Molecular characterization of symbiotic associations between chemoautotrophic sulfur-oxidizing microorganisms and nematodes in shallow marine sediments

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List of abbreviations

(Very common abreviations and units are not listed)

APS	Adenosin-5'-phosphosulfate
AprA	Adenosin-5'-phosphosulfate reductase, alpha subunit
BSA	Bovine Serum Albumin
CARD-FISH	Catalyzed Reporter Deposition FISH
DAPI	4´,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
FISH	Fluorescent *in situ* Hybridization
ISR	Intergenic spacer region
ITS	Internal transcribed spacer
MLSA	Multilocus sequence analysis
PBS	Phosphate Saline Buffer
PHA	poly-β-hydroxyalkanoic acid
PHB	poly-β-hydroxybutyric acid
PHV	poly-β-hydroxyvaleric acid
REP-PCR	Repetitive extragenic palindrome – PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RubisCO	Ribulose-bisphosphate carboxylase/oxygenase
SOB	Sulfur-Oxidizing Bacteria
SRB	Sulfate-Reducing Bacteria
TEM	Transmission Electron Microscopy
tRNA	Transfer RNA
$tRNA^{Ala}$	tRNA for alanine
$tRNA^{Ile}$	tRNA for isoleucine

Summary

Shallow marine sediments are of significant importance for the mineralization of organic matter. The mineralization processes are performed mainly by the microbial communities inhabiting these sediments. Therefore, the understanding of these processes requires investigations of the structure, diversity and dynamics of microbial communities of marine sediments. Molecular biological methods were used to investigate the microbial diversity and community structure in intertidal sandy sediments near the island of Sylt (Wadden Sea). Comparative 16S rRNA sequence analysis revealed a high bacterial diversity. Most sequences retrieved by PCR with a general bacterial primer set were affiliated with *Bacteroidetes*, *Gammaproteobacteria*, *Deltaproteobacteria* and the *Pirellula* cluster of *Planctomycetales*. Fluorescence *in situ* hybridization (FISH) and slot blot hybridization with group specific rRNA-targeted oligonucleotide probes were used to characterize the microbial community structure over depth (0 to 12 cm) and seasons (March, July, October). We found high abundances of bacteria with total cell numbers up to 3×10^9 cells ml^{-1} and a clear seasonal variation, with higher values in July and October versus March. The microbial community was dominated by members of the *Planctomycetes*, the *Cytophaga/Flavobacterium* group, *Gammaproteobacteria*, and bacteria of the *Desulfosarcina/Desulfococcus* group. The high abundance (1.5×10^7–1.8×10^8 cells ml^{-1} accounting for 3–19% of all cells) of presumably aerobic heterotrophic polymer-degrading planctomycetes is in line with the high permeability, deep oxygen penetration, and the high rates of aerobic mineralization of algal biomass measured in the sandy sediments. The high and stable abundance of members of the *Desulfosarcina/Desulfococcus* group, both over depth and season, suggests that these bacteria may play a more important role than previously assumed based on low sulfate reduction rates in parallel cores.

Shallow marine sediments are also preferred environments for nematodes harboring endo- or ectosymbiotic bacteria. In most cases, the symbionts are autotrophic, sulfur-oxidizing bacteria. Considering that each host carries $1–3 \times 10^6$ symbiotic bacteria and the high density of these nematodes, they can significantly contribute to carbon and sulfur cycling in shallow sediments. Another major objective of the present study was to investigate in detail the specificity of the symbiotic association between ectosymbiotic bacteria and gut-bearing nematodes of the genus *Leptonemella* and the identity and phylogeny of the endosymbionts of mouthless nematode, *Astomonema* sp. Sylt sandy sediments were recently described to harbour a very rich population of symbiont-carrying nematodes belonging to the genera *Leptonemella* (subfamily Stilbonematinae). Two species, *L. aphanothecae* and *L. vicina*, carrying morphologically similar ectosymbiotic bacteria, were described as the most abundant nematodes in these sediments. The two

host species were differentiated and characterized by analysis of the 18S rRNA gene. The 18S rRNA sequence analysis showed that the two nematode species were closely related, sharing 96–96.3% sequence identity, and were phylogenetically affiliated with the subfamily Stilbonematinae, family Desmodoridae (94–95% sequence identity). 16S rRNA sequences obtained from symbionts of both host species were nearly identical, (99.5% sequence identity) with only minor nucleotide differences. These differences were not consistent within the symbionts of each host group and did not form a specific pattern that could differentiate between symbionts of the two hosts. Phylogenetic analyses showed that symbiont sequences of *Leptonemella* sp. were clustering together within the *Gammaproteobacteria* and formed a monophyletic clade with sequences of endosymbionts of gutless oligochaetes (*Olavius* sp., *Inanidrilus* sp.) and ectosymbionts of the gut-bearing nematode *Laxus* sp. Internal transcribed spacer (ITS) fragments of symbionts from both hosts had approximately the same length and contained two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. The ITS sequences were highly similar and a species-specific pattern was not observed. In this context, the results obtained in this study strongly suggest that the symbionts of the two *Leptonemella* species are at most sub-species, if not strains of the same species. Alternatively, the possibility that the symbionts of the two *Leptonemella* species are indeed species-specific, but cannot be distinguished by using ITS analysis cannot be ruled out.

Marine nematode worms of the genus *Astomonema* have been described from intertidal sandflats, deep-sea muds, and methane-rich pock marks. All *Astomonema* species lack a mouth and morphological studies show that they are associated with endosymbiotic bacteria, but to date nothing is known about the identity and phylogeny of symbionts from this host group. In this study, we characterized the symbiosis in an undescribed *Astomonema* species from coral reef sediments in the Bahamas. Phylogenetic analysis of the host based on its 18S rRNA gene showed that *Astomonema* sp. belongs to the class Chromadorea with non-symbiotic nematodes of the families Linhomoeidae and Axonolaimidae as its closest relatives. Comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization revealed that the *Astomonema* sp. symbionts belong to the *Gammaproteobacteria* and are most closely related (95–96% sequence similarity) to gammaproteobacterial sulfur-oxidizing symbionts from 2 other host groups: the Gamma 1 endosymbionts of gutless oligochaetes and the ectosymbionts of gut-bearing nematodes. The close phylogenetic relationship of the bacteria from *Astomonema* sp, gutless oligochaetes, and gut-bearing nematodes can not be explained by co-speciation given the distant relationships between these three host groups. Instead, the shared biogeographical distribution of these hosts is assumed to have played a key role in the formation of these symbiotic associations.

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An overview of symbioses

Symbiosis defines a permanent or long lasting association between at least two different species or organisms. The term “symbiosis” was introduced in 1878 by the German botanist de Bary for the living together of two differently named organisms (Smith & Douglas, 1987; Steinert *et al*., 2000). In the present days, the term is often used in the sense of mutualism, which is living together for the benefit of both partners (Smith & Douglas, 1987; Hoffmeister & Martin, 2003). Such symbiotic associations are often established between microorganisms and invertebrates, vertebrates or plants in a variety of terrestrial and aquatic habitats. Thus, from terrestrial plants and insects that form close alliances with fungi or bacteria, to the giant tube worms and shrimps that live together with sulfur-oxidizing bacteria in deep-sea habitats, a great diversity of microbial symbioses were revealed in the past 50 years (Smith, 2001).

The wide acceptance of serial endosymbioses theory (Margulis, 1970; 1981) that the eukaryotic cell structure was symbiotic in origin and that the chloroplasts originated probably from symbiotic aquatic microbes brought aquatic microbial symbioses to the attention of the scientific world. As a result of the rapid and growing interest in aquatic symbioses, many types of associations between microorganisms and a variety of hosts like corals, sponges, giant clams, tube worms, sea anemones, or protozoa were discovered in marine habitats. Intensively studied in the past 25 years, these symbiotic associations have often showed a distinct impact on evolution in terms of speciation and population of new habitats (Steinert *et al*., 2000).

Symbioses in marine habitats

The marine environment, represented by the world oceans and the adjacent seas, covers approximately 71% of the earth’s surface and harbours a great variety of habitats with a wide diversity of plants, animals and microorganisms. Some of these habitats, such as deep-sea hydrothermal vents, cold seeps, anoxic basins or sulfidic sediments are dominated by strong physical (temperature) and chemical gradients (e.g. oxygen, sulfate, sulfide, methane). By using reduced compounds present in excess in these environments as an energy source (sulfide, methane) the microorganisms can provide a nutritional bridge between habitats and the animals that exploit them. Consequently, such habitats are

colonized by a variety of animals which have formed obligate nutritional symbiotic associations with microorganisms, leading to the most unusual physiological and morphological adaptations in the animal world. Such remarkable nutritionally-based symbiotic relationships are those involving chemosynthetic bacteria and marine invertebrates.

Symbiotic associations between bacteria and marine invertebrates

Symbiotic associations involving microbes and marine invertebrates became of high interest at the end of 1970's, when the giant tube worm *Riftia pachyptila* was discovered (Corliss *et al*., 1979; Spiess *et al*., 1980). These gutless and mouthless worms, of up to 2 m in length, living in dense, benthic populations around deep sea hydrothermal vents were the first animals known to obtain their nutrition via chemoautotrophic sulfur-oxidizing symbionts (Cavanaugh *et al*., 1981; Felbeck *et al*., 1981). Now, more than 200 species of invertebrates are known to be involved in similar symbiotic associations. Many of these invertebrates depend on chemoautotrophic sulfur-oxidizing bacterial symbionts to fulfil their organic carbon requirements. However, invertebrates that harbour only heterotrophic symbionts or invertebrates with two nutritional types of symbionts (chemoautotrophic and methanotrophic) have also been discovered. Such examples are the deep-sea polychaetes of the genus *Osedax* and wood-boring bivalves of the family Teredinidae as hosts carrying only heterotrophic bacteria, and some members of the bathymodiolid mussels of the family Mytilidae harbouring both chemoautotrophic (sulfide-oxidizing) and methanotrophic (methane-oxidizing) symbionts.

Symbioses of invertebrates with heterotrophic bacteria

The *Osedax* species were first discovered in 2002, living on the bones of a grey whale carcass at 2891 m depth in Monterey Canyon, off the coast of California (Rouse *et al*., 2004). These mouthless and gutless worms have an inimitable way to obtain nutrition from decomposing mammalian bones. They invade the bones by a highly vascularized root system that originates from the posterior part of a large eggsac (ovisac). Both the roots and the ovisac are packed with bacteriocytes (20–50 μm) which contain large intracellular rod-shaped bacteria (Goffredi *et al*., 2005). The presence of bacterial symbionts in dense populations coupled with the lack of feeding structures (mouth, gut) of the host, supported by stable isotopes and fatty acid analyses demonstrated the reliance of *Osedax* spp. on organic compounds transferred by their endosymbiotic bacteria (Goffredi *et al*., 2005). The close phylogenetic relationship of the symbionts with the members of *Oceanospirillales*, aerobic heterotrophic bacteria able to degrade complex organic compounds, suggested a similar type of metabolism for the *Osedax* symbionts (Goffredi *et al*., 2005). Potential

sources of organic carbon that could be used by the symbionts are the major constituents in bone such as fatty acids, collagen or cholesterol.

Dual symbioses of invertebrates with chemoautotrophic and methanotrophic bacteria

Bathymodiolid mussels of the family Mytilidae are among the dominant constituents of deep sea vents and cold seeps worldwide (Won *et al*., 2003). All species of the genus *Bathymodiolus* contain intracellular symbiotic bacteria in their gills (Fisher, 1990). They rely mainly on these symbionts for their nutrition (Cavanaugh *et al*., 1987; Fisher *et al*., 1993; Pond *et al*., 1998) although a functional gut is still present in many species and filter feeding remains a possible further nutrition source (Page *et al*., 1991). Several *Bathymodiolus* species, such as *B*. *brooksii* and *B. heckerae* from cold seeps in the Gulf of Mexico (Cavanaugh *et al*., 1987; Fisher *et al*., 1993), *B. azoricus* and *B. puteoserpentis* from vents along Mid-Atlantic Ridge (Distel *et al*., 1995; Fiala-Medioni *et al*., 2002), and *Bathymodiolus* sp. from a pockmark area on the Gabon margin (Duperron *et al*., 2005), have been described to harbour two types of symbionts, a chemoautotrophic, sulfur-oxidizing bacterium (thiotroph) and a methanotroph. The co-occurrence of the two symbionts was revealed by using TEM, FISH, 16S rRNA sequencing, enzyme assays and immunohistochemistry analyses. Thus, in all mentioned species, two bacterial morphotypes were identified and the presence of enzymes typical for both autotrophic and methanotrophic bacteria (ribulose-1,5-biphosphate carboxylase/oxygenase and methanol dehydrogenase, respectively) were detected in gill extracts from these species (Cavanaugh *et al*., 1987; Fisher *et al*., 1993; Distel *et al*., 1995; Dupperron *et al*., 2005). The reliance of these mussels on the organic carbon derived from their symbionts was shown by stable isotope analyses of gill tissues (Cavanaugh *et al*., 1987; Fisher *et al*., 1993; Trask & Van Dover, 1999) and lipid biomarkers (Pond *et al*., 1998). Not all *Bathymodiolus* species harbour dual symbionts. Some have only a thiotrophic symbiont (Cavanaugh, 1985; Fisher *et al*., 1987), others only a methanotrophic symbiont (Cavanaugh *et al*., 1987; Childress *et al*., 1986; Fisher *et al*., 1987)**.** This ability of *Bathymodiolus* species to take advantage of different types of bacterial symbionts emphasizes the nutritional adaptability of this genus.

