall, 11 441 (39%) of the assembled ribo-tags classified as Chloroflexi were in this cluster, whereas only two sequences from the clone library (out of 226) were affiliated to this group. The second large cluster depicted on top of the tree (see Fig. 4.3) comprised sequences mainly derived from marine sediment and water and included 9 678 ribo-tags (32.8%) in contigs.

The phylogenetic reconstruction with ribo-contigs from the Poribacteria revealed two subclusters (Fig. S4.4), one of them including two metagenome-derived 16S rRNA sequences from the mediterranean sponge *Aplysina aerophoba*. In this cluster, ribo-contigs had maximally 97% similarity to the

16S rRNA from the draft genome of *Poribacterium* sp. WGA-A3 (Siegl *et al.*, 2010) and 98% similarity to the 16S rRNA located on the fosmid 64K2 (Fieseler *et al.*, 2006), respectively. From the overall 7 761 ribo-tags, 4 228 (54.5%) were in this cluster and 3 533 (45.5%) were in the second subcluster.

Figure S4.2 displays a comparison of the bacterial community structure from the 16S rRNA clone library and the ribo-tags (not contigs) of the metatranscriptome obtained from the same cDNA.

Table 4.3. Matches/mismatches of universal primer set 616V/1492R to respective sequences of seven phyla from the metatranscriptome of *G. barretti**

5' – 3'	**616V** AGA GTT TGA TYM TGG CTC	**1492R** GGY TAC CTT GTT ACG ACT T	**clones**	**ribo-tags**
Proteobacteria	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT ACG ACT T	25%	6.9%
Alpha-	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT ACG ACT T	6.6%	1.8%
Gamma-	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT ACG ACT T	7.6%	3.5%
Spirochaeta	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT Y*CG ACT T * T for 400 tags, C for 322 tags	0.9%	3.8%
Gemmatimonadetes	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT ACG ACT T	5.3%	0.8%
Acidobacteria	AGA GTT TGA TYM C*GG CTC * for 75 tags	AGY TAC CTT GTT T*CG ACT T * for 1162 tags	35.6%	12.2%
Nitrospira	AGA GTT TGA TYM TGG CTC	GGY TAC CTT GTT ACG ACT T	3.6%	1.1%
Chloroflexi	AGA GTT TGA TYM TGG CTC	AGY TAC CTT GTT T*CG ACT T * for 176 reads	8.0%	50.6%
Poribacteria	AGA GTA TGA TAM CGG CTC	A*GY TAC CTT GTT T#CG ACT T * 188 tags # 65 tags	0%	13.2%

*Phyla with primer mismatches (in red) showed a clear shift in their relative abundance in the clone library compared to their relative abundance in the ribo-tag pool.

Sequences of those phyla with a big discrepancy in their relative abundance between clone library and ribo-tag profile (Chloroflexi, Acidobacteria) showed a frequent mismatch on the 5' end of the reverse primer 1492R with only 5 to 8.6% of tags matching (guanine), and 90 and 92% respectively, having an adenine instead of the guanine, indicating potential primer biases in the clone libraries (Table 4.3).

Poribacterial sequences are known to be un-amplifiable by commonly used primers, (Fieseler *et al.*, 2004) and are therefore not represented in the clone library.

4.4.5 Visualization of bacteria and archaea by fluorescence *in situ* hybridisation

In order to verify the presence of certain phyla, which were underrepresented by one of the two analyses (ribo-tags or clone libraries), fluorescent *in situ* hybridisations (FISH) with probes against Archaea, Poribacteria, gamma-Proteobacteria (Fig. 4.4) and Chloroflexi in ultrathin sections of the sponge was performed.

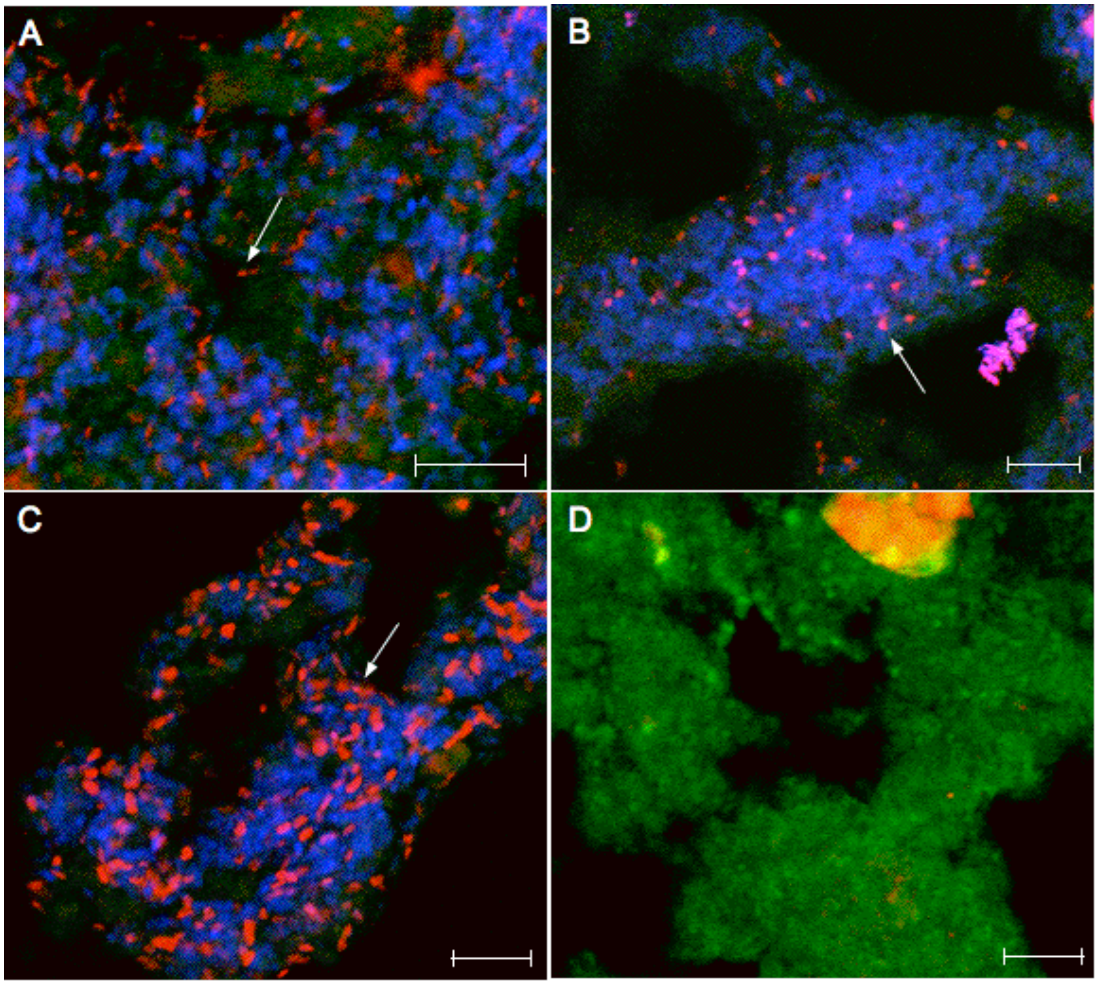

Figure 4.4. Fluorescence *in situ* hybridisations of *Geodia barretti* mesohyl sections, showing a) archaea b) Gamma-Proteobacteria c) Poribacteria and d) autofluorescence (non-EUB probe). Arrows indicate signals of respective cells and bars represent a distance of 5µm.

Due to the high microbial density in *G. barretti* it was unreliable to perform cell counts relative to the amount of total microbial cells. However, we were able to demonstrate the presence of large amounts of archaea and poribacteria as well as the presence of gamma-proteobacteria (Fig. 4.4). Hybridisations for Chloroflexi did not result in clear signals, which could be

tracked down to mismatches of probes used (Table S4.1.) to ribo-contigs in the dominant cluster of uncultured SAR202-related bacteria. While the dominance of Poribacteria over gamma-Proteobacteria as seen in the rRNA-based taxonomic profile was reflected in the FISH analysis, the large amount of archaea was surprising, given their relatively low abundance in the rRNA community profile, but also confirmed by real-time PCR (Radax *et al.*, in review).

An underestimation in the metatranscriptome could be due to a lower amount of ribosomes in archaea compared to bacteria or due to their smaller cell size.

4.4.6 Functional profile of the microbial *G. barretti* community

For a general insight into microbial processes in *G. barretti*, all mRNA tags were compared to a merged database of clusters of orthologous groups (COG, NOG, KOG) and an updated version of archaeal COGs (= arCOG, Makarova *et al.*, 2007). The deduced protein sequences of 3 158 mRNA sequence tags had homology to 1 206 fused COGs. The COG with highest number of mRNA tags was arCOG08647 (89 tags) assigned to a family of hypothetical proteins with PKD domains (suppl. Table S4.2), that are also found in *N. maritimus* and *C. symbiosum* and could represent the surface-layer protein of these archaea.

The second abundant cluster was COG0004 (ammonia permease, 67 sequence tags), followed by COG5267 and NOG78312 with unknown functions and of bacterial origin. Generally, many highly transcribed genes were potentially involved in transport, energy metabolism or were of unknown function. When sorting the mRNAs to the three domains of life according to their best match to the UniRef90 database, we found that most of the functions (COGs) were represented by reads from only one domain, i.e. originating only from archaea, bacteria or eukaryotes, respectively (Fig. 4.5). While, according to the COG category “energy production and conversion”, archaea clearly gain

their energy from ammonia oxidation, bacterial key enzymes involved in a variety of metabolisms, including nitrite oxidoreductase (COG1140) and aerobic-type carbon monoxide dehydrogenase (COG1529, COG1319, COG 2080), were transcribed.

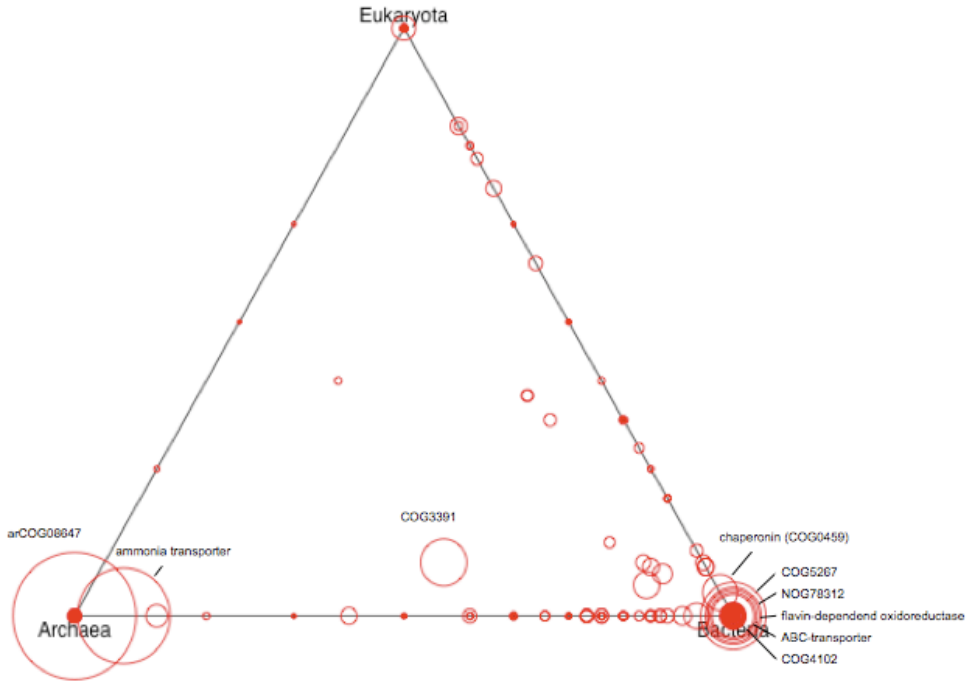

Figure 4.5. Depiction of mRNA tags with matches to clusters of orthologous groups (COG, NOG, KOG and arCOG), and their affiliation to the three domains of life in the metatranscriptome of *Geodia barretti*. Number of tags in a certain cluster corresponds to the size of the circle. The localisation of the circles is determined by the number of tags classified as bacteria, archaea and/or eukarya. Clusters in the vertices are specific, while clusters inside or on the sides of the triangle have matches to more than one domain.

Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase related proteins (COG2141) were among the more abundant bacterial gene transcripts in this category, indicating an oxidation of aldehydes. Furthermore, oxidoreductases potentially related to alcohol dehydrogenases (COG0667, COG1062), pyruvate/2-oxoglutarate dehydrogenases (COG0022) and isocitrate dehydrogenases (COG2838) were found in this category. Eukaryotic transcripts (for top ten eukaryotic transcripts see Table S4.4) related to proteins involved in signal transduction and secretion, including fibrillins, TNF-receptor-associated proteins, clathrin-associated proteins and phosphatidylinositol kinase (KOG1217, KOG0297, KOG2740, KOG0985, COG5032) were dominating, together with proteins from the cytoskeleton, like actin and tubulin (COG5277, COG5023).

In order to better characterize the distribution of COGs in our dataset, we compared mRNA reads from *G. barretti* to other marine metagenomic and metatranscriptomic datasets. Interestingly, the majority of COGs identified in *G. barretti* (244 COGs) were shared with a metagenomic dataset from an Australian sponge (*Cymbastela concentrica*) living in the photic zone (Thomas *et al.*, 2010), while COGs in the *G. barretti* metatrasncriptome had 49 COGs in common with seawater sequences (Shi *et al.*, 2009; Vila-Costa *et al.*, 2010). The COGs exclusively found in both sponge datasets included, among others, transposases (COG0675, COG3335, COG2801, COG3293) that were implicated earlier to be abundant in sponge-symbiont interactions (Thomas *et al.*, 2010; Siegl *et al.*, 2010). Transcripts of the *G. barretti* dataset that pointed to specific activities of beneficial symbionts encoded cobyrinic acid a,c-diamide synthase (COG1797), involved in biosynthesis of cobalamin/vitamin B12, because the cofactor Vitamin B12 is essential for many animals and is primarily produced by symbiotic bacteria. Bacterial transcripts for phytanoyl-CoA dioxygenase (KOG3290, NOG81762) could point to a bacterial consumption of phytanic acid produced by the sponge. Transcripts of proteins involved in cell recognition, adhesion and/or signaling, like ankyrin repeat proteins (COG0666, KOG0522) Concanavalin A-like lectin/glucanase (arCOG07813), TonB dependent receptors and collagen-binding surface proteins (COG1629, COG0811, COG0543, COG0810, NOG133096, NOG138886, NOG69105, NOG69934, NOG68430) are also present in the *G. barretti* metatranscriptome. The occurrence of a subunit of the sup-operon (*sup*D, sponge symbiont ubiquitous PKS) with ~50% identity to the *Aplysina aerophoba* metagenome fragment pAPKS18 (Siegl *et al.*, 2010), suggests the production of bioactive compounds for chemical defense.

4.4.7 Taxonomic classification of mRNA sequences

Simultaneously obtained rRNA and mRNA provide the opportunity to obtain taxonomic profiles from both molecules (Urich *et al.*, 2008). Whereas the taxonomic classification of mRNA-tags to the 3 domains, Archaea, Bacteria

and Eukarya using the best Blast match to Uniref90 reflected approximately the taxonomic composition obtained by the SSU ribo-tags, strong differences were observed at higher resolution, in particular for the bacterial phyla. For example, mRNA from Proteobacteria was most abundant, whereas only a small fraction of mRNAs was assigned to Chloroflexi and Acidobacteria (> 5% and >2%), the most abundant phyla reported by SSU rRNA. We tested if this incongruency resulted from the lack of suitable reference genomes. By running a simulation with 5 different classification programs – TACOA (Diaz *et al.*, 2009), MEGAN (Huson *et al.*, 2007), CARMA (Krause *et al.*, 2008), SOrtITEMS (Monzoorul Haque *et al.*, 2009), PhymmBI (Brady and Salzberg, 2009). The simulation included 100 250 bp long randomly generated genome tags of three species from three different phyla and two different domains, i.e. *Dehalococcoides* sp. VS (phylum Chloroflexi), *Nitrospira defluvii* (phylum Nitrospirae) and the archaeal species "*Candidatus* Nitrososphaera gargensis" (phylum Thaumarchaeota). The latter two genomes were not yet in the reference databases at the time of simulation, reflecting a similar situation as in the *G. barretti* metatranscriptome dataset. Whereas most sequences of *Dehalococcoides* sp. VS were correctly assigned by all programs, comparably few correct assignments were obtained for *N. defluvii* and *N. gargensis* (see suppl. text: Optimisation of ribo-contig assembly). Only the program MEGAN resulted in exclusively correct assignments, although the majority of sequences (~75%) remained un-assigned. This highlights the importance for more references genomes from representatives of yet poorly characterised phyla. Initiatives like the Genomic Encyclopedia of Bacteria and Archaea (Wu *et al.*, 2009) will be of special value. In this metatranscriptomic study no close reference genomes were available for SAR202-Chloroflexi and Acidobacteria (< 86% identity between sponge SSU rRNAs to SSU rRNA genes of closest related genome sequence), which however represented dominant groups in the microbial community. We therefore refrained from performing a general taxonomic classification of all mRNAs to the different bacterial phyla.

4.4.8 Highly transcribed genes from archaea are involved in ammonia oxidation

Despite the limited success to taxonomically classify bacterial mRNA, we were able to identify mRNAs from the archaeal population in *G. barretti* due to the availability of two genomes from the closely related species of the thaumarchaeal group I.1.a (Fig. 4.2a.), "*Candidatus* Cenarchaeum symbiosum" (Hallam *et al.*, 2006) and *Nitrosopumilus maritimus*, respectively (Walker *et al.*, 2010).

12% of all annotated mRNA tags (UniRef90) were from archaea, although the relative archaeal abundance measured on the level of ribosomal RNA was lower (2 %, Fig. 4.1.). High transcriptional activity of archaea has, however, been confirmed in a quantitative study of *amo*A genes using qPCR (Radax *et al.*, in review) and by their high abundance in FISH analysis (Fig. 4.4.).

All suspected archaeal key enzymes for ammonia oxidation were present among the highest transcribed genes (Table 4.4.), i.e. transcripts of the ammonia monooxygenase subunits A (Nmar_1500, CENSYa_0402), B (Nmar_1503, CENSYa_0394) and C (Nmar_1502, CENSYa_0670), as well as of NirK-like multicopper proteins (Nmar_1259, Nmar_1667, see (Bartossek *et al.*, 2010) were found as main constituents in the archaeal mRNA-tag pool.

Interestingly, also ammonia transporters (COG0004) were among the most highly transcribed genes suggesting that an effective transport system is needed for ammonia oxidation or perhaps ammonia assimilation in archaea. In parallel, also a urea transporter (COG0591) was transcribed (4 archaeal plus one bacterial tags, which might point to the capability of the archaeal population to use both ammonia and urea as substrates. Interestingly, genes of archaea involved in ammonia oxidation and transport were also found in high abundance in a metatranscriptomic study in a coastal bacterioplankton pointing to highly active archaeal populations (Shi *et al.*, 2009; Hollibaugh *et al.*, 2010).

Table 4.4. Ten most abundant archaeal transcripts in the *G. barretti* metatranscriptome*

Coverage*	Accession no.	Bit score	Predicted function	No. of hits	Closest ortholog in
7.88	A9A2R7 & A0RXK8 & B3T383	108.3	ammonium transmembrane transporter	62	Thaumarchaeota
4.10	*A0RX84 & A9A3F6*	*84.7*	*Putative uncharacterized protein*	*8*	*Thaumarchaeota*
4.05	A9A3F7	91.1	Transcriptional regulator, AbrB family	7	Thaumarchaeota
3.85	A9A5U2 & A9A4Y9 & A0RZ96 & A0RTY9 & A0RTY7 & A0RZ95	78.15	Putative uncharacterized protein	86	Thaumarchaeota
3.72	A9A4U4	147.1	Ammonia monooxygenase, subunit C (AmoC)	10	Thaumarchaeota
3.15	B5LRU4 & B3Y8E4 & C8BMD6 & A0RUM1 & C9K7J9 & C0KGQ9	144.8	Ammonia monooxygenase, subunit A (AmoA)	9	Thaumarchaeota
2.14	A9A1H6	113.9	multicopper protein/nitrite reducatase (NirK)	14	Thaumarchaeota
2.05	A0RUL3 & A9A4U5	108.9	Ammonia monooxygenase, subunit B (AmoB)	6	Thaumarchaeota
1.50	*A9A4X9*	*163*	*4Fe-4S ferredoxin iron-sulfur binding domain protein*	*2*	*Thaumarchaeota*
1.14	C3MKY5	105.7	Thiamine pyrophosphate protein domain protein TPP-binding	5	Crenarchaeota

*coverage was calculated with the mean read length of mRNA tags over the length of the protein (closest known ortholog).

Within the highly transcribed archaeal genes were five proteins with unknown function but specific for group I.1a of Thaumarchaeota (Nmar_0992/CENSYa_1328, Nmar_0505/CENSYa_1398, Nmar_1506/ CENSYa_0391, Nmar_1282/ CENSYa_0064, Nmar_1498/CENSYa_0404), both subunits of the cell-division protein cdvAB (Nmar_700/CENSYa_0268 and Nmar_0816/CENSYa_0986), a highly conserved 4Fe-4S ferredoxin iron-sulfur binding domain protein (Nmar_1537/CENSYa_1647) and a blue copper protein (Nmar_1650, 1273/CENSYa_1796), which are probably involved in electron transfers and part of the energy metabolism in archaea (Table 4.4).