Symbioses of invertebrates with chemoautotrophic sulfur-oxidizing bacteria

Symbiotic associations between marine invertebrates and chemoautotrophic sulfur-oxidizing bacteria are present among members of seven animal phyla: Platyhelminthes, Nematoda, Echiurida, Annelida, Mollusca, Arthropoda, and Echinodermata (Ott *et al*., 2004a). Initially discovered at deep-sea hydrothermal vents (Cavanaugh *et al*., 1981; Felbeck, 1981; Corliss *et al*., 1979), such symbiotic associations are common in a variety of habitats ranging from

cold seeps and continental slope sediments to shallow, sheltered intertidal and subtidal sediments, organic rich mud and mangrove peat (Cavanaugh, 1994; Ott *et al*., 2004a, b; Bright & Giere 2005). Chemoautotrophic symbioses occur in habitats where sulfide is produced either geothermally (hydrothermal vents) or biologically (hydrocarbon cold seeps, reducing shelf sediments) (Bright & Giere, 2005). The spatial fluctuations of oxygen and hydrogen sulfide in these habitats create a redox boundary where the two compounds are in close vicinity (Ott *et al*., 2004a, b; Bright & Giere, 2005). Thus, the invertebrates using various strategies provide their symbionts with an oxidant and an energy source. Such examples are the vestimentiferan tube worms *Riftia pachyptila* which supplies the symbionts with inorganic carbon, O_2, sulfide and nitrate using a well developed circulatory system. The uptake of these substances takes place in a highly vascularized area, the plume, the only part of the worm which is in contact with the venting waters. The worm blood contains specialized extracellular haemoglobins with high affinities to bind and transport O_2 and sulfide to the trophosome where the symbionts are located (Arp & Childress, 1981, 1983; Childress *et al*., 1984; Goffredi *et al*., 1997a). Also the celomic fluid and to lesser extent the blood of *Riftia* is characterized by a pronounced base excess that permits retention and storage of large quantities of CO_2 (Weber & Vinogradov, 2001). Due to the alkaline pH, these worms can “trap” bicarbonate by the rapid conversion of CO_2 (Childress *et al*., 1993). Thus, the dissolved inorganic carbon is transported via the vascular system either as CO_2 or as HCO_3^- to the symbiont-containing trophosome (Goffredi *et al*., 1997b). In contrast to the tube worms which live bond to a substratum, most of the symbiont-containing bivalves are burrowers. They draw oxygenated water through their burrows, where it mixes with reduced inorganic compounds from the sediment (Southward, 1987). In bivalve families of Solemyidae, Lucinidae, Vesicomyidae and Mytilidae the transport of O_2 and sulfide to the symbionts was also attributed to the haemoglobins from the highly vascularized gills. The gills represent the body region where the symbionts occur. A different strategy is used by the nematodes and gutless oligochaetes harbouring ecto- or endosymbionts. They move along the chemocline between oxic and anoxic layers of the sediment providing their symbionts with O_2 and sulfide. The nematodes do not posses a well developed vascular system, their haemoglobin having a much reduced binding capacity than in other invertebrates. Therefore, the transport of sulfide and oxygen to the endosymbionts is mostly by diffusion. The energy obtained by oxidation of reduced sulfur compounds (e.g. sulfide, thiosulfate or elemental sulfur) with oxygen or nitrate as terminal electron acceptors is used by the symbionts to fix CO_2 into organic compounds. The animals gain nutrition either by the transfer of these organic compounds from the bacterial symbionts or by digestion of the symbionts.

Chemoautotrophic symbioses are well represented among marine invertebrate phyla in particular in annelids, molluscs and nematodes. More and more species from these groups are being discovered that harbour sulfur-oxidizing chemoautotrophic symbionts but also other types of symbionts (see *'Chemoautotrophic symbioses in oligochaetes'*) emphasising the capacity of these hosts to acquire and establish relationships with new partners. In shallow intertidal and subtidal sulfidic sediments members of two invertebrate groups, Oigochaeta and Nematoda are the most common metazoans involved in symbiotic associations with chemoautotrophic microorganisms.

Chemoautotrophic symbioses in oligochaetes

The annelid subtaxon Oligochaeta includes two genera of marine Tubificidae, *Olavius* and *Inanidrillus* which are associated with sulfur-oxidizing microorganisms. Since 1981, when the first chemoautotrophic symbiosis between *Inanidrilus leukodermatus* (*Phallodrilus leukodermatus*) and thiotrophic bacteria was described, more than 30 species of gutless oligochaetes have been shown to harbour such symbionts (Giere, 1981; Bright & Giere, 2005; Dubilier *et al*., 2005). Probably this type of association extends to all of the 80 species described so far belonging to these two monophyletic genera of gutless oligochaetes, but this requires further investigations (Erseus *et al*., 2003; Bright & Giere; 2005).

Gutless oligochates are small worms of about 0.5 mm diameter and 2–5 cm length, characterized by the lack of gut, anus, or nefridia (excretory system). As other symbiotic gutless marine invertebrates, they gain nourishment via chemosynthesis from their symbiontic partners. They occur worldwide in shallow, muddy or sandy marine sediments (Dubilier *et al*., 2005). The preferred habitats are tropical and subtropical coral reefs, where high abundances, of up to 85×10^4 individuals m^{-2}, and a high diversity has been found (Giere *et al*., 1982; Erseus, 2003). Considered to be part of the thiobiotic meiofauna, gutless oligochaetes are most dominant in deeper sediment layers (5–15 cm below sediment surface) where they are regularly exposed to low oxygen concentrations and to sulfidic conditions (Giere, 1993; Bright & Giere, 2005). They can survive short periods in the absence of oxygen by switching to an anaerobic metabolism like many other marine invertebrates (Dubilier *et al*., 1995; Grieshaber *et al*. 1992). Oxygen is required not only for their own respiration, but also as a terminal electron acceptor for the thiotrophic symbionts. When oxygen becomes limiting, the worms migrate upwards and they can be found in the upper sediment layers. Such an example is *Olavius crassitunicatus* from the up-welling site off the cost of Peru, which is most abundant in the upper 1–5 cm of the sediment due to the extremely low oxygen concentrations (< 1 μM) just above the sediment surface (Levin 2002,

2003; Blazejak *et al*., 2005; Dubilier *et al*., 2005). In general, purely oxic or highly sulfidic (> 500 μmol H_2S) sands are avoided by the gutless oligochaetes (Bright & Giere, 2005).

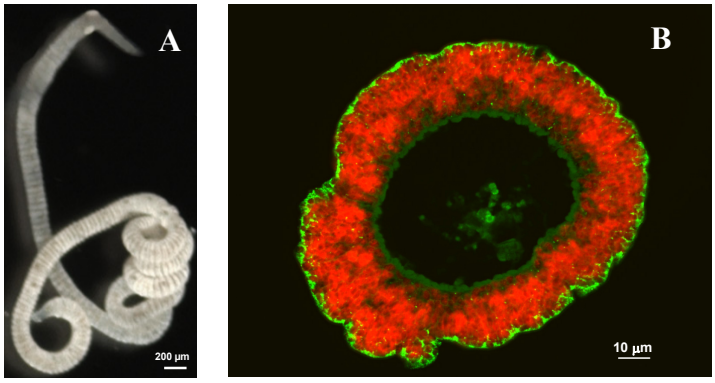

Fig. 1. The gutless oligochaete *Olavius crassitunicatus*, general aspect (A) and FISH on cross-section evidencing the presence of Gammaproteobacterial (in red) and sulfate-reducing (in green) endosymbionts (B). From Blazejak *et al*., unpublished and Blazejak *et al*., 2005.

Bacterial symbionts of the gutless oligochaetes occur in a multicellular layer below the cuticle of the worm in the so called symbiotic region (Dubilier *et al*., 2005). The bacteria in the apical part of the symbiotic region are extracellular, located between extensions of the epidermal cells, in contrast to bacteria from the basal area which are enclosed in vacuoles of the epidermal cells and appear to be in various stages of lysis (Giere, 1981; Giere *et al*., 1995; Dubilier *et al*., 2005). Up to 10^6 bacterial cells, corresponding to 25% of the worm's volume were assumed to occur in one individual (Giere *et al*., 1995). Due to the permeability of the worm cuticle for molecules as large as 70 kDa the symbionts have access to most substances in the pore water where the worms live (Dubilier *et al*., 2005).

Ultrastructural studies showed that the bacterial symbionts are morphologically similar in all *Inanidrilus* and *Olavius* species examined to date. All gutless oligochaetes harbour at least two gram-negative bacterial morphotypes: large, oval bacteria of 2–7 μm in length filled with sulfur and poly-β-hydroxybutyrate (PHB) vesicles, and small rod-shaped or slightly bent bacteria of 0.7–1.5 μm length without any conspicuous inclusions (Bright & Giere, 2005; Dubilier *et al*., 2005). In some of these hosts, a very long and thin morphotype of 10 μm length and only 0.3 μm diameter was described as a third morphotype (Giere 1995; Giere & Krieger 2001).

The capacity of the large oval bacteria to store sulfur in intracellular globules and the presence of form I ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) in these symbionts demonstrates their chemoautotrophic sulfur-oxidizing nature (Dubilier *et al*., 2001; Krieger *et al*., 2000). RubisCO is the enzyme which catalyzes the first step in the autotrophic CO_2 fixation via Calvin-Benson-Bassham (CBB) cycle and therefore its presence is an indicator of chemoautotrophy. Of the two structurally distinct forms of RubisCO, form I and form II, RubisCO form I is more suitable for CO_2 fixation under aerobic conditions (Tabita, 1988). Due to the dominant presence in almost all gutless-oligochaetes,

the large oval shaped bacteria are considered “primary” symbionts. Based on 16S rRNA sequence analyses they have been identified as *Gammaproteobacteria* and designated Gamma 1 symbionts.

The small bacterial morphotypes have been identified based on 16S rRNA sequence analysis and FISH and can belong to *Alpha* -, *Gamma* -, or *Deltaproteobacteria* (Dubilier *et al*., 2005). The third morphotype described for the first time in *O. crassitunicatus* from Peru margin has been identified as a spirochete (Giere & Krieger 2001; Dubilier *et al*., 2005). In some oligochaete species, such as *O. loisae* (Dubilier *et al*., 1999) or *O. algarvensis* (Giere *et al*., 2002), a fourth morphotype of intermediate size between the large oval and small bacteria was observed but the identity of this symbiont remains uncertain (Dubilier *et al*., 2005). Although two to four different bacterial morphotypes have been described in gutless oligochaetes, even more phylotypes were detected based on molecular analyses. Such examples are *O. crassitunicas* (Peru) where five to six distinct bacterial phylotypes were identified (Blazejak *et al*., 2005), and *I. leukodermatus* (Bermuda) and *I. makropetalos* (Bahamas) with up to five symbiont phylotypes co-occurring in the same host (Blazejak *et al*., 2006).

Chemoautotrophic symbioses in nematodes

An overview of the phylum Nematoda

The nematodes are the most abundant animal group on earth, accounting for 80% of all animal individuals (Lorentzen, 1994). They have a high ability to exploit various ecological niches, from terrestrial to marine environments (Lorentzen, 1994; Mitreva *et al*., 2005). The nematode phylum includes free-living terrestrial and marine microbivores, meiofaunal predators, herbivores, and animal and plant parasites (Parkinson *et al*., 2004; Mitreva *et al*., 2005). They can be found in very high numbers (e.g. several millions per square meter in marine sediments and terrestrial soils; Lorentzen, 1994) and high diversity. It was estimated that the species number ranges from 100.000 to 1 million (Lambshead, 1993).

In the marine environment, the nematodes are the most numerous Metazoa, representing 60–90% of the total meiobenthos individuals (Nicholas, 2001). They are usually strictly bound to a substratum, living attached to sediment particles or aggregates. The length of marine nematodes can vary between 1–3 mm but sizes over 10 mm or shorter than 1 mm have been described (Riemann, 1988).

In marine sediments, the nematodes colonize every type of sediment, from muddy to fine or coarse sandy sediments of intertidal, subtidal or deep sea areas (Hope & Murphy, 1969, 1977; Ott *et al*., 1982, Ott & Novak 1989; Ott *et al*., 2004; Austen *et al*., 1993; Riemann 1993, 2003; Buchholz & Lampadariou, 2002). Cold arctic waters as well as hot

springs harbour these worms and nematodes have been found even in hypersalinic brines where salt is crystallizing (Riemann, 1988). While in muddy sediments most nematodes (up to 90%) are found in the upper 5 cm, in fine and coarse sands they occur deeper, with a population maximum at about 10 to 20 cm depth (Riemann, 1988). Many nematodes do not need constant oxygen supply and have a high tolerance for anoxic conditions. Thus, certain groups are regular inhabitants of anoxic sediment layers where hydrogen sulfide accumulates through bacterial action (Riemann, 1988; Anderson, 2001; Jorgensen & Fenchel, 1974; Giere, 1992; Jorgensen, 1989). Among the nematode species that benefit from the exploitation of sulfide and the advantages offered by this ecological niche (e.g. low grazing pressure, decreased competition) are those involved in symbiotic associations with chemoautotrophic sulfur-oxidizing microorganisms.

Symbiotic nematodes

Marine nematodes involved in symbiotic associations are members of the class Chromadorea of the phylum Nematoda (De Ley & Blaxter, 2002; Fig. 2). Two types of marine nematode are generally recognized to form symbiotic associations with microbes: the Stilbonematinae that have a fully functional gut and the Siphonolaimidae that have no mouth and a reduced gut. These host groups are phylogenetically distinct (Lorentzen, 1981; 1994; De Ley & Blaxter, 2002) as they are members of two distantly related orders, order Monhysterida (mouthless nematodes) and order Desmodorida (gut-bearing nematodes) (Fig. 2). Also, the two host groups are engaged in two different types of symbioses: ecto- and endosymbioses. Thus, gut-bearing nematodes have ectosymbiotic bacteria attached to their body, and mouthless nematodes harbour endosymbiotic microorganisms inside of their body cavity. Frequently, both host groups share the same habitats and can be found in high abundance in shallow intertidal and subtidal sediments. While symbioses between gut bearing nematodes and bacteria have been intensively studied in the past 15 years (reviewed in Ott *et al*., 2004a, b), less attention has been paid to the mouthless nematodes and their endosymbionts.

Gut bearing nematodes with ectosymbiotic bacteria

The gut-bearing nematodes with ectosymbiotic bacteria are members of closely related genera within the subfamily Stilbonematinae of the family Desmodoridae (order Desmodorida) (Ott *et al*., 2004b). The subfamily Stilbonematinae contains 30 described species belonging to 8 genera (Riemann *et al*., 2003; Ott *et al*., 2004b). Based on morphological characters (e.g. unique glandular sense organs; Bauer-Nebelsick *et al*., 1995), and 18S rRNA gene sequence analyses (Kampfer *et al*., 1998) the Stilbonematinae form a monophyletic clade within the Desmodoridae.