No bacterial *amo* transcripts or any other dominant bacterial funtional genes were identified in our dataset (Table S4.3) indicating that ammonia oxidizing archaea are the major drivers of nitrification. Nitrite oxidizers performing the second step in nitrification (oxidation of nitrite to nitrate) could similarly be identified on the SSU RNA (Fig. 4.2b.) and functional gene level, as transcripts of the potential nitrite oxidoreductase *nxr*, subunit A and B with high similarity to the genes of *Nitrospira defluvii*, (Lücker *et al.*, 2010) are found. Bacterial transcripts indicative of anaerobic nitrogen processes (i.e.

denitrification, anammox) could not be identified, whereas aerobic nitrification seemed to be active, indicating that the sponge was oxygenated at the time of sampling by actively pumping water.

4.5 Conclusion

We have used the marine cold-water sponge *G. barretti* as a case study to explore the applicability of metatranscriptomics for the characterization of a complex and little studied microbial community both on its structural and functional level. Different from other approaches the random reverse transcription of total RNA allows qualitative and quantitative assessment of both rRNA and mRNA markers in parallel.

This allowed us (1) to characterize the sponge "holobiont" (the host and its symbiotic microbial community) in a single RNA analysis and independent of PCR primers, (2) to use the large rRNA tag dataset for taxonomic profiling and quantitative assessment of eukaryota, archaea and bacteria, (3) to reassemble the full length rRNA genes of the most abundant OTUs and thus to obtain detailed phylogenetic characterization of the respective organisms, and (4) to analyse the most abundant mRNA species of the holobiont in the context of a detailed community profile and to identify archaeal genes of ammonia oxidation as the most abundantly transcribed genes in the community.

The limitations of our approach (and similar approaches) are given through the quality of reference databases and reference genome sequences as well a by the quality of deep sequencing (all of which are, however, constantly improving). For example, the assembly of rRNA genes from the complex datasets needs to take into account the error rates of the sequencing and thus is not suitable for the study of "species-level" diversity. Our comparison of *in silico* assembled rRNA genes and genes from clone libraries shows, however, that the approach is well suited to even reconstruct complete or nearly complete rRNA genes. Additionally, different from PCR-based approaches it is less biased and allows detailed study of groups that are not or only

insufficiently captured in other analyses, as e.g. the Chloroflexi and Poribacteria.

4.6 Acknowledgements

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4.8 Supplementary Material

4.8.1 Supplementary Figures

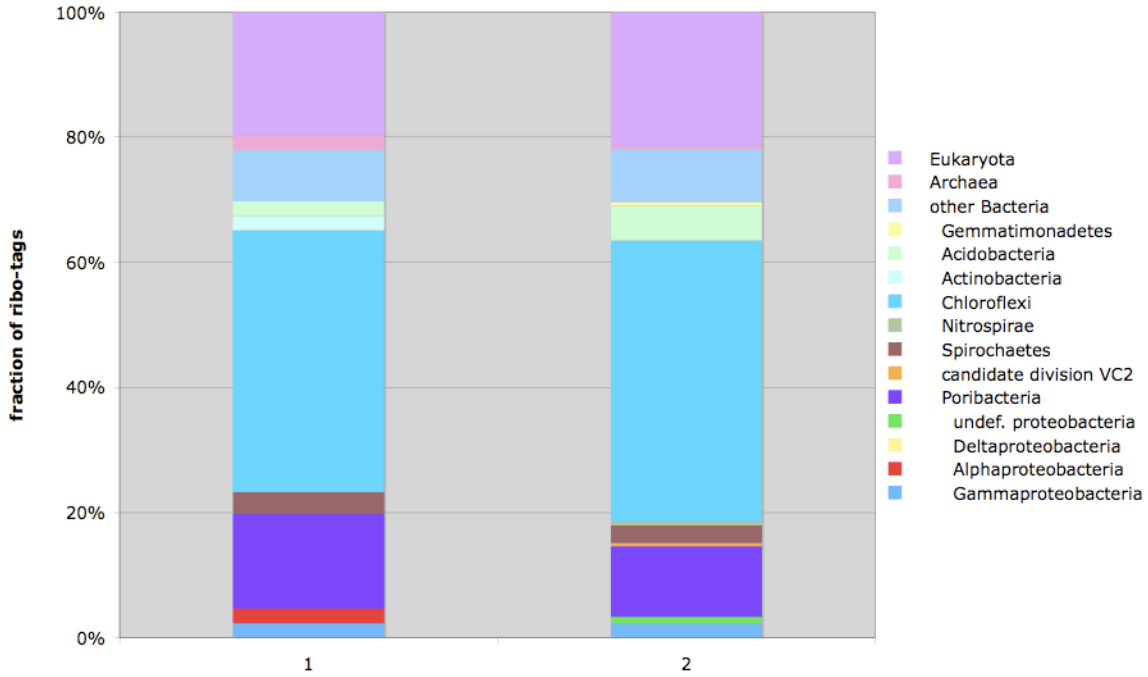

Figure S4.1. SSU ribo-tag community profile of two *G. barretti* samples

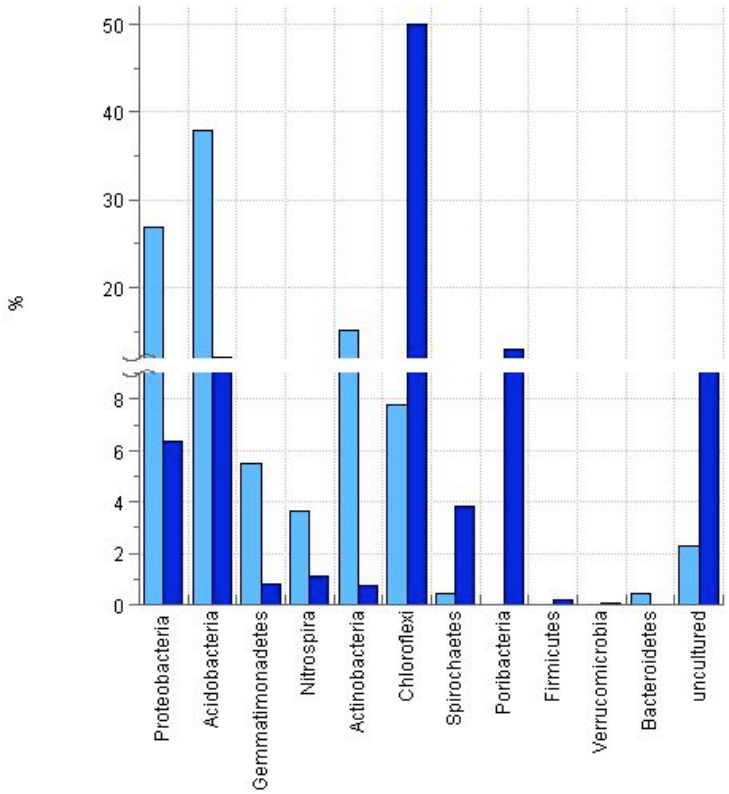

Figure S4.2. Discrepancy between number of 16S rRNA sequences in clone library and ribo-tags in metatranscriptome in the abundant phyla with > 0.5% of all sequence tags.

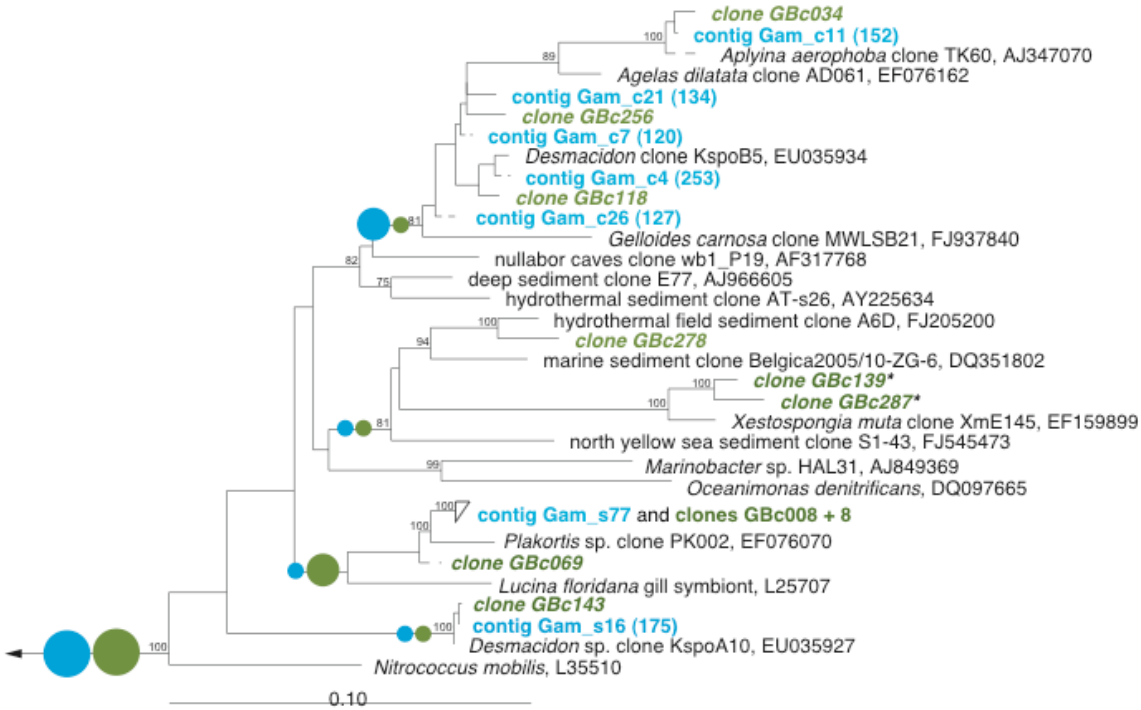

Figure S4.3. Maximum likelihood tree of a section of subdivision Gammaproteobacteria. Assembled contigs from *Geodia barretti* metatranscriptome ribotags with more than 100 reads are depicted in blue, clones from the cDNA 16S rRNA clone library are shown in green. Respectively, the colored circles for major branches indicate the relative amount of contigs vs clones. Filled circles indicate >90% parsimony bootstrap support whereas empty circles indicate >75% bootstrap support. Size bar indicated 10% sequence divergence. Other proteobacterial sequences are used as an outgroup.