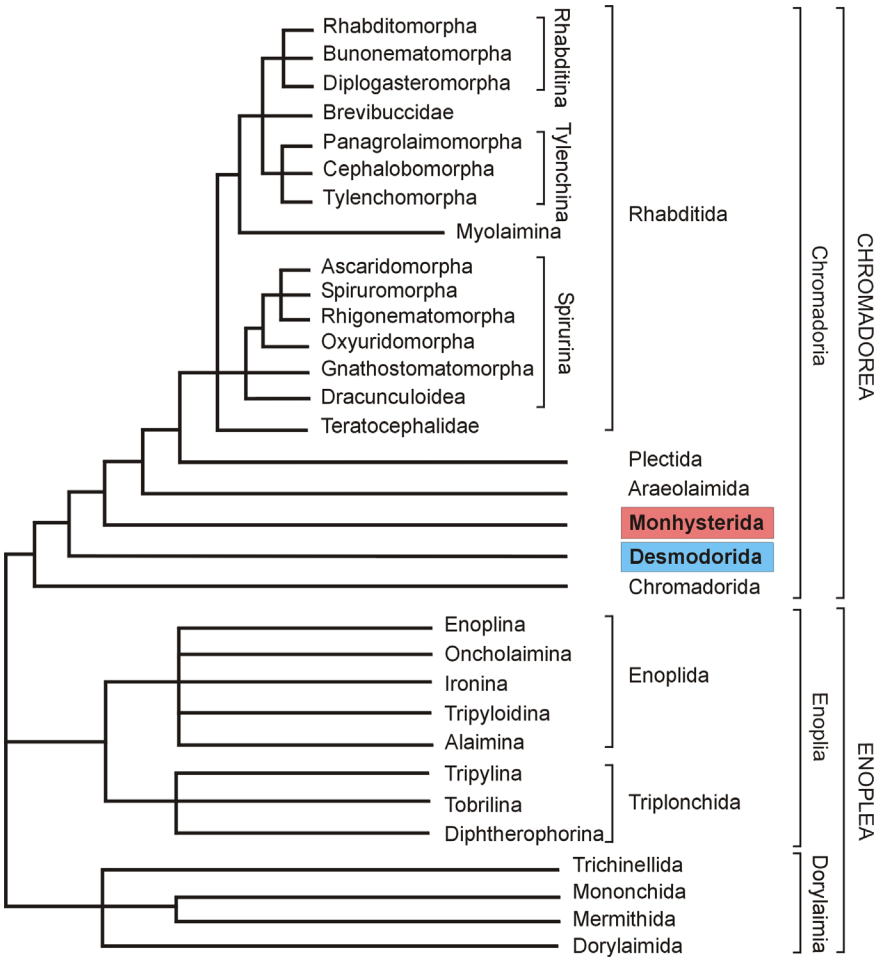

Fig. 2. Overview of the phylogenetic relationships among nematodes based on 18S rRNA gene sequence analyses. Nematode orders with members that are involved in symbiotic associations are highlighted. Modified from De Ley & Blaxter, 2002.

These nematodes were first described by Greef (1869) who considered the symbiotic bacterial coat as part of the cuticular ornamentation. Later descriptions regarded the symbionts as fungal spores or “blue-green algae” (cyanobacteria) (reviewed in Ott *et al*., 2004b). Powell and co-workers (1979) suggested for first time that the symbionts may by sulfur-oxidizers based on incubation experiments in $Na_2{}^{35}S$ of symbiotic nematodes of the genus *Eubostrichus.* Thus, the analysis of autoradiographs has showed that the uptake of radioactively labelled sulfur was mostly confined to the symbionts (Powell *et al*., 1979). Other studies such as microrespiration experiments of *Stilbonema* sp., *Catanema* sp., and *Robbea* sp. which showed that overall consumption of oxygen of these nematodes and their symbionts is enhanced following exposure to either thiosulfate or sulfide further supported the sulfur-oxidizing nature of the symbionts (Schiemer *et al*., 1990). In addition, stable carbon isotope measurements ($\delta^{13}C$) of the worms and their symbionts have showed low values of −24.9 to −27.5, values that were similar to those known from invertebrate tissues containing endosymbiotic chemoautotrophic bacteria (Ott *et al*., 1991). Also, the detection

of enzymes associated with sulfur metabolism (e.g. ATP sulfurylase) and of key enzyme for autotrophic carbon fixation, RubisCO, as well as the presence of elemental sulfur within the bacterial cell indicate a chemolithoautotrophic nature of the symbionts (Polz *et al*., 1992). Moreover, TEM cross sections of *Catanema* sp. revealed polyhedral, electron dense bodies scattered throughout the cytoplasm of the bacterial symbionts that resemble carboxysomes (Polz *et al*., 1992).

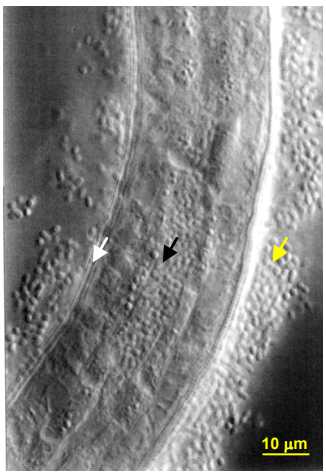

Fig. 3. Ectosymbionts of the gut-bearing nematode *Leptonemella aphanothecae* (yellow arrowhead), attached to the cuticle of the worm (white arrowhead); bacterial cells with a similar morphology to that of symbionts can be observed inside of the worm's gut (black arrowhead). Phase contrast micrograph courtesy of Dr. Franz Riemann.

The chemoautotrophic sulfur-oxidizing symbionts coat the nematodes surface in a species-specific pattern (Ott *et al*., 1991). Their size, morphology and arrangement vary between genera and/or between host species (Table 1). The symbionts cover almost the whole body of the nematode except the head and the tip of the tail (Fig. 3). Due to the elemental sulfur and the poly-β-hydroxy alkanoic acids (PHAs) inclusions the symbiotic bacteria have a shiny white colour under incident light (Ott *et al*., 2004a). The PHAs were identified as poly-β-hydroxybutyric acid (PHB) and poly-β-hydroxyvaleric acid (PHV) in symbionts of *Catanema* sp., *Stilbonema* sp., and *Robbea* sp. (Polz *et al*., 1992).

Molecular characterization based on 16S rRNA gene sequencing and FISH of the ectosymbionts of several Stilbonematinae, *Laxus oneistus* (Poltz *et al*., 1994), *L. cosmopolitus*, *Catanema* sp. *Stilbonema majum* and *Eubostrichus toparius* (Vanura, 2001), showed that the symbionts belong to the *Gammaproteobacteria* (see '*Phylogeny of chemoautotrophic symbionts'*). However, in some of these species such as *S. majum*, *E. toparius* (Vanura, 2001) and *E. dianae* (Poltz *et al*., 1999) several other 16S rRNA sequences were retrieved belonging to the *Cytophaga*, *Caulobacter*, *Alpha- Gamma-* and *Deltaproteobacteria* (Ott *et al*., 2004b). It is not known whether these sequences belong to symbionts or whether they are from contaminants, since their identification based on FISH has not yet been described.

The benefit of both partners from this symbiotic association has been shown in numerous studies. There is a general agreement that the symbiosis between Stilbonematinae and their ectosymbionts is essentially a nutritional symbiosis (Ott *et al*., 2004a, b). Thus, the first studies based on TEM showed a high morphological and structural

similarity between the ectosymbionts and the bacteria observed in the gut of the nematode suggesting that the host may feed on their own symbionts (Fig. 3) (Ott *et al*., 2004b). Although ingestion of the bacteria was never been observed in experiments, it was assumed that due to the ability of the worms to coil and reach any region of their body with the mouth they can graze on their own symbionts (Ott *et al*., 2004b). This assumption was sustained by the observation of patches without symbionts on the worm cuticle, patches which could have been produced by recent grazing. Around patches, dividing bacterial cells were observed, implying grazing as a stimulating factor of bacterial grows and division (Poltz *et al*., 1992; Ott *et al*., 2004b). In addition, the analysis of $\delta^{13}C$ values of symbiotic nematodes from which the symbionts were removed showed a low value of –25.9 % (Ott *et al*., 1991). This value was in contrast with $\delta^{13}C$ values (–10.5%) of non-symbiotic nematodes from the same habitat and very similar with $\delta^{13}C$ values of free living sulfur-oxidizing bacteria and of chemoautotrophic symbionts of invertebrates from hydrothermal vents and subtidal sediments (Rau, 1981; Ott *et al*., 1991; 2004b).

Nematode host	**Symbiont**			
	Size (μm)	Morphology	Arrangement	Reference
Stilbonema spp.	1-2	cocci	Multilayered (up to 10 layers) with no apparent order; embedded in a mucous matrix	Ott *et al*., 1991; 1995; 2004a, b; Polz *et al*., 1992
Laxus spp.	2-5	rods	Monolayer, ordered	Ott *et al*., 1991; 1995; 2004a, b
Catanema spp.	2-5	rods	Monolayer, ordered, embedded in a mucous matrix or appear free	Ott *et al*., 1991; 1995; 2004a, b; Polz *et al*., 1992
Robbea spp.	2-5	rods	Monolayer, ordered perpendicular or parallel on the body	Ott *et al*., 1991; 1995; 2004a, b
Eubostrichus spp.	up to 120	Non-septate filaments	Highly ordered, monolayered; attached with one or both ends to the cuticle; aspect of fur-like coat	Ott *et al*., 1991; 1995; 2004a, b; Polz *et al*., 1992
Leptonemella spp.	up to 40	Coccoid or rod like	Multilayered or single layer with no apparent order; embedded in a mucous matrix	Ott *et al*., 1991; 1995; 2004a, b; Polz *et al*., 1992; Riemann *et al*., 2003

Table 1. Size, morphology and arrangement of bacterial symbionts of various Stilbonematinae species

There is also the possibility that the symbionts are involved in sulfide detoxification of the worms, since freshly collected symbiotic nematodes from the sulfidic sediments have showed much lower internal sulfide and thiosulfate concentrations than non-symbiotic nematodes (Hentschel, 1999). When the bacterial coat was removed, the nematodes died

quickly in 200 μM sulfide as a consequence of accumulation of high concentrations of sulfide and thiosulfate, whereas similar concentrations did not affect the symbiotic worms. Moreover, it has been showed that the symbiotic bacteria were able to increase the heat tolerance in *Stilbonematinae* under sulfide stress (Ott, 1995; Ott *et al*., 2004a).

The benefit for the bacterial ectosymbionts from this association lies in the access to both oxygen and reduced sulfur compounds provided by a motile host. In marine sediments, oxygen and sulfide coexist in a narrow microzone for short time periods, only when sulfide concentrations in deeper sediment layers are high. When sulfide concentrations are low, there may be no overlap at all (Powell, 1989). The Stilbonematinae occur exclusively in sulfidic sediments usually concentrated around this oxygen-sulfide microzone, but they can be found in deeper sediment layers as well (Ott & Novak, 1989; Ott *et al*., 20004b). Due to their small size the nematodes move through the sediment's pore-space following the changes of chemical gradients, thus alternately providing their symbionts with reduced sulfur compounds and oxygen (Ott and Novak 1989; Ott *et al*., 1991; Ott *et al*., 2004b). Also, it has been shown that when the symbionts possess internal stores of elemental sulfur and polythionates, nitrate can serve as alternative terminal electron acceptor (Hentschel *et al*., 1999; Ott *et al*., 2004b). Nitrate respiration is probably important when the nematodes spend more time in deeper sediment layers rich in sulfide (Hentschel *et al*., 1999). Nitrate respiration has been also described in the chemoautotrophic sulfur-oxidizing symbionts of other invertebrates from sulfidic-rich habitats, for instance the bivalves *Lucinoma aequizonata* and *Solemya reidi* (Hentschel *et al*., 1993; Hentschel & Felbeck, 1995) and the annelids *Riftia pachyptila* and *Inanidrilus leukodermatus* (Hentschel & Felbeck, 1993; Giere, 1991).

Partner acquisition, recognition, and loss have been studied in symbiotic gut-bearing nematodes in laboratory experiments as well as through field observations. The nematodes moult four times during their development to adults and every time loose their symbionts together with the old cuticle. Nevertheless, aposymbiotic animals were rarely observed in field investigations, and even the juveniles in their first stages of development had a symbiotic coat. These observations led to the conclusion that newly hatched and recently moulted worms are rapidly colonized by symbionts. However, such a rapid colonization process was never observed experimentally (Ott *et al*, 2004b). On the other hand, it was hypothesised, and for some nematode species (e.g. *Laxus cosmopolitus* and *L. oneistus*) proved experimentally, that recognition between partners involves highly specific mechanisms based on lectine-sugar interactions (Nussbaumer *et al*., 2004; Ott *et al*., 2004b). This hypothesis implies that after hatching or moult, the symbionts are acquired from the environment through such specific interactions and that the nematode cuticle is colonized *de novo* (Ott *et al*., 2004b). Of course, this could only be envisioned if the

symbionts occur as free-living bacteria within the microbial communities of the worm's habitats, a presence that has not been confirmed up to now.

Gutless nematodes with endosymbiotic bacteria

Nematodes with endosymbiotic microorganisms belong to the genera *Astomonema* (Ott, 1982; Austen *et al*., 1993), *Parastomonema* (Kito 1989), and *Rhaptothyreus* (Hope & Murphy, 1969; Riemann, 1993). The genus *Astomonema*, contains four described species, *A. jenneri* (Ott *et al*., 1982), *A. otti*, *A. obscura* (Vidacovic and Boucher 1987), and *A. southwardorum* (Austen *et al*., 1993; Giere *et al*., 1995), and the genus *Parastomonema* one described species, *P. fijiense* (Kito, 1989). Both genera are placed together within the family Siphonolaimidae (Order Monhysterida, Class Chromadorida) (Ott, 1982; Lorentzen, 1994; De Ley & Blaxter, 2002). For the genus *Rhaptothyreus*, which consists of two described species, *R. typicus* (Hope & Murphy, 1969) and *R. minor* (Riemann, 1993) a new family, Rhaptothyreidae was created (Hope & Murphy, 1969). Although they live in different habitats, with *Astomonema* and *Parastomonema* species dominating shallow water intertidal and subtidal sediments, while *Rhaptothyreus* species prefer deep sea sediments, all these nematodes were described to harbour bacteria-like structures inside their body (Ott *et al*., 1982; Kito 1989; Giere *et al*., 1995; Riemann, 1993). There are only two microscopical investigations of *A. jenneri* (Ott *et al*., 1982) and *A. southwardorum* (Giere *et al*., 1995) that provide a detailed characterization of the bacterial endosymbionts in nematodes. The other studies just cited, focused mainly on the taxonomic characterisation of the nematode and barely mentioned the presence of the symbiotic microorganisms.

Members of the genus *Astomonema* have a long, slender, and cylindrical body of 3–4 mm length and 25–90 μm width for adults, and 1–2 mm length and 15–25 μm width for juveniles. Longer (up to 16 mm) or thinner (14 μm) dimensions have been observed (Ott, 1982; Austen *et al*., 1993; Giere, 1995). All adults lack a mouth opening, a functional gut and an anus; in some very small juveniles, a mouth opening was observed (Ott *et al*., 1982; 2004b).