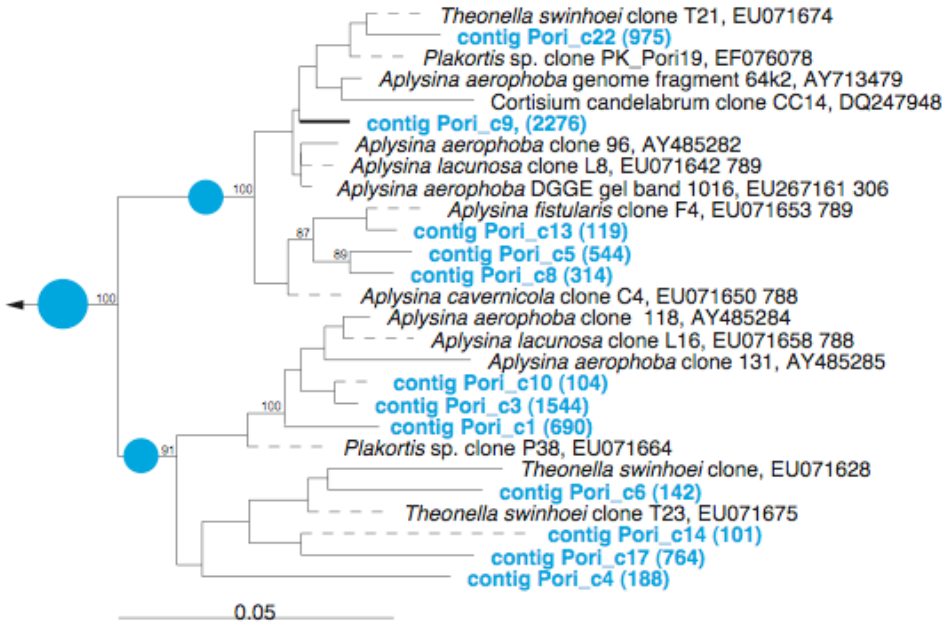

Figure S4.4. Maximum likelihood tree of the phylum Poribacteria. Assembled contigs from *Geodia barretti* metatranscriptome ribotags with more than 100 reads are depicted in blue, clones from the cDNA 16S rRNA clone library are shown in green. Respectively, the relative

amount of contigs vs clones is indicated by the colored circles for major branches. The number of reads in a contigs is written in brackets for each contig. Full-length sequences were used for treeing calculation and shorter sequences are added via the parsimony interactive tool in ARB. Parsimony bootstrap numbers are given if branch support is more than 75% .Size bar indicated 10% sequence divergence. Various sequences from other bacterial phyla were used as an outgroup.

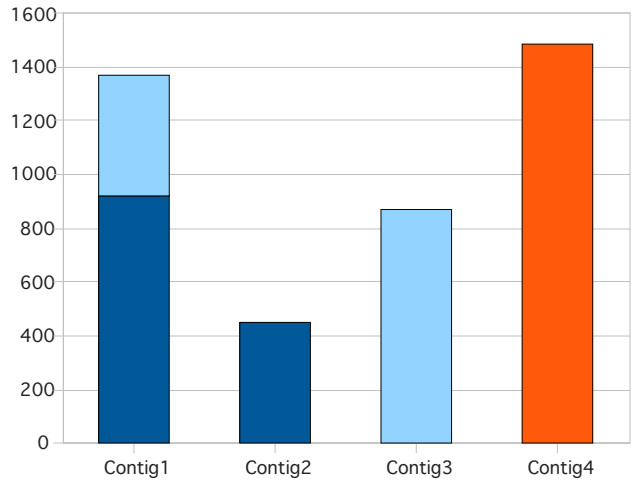

Figure S4.5. *In silico* test results of optimized parameters (o 150 p 99 n -130 –g 150) used for contig assembly of ribo-tags. Red bar shows the clear sepapration of the full-length outlier sequence, whereas closely related sequences (light and dark blue) are not separated totally.

4.8.2 Supplementary Tables

Table S4.1. FISH probes used in this study

	specificity	**FA conc.**	**dye**
dArch915*	Archaea	20	Cy3
dCren569*	Cren-/Thaumarchaea	20	Cy3
EUBmix	Bacteria	0-50	Cy5
dGam42a*	Gammaproteobacteria	35	Cy5
Por1130	Poribacteria	35	Cy3
GNSB-941	Chloroflexi	35	Cy3
CFX1223	Chloroflexi	35	Cy3

* indicates labeling on both 5' and 3'- end (DOPE-FISH, Stoecker *et al.*, 2010)

Table S4.2. Ten most abundant mRNA transcripts in *G. barretti* showing a match to clusters of orthologous groups

OG	functional description	Protein description	A	B	E	ABE
arCOG08647	Function unknown	Uncharacterized conserved protein	89	0	0	89
COG0004	Inorganic ion transport and metabolism	Ammonia permease	62	5	0	67
COG5267	Function unknown	Uncharacterized conserved protein	0	47	0	47
NOG78312	Function unknown	Uncharacterized conserved protein	0	40	0	40
COG2141	Energy production and conversion	F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related oxidoreductases	0	37	0	37
COG3391	Function unknown	Uncharacterized conserved protein	13	17	3	33
COG0747	Amino acid transport and metabolism	ABC-type dipeptide transport system, periplasmic component	0	33	0	33
COG4102	Function unknown	Uncharacterized conserved protein	0	30	0	30
COG0459	Posttranslational modification; protein turnover; chaperones	Chaperonin GroEL (HSP60 family)	0	24	1	25
COG1028	Secondary metabolites biosynthesis; & General function prediction & Lipid transport and metabolism	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	2	16	1	19

A = number of archaeal sequence tags, B = number of bacterial sequence tags, E = number of eukaryotic sequence tags, ABE = number of sequence tags from all three domains

Table S4.3. Ten most abundant bacterial gene transcripts, best match to UniRef90

coverage	Accession no.	bits	Protein annotation	hits	read length	protein length
30.68	B7RDP9	55.6	Putative uncharacterized protein	70	239.3	182
8.50	D2MKQ0	60.4	Putative uncharacterized protein	6	225.2	53
7.38	Q1MYB0	65.2	Putative uncharacterized protein	11	239.5	119
7.32	Q73YI1	58.7	Putative uncharacterized protein	10	241.7	110
7.05	D2MLE1	87.3	Putative uncharacterized protein (Fragment)	13	224.5	138
6.58	D2MHA0	67.9	Secreted protein (Fragment)	6	217	66
4.97	UPI0001694AF3	60.3	hypothetical protein Xoryp_04050	7	253.6	119
4.83	A6P143	51.3	Putative uncharacterized protein	6	200.3	83
4.70	Q1YSE1	62.2	Putative uncharacterized protein	7	233.7	116
4.38	Q5ZUQ1	67.4	Putative uncharacterized protein	8	243	148

Table S4.4. Ten most abundant eukaryotic gene transcripts, best match to UniRef90

coverage	Accession no.	bits	Protein annotation	hits	read length	protein length
6.65	B5DX74	50.5	GA26398	8	244.4	98
2.10	A8DUN7	51.8	Predicted protein (Fragment)	2	186	59
1.85	B2LUN0	69.7	Thymosin beta	1	227	41
1.68	O62543	100.4	Serum response factor	9	214.1	383
1.67	O99359	135.1	Cytochrome c oxidase subunit 2	5	246.4	246
1.48	A7TCN0	50.8	Predicted protein (Fragment)	1	236	53
1.44	Q5MWS1	141	Cytochrome c oxidase subunit 1 (Fragment)	4	253.5	235
1.43	B8YCQ7	56	Ferritin	3	245.3	172
1.18	UPI00019245DA	102	similar to Histone H4 replacement CG3379-PC	2	212.5	120
1.02	B4G2W4	127.8	GL23535, Actin (EST library of L.baicalensis)	5	231	379

4.8.3 Supplementary methods

4.8.3.1. Optimisation of parameters for ribo-contig assembly

The reverse transcription yielded random regions on the 16S rRNA and therefore allows for *in silico* reassembly of long, ideally full length rRNA for certain abundant taxa. However, the alteration of variable and conserved regions on the SSU rRNA molecule poses challenges to a reliable assembly without chimeric rRNA formation. We have therefore performed pilot studies on model "communities" *in silico* to evaluate the assembly of SSU rRNA sequences of closely and more distantly related species from the six most abundant bacterial higher taxa (phyla, classes) found in *G. barretti*. Three full-length 16S rRNA sequences of the taxa *Alphaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Chloroflexi*, *Spirochaeta* and the candidate division Poribacteria were downloaded from GenBank. Each of the test communities consisted of three known rRNA genes with one (the "outlier") being 87- 95% identical to the other two, which were more closely related to each other (97-99% identity). The rRNAs were randomly cut into 250bp and 400 bp fragments, mimicking the lengths of Roche FLX and FLX-Titanium pyrosequences, respectively. These were mixed and reassembled using the program CAP3 (Huang and Madan, 1999). Quality files with an average Phred quality score of 33 (as determined from the *Geodia barretti* transcriptome data) were produced and served as input for the assembly.

Different parameters were varied in the assembly protocol.

1. sequence coverage: 8x, 16x, 100x
2. minimum sequence overlap: (-o): 50bp, 100bp, 150bp, 200bp
3. minimum percent sequence identity in overlap: (-p): 90%, 95%, 97%, 99%
4. mismatch score (-n): -5, -130
5. gap penalty (-g): 6, 150

Different combinations of these parameters were tested and evaluated for their success to reassemble the "original" full length 16S rRNA. These tests showed that especially assemblies with the longer sequence input (of 400 bp) yielded considerably better results (see for example Fig. S4.5.).

Because of this result, we performed a highly stringent assembly with parameters set to –o 150 –p 99 –n 130 –g 150 to obtain longer reads from the 250bp FLX sequences which then serve as input for the further assembly in a second round. With these parameters sequence reads are assembled into the same contigs if they have no more than 2 mismatches over an overlap of 150bp. Resulting contigs were no technical chimeras. The second assembly round was subsequently performed with

less stringent parameters (-o 150 –p 97). Allowing 3% deviation of the sequences in the overlap reflects the technical error rate of pyrosequencing (Kunin *et al*., 2008)
The second assembly round increased contig lengths to 300-1500 bp, with an average of 698bp. The tests showed good separation of the ”outlier” rRNA in all “communities”, whereas a clear separation of the more similar sequences was not always achieved.
The application of this method onto the *Geodia barretti* dataset led to contigs with an average length of 347 base pairs from the first assembly round and an average length of 729 bp after the second round. (for more details see Table 4.2).
Those 16S rRNA sequences were introduced in phylogenetic analysis together with Sanger sequenced 16S rRNA sequences of the same nucleic acid preparation (see Fig. 4.2, 4.3, S4.3, S4.4).

4.8.3.2. Software comparison for mRNA binning

Contemporary sequencing technology made sequencing less costly and quicker but on the other hand generates short sequence reads which are difficult to assemble and classify taxonomically. Taxonomic binning is especially challenging when the dataset contains genome fragments of novel taxa, that lack reference (meta)genomes in public databases. Different approaches have been developed in recent years. Ideally, they should be evaluated on large-scale test data, but so far, no critical assessment has been published. Therefore, we evaluated methods for taxonomic classification of metatranscriptome tags using a small-scale test dataset, representing the main challenges for the classification of mRNA sequence reads from environmental samples, like a marine sponge. An artificial metatranscriptome, stemming from 3 different microbial (2 bacterial and 1 archaeal) genomes (two thereof unpublished; not present in the reference database), was simulated and different computational approaches benchmarked with regard to the correct taxonomical classification of the sequence reads.
Software programs tested for this purpose (see list below) are either based on sequence composition (e.g. oligonucleotide frequencies, GC-content, etc.) and/or on similarity to other sequences, employing evolutionary conservation of sequences. Both approaches are either supervised (dependent on a reference sequence database) or unsupervised (not dependent on a reference sequence database and directly learning from the analyzed dataset).

In this study, programs with different before mentioned approaches were evaluated with a small-scale simulated metatranscriptome, containing three important phyla represented in the *Geodia barretti*-transcriptome: Chloroflexi, Thaumarchaea and Nitrospira. The majority of sequence input (genome of archaea and nitrospira) was unpublished at the time of analysis, which reflects the metatranscriptome dataset of *G. barretti* in some respect, as this sponge harbours many microorganisms without closely related, genome sequences (Table 4.5.).
For homology-based approaches that means, the nearest sequence match might be from a distantly related organism and consequently, has only low similarity. The application of stringent parameters to avoid unspecific classifications would therefore omit the detection of novel organisms.

In total, the test set was composed of 100 simulated mRNA-tags from 3 proteomes (34 sequence reads of *Nitrospira defluvii* (Lücker *et al.*, 2010), 13 of *Dehalococcoides* sp. (CP001827) and 53 sequences of *Nitrososphaera gargensis* (Hatzenpichler *et al.*, 2008)) with mean length of 250bp. The proteomes of those genomes were downloaded and DNA sequences randomly fragmented with the software Metasim (Richter *et al.*, 2008).

Results

1. Sequence composition-based approaches

TACOA (Diaz *et al.*, 2009) (TAxonomic COmposition Analysis method); supervised; applies the *k*-nearest neighbor (*k*-NN) approach.

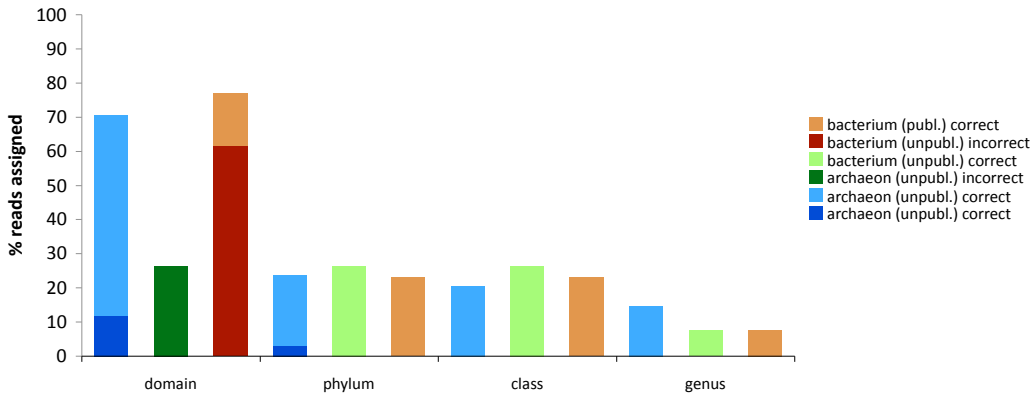

Figure S4.6. Results of taxonomic classification of mRNA-tags from a simulated metatranscriptome with the software TACOA. The simulated dataset included randomly fragmented nucleotide sequences of genes in Thaumarchaea (blue), Nitrospira (green) and

Chloroflexi (red), whereby the genome sequences of both the archaeon and the Nitrospira used were not deposited in a public database at the time of analysis. The percentage of correct classification is indicated by darker shades of the respective color and lighter shade indicates the percentage of incorrect classifications on the taxonomic levels given on the x-axis. More than half the genes from a novel archaeal genome are classified as bacteria or eukarya, while around a quarter of genes from a novel bacterial genome are classified at all, but correctly. A taxonomic classification of 250 bp sequences of genes into different phyla/classes or genera seems generally inaccurate with this approach.

In this simulation, the majority of reads were incorrectly assigned from phylum to genus level. Also on domain level the novel archaeal genome was incorrectly classified into bacteria or eukaryotes, while the bacterial sequence reads were correctly assigned to the bacterial domain.

2. Homology-based approaches

MEGAN (MEtagenome ANalyzer) assigns each read (or contig) of the dataset to a lowest common ancestor of the hits of a BLAST query using the NCBI taxonomy (Huson *et al.*, 2007).

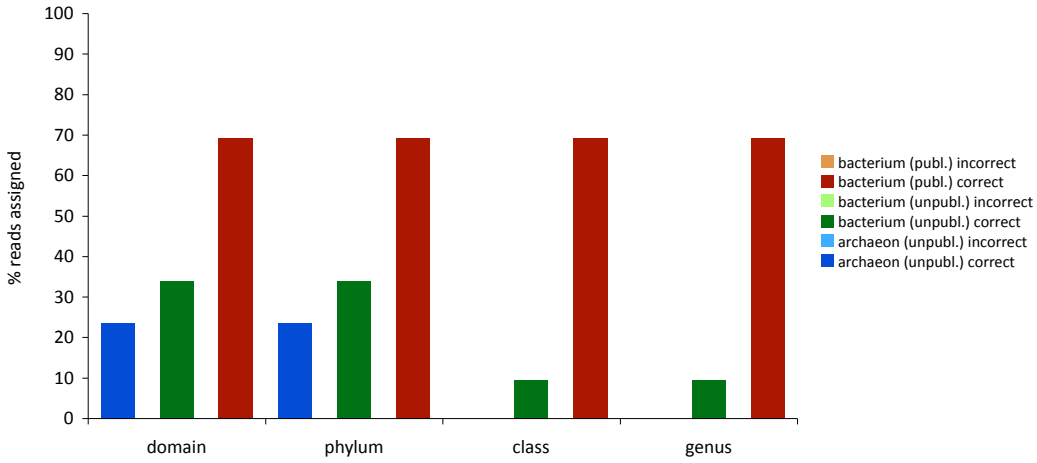

Figure S4.7. Results of taxonomic classification of mRNA-tags from a simulated metatranscriptome with the software MEGAN. For color coding see Fig S4.6. While more than two thirds of gene tags from a published genome are classified correctly on all 4 taxonomic levels, a third of tags from a novel bacterial genome are correctly classified on domain and phylum level and novel archaeal genes are correctly classified exclusively on domain level with this approach.

MEGAN produced little incorrect assignments with the parameters used, but for the novel archaeon, a wrong classification on phylum level. On domain level, the results seem to be reliable if keeping in mind that only approximately 20% of novel archaeal and 30% of novel bacterial sequence reads are binned.

CARMA (Computational Analysis of Replicate Measures for Arrays) first identifies Pfam domain and protein family fragments in unassembled reads using Pfam profile hidden Markov models (pHMMs). A phylogenetic tree is reconstructed for each matching Pfam family. The reads containing identified domains are then classified into a higher taxonomy order according to their phylogenetic relationships (Krause *et al.*, 2008).

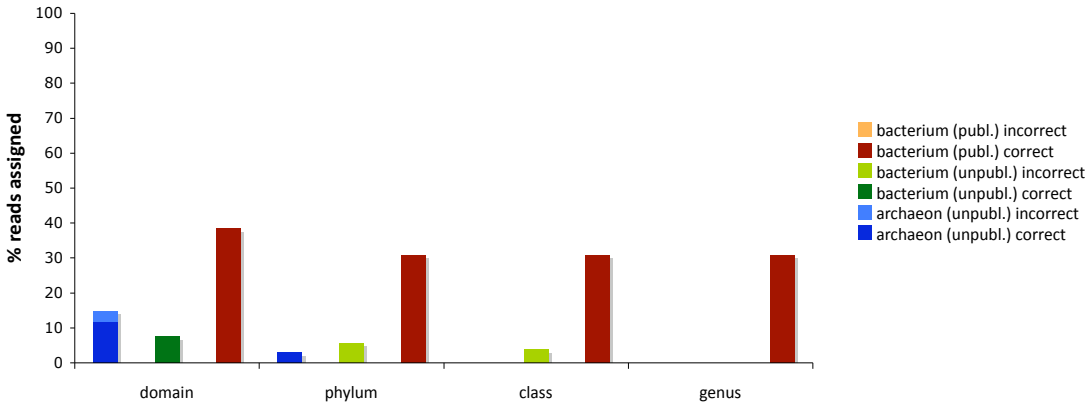

Figure S4.8. Results of taxonomic classification of mRNA-tags from a simulated metatranscriptome with the software CARMA. For color coding see Fig. S4.6. Around a third of tags stemming from the deposited Chloroflexi genome were correctly classified, while the results for novel genomes were insufficient, especially on taxonomic levels deeper than domain.

A third of sequences from *Dehalococcoides*, with references deposited in the NCBI database, were binned correctly. 10% of reads stemming from genomes lacking references in the database were correctly binned on kingdom level (with 4%) wrong assignments for the new archaeon), on any taxonomic level below no binning is possible.

SOrt-ITEMS (Sequence Ortholog based approach for binning and Improved Taxonomic Estimation of Metagenomic Sequences) starts with a BLAST alignment of the reads of the dataset followed by an assessment of the alignment using bit-score, alignment length, percentage of identities, and positives. Assuming a uniform rate of evolution and based on thresholds for these parameters reads are either ranked into and assigned in a appropriate taxonomical level or binned as unassigned (Monzoorul Haque *et al.*, 2009).