The description of *A. jenneri* and *A. southwardorum* (Ott *et al*., 1982; 2004b; Giere, 1995) revealed a number of differences between these species in their morphology and regarding the position, number, size and morphology of the symbionts. Thus, *A. jenneri* collected from an intertidal mud flat (Banks Channel, North Carolina,) harbours two different morphotypes of bacteria inside of the body cavity (Ott *et al*., 1982). Both morphotypes are considered intracellular due to their location within the cytoplasm of the host intestinal wall cells (Ott *et al*., 1982; 2004). The dominant bacterial morphotype were large elliptical cells of about 3 × 1 μm, filled with dark globules (probably PHB granules) and large membrane

bound vesicles (probably sulfur). The less common morphotype was small (0.1–0.5 μm) and rod shaped, and occurred at the beginning of the gut rudiment (Ott *et al*., 1982; 2004). In *A. southwardorum,* found in silt sediments of a methane seep pockmark (North Sea), only one large bacterial morphotype, of 5 × 10 μm was observed (Fig. 4) that is located extracellularly and completely fills the lumen of the gut rudiment (Austen *et al*., 1993; Giere *et al*., 1995). Apparently, these bacteria have a similar cellular organization as the large bacteria described in *A. jenneri*.

The identity of these microorganisms and their possible role in the host nutrition remains to be clarified. These bacteria constitute about 25 to 50% of the body mass of *Astomonema* spp. and have been characterized as thiotrophs based on their structural similarity with thiotrophic symbionts of other invertebrates (e.g. the granular aspect of the cytoplasm, the shiny white colour which indicates storage of elemental sulfur). The lack of feeding structures of the nematode host and the absence of specialisation of the body surface for direct uptake of organic molecules from the environment, strongly suggests a nutrient transfer from the prokaryotes to the host (Ott *et al*., 2004b). The nature of the habitat occupied by these worms, reduced sulfidic sediments (*A. jenneri*), or soft subtidal sediments, where reduced sulfur compounds are available from below and oxygen is accessible in the superficial layers (*A. southwardorum*, *A. otti*, *P. fijiese*), support the hypothesis that the symbionts of *Astomonema* are thiotrophs (Ott *et al*., 1982; 2004b; Giere *et al*., 1995).

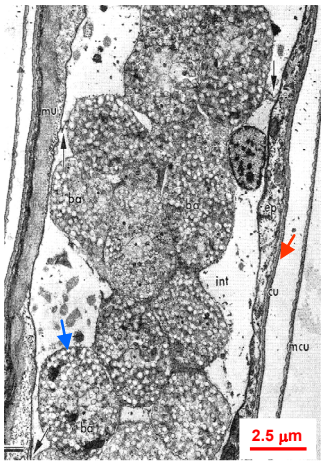

Fig. 4. Endosymbionts of the gutless nematode *Astomonema southwardorum* (blue arrowhead) filling almost the entire body cavity of the worm. The large bacterial cells contain numerous globules and clear voids, presumably PHB inclusions and elemental sulfur granules. The bacteria are lined by the worm cuticle (*cu*, red arrowhead). Transmission electron micrograph, from Giere *et al*., (1995).

Another member of the family Siphonolaimidae possibly involved in a symbiotic association is *Siphonolaimus tubicen* (Ott, 1972), most likely a close relative of *Astomonema* spp. This siphonolaimid has a functional digestive tract and rod shaped microorganisms were observed under the cuticle, inside the epidermal cells of the nematode (Ott *et al*., 1982). Since no other investigations were performed on *Siphonolaimus tubicen* it is difficult to comment on the nature of this association.

The pathways of symbionts acquisition and transmission in gutless nematodes are not fully understood at this time. It has been hypothesized that the symbionts are acquired from

the environment by juveniles in their first stage of life when they have a mouth opening (Ott *et al*., 2004b).

Phylogeny of chemoautotrophic symbionts

A variety of invertebrate hosts form symbioses with chemoautotrophic bacteria (Southward, 1987; Fisher, 1990; Cavanaugh, 1994; Ott *et al*., 2004a, b; Bright & Giere, 2005). In general, the symbionts vary in shape, size, and ultrastructure between different hosts indicating that they may be different species. Since none of these symbionts have been cultured to date, their identification and characterization is dependent on molecular methods. These have been intensively used in the past 20 years to determine the phylogenetic relationships among bacteria, including uncultured microorganisms (Woese, 1987). In chemoautotrophic symbioses, the phylogenetic analyses based on 16S rRNA sequences have been applied with success for microorganisms of various invertebrates such as bivalves (Cavanaugh, 1994; Distel *et al*., 1994, 1995; Krueger *et al*., 1996; Fujiwara *et al*., 2001; Duperron *et al*., 2005), oligochaetes (Dubilier *et al*., 1995; Dubilier *et al*., 1999; Dubilier *et al*., 2001; Blazejak *et al*., 2005), nematodes (Poltz *et al*., 1994) or vestimentiferans (Di Meo *et al*., 2000: Elsaied *et al*., 2002).

There are some common findings in all these phylogenetic studies involving chemoautotrophic sulfur-oxidizing symbionts. One common aspect is that the chemoautotrophic sulfur-oxidizing symbionts of marine invertebrates, with few exceptions such as *Alvinichocha* spp. symbionts (Gasteropoda; Urakawa *et al*., 2005; Suzuki *et al*., 2005) or *Alvinella pompejana* symbionts (Polychaeta; Campbell *et al*., 2003) that belong to *Epsilonproteobacteria*, all belong to the *Gammaproteobacteria*. Their closest relatives in most cases are free living sulfur-oxidizing bacteria. The symbionts are species-specific, i.e. individual members of a given invertebrate host have the same symbionts (Cavanaugh *et al*., 1994) (Fig. 5), as a rule they are specific to a given host and not found in other host species. Often they are clustered together, such as the vestimentiferan tubeworm's symbionts that consistently fall in a cluster with the symbionts of bivalves from shallow water habitats (Cavanaugh, 1994) (Fig. 5). This clade of symbiotic bacteria is most closely related to the sulfur-oxidizing bacteria *Leucothrix mucor* (Fig. 5). The symbionts of the gutless oligochaetes are consistently affiliated with those of gut-bearing nematodes, and cluster together with a clade of free-living phototrophic sulfur-oxidizing bacteria of the family *Chromatiaceae* (Dubilier *et al*., 2005; Blazajek *et al*., 2005; Fig. 5). Other chemoautotrophic symbionts reported for their constant monophyletic affiliation are those of deep sea mussels (*Bathymodiolus* spp.) and clams (*Calyptogena* and *Vesicomya* species) (Distel & Cavanaugh, 1994; Distel *et al*., 1995; Fujiwara *et al*., 2001; Fig. 5). In contrast to these symbiont clusters, few other symbiotic chemoautotrophs such as symbionts of

Pogonophora (i.e *Oligobrachia mashikoi* symbiont), Gasteropoda (i.e *Ifremeria nautilei* symbiont) or Oligochaeta (i.e *Olavius crassitunicatus* Gamma 2 symbiont) have as closest relatives clone sequences from deep sea (cold seeps or hydrothermal vents) and shallow water communities rather than other chemoautotrophic symbionts (Fig.5; Blazajek *et al*., 2005).

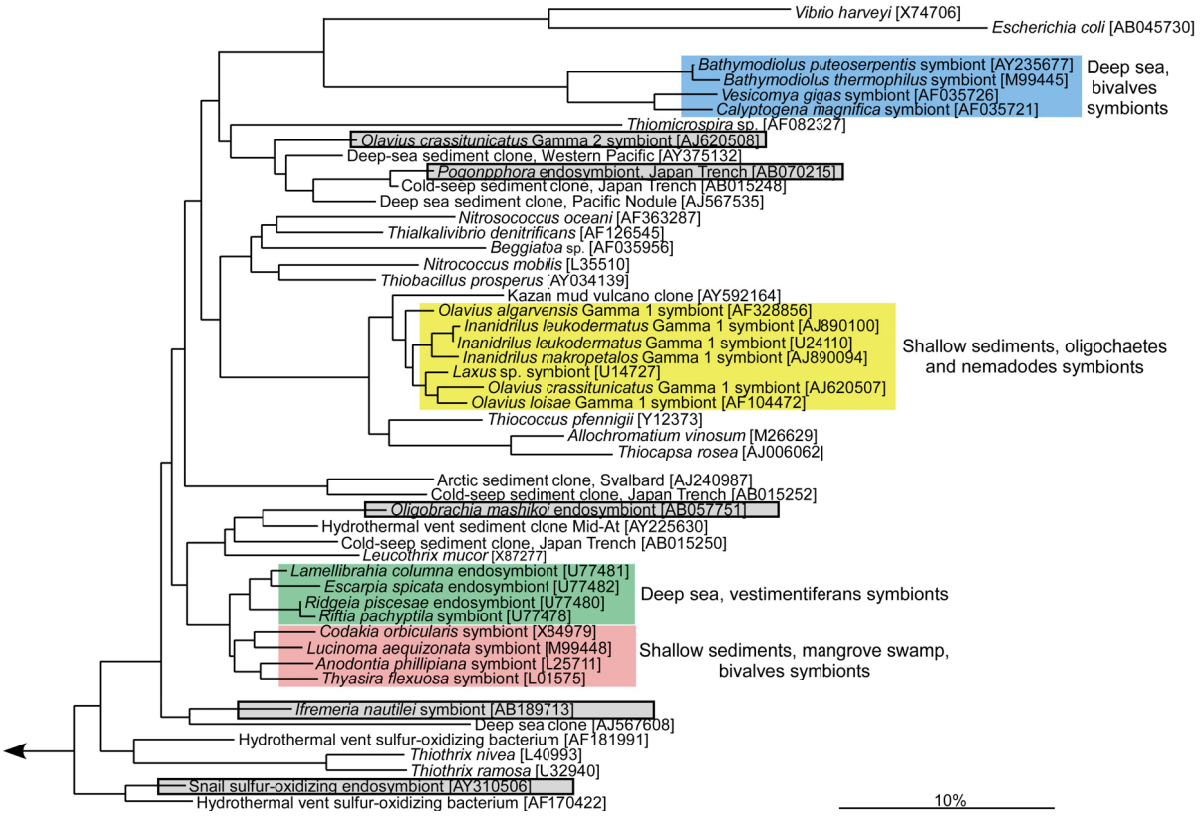

Fig. 5. Phylogenetic relationships of the chemoautotrophic sulfur-oxidizing symbionts (*Gammaproteobacteria)* of various invertebrate groups. 16S rRNA sequences of deep-sea mussels and clams (blue highlight), oligochaetes and nematodes (yellow), tube worms (green) and shallow water clams symbionts (pink) form distinct clusters. The scale bar represents 10% estimated sequence divergence.

In many bacterial symbioses, it seems that the symbionts have a monophyletic origin within individual host lineages suggesting that these symbioses evolved from a single bacterial species associated with an ancestral host (Krueger *et al*., 1996). Such symbioses were described between the bivalve families Lucinidae and Thyasiridae from shallow water environments and their chemoautotrophic symbionts (Distel *et al*., 1994). In these cases, co-speciation is observed with the diversification of the symbiont lineage in parallel with that of the host group, evidenced by the congruence between host and symbiont phylogenies. Likewise, the monophyletic origin of the symbionts and co-speciation with their hosts are suggested for the deep sea bivalve family Vesicomyidae (*Calyptogena* and *Vesicomya* species) (Distel *et al*., 1994). The close affiliation of gutless oligochaetes of the genera *Olavius* and *Inanidrillus* and gut-bearing nematode symbionts of *Laxus* sp. which form a

monophyletic clade despite the large evolutionary distance between oligochaetes and nematodes rules out co-speciation as an explanation for the monophyly of their symbionts. Instead, the shared biogeographical distribution of the two hosts is considered to have played an important role in the establishment of these symbioses (Dubilier *et al*., 2005).

Marine Sediments

Marine sediments cover about 70% of the Earth's surface. Most of the primary production that settles through the water column is mineralized in sediments. In consequence, marine sediments play a significant role in the global recycling of carbon and nutrients. Marine sediments are most often stratified habitats characterized by three major zones of organic matter oxidation: an oxic surface zone, in which the oxygen is the major oxidant, a suboxic but oxidized zone, in which nitrate, nitrite, manganese and iron oxides are the main electron acceptors, and an anoxic zone in which sulfate reduction and methanogenesis prevail (Alongi, 1997). These zones are subjected to temporal and spatial changes depending on various factors. For instance, the borders between oxic, suboxic and anoxic zones fluctuate according with the input of organic matter, with the light which drives photosynthesis, or with bioturbation produced by diverse macrofauna animals. Shelf seas characterized by high nutrient concentrations generate up to 30% of the total primary production in the ocean and from this portion, as much as 50% enter the sediment (Jørgensen *et al*., 1990). In these high productivity areas, the suboxic and sulfidic zones can extend close to the sediment surface (Jørgensen & Revsbech, 1989). In contrast, in the open oceans productivity is much lower and only 1% of the primary production of the surface waters reaches the bottom. Usually in these sediments, the oxic and suboxic zones are much thicker (Jørgensen, 1983).

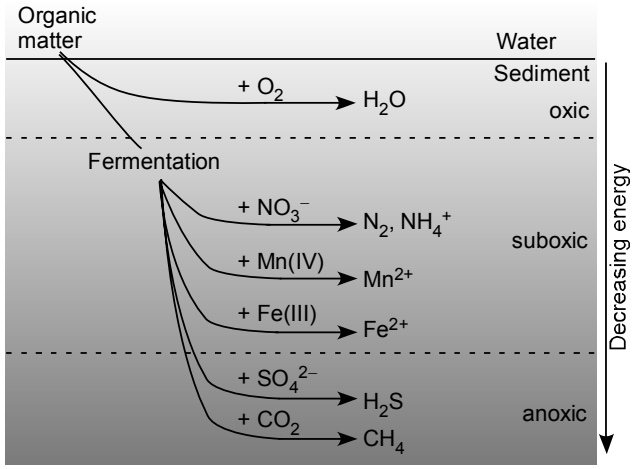

Fig. 6. The major oxidation reactions in the decomposition of the organic matter in marine sediments (modified after Alongi, 1997).

The decomposition and recycling of organic matter in sediments is performed by a variety of bacteria which use different electron acceptors. In accordance with the decreasing energy yields per mol of organic carbon oxidized, a vertical succession of the oxidants is usually observed in the sediment, with O_2 in the top layers, followed by NO_3^-, NO_2^-, Fe (III), Mn (IV), SO_4^{-2} and CO_2 (Fig. 6). Thus, the organic matter entering the sediments is initially

broken down, by extracellular and membrane-bound hydrolytic enzymes produced by numerous bacteria, from large macromolecules or polymers (e.g. carbohydrates or proteins) to soluble compounds (e.g. monosaccharides, aminoacids, alcohols). These soluble compounds represent the dissolved organic matter (DOM) which is decomposed by fermenting and acetogenic bacteria to simpler compounds, such as acetate, lactate and molecular hydrogen. Such simpler compounds are further oxidized by various bacteria that use nitrate, sulfate, and metal oxides as terminal electron acceptors (Alongi, 1997; Fig. 6).