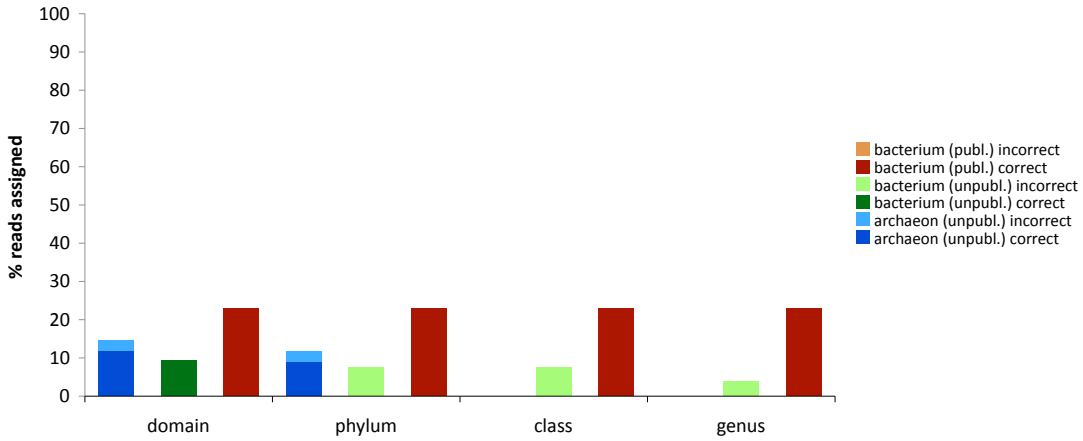

Figure S4.9. Results of taxonomic classification of mRNA-tags from a simulated metatranscriptome with the software SOrt-Items. For color coding see Fig. S4.6. A taxonomic classification of sequence tags from bacterial and archaeal genes was correct for up to a fifth of the input sequences, while the taxonomic information received for novel gene sequences was for about 10% correct. No reliable classification was made for novel gene sequence tags on class or genus level.

This new software program gave results akin those of CARMA, showing correct binning only with sequences having references in the database.

In homology-based approaches, the result depends on the presence of a closely related gene sequence in the database. Therefore, new genomes will not be binned correctly.

3. Combined approaches

PhymmBl (Brady and Salzberg, 2009) to identify putative prokaryotic ORFs, Phymm applies interpolated Markov models (IMMs) to characterize variable-length oligonucleotides typical of a phylogenetic grouping. In addition the program was combined with the BLAST algorithm to improve results (PhymmBl).

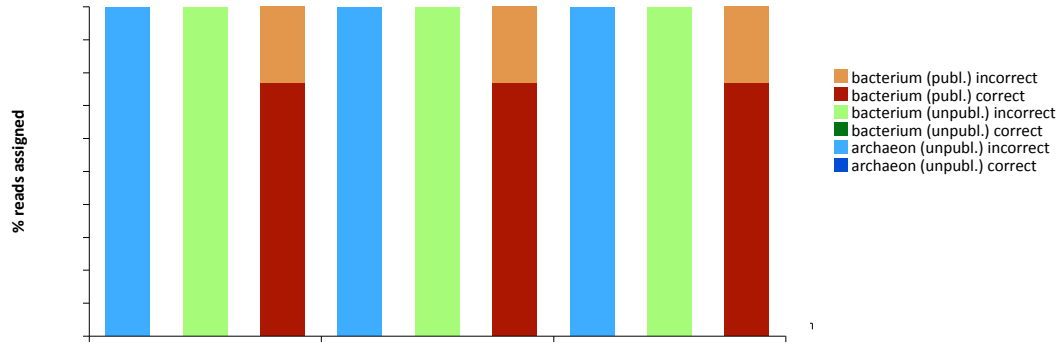

Figure S4.10. Results of taxonomic classification of mRNA-tags from a simulated metatranscriptome with the software PhymmBL. For color coding see Fig S4.6. This software classified all sequence tags on 3 taxonomic levels, while all assignments for novel bacterial and archaeal gene tags were incorrect. Gene sequences from the published Chloroflexi genome were classified correctly to almost 80% constantly over the taxonomic levels from phylum to genus.

PhymmBL classified all input sequences, with 100% wrong assignments for novel sequences. Known sequences are binned correctly to almost 80% on all 3 phylogenetic levels they allow, nevertheless the resuming >20% are binned incorrectly on all levels of assignment.

The result of the binning attempt of 250 base pair sequences with five programs shows difficulties in reliable separation of sequences based on both composition and homology. In terms of correct read assignments in total, the program MEGAN is promising, but the majority of mRNA-tags (3/4) were not assigned at all due to their low bit-score and stringent parameters used.

As most of the evaluated methods depend on publicly available sequence data, the challenge for them was how to deal with sequences that stem from organisms not present in the databases, as this is common in environmental microbial community analysis. Thus, we chose one organism present in the NCBI nr dataset, *Dehaloccocoides sp. VS* and two organisms that were not present in the NCBI nr dataset, *Nitrospira defluvii* and *Nitrososphaera gargaensis.*

Although *Dehalococcoides* reads made up only 13% of the whole test-s*et a*l approaches yielded most correct assignments therewith. In terms of total correct read assignments (including simulated reads from the other two genomes), Megan was the program performing best. More importantly, the number of wrong assignment with MEGAN was zero. We want to emphasize at this point that it is

better not to assign a read than to get a wrong characterization of the present community. CARMA and Sort-ITEMS both had less sensitivity and an increased false positive rate compared to MEGAN. Remarkably, TACOA and phymmBl showed a huge increase in sensitivity, however most of the reads got wrongly classified.
According to those tests, MEGAN was chosen for taxonomical binning of SSU/LSU rRNA and mRNA based on BLAST against the UniRef database.

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5 GENERAL DISCUSSION

Since the mid-1980s many new insights have been gained into the composition of naturally occurring microbial communities. Comparative analysis of ribosomal RNA (rRNA) sequences revealed the vast diversity of bacteria and archaea, reducing plants and animals to a peripheral branch in the phylogenetic Tree of Life (Olsen *et al.*, 1986; Woese, 1987; Woese *et al.*, 1990; Pace, 1997). As environmental surveys were initiated, microbiologists were confronted with a natural diversity which far exceeds that represented by pure cultures, revealing major lines of descent that were previously unrecognized (Staley and Konopka, 1985; Fieseler *et al.*, 2004). One of the important findings in this microbial revolution was the discovery of mesophilic archaea (DeLong, 1992; Fuhrman *et al.*, 1992; Preston *et al.*, 1996), abolishing the paradigm of archaea being restricted to extreme environments. Studies thereafter (Francis *et al.*, 2005; Leininger *et al.*, 2006; Wuchter *et al.*, 2006) unraveled a widespread occurrence and high abundance of archaea on land and in the ocean. Recent studies indicated that Thaumarchaea are deep branching in the evolutionary tree of archaea (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010) and might even be the most closely related ancestors of Eukaryotes (Kelly *et al.*, 2011). This makes them interesting candidates as sponge symbionts as their hosts are on the phylogenetic basis of eukaryotes with the next living relative being the protists choanoflagellata (Borchiellini *et al.*, 2001). The association of those evolutionary ancient organisms could date back to early Cambrian times when sponges first appeared and this old interaction might have influenced the evolution of archaeal and eukaryotic metabolism and niche adaptation.

5.1 Diversity of Sponge-Microbe associations

The mesohyl of sponges harbors a huge diversity of organisms spanning over all three domains of life. In particular, a large amount of heterotrophic and

autotrophic bacteria are present, which can contribute to the sponge energy demands by uptake as food and through the fixation of carbon or nitrogen (reviewed in Taylor *et al.*, 2007).

A recent review of all sponge-derived 16S rRNA sequences available (Webster and Taylor, 2011) outlined more than 25 phyla within more than 10 000 sequences - excluding around 259 000 V6-tags and 80 000 16S rRNA tags from pyrosequencing studies (Webster *et al.*, 2009, chapter 4). This demonstrates the enormous effort made in the last 30 years to describe the microbial diversity in marine sponges and discern sponge-specific patterns in their phylogeny (reviewed in Taylor *et al.*, 2007).

Nevertheless, those studies were almost exclusively based on cloning of PCR products, which might have lead to a severe bias in the assessment of microbial community compositions inclusively resulting in whole phyla and domains undetected, as in the case of Poribacteria and Archaea. In addition, the mere taxonomical identification of microbial DNA in a complex system like a marine sponge seldomly indicates whether the microorganism originated from the seawater pumped through the sponge channels, or represented food particles, parasites or symbionts.

In this study a metatranscriptomic approach was used as an attempt to overcome some of the limitations outlined above, allowing (i) the identification of active sponge associated microbial groups on RNA level independently of PCR amplifications and (ii) to simultaneously tackle functional information by analysing transcribed protein genes. In *G. barretti*, at least 10 bacterial phyla and thaumarchaea were present that were identified by analysing approximately 110 000 small subunit (SSU) rRNA sequence tags (chapter 4). The subsequent implementation of ribo-tag assembly into long 16S rRNA sequences (= ribo-contigs) allowed phylogenetic analyses and thus identification of potential sponge-specific clusters of the most abundant bacterial and archaeal phyla. Interestingly, the ribo-contig with the majority of bacterial ribo-tags was assigned to the bacterial phylum Chloroflexi, related to an uncultured cluster, which had potentially been underestimated in sponges so far. This cluster includes the seawater-derived

sequence SAR202, which was found to be more abundant in deeper waters of the Atlantic Ocean (Morris *et al.*, 2004; Varela *et al.*, 2008). Ribo-tags from the phyla Acidobacteria, Proteobacteria, Candidate Phylum Poribacteria and Actinobacteria were also abundant in the community profile of *G. barretti* whereas tags from Bacteroidetes, Gemmatimonadetes as well as Archaea were found less frequently (see Fig. 4.1.). This diversity is in line with the microbial community found in other sponges (see section 1.3.). While the relative abundances of most bacterial phyla appear to reflect their occurrence within the sponge tissue (as also confirmed by FISH, chapter 4), the relative abundance of archaea found in the metatranscriptomic profile is likely an underestimation due to the cell size and ribosomal content of these organisms (own observation, Odaa *et al.*, 2000). Furthermore, quantitative PCR of archaeal ammonia monoxygenase subunit A gene (*amo*A) in *G. barretti* (chapter 2) revealed high archaeal abundance, exceeding numbers found in any other habitat investigated. A high number of archaea could also be confirmed by FISH analysis (chapter 3 and 4), in which cell density of archaea was clearly higher than for Gammaproteobacteria (chapter 4), although their relative fraction in the taxonomic profile of the metatranscriptome was comparable (see chapter 4). In contrast to archaea, the abundance of Chloroflexi might have been overestimated because the organisms seem to occur as filaments in nature and therefore the cell size (and ribosomal content) might be notably bigger than in other bacterial cells (Miura and Okabe, 2008). Thus we conclude, that PCR-independent analyses via metatranscriptomics allows to identify the presence of organisms from all three domains simultaneously including organisms that are not identified in PCR-based studies. However, relative abundances deduced from ribosomal content of the cells might not always directly relate to the relative abundance of cells, underlining the importance of coupling different analytical methods.