Intertidal sediments

Intertidal sediments are complex environments located at the boundary between land and sea. These environments include shingle banks, sandy beaches, mud and sandflats, saltmarsh and mangrove communities (Jickelles & Rae, 1997). By their unique position, the intertidal sediments are responsible for the transfer and recycling of the nutrients between the land and the sea (Malcolm & Sivyer, 1997). These sediments are permanently exposed to a complex combination of variable environmental factors such as tides, currents, air exposure, storms, bioturbation, light intensities or temperature ranges which may induce major changes in the transport of substances to and from the sediment. Such an example is the diurnal drying and flooding of the sediment induced by tides. Thus, when the tide has ebbed, the dissolved substances produced in the sediment (e.g. ammonium) cannot be released due to the absence of the overlying water and consequently they accumulate in the sediment. Such pools of concentrated nutrients are released when the water returns. In contrast, the substances originating in the water column and consumed in the sediment became depleted when the water is not present (Malcom & Sivyer, 1997). The intertidal areas are generally characterized as dynamic environments ranging from high-energy sandy beaches to low-energy sand- or mudflats where physical forces are less dominant.

Intertidal sandy sediments

Biogeochemistry

Sandy sediments cover about 70% of the costal zones. Composed mainly by sand grain of large size with relatively low specific surface area, low adsorption capacity and consequently low organic matter content, these sediments were considered until recently “geochemical deserts”(Keil *et al*., 1994; Jickells & Rae, 1997; Boudreau *et al*., 2001). For this reason, their study was neglected many years. Once it was showed that the organic matter remineralization in low carbon sands can have turnover rates comparable to those in organic rich muds (Rowe *et al*., 1988; Cammen, 1991; Grant *et al*., 1991) the sandy sediments became of high scientific interest. Therefore, in the last decade, many studies

have focused on the geology and biochemistry of these sediments (e.g. Rusch & Huettel, 2000; Rusch *et al*., 2001, 2003; de Beer *et al*., 2005). Although poor in organic carbon, these sands are characterized by a high input of organic matter, solutes and particles which are transported across the water sediment interface. The high permeability of sandy sediments allows advection as the main transport process (Malcolm & Sivyer, 1997). Also, it has been showed that the sandy sediments act as filters, trapping into the sand pores algae, bacteria or suspended particles transported with the penetrating water into the sediments (Huettel & Rusch, 2000; Rusch & Huettel, 2000). The input of organic carbon in these sands is not limited only to production and trapping from the overlaying waters but also from benthic production, since light is sufficient in these sediments to support growth of algae and phototrophic bacteria. Additionally the deep oxygen penetration of up to 5–9 cm (de Beer *et al*., 2005), induced by continuous mixing and flushing of the sediment produced by hydrodynamic forces (e.g. waves, tides, currents) and bioturbation events, result in a rapid oxidation of the organic matter in sandy sediments. Due to this rapid transport of organic material and electron acceptors, intertidal sandy sediments are currently considered hot spots of aerobic and anaerobic degradation of organic matter (Kerner, 1993; Kerner & Yasseri, 1997; D' Andrea *et al*., 2002).

Microbial communities of sandy sediments

Molecular ecology studies have recently showed that marine sandy sediments are characterized by diverse microbial communities, but with overall cell abundances lower than those encountered in organic rich sediments (e.g Llobret-Brosa *et al*., 1998; Wieringa *et al*., 2000; Rusch *et al*., 2001, 2003; Ishii *et al*., 2004; Bühring *et al*., 2005). Due to the high permeability and dynamics of these sediments, the microbial communities do no display the strong stratification typically encountered in organic-rich sediments and show little variation of the total cell number over depth and seasons (Rusch *et al*., 2003; Ishii *et al*., 2004; Musat *et al*., 2006). With respect to microbial activity, recent biogeochemical studies have showed that sandy sediments are microbiologically highly active, comparable with organic-rich sediments (Shum & Sundby, 1996; Rusch *et al*., 2001, 2003; D' Andrea *et al*., 2002; de Beer *et al*., 2005).

Culture-independent molecular methods are the most appropriate tools for the study of microbial communities (Amann *et al*., 1995; see p. 22). Such methods refer to the description of the community by using non-quantitative or semi-quantitative methods based on 16S rRNA gene sequencing and secondly to the composition and structure of the community by making use of quantitative methods, such as fluorescence *in situ* hybridization.

Phylogenetic microbial diversity

Few studies have investigated the phylogenetic microbial diversity of marine sandy sediments (Rochelle *et al*., 1994; Gray & Herwig., 1996; Cifuentes *et al*., 2000; Bowman *et al*., 2003), while others focused only on phylogenetic diversity of certain bacterial groups such as sulfate-reducing bacteria (Devereux *et al*., 1994; Purdy *et al*., 2003) or sulfur-oxidizing bacteria (Brinkhoff *et al*., 1998). Using the 16S rRNA gene as a phylogenetic marker, these studies have revealed a great phylogenetic diversity with vast fractions of the 16S rRNA sequences affiliated to *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*, *Planctomycetes*, *Bacteroidetes* (formerly *Cytophaga/Flavobacterium/Bacteroides* phylum), *Verruco-microbiales*, and *Actinobacteria* groups. Within *Gammaproteobacteria* a relatively high number of sequences were closely affiliated with those of free-living and endosymbiotic sulfur-oxidizing bacteria (Gray & Herwig, 1996; Cifuentes *et al*., 2000; Bowman *et al*., 2003; Musat *et al*., 2006). The majority of deltaproteobacterial sequences were related with those of sulfate-reducing bacteria, and within the *Planctomycetes* group, the sequences were mainly related to those of *Pirelulla* spp and *Planctomyces* spp.. (Rochelle *et al*., 1994; Gray & Herwig, 1996; Cifuentes *et al*., 2000). Nevertheless, the greatest numbers of sequences retrieved from sandy sediments were usually more closely related with sequences of uncultured microorganisms rather than with those of cultured microorganisms.

Microbial community structure

Quantitative molecular methods have shown that the abundance of microorganisms in sandy sediments is about one order of magnitude lower that in organic-rich sediments, of up to 10^8 cells ml^{-1} sediment (Llobet-Brossa *et al*., 1998; Goni-Urriza *et al*., 1998; Wieringa *et al*., 2000; Rusch *et al*., 2001; 2003). Nevertheless, recent studies conducted on sandy sediments of the Wadden Sea revealed total cell numbers comparable with those found in muddy sediments (Ishii *et al*., 2004; Musat *et al*., 2006). The highest number of cells is usually recorded at depths of 1–3 cm, probably a consequence of the active mixing and washing of the sediment surface (Llobret-Brossa *et al*., 1998; Ishii *et al*., 2004; Rusch *et al*., 2001). Another consequence of the active mixing, deep-oxygen penetration and high bioturbation activities in sandy sediments is the lack of stratification reflected by a relatively constant depth distribution of bacterial community below the maximum (Llobret-Brossa *et al*., 1998; Rusch *et al*., 2001, 2003; Ishii *et al*., 2004; Musat *et al*., 2006).

The most abundant microbial groups in sandy sediments are *Cytophaga-Flavobacterium* and *Planctomycetes*. These groups contain aerobic heterotrophic organisms which are able to degrade polymeric substances but also facultative anaerobes, capable of fermenting carbohydrates. Both groups were detected in the upper layers

(Llobet-Brossa *et al*., 1998; Rush *et al*., 2003; Ishii *et al*., 2004; Bühring *et al*., 2005; Musat *et al*., 2006) as well as in the anoxic regions (Ravenschlag *et al*., 2001) of the sediments. Often found attached to the sand grains or in association with phytodetrital macroaggregates ("marine snow", DeLong *et al*., 1993; Rath *et al*., 1998), *Planctomycetes* may prevail in the high energy sandy sediments over the *Cytophaga-Flavobacterium* species that are easily removed by waves and currents from the sediment.

Other bacterial groups detected in high abundances in sandy sediments are *Alpha*- and *Gammaproteobacteria*. These groups are morphologically, metabolically and ecologically highly diverse and their presence in sediments is difficult to correlate with certain physiological processes, unless specific subgroups within *Alphaproteobacteria* or *Gammaproteobacteria* are targeted (e.g *Roseobacter* clade or sulfur-oxidizing bacterial species).

Betaproteobacteria is less abundant in sandy sediments than other bacterial groups. Typically, members of the *Betaproteobacteria* are found in high numbers in fresh water habitats (Glöckner *et al*., 1999) and only in low abundances in marine bakterioplankton and benthos (Llobet-Brossa *et al*., 1998; Rusch *et al*., 2003; Ishii *et al*., 2004; Musat *et al*., 2006). Their presence in sandy sediments is probably accidental and results from the washing and mixing of the sediment correlated with the sediment property to act as filter, capturing different particles that enter from the water column. This hypothesis is also sustained by their low abundances and presence only in the top layers (1–2 cm) of the sediment.

Deltaproteobacteria is represented in sandy sediments by members of the *Desulfosarcina-Desulfococcus* group. This group of sulfate-reducing bacteria contains strictly anaerobic and nutritionally versatile organisms able to completely oxidize compounds such as formate, acetate propionate, butyrate or alcohols (Kuever *et al*., 2005). Their presence in sandy sediments, especially at the sediment surface (Bühring *et al*., 2005; Ishii *et al*., 2004; Musat *et al*., 2006), raised numerous questions about how they survive in the presence of oxygen and if they are active or can use oxygen as terminal electron acceptor. Other sulfate-reducing bacteria detected in sandy sediments are members of the *Desulfovibrio*, *Desulfobulbus* and *Desulfomicrobium* groups (Devereux *et al*. 1994; Wieringa *et al*., 2000; Rusch *et al*., 2003; Bühring *et al*., 2005).

Methods

The rRNA approach

The application of molecular methods based on analysis of ribosomal RNA genes for studies of microbial communities has greatly increased our knowledge on the diversity, distribution and abundance of microorganisms in various environments (Fig. 7). The 16S rRNA sequences retrieved from gene libraries offer information about the identity of the microorganisms and the estimation of the genetic diversity of the sample. Although the diversity of retrieved sequences dose not reflects the real diversity in the sample mainly due to the biases introduced by PCR, the 16S rRNA sequences analysis is one of the most used method in studies of microbial diversity. In addition, phylogenetic analyses of the 16S rRNA sequences offer information about the possible metabolic capacities of these bacteria by comparing their 16S rRNA sequences with the rRNA sequences of cultured microorganisms in the data-base. The 16S rRNA sequences retrieved can be used for the selection or design of sequence- or group-specific oligonucleotide probes, that can be applied back in the environmental sample to reveal the structure of the microbial community. This later step is carried out by using fluorescence *in situ* hybridization, or the more sensitive Catalyzed Reporter Deposition (CARD)-FISH (Fig. 7). Also, the abundance and, to a certain extent, the physiological state of the microbial community can be quantified by hybridization of extracted nucleic acids (RNA) using similar sets of probes (slot-blot hybridization).

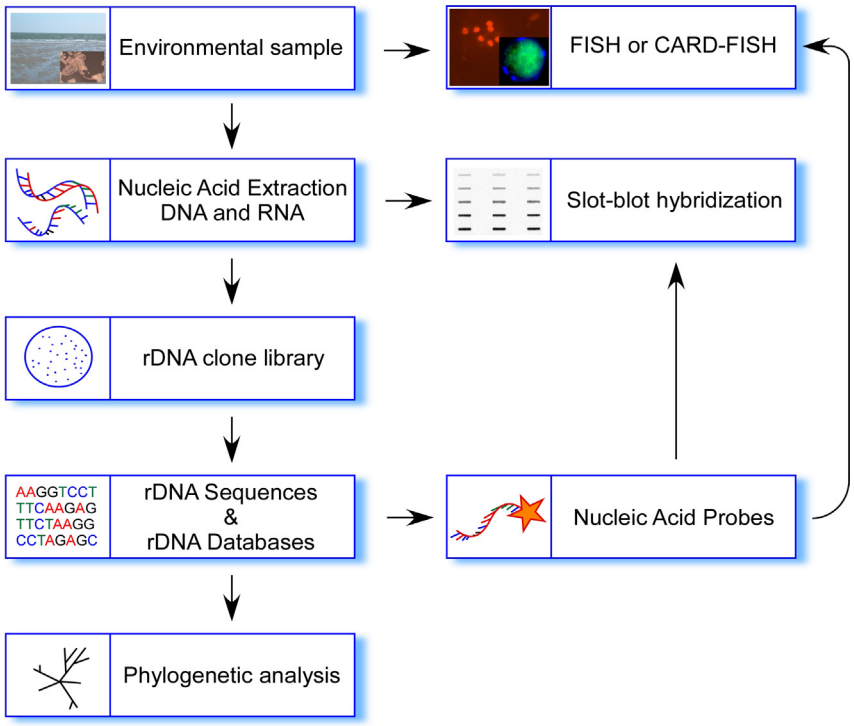

Fig. 7. The major steps of the rRNA approach used to study the diversity and structure of microbial communities or particular interactions between microbes and invertebrates such as the symbiosis of SOB and nematodes (modified from Amann *et al*., 1995).

Outline of the experimental work

1. The Wadden Sea is a costal area along the Dutch, German, and Danish North Sea coast. Most of the Wadden Sea is protected from the North Sea by barrier islands and is strongly influenced by tides, with about 50% of the sediments diurnally exposed during low tide (de Beer *et al*., 2005). The Wadden Sea represents the largest tidal flat worldwide and is of great importance for the interactions between the land and the sea (Grossart *et al*., 2004). Numerous biogeochemical and microbiological studies have been conducted on these tidal flats in the past years. The most studied sites were Jadebusen Bay (site Dangast) (Llobet-Brossa *et al*., 1998, 2002; Brinkhoff *et al*., 1998; Böttcher *et al*., 2000; Mussmann *et al*., 2005) situated in the costal area to the west of the Weser estuary, Königshafen Bay of the island Sylt (Huettel, 1990; Rusch & Huettel, 2000; Rusch *et al*., 2001), and the back barrier areas of the Frisian island of Spiekeroog (e.g. Janssand, Gröninger plate) (Freitag *et al*., 2003; Grossart *et al*., 2004; Ishii *et al*., 2004; Bühring *et al*., 2005).