5.1.1 Sponge-specific sequence clusters

The work described in chapter 4 includes a new analysis method, with the assembly of ribosomal RNA reads obtained by pyrosequencing of approximately 250 base pairs in length into up to full-length 16S rRNA contigs for phylogenetic analysis and assessment of ITS (internal transcribed spacer) residues. We found contigs with a good coverage (a high fraction of reads) in sponge-specific sequence clusters. Deep sequencing techniques applied on sponges and seawater have the potential to break up sponge-specific sequence clusters, if rarely detected sequences are taken into account. Nevertheless, the detection of similar microbes in various unrelated sponge species should indicate a related function and ecological niche, as shown by of the mutualistic cyanobacterial symbiont "*Candidatus* Synechococcus spongiarum". This symbiont provides 50% of the host's nutrition by carbon fixation. Akin, sponge-derived Nitrospira (nitrite-oxidizing bacteria) are exclusively related to the cluster IV of the phylum Nitrospira, with a growing sponge-specific cluster, called cluster IVb (Fig 4.2b.). For archaea, sequence information is sparse, but nevertheless, a sponge-specific cluster related to "*Cand.* C. symbiosum" is being recognized (chapter 2), including 80% of ribo-tags in a contig related to this cluster (chapter 4). This phylogenetic pattern might point to these archaea being key ammonia oxidisers in certain sponges but further investigations are needed to prove this hypothesis.

The dominant ribo-contig related to the subcluster 3 of the uncultured SAR202 cluster of Chloroflexi (Morris *et al.*, 2004) is part of a sponge-specific sequence cluster (Fig. 4.3), encompassing sequences from seven different sponge species (including short sequences only shown in Taylor *et al.* 2007). Furthermore, a second and new sponge-specific cluster was composed by 16S rRNA sequences from *G. barretti* and two other sponges. The function of these Chloroflexi is not known, due to the lack of isolates from this cluster. A study by Morris and colleagues (2004) showed that they are abundant in meso- and bathypelagic waters and in 2008, fluorescence *in situ* hydbridisations in combination with microautoradiography analyses showed the preferential uptake of L-aspartic acids of SAR202-related Chloroflexi, in

contrast to other bacteria and Thaumarchaea in the water column, which take up D-aspartic acids (Varela *et al.*, 2008). This adaptation to the ocean, in which the majority of amino acids is available as L-enantiomeric form, also indicates an important role of those Chloroflexi in biochemical cycling, which exactly stays elusive for now.

Gammaproteobacteria were the most abundant proteobacterial class in *G. barretti*, and phylogenetic analysis with ribo-contigs affiliated with this class showed that the contig harboring the largest number of sequence tags was in a sponge-specific sequence cluster comprising sequences of at least four other sponge species (Fig. S4.3). The respective organisms might be involved in nitrite oxidation due to their phylogenetic relationship to *Nitrococcus mobilis*. Furthermore, ribo-contig sequences from abundant lineages in the metatranscriptome dataset of *G. barretti* were related to sponge-specific sequence clusters recognised before in the phyla Acidobacteria, Gemmatimonadetes, Spirochaeta, and Deltaproteobacteria.

5.1.2 Nitrogen cycling in marine sponges

The release of high amounts of nitrate from tropical sponges was discovered already in the 1980s (Corredor *et al.*, 1988; Diaz and Ward, 1997), but only recently the identity and abundance of the potential key players in this processes were brought to light (chapter 2 – 4, Bayer *et al.*, 2008; Schläppy *et al.*, 2010). Although sponge cells tolerate ammonia to certain concentrations (25 mM, Sipkema *et al.*, 2004), ammonia is generally toxic to aquatic animals and is produced in large amounts by eukaryotes as a waste product. However, for microorganisms it can serve as a nitrogen and energy source. The presence of large amounts of microbes in many marine demosponges and the presence and high abundance of ammonia oxidizers in their mesohyl (Bayer *et al.*, 2008; Steger *et al.*, 2008; Mohamed *et al.*, 2009, chapter 2 and 3) indicate a considerable production and turnover of this compound. Furthermore, the measurement of anaerobic ammonia oxidation and denitrification in *G. barretti* (Hoffmann *et al.*, 2009, chapter 2), denitrification

in mediterranean sponges (Schläppy *et al.*, 2010) and the occurance of bacteria putatively involved in anaerobic ammonia oxidation in *Mycale laxissima*, (Mohamed *et al.*, 2009) indicated that nitrogen turnover occurs also under anaerobic conditions in sponges, e.g. in phases of non-water-pumping.

The conversion of inorganic nitrogen compounds like ammonia and nitrate back to atmospheric nitrogen has great impact on the marine ecosystem. Since the turnover rates of nitrogenous compounds in sponges can exceed rates known from the water column and ocean sediments up to 10-fold, these animals might be nitrogen sinks in areas with high sponge biomass.

The evidence for archaeal ammonia oxidation in marine cold-water sponges shown here (chapter 2-4) emphasizes the importance of these symbioses in ecosystem functioning and marine nitrogen cycling in deep and cold oceanic environments.

5.2 Archaea as main ammonia oxidisers in marine cold-water sponges

Given the frequent association of Thaumarchaeota group I.1a with marine sponges and the indications for nitrification within these host organisms (Corredor *et al.*, 1988; Diaz and Ward, 1997; Diaz *et al.*, 2004; Bayer *et al.*, 2008, chapter 2 and 3), evidence suggests that ammonia-oxidizing archaea (AOA) frequently thrive in sponges. Nevertheless, AOB have also been found in marine sponges, albeit only identified by on PCR amplification (Diaz *et al.*, 2004; Bayer *et al.*, 2008; Mohamed *et al.*, 2009). The sponges investigated in this study showed nitrification activity in incubation experiments and a higher relative abundance of AOA in contrast to AOB on genomic as well as transcript level (chapter 2). Furthermore, the activity of AOA in *G. barretti* was supported by a metatranscriptomic study, in which genes potentially involved in ammonia oxidation of archaea were among the most abundant of all transcripts identified (chapter 4).

Growth of the only cultivated strain from marine thaumarchaea, *N. maritimus*, (group I.1a) is adapted to extreme nutrient-limited conditions (Martens-Habbena *et al.*, 2009), with ammonia concentrations below 10 nM. This is more than 100-fold lower than the minimum nutrient requirements for growth of AOB in culture (Prosser, 1989; Bollmann *et al.*, 2002).

Although some thaumarchaea recovered from soil (in group I.1b) can grow on slightly higher ammonia concentrations (up to 15-20 mM, Tourna *et al.*, 2011) these are still below the optimum for AOB, which can grow in environments with up to 1000 mM ammonia (Koops *et al.*, 2003). Sponges seem to be a rich source in ammonia, as they release it either constantly (chapter 2), or only in autumn (Bayer *et al.*, 2008). In times of active pumping activity, however, the conditions inside the sponge might resemble the conditions of the surrounding seawater, i.e. might be very low on nutrients. Nevertheless, ammonia accumulation could occur in phases of non-pumping, which would favour the activity of organisms that are adapted to higher concentrations. The differences in nutrient availability, oxygen concentration and temperature might contribute to shape the microbial community, including that of AOA and AOB associated with particular marine sponges.

Table 5.1 indicates, that thaumarchaeal 16S rRNA sequences from sponges with stable nitrification activity phylogenetically cluster with other sponge-derived thaumarchaea. AOA from *G. barretti* and *P. ventilabrum* are related to a sponge-specific cluster (ssc 1) together with 16S rRNA from sponge species living in warm- and cold-water habitats, including "*Cand.* C. symbiosum". AOA sequences from the mediterranean sponge *A. aerophoba* cluster with archaea derived from sponge species sampled in Korea (ssc 2). It is possible that *A. aerophoba* harbours AOA with different affinity to distinct ammonia concentrations than in *G. barretti* and *P. ventilabrum*, because of the seasonal ammonia limitation in *A. aerophoba* in spring (Bayer *et al.*, 2008). In addition to the sponge-specific AOA lineage (ssc 1), all sponge species analysed in this study possessed a second lineage of Thaumarchaea, related to 16S rRNA sequences from cold and deep-water samples (chapter 2). However, the activity of AOA could be assigned to the sponge-specific lineage

(ssc 1) by the metatranscriptomics analysis of *G. barretti*, in which the majority of archaeal rRNA transcripts were related to this cluster (chapter 4).

Table 5.1. Environmental factors of nitrifying sponges, possibly correlating with AOA and AOB abundance and phylogenetic patterns

	G. barretti	*P. ventilab*	*A. dichotoma*	*T. semisub*	*A. aerophoba*
Temperature	Cold	Cold	Cold	Cold	moderate
Nitrification	+	+	NH_3 oxidation	unstable	+
AOA 16S rRNA pattern	ssc 1 & cold water cluster	ssc 1 & cold water cluster	cold water cluster	cold water cluster	ssc 2
qPCR DNA	AOA	AOA + AOB	AOA ± AOB	AOA	AOA + AOB
qPCR RNA	AOA	AOA	± AOA ± AOB	± AOA	n. determined
NH_3 availability	release	release	release	release	release/ limited
Oxygen	oxic/anoxic	oxic	oxic	oxic/anoxic	oxic/anoxic
Distribution of AOA	equally	upper layer	outer mm	equally	n. determined
Morphology	bulky	fan	branched	mushroom	tubes
Reference	this study	this study	this study	this study	Bayer *et al.*, 2008

ssc 1 and ssc 2 = two different thaumarchaeal sponge-specific cluster (see boxes in Fig. 2.3)
P. ventilab = *P. ventilabrum*, *T. semisub* = *T. semisuberites*

Furthermore, it is yet unclear to what extent also ammonia oxidizing bacteria (AOB) contribute to the nitrification observed in marine sponges. AOA and AOB might be differentially adapted to changes in ammonia or oxygen supply. Therefore, sponges with fluctuating ammonia secretion, like *A. aerophoba* could harbour more complex populations of ammonia oxidizers, which are adapted to the differences in ammonia concentrations.

In this study, bacterial *amo*A was detected only in some individuals of *Antho dichotoma* also on the transcription level (but in far lower levels than AOA, chapter 2), inferring at least a partial involvement of bacterial ammonia oxidation activity in this sponge. *A. dichotoma* has thin branches with a core of spicules and a relatively thin layer of mesohyl (Table 5.1). Temperatures of 15°C and nutrient availability from the surrounding seawater is comparable to the other sponges (showing no bacterial *amo*A transcription), so a

correlation with constant oxygen availability due to the diffusive boundary layer of 1 mm (Hoffmann *et al.*, 2008) is possible. However, the distribution of AOA in the fan-shaped sponge *P. ventilabrum*, indicates that also AOA might be dependent on specific oxygen and ammonia levels. By FISH, 100 times more archaea were detected in the outer 50 µm of the mesohyl (chapter 2), where oxygen and ammonia concentrations seem more favourable (Table 5.1.).