An extensive investigation, involving the study of main microbiological process (primary production, aerobic and anaerobic carbon degradation) and transport phenomena corroborated with microsensor measurements and description and quantification of microbial community over a 2-year time period, was initiated in 1999 on an intertidal sandflat located in List (Island of Sylt). One of the main objectives of the present work focused on the microbial diversity and community structure of these sediments, as an integrate part of this comprehensive investigation. The description of the microbial community composition over depth (0–12 cm) and seasons (March, July, October) was performed by using two quantitative rRNA-based methods, fluorescence *in situ* hybridization (FISH) and slot blot hybridization. In addition, the bacterial phylogenetic diversity was obtained by comparative sequencing of the 16S rRNA genes. By combining two different quantitative methods – one based on single cell detection (FISH), the other on extraction of total rRNA, it has been intended to achieve a more complete description of the microbial community in these heterogeneous sands.

2. A rather new and interesting aspect of the Sylt intertidal sandflat is the presence of high abundances of symbiont-carrying nematodes. These nematodes were first reported in Sylt in 1991 by Dr. Olav Giere. Few years latter, an extensive study conducted by Riemann *et al*. (2003) revealed that all symbiont-carrying nematodes identified in these sediments belong to the genus *Leptonemella* (subfamily Stilbonematinae). Thus, three co-occurring species, *L. aphanothecae*, *L. vicina*, and *L. gorgo* were described to live preferentially in the vicinity of polychaete *Arenicola marina* burrows (Riemann *et al*., 2003). To date seven *Leptonemella* species are known from shallow sediments of the Adriatic Sea, North Sea and Atlantic Ocean (Ott & Schiemer, 1973, Ott *et al*., 1991; Hoschitz *et al*., 1999; Riemann

et al., 2003). Likewise, with other members of the subfamily Stilbonematinae, *Leptonemella* species carries chemoautotrophic sulfur-oxidizing bacterial symbionts on their cuticle. These symbionts cover almost the entire body of the worm and are arranged in a species-specific pattern (reviewed in Ott *et al*., 2004a, b) (table 1).

Of the three co-occurring species of the genus *Leptonemella*, *L. aphanothecae* and *L. vicina* are the most abundant inhabitants in Sylt sediments. These worms reach a maximum abundance of approximately 7 individuals ml^{-1} sediment at a sediment depth of 5–10 cm depth. Their distribution in the sediment is patchy, and was assumed to be correlated with the sulfide concentrations (patches of 100–150 μM sulfide were measured; Riemann *et al*., 2003). The symbiotic coat consists of a multilayer of coccoid bacteria embedded in a mucous matrix and forms a sheath around the nematode body excepting the head and the tail. The thickness of this symbiotic coat was estimated to be 5–10 μm on a *Leptonemella* with 25 μm body diameter (Riemann *et al*., 2003). Thus, it was calculated that a worm of 2000 μm length and 25 μm body diameter is inhabited by approximately 1.25 to 2.93 million symbiotic bacteria (Riemann *et al*., 2003).

The two dominant species, *L. aphanotecae* and *L. vicina* are very similar morphologically which makes their identification difficult (Riemann *et al*., 2003). Also, the morphology and arrangement of the bacterial symbionts are nearly identical. These aspects, together with the shared habitat, the loss of the symbionts through moulting, and the rapid acquisition of a new symbiotic coat make these nematode species ideal candidates to study the species-specific interactions between host and symbiont. Thus, a second objective of the present work was to determine whether the symbionts of these hosts are identical or whether each species carries its own species-specific symbionts. Trying to answer to this question, the whole 16S rRNA gene together with the 16S-23S rRNA internal transcribed spacer (ITS or ISR) of the symbionts of both species were amplified and compared. Fluorescence *in situ* hybridization with group- and species-specific oligonucleotide probes was intended to confirm that the sequences obtained belong to the symbiotic bacteria. To differentiate between the two host species, the 18S rRNA gene was amplified using specific primers, cloned and sequenced.

3. Besides the free living nematodes of the subfamily Stilbonematinae that harbour ectosymbionts, marine nematodes with endosymbiotic bacteria, apparently replacing their unfunctional gut, have been described (Ott *et al*., 1982; Austen *et al*., 1993; Giere *et al*., 1995;). These nematodes are mouthless and possess a gut rudiment and no anus. Based on microscopical observations (TEM) they were described as having most of the body cavity filled with large bacteria. Few species of mouthless nematodes belonging to the

genera *Astomonema*, *Parastomonema*, and *Rhaptothyreus* have been described to date from shallow and deep sea sediments.

Nematode worms of the genus *Astomonema* (family Siphonolaimidae) contain four species described from intertidal sandflats, mudflats, and methane-rich pock marks. The lack of feeding structures in all *Astomonema* species and observation of large bacterial cells filling nearly the entire body of the worm suggested the engagement of *Astomonema* spp. in symbiotic associations with these microorganisms (Ott *et al*., 1982; Giere *et al*., 1995). Moreover, the preferred occurrence of the nematode host in deeper sediment horizons, at the boundary between oxic and sulfidic layers, and the ultrastructural aspects of the bacterial cells (e.g. the presence of large intracitoplasmatic vesicles, presumably containing sulfur) pointed out to the thiotrophic nature of the symbionts (Ott *et al*., 1982, 2004b; Giere *et al*., 1995). Nevertheless, besides morphological description of the nematode species and their bacterial symbionts based on electron microscopy, there are no molecular data available that would permit an accurate identification and characterization of these microorganisms and their relationship with other bacterial symbionts.

The third objective of my work was to investigate the symbiotic association of *Astomonema* sp. from coral reef sediments in the Bahamas. The ultrastructure of the symbiosis was examined with electron microscopy. The bacterial symbiont and the nematode host were characterized using comparative 16S rRNA and 18S rRNA sequence analyses. Thus, the phylogenetic position of the symbiont and the host were determined. Fluorescence *in situ* hybridization with specific probes was applied to demonstrate that the 16S rRNA sequences obtained correspond to the endosymbionts.

4. The investigation of symbiotic associations between invertebrate hosts and microorganisms are principally based on the use of molecular methods. For a complete understanding of the mechanisms that drive these associations, an ideal situation will be to separate the host and the symbiont from each other and to study them independently. Up to date none of the bacterial symbionts could be separated from its particular host and maintained viable in enrichments or pure cultures.

The fourth objective of the present work was to cultivate the sulfate-reducing endosymbionts of the gutless oligochaete *Olavius algarvensis*. *O. algarvensis* is a small tubificid worm that lives in the Mediterranean in coarse-grained sediment at depths of 5–15 cm. This oligochaete harbours sulfur-oxidizing and sulfate-reducing symbionts just below the cuticle, between extensions of the epidermal cells (Dubilier *at al*., 2001). It has been showed that these symbionts are involved in a syntrophic sulfur-cycle in which oxidized and reduced sulfur compounds are recycled between the two symbionts (Dubiler *et al*., 2001). Phylogenetic analysis of the 16S rRNA sequence placed the sulfate-reducing symbiont

within a subgroup of free-living sulfate-reducing bacteria of the *Dessulfosarcina/Desulfococcus* group. The closest cultivable relative of this symbiont was *Desulfosarcina variabilis* with 93% sequence identity. Cultivation was attempted by using the agar dilution series method with a broad range of substrates combined with different reductants and complex nutrient mixtures (sediment, oyster and yeast extract).

Results and Discussions

1. Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea

Marine sediments play a major role in the global recycling of carbon and nutrients. The decomposition and recycling of organic matter in sediments is performed mainly by microorganisms. The identification and quantification of these microorganisms is of high importance since it provides information regarding the biochemical processes within the sediment and the metabolic abilities of the microbial community to degrade the organic matter that enters the sediment.

Despite the fact that sandy sediment cover 70% of costal zones, the study of microbial communities in these sediments was neglected for many years. The main reason was that sandy sediments have low organic carbon content and it was assumed that they have also low microbial activities. The study of microbial communities of sandy sediments became of significant importance once it has been showed that the rates of organic matter re-mineralization and oxygen uptake of these sediments are comparable with those of organic-rich muddy sediments and therefore are microbiologically highly active.

The present study had as a major objective the extensive description of the microbial community of a costal marine sandy sediment by combining two quantitative methods, fluorescence *in situ* hybridization (FISH) and slot-blot hybridization with group-specific rRNA-targeted oligonucleotide probes, together with comparative 16S rRNA sequence analysis. The sampling area was located on an intertidal sand flat near List, on the island Sylt, in the northern Wadden Sea. The sediment at the sampling site was mainly composed of coarse silicate sand with a grain size of 200–400 μm and a permeability of 1.5–7 × 10^{-11} m^2. The sediment colour was yellow from surface to 4 cm depth, gray below 4 cm, and changed to black below 10 cm indicating high sulfide concentrations in this region (for a detailed site description see de Beer *et al*., 2005). The environmental conditions

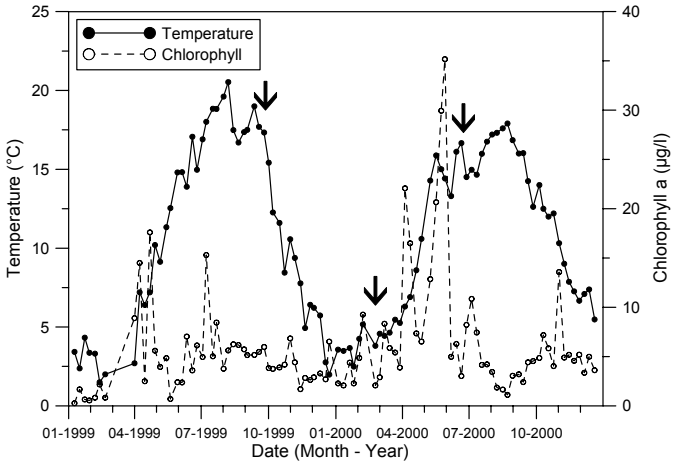

Fig.1. Seasonal variation of chlorophyll *a* concentrations and temperature in the water column in the main tidal channel near the sampling site. The values are weakly means. The sampling times are indicated by arrows.

in the water column at two nearby stations during the period of investigation are summarized in Fig 1. Temperature showed a clear seasonal cycle with maximum water temperatures of about 18–20 °C in August and minimum temperatures of about 1 °C in winter. Phytoplankton biomass (measured as chlorophyll *a*) showed a clear diatom-dominated spring bloom in April of 10–20 μg chlorophyll l^{-1}, sometimes followed by a second bloom of the Haptophycea *Phaeocystis globosa* (e.g. in May 2000). The sediment samples were collected at low tide in the middle of the flat, approximately 40 m in the offshore direction from the high water line on June 22, 1999, October 6, 1999, March 7, 2000, and July 5, 2000. Three parallel cores were collected per season from the upper sediment layers (0–12 cm), sub-sampled and preserved for molecular analyses (–80 °C for nucleic acid extractions and fixed in formaldehyde for FISH).

Comparative 16S rRNA sequence analysis

In order to have a first view into the bacterial diversity of Sylt sandy sediments two 16S rDNA clone libraries were constructed from the sediment cores taken in June 1999 and July 2000 using the bacterial-specific primers 8f (Hicks *et al*., 1992) and 1492r (Kane *et al*., 1993). The clone libraries were screened by amplified rDNA restriction analysis (ARDRA), divided into similarity groups and representatives of each group were sequenced and used for phylogenetic tree reconstruction. Using only full-length sequences (≥ 1300 bp) phylogenetic trees were constructed by performing neighbour-joining, maximum likelihood and maximum parsimony analysis. Topologies derived by these different approaches were compared with each other to construct a consensus tree. Partial sequences were then inserted into the reconstructed tree by applying parsimony criteria, without allowing changes in the overall topology.

Comparative 16S rRNA sequence analysis revealed a high phylogenetic diversity, with numerous cultivated as well as uncultivated bacterial lineages (Fig. 2). Sequence similarity to cultivated species was lower than 97%. In contrast, sequence similarity to other environmental 16S rRNA sequences was often high, up to 99%. Most sequences belonged to four bacterial groups: *Bacteroidetes* (formerly *Cytophaga/Flavobacterium/Bacteroides* phylum), *Planctomycetes*, *Deltaproteobacteria*, and *Gammaproteobacteria*. A high number of phylotypes (11) were detected within the *Bacteroidetes*. These phylotypes were widely distributed throughout the phylum, did not form specific clusters and the similarity to known species was lower than 95%. The *Planctomycetes* group was represented by 9 different phylotypes affiliated with *Pirellula* spp. A total number of 12 phylotypes were found within the *Deltaproteobacteria* group and formed separate clusters: 5 phylotypes were related with *Desulfosarcina/Desulfoccocus* species, 4 phylotypes were closely affiliated with the genera

Desulfocapsa and *Desulfobulbus*, and 3 phylotypes were not affiliated with any known deltaproteobacterial species but rather with so far uncultivated species.

Fig. 2. 16S rRNA-based phylogenetic reconstruction showing the affiliation of Sylt sequences (in bold) with selected reference sequences. Partial sequences were added to the existing tree without allowing changes in the overall tree topology. The scale bar represents 10% estimated sequence divergence.

Within the *Gammaproteobacteria,* the 10 phylotypes were affiliated with symbiotic and free-living sulfur-oxidizing bacteria. A few other sequences were affiliated with the *Holophaga-Acidobacteria* (2), *Verrucomicrobiales* (2), *Deinococcales* (2), *Actinobacteria* (1), *Deferribacterales* (1) and Candidate division OP3 (1). The libraries analysis was not intended to yield an in-depth description of the presumably high bacterial diversity but rather to assist in the selection of group-specific hybridization probes for further quantitative methods. The high phylogenetic diversity retrieved from the rather low number of 106 clones indicates that the bacterial diversity in Sylt sediments is high. A true diversity study would have required the screening of a larger number of clones. This is supported by the absence of representatives belonging to A*lpha-* and *Betaproteobacteria* from the clone libraries although their presence in Sylt sediments was showed by FISH and slot-blot hybridization (see below).

The high 16S rRNA diversity of bacteria detected in Sylt sandy sediments was comparable with that previously described in more detail for organic rich muddy sediments (e.g. Ravenschlag *et al*., 1999; Bowman *et al*., 2003; Bowman & McCuaig, 2003). The dominance of diverse representatives of *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, and *Planctomycetes* has also been described from muddy sediments. Such a high number of phylotypes within the *Bacteroidetes* and *Planctomycetes* clusters has not been observed previously in sandy sediments. In a previous study of phylogenetic diversity of bacteria from sandy sediments in Puget Sound (USA), sequences related to *Bacteroidetes* were not found and the number of phylotypes within the *Planctomycetes* was limited to two (Gray & Herwig, 1996). The contamination of the Puget Sound site with creosote may have contributed to the limited representation of the *Bacteroidetes* and *Planctomycetes* groups. The sandy sediments in Sylt are not strongly influenced by contaminants, and the high diversity observed in this study can be considered as a general characteristic for such environments. The affiliation of all *Planctomycetes* sequences with those of *Pirellula* spp. suggests an aerobic heterotrophic metabolism and involvement in the degradation of algal polymers (Glöckner *et al*., 2003; Schlesner *et al*., 2004).