Calculation of archaeal cell specific nitrification rates in *P. ventilabrum* resulted in up to 6 fmol per cell and day, which were in the same range as for planktonic AOA (Wuchter *et al.*, 2006; de la Torre *et al.*, 2008). This rate is in the lower range of AOB in seawater (Ward, 1987; Ward *et al.*, 1989). Tropical sponges exhibit higher nitrification rates than cold-water sponges, therefore it might be possible that AOB are the responsible nitrifyers in those sponges, because AOB have generally higher conversion rates per cell (Ward *et al.*, 1989). However, the relative abundance of AOA and AOB has not yet been determined in sponges from warmer waters.

This was the first quantitative analysis on AOA and β-AOB in marine sponges and more studies on the relative abundance and activity of those microbes in warm- and cold-water sponges and with different oxygen and ammonia availability would help to determine the factors involved in niche differentiation between AOA and AOB in sponges.

5.3 Metatranscriptomics for the characterization of sponge microbiota

Facing the challenges in sponge microbiology, a method combining in-depth diversity and insight in metabolic pathways of active symbionts was used to study the marine cold-water sponge *G. barretti* with its microbiota. The so-called “double-RNA” approach, based on the sequencing of cDNA without previous depletion of rRNA, was applied only once before (Urich *et al.*, 2008), and the analysis protocol was validated and further developed in this study. The approach allows the assessment of phylogenetic and genetic information

of active populations from all three domains of life in the holobiont sponge. However, the annotation of functional genes strongly depends on the availability of reference genomes, and since the largest fraction of sponge-associated microbes defers isolation genes matching to sequenced genomes in the database were the minority in this study. This became especially apparent for the most abundant group, the Chloroflexi. According to the taxonomic profile, around half of the bacterial sequences were affiliated with this phylum, still a prevalence of chloroflexi genes was not seen in the functional profile. It is important to know the limitations of this kind of data analysis, as it can lead to incorrect results if not examined critically. After evaluating different methods (supplementary text 4.8.3.2), we used a rather conservative analysis protocol for the taxonomic binning of functional genes. Important information was obtained for active nitrifyers. Transcripts from genes encoding ammonia transporters and the ammonia monooxygenase (*amo*A, B and C) were related to archaea, as well as transcripts of genes encoding archaeal *nir*K, a gene expressed in bacteria only under denitrifying conditions, and an uncharacterised gene (Nmar_1506), that is highly conserved throughout thaumarchaeal genomes, were found in high abundance. As the mechanism of ammonia oxidation in archaea is unknown (see section 1.6.1), the involvement of the latter genes in the pathway is suggested. In the bacterial mRNA pool, functional genes for nitrite oxidation (*nxr*A and B) with similarity of the *Nitrospira defluvii* genome (Lücker *et al*., 2010) were detected, confirming the identification of Nitrospira as the only NOB in the taxonomic analysis. This nitrite oxidoreductase gene was only identified because the reference genome mentioned above was published at the time of analysis, as was the first genome of a marine sponge (*Amphimedon queenslandica*, Srivastava *et al*., 2010) and a metagenome of the Australian shallow-water sponge *C. concentrica* (Thomas *et al*., 2010). Furthermore, genomic fragments of sponge-associated Poribacteria, Chloroflexi (Siegl and Hentschel, 2010; Siegl *et al*., 2010) and Deltaproteobacteria (Liu *et al*., 2010) were published. Although this sequence information was invaluable for the analysis of the metatranscriptome, the

Chloroflexi genome was unfortunately not from a representative of the uncultured SAR202 cluster, of which the abundant symbionts of *G. barretti* were related to. So this work emphasizes the importance of new methods to gain genome information of uncultured microorganisms.

Originally, it was planned to perform comparisons of rRNA and mRNA transcripts between *G. barretti* in oxic and anoxic condition. However, that goal did not prove feasible due to techniqual difficulties. In future experiments, a comparison of different metatranscriptomes as well as the analysis of datasets with more mRNA (e.g. after depletion of rRNA) would allow a better coverage of less abundant transcripts, providing further insights into the active microbial processes. Although the dataset generated in this study harbors great potential, it can currently not be analyzed in the desired detail. However, it comprises a resource for future analyses (e.g. for studies on symbiotic interactions between eukaryotes and microbes) as more reference genomes become available.

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6 CONCLUSIONS

This thesis enhances the knowledge of nitrogen cycling in marine sponges, and in particular of microbially mediated nitrification. It gives evidence that species of Thaumarchaeota group I.1a are key ammonia oxidizers in these animals and that a full nitrogen cycle, including even anaerobic processes may be present, as shown for *Geodia barretti*. The high numbers of ammonia oxidizing archaea (AOA) and their high activities indicate that ammonia oxidation is generally a dominant process in the sponge. Although this study gives further evidence that the presence of these symbionts is beneficial for the sponge, it is still possible that AOA simply occupy a favorable niche with regular supply of ammonia.

The high abundance of AOA also means that sponges are particularly suited to study archaeal physiology and activity as they represent natural fermentors for these archaea. The metatranscriptomic study performed on *Geodia barretti* was a first attempt in this direction and allows raising hypothesis on specific gene functions and activity of AOA. In more general terms sponges can serve in identifying the physiological activities of many organisms that are naturally enriched in these animals and that have resisted cultivation in pure laboratory cultures. Sponges are regarded as living fermenters for aerobic and anaerobic, photic and aphotic, autotrophic and heterotrophic and psychro- and mesophilic organisms. There are many things to be learned from sponges in microbial ecology, biotechnology and symbiotic interactions.

7 ABSTRACT

Thaumarchaeota have been discovered not only in a diverse range of moderate terrestrial and marine habitats but also as frequent inhabitants of marine sponges. Based on metagenomic and cultivation studies, it has become evident over the past years that some (or all) thaumarchaea have the capability of oxidizing ammonia using the enzyme ammonia monooxygenase (Amo), a homologue of the well-known bacterial counterpart. Here we explore the activity and diversity of *Thaumarchaeota* in marine cold-water sponges of the Northern hemisphere (Norway) by combining quantitative physiological data and molecular analyses. By monitoring the production and consumption of nitrogen compounds in defined incubation experiments we have demonstrated and quantified nitrification in *Geodia barretti*, *Phakellia ventilabrum*, *Tentorium semisuberites* and *Antho dichotoma*. In parallel, large numbers of Amo-encoding archaea were detected by quantitative PCR (up to $6*10^8$ archaeal *amo*A gene copies per µg of nucleic acids) and fluorescence *in situ* hybridisation, with bacterial *amo*A genes mostly being under the detection limit. We report denitrification and anammox rates in the sponge *Geodia barrett*i beside nitrification activity by employing stable isotope labelling techniques, thus closing the nitrogen cycle in a marine sponge for the first time. We also identified the potential microbial lineages that are responsible for the activities.

To obtain insights into the *in situ* diversity and function of active microbes in *Geodia barretti* we employed the "double-RNA" approach, which involved analysis of reverse-transcribed total RNA. Of the approximately 260,000 RNA-tags obtained by pyrosequencing, we assigned ≈110,000 tags to small subunit rRNA and derived a detailed community profile of all three domains of life. Around 50% of all 16S rRNA-tags were assembled and phylogeny of the abundant taxa was performed and compared to sequences of a 16S rRNA clone library of the same cDNA. Within the expressed sequence tags (mRNA), we identified a large number of archaeal genes that are potentially involved in transport and oxidation of ammonia. Some of these highly expressed genes are conserved in thaumarchaeal genomes but their potential function in ammonia oxidation was not previously recognized. From our studies we infer a key role for archaea in the nitrogen metabolism in marine sponges.

8 ZUSAMMENFASSUNG

Thaumarchaea (Archaea) kommen in vielen terrestrischen und marinen Lebensräumen vor und sind oftmals mit marinen Schwämmen assoziiert. Basierend auf Metagenom- und Kultivierungsstudien hat sich gezeigt, dass viele (wenn nicht sogar alle) Thaumarchaea die Fähigkeit besitzen, mittels dem Enzym Ammoniummonooxygenase (AMO), Ammonium zu oxidieren. In dieser Studie untersuchen wir die Aktivität und Diversität der Thaumarchaea in Kaltwasserschwämmen aus Norwegen. Durch die Kombination von quantitativ-physiologischen und molekularen Analysen und die Messungen von Produktion und Verbrauch bestimmter Stickstoffverbindungen in Inkubationsexperimenten wurde Nitrifikation in *Geodia barretti*, *Phakellia ventilabrum*, *Antho dichotoma* und *Tentorium semisuberites* nachgewiesen und quantifiziert. Zugleich konnte mittels quantitativer PCR und fluoreszenter *in situ* Hybridisierung eine hohe Anzahl an AMO-kodierenden Archaea (bis zu $6*10^8$ archaeale *amo*A Genkopien pro µg Nukleinsäure) detektiert werden. Im Schwamm *G. barretti* wurden zum ersten Mal Raten von Denitrifikation und anaerober Ammoniumoxidation nachgewiesen und somit wurde gezeigt, dass der gesamten Stickstoffkreislauf in marinen Schwämmen ablaufen kann.

Um die Diversität und Funktion der aktiven Archaea (und Bakterien) in *G. barretti* genauer zu untersuchen, haben wir den "Doppel-RNS" Metatranskriptomik Ansatz angewandt, wobei revers-transkribierte RNS direkt sequenziert und anschließend bioinformatisch ausgewertet wurde. Von den ca. 260.000 RNS-tags der Pyrosequenzierung, waren etwa 110.000 von der kleinen Untereinheit der ribosomalen RNS, woraus ein detailliertes taxonomisches Profil von den drei Domänen des Lebens des Schwammsystems erstellt wurde. Innerhalb der transkribierten mRNS-tags haben wir eine große Anzahl an archaealen Genen identifiziert, die wahrscheinlich in den Transport und die Oxidation von Ammonium involviert sind. Einige dieser hoch transkribierten Gene sind in thaumarchaealen Genomen konserviert, aber deren eventueller Beitrag in Ammoniumoxidation war bisher noch nicht bekannt. Aus den Messungen von Nitrifikationsraten, zusammen mit hoher Transkription von *amo*A Genen in mehreren Schwammarten aus der mesopelagischen Zone des Nordatlantiks, schließen wir eine Schlüsselrolle der Archaea für den Stickstoffmetabolismus mariner Schwämme.

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sponges: evidence from physiological studies and metatranscriptomics. Symposium for aquatic microbial ecology, Piran, Slovenia, 28th August – 4th. September 2009

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T. Urich, Lanzen A, **Radax R.**, Schleper C. Simultaneous assessment of (soil) microbial community structure and function through analysis of the metatranscriptome. Bodenkundliches Kolloquium, Institut für Bodenkunde und Standortslehre, University Hohenheim, Germany, 8th December 2008.

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