Size, structure and activity of the microbial community

Community structure of bacteria in Sylt sandy sediments was investigated by FISH and slot-blot hybridization. FISH and total cell counts analysis was performed on two parallel sediment cores for each season. Sediment cores were sectioned in 1 cm layers, fixed, treated by sonication, and filtered. Filters containing microbial cells were hybridized, stained with DAPI and microscopically counted as described (Snaidr *et al*., 1997). For a list of the oligonucleotide probes and formamide concentrations at which these probes were hybridized see Manuscript 1. Slot-blot hybridization was performed on a single sediment

core per season. Each core was sliced in 1 cm thick layers. Total RNA was extracted from each layer by bead beating, phenol extraction, and isopropanol precipitation as described previously (Stahl *et al*., 1988; MacGregor *et al*., 1997). The RNA was blotted onto nylon membranes in triplicates and hybridized with ^{33}P-labelled oligonucleotide probes as described by Stahl *et al*. (1988). After hybridization, the membranes were washed at different temperatures depending on the dissociation temperature (Td) of each probe. The probes and the dissociation temperatures used are given in Manuscript 1. Hybridization signal intensity was quantified as described previously (Sahm *et al*., 1999).

Community structure

The contribution of *Bacteria*, *Archaea* and *Eukarya* to the overall microbial community was determined by slot-blot hybridization with probes specific for these domains. Bacterial rRNA (using the EUB338-I probe) accounted for up to 77% of total rRNA in March, 58% in July and 83% in October (Table 1). These percentages did not increase significantly after probing with EUB338-II (fully complementary to the 16S rRNA of some *Planctomycetes* species which are missed by probe EUB338-I) and EUB338-III (designed for *Verrucomicrobiales* species), as their rRNA accounted for only 0.5–1.8% of total rRNA (data not shown). Archaeal and eukaryotic rRNA amounts were also very low, with highest values at only 3% archaeal and 1% eukaryotic rRNA (Table 1). Total bacterial, archaeal, and eukaryotic rRNA (EUB338-I + EUB338-II + EUB338-III, ARCH915, and EUK1379) was never summing up to the total amount of rRNA as determined with the UNI1390 probe, despite the fact that the measurements were repeated several times. This might, at least in part, be a consequence of eukaryotic rRNA underestimation. We checked both probes EUK1379 and UNI1390 against the 18S rRNA sequences of several species of planktonic and benthic ciliates and flagellates which are known to be abundant in the North Sea (Berninger & Epstein, 1995; MURSYS; Wickham *et al*., 2000). Some of these species (e.g. *Myrionecta rubra*, *Chlamydodon excocellatus*, *Masodinium pulex, Cyclidium plouneouri, C. porcatum*) are targeted only by the universal probe but not by the eukaryotic probe. Since the detection of *Archaea* was also consistently low, we focused in more detail on the domain that clearly dominated the microbial community of the Sylt sandy marine sediment, *Bacteria*.

Microbial cell numbers and seasonal activity

Total microbial cell numbers was determined in two parallel cores for each sampling season (March, July and October) using DAPI staining. For all seasonal comparisons, it should be considered that the cell detachment protocol for the October 1999 sample was different to that of March and July 2000. Cell numbers were relatively high in all samples, ranging from

0.4–3.3 × 10^9 cells ml^{-1} sediment. Cell numbers were two-fold lower in March than in July and October, with up to 1.1 × 10^9 cells ml^{-1} sediment in March, and 2.0–3.3 × 10^9 cells ml^{-1} sediment in July and October. Single peaks in cell numbers always occurred in the first 6 cm, but overall there was no obvious decrease of total cell numbers with increasing depth. Little variation was observed between the two parallel cores from each season (Table 1), despite the fact that the distance between each core was as large as 10 cm.

The total microbial cell numbers determined for the Sylt sandy sediments were significantly higher than those previously reported for other sandy sediments which were often around 10^8 cells ml^{-1} (Wieringa *et al*., 2000; Rusch *et al*., 2001; 2003), and rather comparable with those reported for muddy sediments (1–5 × 10^9 cells ml^{-1}; Llobet-Brossa *et al*., 1998, 2002; Sahm *et al*., 1999; Ravenschlag *et al*., 2000; Mussmann *et al*., 2005). Initial experiments on Sylt sandy sediments clearly showed that total cell numbers strongly depend on the cell detachment protocol. Recently, elevated cell numbers, comparable with those obtained in the present study were also reported for another sandy intertidal flat in the Wadden Sea (Ishii *et al*., 2004). In contrast with muddy sediments where cell numbers were reported to decrease steeply with depth in top 5-10 cm from 3–5 × 10^9 cells ml^{-1} to about 1–2 × 10^9 cells ml^{-1} the sandy sediments investigated in this study did not show such a significant decrease with depth. This is probably a result of the higher permeability and the resulting high availability of both substrates and electron acceptors in deeper sediment layers (de Beer *et al*., 2005). An alternative explanation would be a continuous mixing of the upper sediment layer which is, however, not supported by other data from this study and that of de Beer *et al*. (2005).

The seasonal and vertical distribution of “prokaryotic rRNA”, defined as the sum of slot blot values obtained with probes EUB338-I–III + ARCH915, corresponded well with total prokaryotic cell numbers as determined by DAPI staining for the top 6 cm (Fig. 3, panels A & B). Total prokaryotic rRNA in March was at most half of the amount measured in July and October in the upper 6 cm. As with total prokaryotic cell numbers, the highest rRNA amounts were always in the top layer. Below 6 cm, there were no obvious seasonal differences in total prokaryotic rRNA. Average cellular rRNA contents were calculated by dividing the amount of “prokaryotic rRNA” by the DAPI counts. The winter values (March 2000) were not lower than the summer and fall values (Fig. 3, panel C). The values ranged quite strongly from 0.1 to 2.2 fg rRNA $cell^{-1}$, with peaks occurring at 2 and 8 cm sediment depth. Cell numbers and rRNA concentrations were generally higher in July and October than in March. This is expected and likely caused by the higher availability of degradable organic carbon in summer and fall (Fig. 1, also see estimates in de Beer *et al*., 2005).

Table 1. Quantification of different microbial groups using FISH (core A/B) [% of DAPI] and rRNA slot blot hybridization (core C) [% of total RNA].

Depth [cm]	Absolute prok. cell counts [10^9 ml^{-1}]			Total RNA (probe UNI1390)* [ng RNA ml^{-1}]			Bacteria (probe EUB338-I)						Archaea (probe ARCH915)			Eukarya (probe EUK1379)		
	OCT	MAR	JUL	OCT	MAR	JUL	OCT		MAR		JUL		OCT	MAR	JUL	OCT	MAR	JUL
							% DAPI	% RNA*	% DAPI	% RNA*	% DAPI	% RNA*	% RNA	% RNA	% RNA	% RNA	% RNA	% RNA
0.5	2.0/2.0[a]	0.6/0.6	2.0/1.3	1814	880	3288	35/34	83	63/56	47	83/79	36	1	1	1	1	1	1
1.5	1.3/1.4	0.5/0.6	1.4/1.1	3474	1478	4420	49/47	77	51/52	38	85/87	36	1	1	2	1	1	1
2.5	1.3/1.4	0.5/0.6	3.3/1.6	1594	1460	ND	42/38	48	65/45	26	65/65	ND	ND	1	ND	1	1	ND
3.5	1.6/1.6	0.6/1.0	1.6/1.1	1906	1318	ND	ND/33	63	55/57	35	81/72	ND	1	2	ND	1	1	ND
4.5	1.1/1.2	1.0/1.0	2.0/1.9	1898	1124	2264	36/33	71	51/46	56	70/76	57	1	1	2	1	1	1
5.5	1.4/1.4	1.1/0.9	1.8/1.7	1365	1145	2252	30/31	53	42/51	34	63/69	53	1	2	ND	0	1	1
6.5	1.1/1.1	0.7/0.5	ND/1.1	1654	1125	1924	31/33	50	61/56	77	ND/80	50	0	2	1	0	1	0
7.5	1.2/1.2	0.4/0.5	1.0/0.9	2087	1401	3291	30/24	53	63/49	66	62/58	55	1	3	1	0	1	0
8.5	1.2/1.2	0.4/0.6	1.2/1.2	1779	828	1847	ND/27	45	24/58	41	65/76	32	1	1	2	0	1	0
9.5	1.1/1.2	0.6/0.5	1.2/1.3	832	620	1034	32/22	43	14/57	37	74/66	38	1	ND	1	0	0	0
10.5	1.1/1.1	0.6/0.5	1.6/1.5	320	1211	1380	26/17	55	14/45	43	76/72	38	1	2	1	1	0	0
11.5	1.2/1.2	0.6/0.5	0.7/1.6	165	1193	2561	16/25	63	11/56	50	73/78	58	ND	2	1	1	0	0

Depth [cm]	*Alphaproteobacteria* (probe ALF968)						*Betaproteobacteria* (probe BET42a)					*Gammaproteobacteria* (probe GAM42a)					
	OCT		MAR		JUL		OCT		MAR		JUL	OCT		MAR		JUL	
	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA
0.5	5/4	15	11/8	8	11	10	0/1	0	3/2	1	1	2/4	18	10/12	8	7	13
1.5	3/4	15	8/15	15	7	12	0/1	0	8/1	1	1	2/4	17	15/9	8	5	16
2.5	5/4	ND	8/10	4	7	ND	ND/1	0	5/1	1	ND	2/4	13	10/9	6	3	ND
3.5	ND/3	12	7/4	7	10	ND	ND/0	0	2/2	1	ND	ND/3	15	5/10	8	4	ND
4.5	2/3	11	7/4	7	7	7	ND	0	1/1	1	1	1/2	13	5/8	8	4	16
5.5	1/2	11	7/2	6	4	ND	ND	0	1/1	0	1	0/2	11	4/10	9	7	21
6.5	2/1	10	5/4	5	3	7	ND	0	0/5	1	0	1/2	23	11/7	9	6	17
7.5	1/3	9	3/4	5	6	8	ND	0	1/3	1	0	0/2	13	10/6	10	9	18
8.5	ND/1	9	9/4	5	3	4	ND	0	1/2	0	0	ND/0	11	ND/9	9	5	17
9.5	1/1	12	8/2	ND	2	5	ND	0	2/3	0	0	0/ND	10	ND/6	8	4	15
10.5	0/0	8	8/3	5	4	6	ND	0	1/3	0	ND	ND	11	ND/8	10	2	18
11.5	0/0	9	5/3	11	4	7	ND	0	3/3	1	0	ND	9	ND/ND	12	3	11

Table 1. Continued

Depth [cm]	*Gammprot* .(probe GAM660) OCT % DAPI	MAR % DAPI	JUL % DAPI	*Desulfosarcinales* (probe DSS658) OCT % DAPI	OCT % RNA	MAR % DAPI	MAR % RNA	JUL % DAPI	JUL % RNA	*Desulfovibrio* (probe DSV698) OCT % DAPI	OCT % RNA	MAR % DAPI	MAR % RNA	JUL % DAPI	JUL % RNA
0.5	2/2	13/8	1	3/3	20	4/5	16	1	23	0/1	1	0/7	1	2	1
1.5	2/2	14/3	1	2/2	15	5/7	14	1	19	0/1	2	2/12	1	1	2
2.5	1/2	1/11	4	2/2	20	1/5	14	1	ND	0/1	1	ND/13	1	1	ND
3.5	ND/1	1/1	4	ND/3	17	1/5	16	0	ND	ND/1	2	ND/1	1	1	ND
4.5	1/1	2/2	1	3/4	17	2/6	17	0	20	1/1	1	ND/1	1	2	2
5.5	1/0	6/3	1	2/5	15	1/4	14	2	17	0/0	2	0/1	1	1	2
6.5	0/0	11/6	0	2/3	16	2/5	17	1	19	0/0	2	1/2	2	1	2
7.5	ND	5/3	1	3/3	11	3/6	14	2	17	0/1	2	1/3	2	0	2
8.5	ND	ND/2	ND	ND/4	18	1/10	20	4	15	ND/1	2	1/3	2	ND	2
9.5	ND	ND/4	ND	3/6	16	1/8	21	5	19	0/0	2	1/4	2	ND	2
10.5	ND	ND/4	ND	2/3	20	1/7	19	5	19	0/0	1	1/4	2	ND	2
11.5	ND	ND/4	ND	2/3	15	3/6	17	6	19	0/0	1	1/4	2	ND	2

Depth [cm]	*Desulforhopalus* (DSR651) OCT % DAPI	MAR % DAPI	*Planctomycetales* probe (PLA886) OCT % DAPI	OCT % RNA	MAR % DAPI	MAR % RNA	JUL % DAPI	JUL % RNA	*Cytophaga - Flavobacterium* (probe CF319a) OCT % DAPI	OCT % RNA	MAR % DAPI	MAR % RNA	JUL % DAPI	JUL % RNA
0.5	1/1	7/8	7/4	70	7/19	33	13	29	4/7	3	11/7	5	6	7
1.5	1/1	9/1	7/6	48	10/13	17	15	31	8/8	5	9/9	5	9	8
2.5	1/2	8/1	6/6	42	5/13	6	9	ND	9/11	5	9/2	3	2	ND
3.5	ND/2	1/2	ND/6	39	4/6	12	11	ND	ND/7	4	6/5	3	2	ND
4.5	1/1	1/0	6/5	27	9/6	24	10	31	4/5	4	5/4	2	2	6
5.5	1/2	1/1	5/5	20	17/4	16	9	27	4/6	2	8/3	2	2	5
6.5	1/1	2/3	7/4	20	19/6	26	9	28	2/4	2	7/3	2	2	5
7.5	1/0	3/2	5/5	27	18/4	30	13	18	3/2	2	3/3	2	1	6
8.5	ND/1	2/3	ND/7	31	6/7	16	9	8	ND/2	2	4/3	2	1	4
9.5	1/1	1/2	3/5	18	7/5	10	5	26	2/6	2	2/1	2	1	4
10.5	1/1	2/1	3/3	37	8/5	18	7	30	6/0	2	6/1	2	1	5
11.5	0/1	1/1	4/5	46	8/4	23	8	43	4/0	2	2/2	2	1	4

[a] core A value/core B value

ND: Not determined

* From de Beer *et al*. [11]

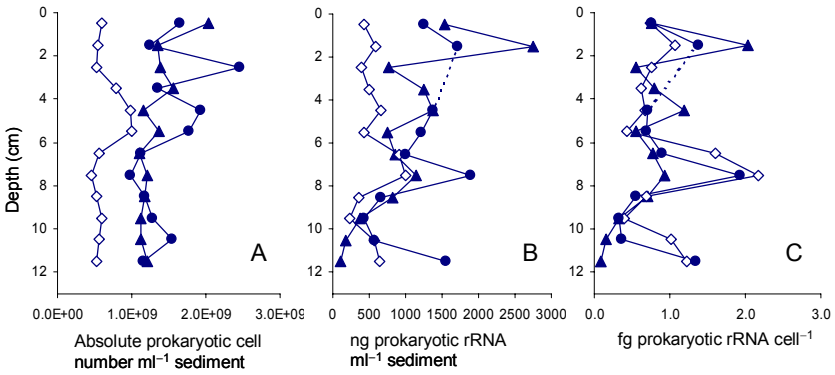

Fig. 3. Vertical profiles of total cell counts (panel A, mean of two parallel cores), prokaryotic rRNA recovery (panel B), and prokaryotic cellular rRNA content (panel C) in sandy Sylt sediments. The samples were collected in October 1999 (▲), March 2000 (◇) and July 2000 (●).

During summer, the main source of organic carbon was from phytoplankton. Just before the July 2000 sampling, there was a strong phytoplankton bloom as indicated by an elevated chlorophyll *a* concentration (Fig. 1). In October 1999 the temperature was still high but the phytoplankton abundance was much lower (Fig. 1). At that time, the abundant benthic diatoms were most likely the main source of organic carbon. The March 2000 sampling is characteristic for an end-of-winter situation. It should be noted that, although both the abundance of prokaryotic cells as well as rRNA concentrations were lower in winter, the average cellular rRNA content calculated from these two parameters remained similar. It seems that over the winter months the overall size of the microbial community is reduced rather than the average cellular ribosome content. However, the strong variability of average cellular ribosome contents (0.1–2.2 fg rRNA cell^{-1}) over depth suggests that only limited information is provided by averaging for total prokaryotic rRNA. It is possible that different bacterial populations with different cellular rRNA contents dominate during different seasons.

Quantification of the bacterial groups

Planctomycetes

Planctomycetes is an abundant group in marine systems, inhabiting the water column and the sediment. In marine sediments members of the *Planctomycetes* are present in the upper layers (Rusch *et al*., 2003; Ishii *et al*., 2004) as well as in the anoxic regions (Ravenschlag *et al*., 2001) often found attached to the sand grains or in association with phytodetrital macroaggregates (“marine snow”, DeLong *et al*., 1993; Ravenschlag *et al*., 2001). In Sylt sandy sediments *Planctomycetes* was the most abundant bacterial group based on slot blot hybridization (Table 1 and Fig. 4, panel A). Specific cell counts with probe PLA886 ranged between 1.5×10^7 and 1.8×10^8 cells ml^{-1} (3–19% of total cells). They were highest in the upper 8 cm. A summer peak of *Planctomycetes* was observed with FISH over the full length of the core, whereas slot blot hybridization values of probe PLA886 were higher only in the upper 6 cm of the sediment in summer and fall. Particularly at the sediment surface very high slot blot values were measured with PLA886 (up to 70% of total

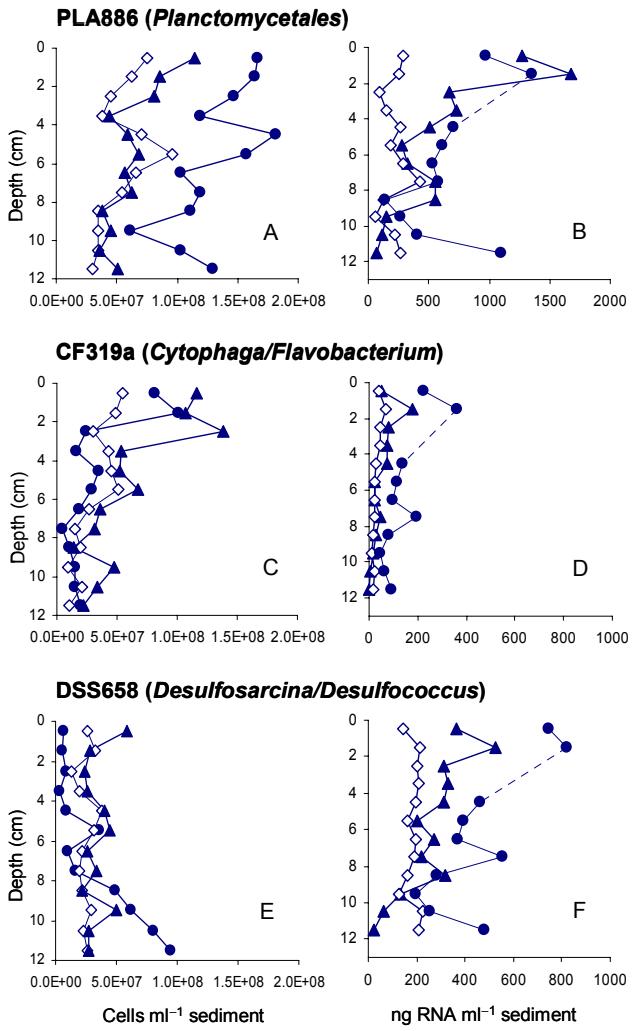

Fig. 4. Vertical profiles of bacterial groups in Sylt sediments as detected by FISH (A, C, E) and rRNA slot-blot hybridization (B, D, F) in October 1999 (▲), March 2000 (◇) and July 2000 (●). Note the different horizontal scales.

rRNA). These extremely high values and their inconsistence with the seasonal variations of the *Planctomycetes* cells determined by FISH were most likely caused by cross-hybridization of PLA886 to the 18S rRNA of eukaryotes (e.g. ciliates and unicellular algae) that also occur in coastal marine sediments. It was already known at the time of its design that PLA886 could also bind to the 18S rRNA of diatoms, in addition to that of some fungi and protists (Neef *et al*., 1998; Ishii *et al*., 2004). We checked the probes PLA886 and EUK1379 against the 18S rRNA sequences of abundant species of benthic and planktonic diatoms, ciliates, and flagellates in the North Sea (Berninger *et al*., 1995; Wickham *et al*., 2000). Some of them (e.g. *Cyclidium porcatum, Peridinium* sp., *Chlamydodon excocellatus, Zoothamnium* sp., *Uronema* sp.) were found to be targeted only by the PLA886 but not by the EUK1379, leading us to the assumption that the *Planctomycetes* rRNA might be overestimated. The slot blot values measured with PLA886 should therefore not be used to quantify their abundance. FISH counts are less biased since the morphology of *Planctomycetes* is quite specific. The cells detected by PLA886 were usually large cocci of different sizes (up to 1 μm diameter), occurring as single cells or in disordered clusters (Fig. 5, panels A & B). This morphology is significantly different from that of ciliates and unicellular algae so that we can exclude the possibility of false-positive FISH signals from these members of the Wadden Sea ecosystem. Reliable FISH quantification with PLA886 (which was originally designed for this purpose) is, however, possible because of clear microscopic differentiation between prokaryotes and eukaryotes based on cell size and organization.

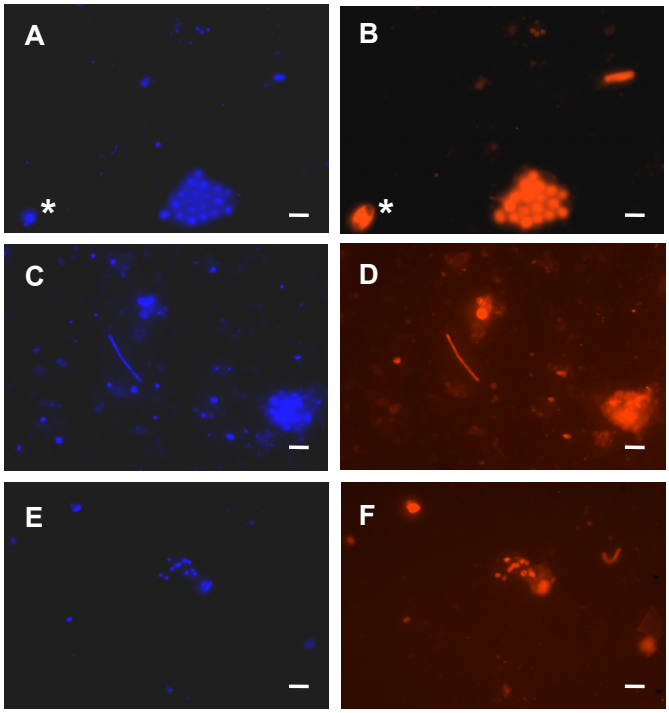

Fig. 5. Epifluorescence micrographs of bacteria in sediment samples from Sylt. Left panels show DAPI staining (blue) and right panels the corresponding FISH signal with the different probes (red). (A-B) Probe PLA886 (C-D) probe CF319a and (E-F) probe DSS658. The scale bar is equivalent to 5 µm.
* Diatom cell, not counted.

The high abundance of *Planctomycetes* in the summer core, where-except for one depth layer-this group made up more than 1.0 × 10^8 cells ml^{-1}, is correlating with the higher abundance of phytoplankton in July. Thus, we postulate that *Planctomycetes*, especially the *Pirellula/Blastopirellula/Rhodopirellula* group (Schlesner *et al*., 2004), play an important role in the main microbial process occurring in the upper layers of sandy sediments, the aerobic heterotrophic mineralization of algal biomass (de Beer *et al*., 2005). This hypothesis is well sustained by the genome analysis of *Rhodopirellula baltica*, a coastal isolate of *Planctomycetes*, which revealed the presence of a high number of sulfatases involved in the degradation of polysaccharidic algal cell wall components (Glöckner *et al*., 2003).

Cytophaga/Flavobacterium

High abundance of members of the *Cytophaga/Flavobacterium* (CF) group of the *Bacteroidetes* was first reported from marine sediments by Llobet-Brossa *et al*. (1998). Since then, CF have often been found to be abundant in oxic and anoxic regions of marine sediments (e.g. Ravenschlag *et al*., 2001; Rusch *et al*., 2003; Ishii *et al*., 2004; Bühring *et al*., 2005), but also in coastal marine bacterioplankton (e.g. Cottrell *et al*., 2000; Eilers *et al*., 2000, 2001). Similar to *Planctomycetes*, many members of this group are specialized in the degradation of complex macromolecules. Even though they occur in enormous numbers in habitats rich in organic material, especially those involved in the aerobic or anaerobic degradation of algae blooms (Rossello-Mora *et al*., 1999; Riemann *et al*. 2000; Fandino *et al*., 2001), some species can adapt to low nutrient levels as well (Höfle 1982, 1983).

In the present study probe CF319a (Manz *et al*., 1992) was used to quantify members of the *Cytophaga/Flavobacterium* group (CF) of the phylum *Bacteroidetes*. These were abundant in the Sylt sand flat, but only at the sediment surface, based on both slot-blot and FISH (Table 1 and Fig. 4, panels C & D). The CF were most abundant in the top 2 cm, with

cell numbers ranging between 1.2×10^6–1.8×10^8 cells ml^{-1} (1–11% of total cells). Most of the cells identified within this cluster were morphologically diverse and included long and short rods (0.5 to 1.5 μm in length) and short filaments (Fig. 5, panels C & D). Up to 8% of total rRNA was detected by slot blot hybridization with probe CF319a. Seasonal differences were observed also in the upper 2 cm. Corresponding to the general trend, cell numbers and rRNA values were lower in March than in July and October (Fig. 2A, B). For this probe, the two quantification methods agreed well, supporting a relative contribution of CF319a-positive cells to the microbial community of sandy sediments of 1–10%. The higher abundance in surface layers was also reported in previous studies on sandy sediments (Llobet-Brossa *et al*., 1998; Ishii *et al*., 2004). It seems that certain populations of the *Cytophaga/Flavobacterium* group dwell in this mostly fully aerobic zone receiving relatively high amounts of organic matter from the overlaying water column (de Beer *et al*., 2005). Probing with CF319a showed also a relatively constant depth distribution down to 12 cm, supporting a recent study on 40-cm cores (Ishii *et al*., 2004). The relatively constant CF abundance below the maximum might be due to the presence of aerobic species sustained by occasional oxygen penetration to depths up to 8 cm based on water flow or to even higher depth based on bioturbation (de Beer *et al*., 2005). Alternatively, the presence of anaerobic, fermentative members of the *Cytophaga/Flavobacterium* group in deeper sediment layers is also a possible explanation (Rossello-Mora *et al*., 1999; Ravenschlag *et al*., 2001). The *Bacteroidetes* 16S rRNA sequences have a much lower similarity to each other than, e.g., to those of the *Planctomycetes*, supporting the assumption of different metabolic functions of specific CF populations in sandy sediments.

Deltaproteobacteria

Previous studies on marine sediments have shown that SRB constitute an important part of the microbial community (Sahm *et al*., 1999; Ravenschlag *et al*., 2000; Wieringa *et al*., 2000; Llobet-Brossa *et al*., 2002; Bowman *et al*., 2003). Within the polyphyletic group of sulfate-reducing bacteria, the deltaproteobacterial *Desulfosarcina*/*Desulfococcus* group was described as the most abundant in muddy (e.g. Ravenschlag *et al*., 2000; Llobet-Brossa *et al*., 2002; Mussmann *et al*., 2005) and sandy sediments (Ishii *et al*., 2004; Bühring *et al*., 2005). The present study confirms these findings. Of the three probes used to detect deltaproteobacterial SRB, the probe for the *Desulfosarcina*/*Desulfococcus* group (DSS658) detected the highest cell numbers (3.2×10^6 – 9.5×10^7 cells ml^{-1}; 0.4–10% of DAPI) and rRNA concentrations (11–23% of total rRNA). Using FISH, probe DSS658 detected up to 81% of all cells hybridizing with a set of deltaproteobacterial probes and using slot-blot hybridization, up to 96% of the rRNA was detected with the same probe set. This corresponds well with our phylogenetic analyses which showed a cluster of *Desulfosarcina*-

related sequences in the Sylt clone library. *Desulfosarcina/Desulfococcus* was abundant in all sediment layers examined. As with all other bacterial groups, the rRNA concentrations were lower in March than in July and October, but this trend was not observed in the FISH data. The rRNA fractions detected were generally higher than relative cell abundance (Table 1 and Fig. 4, panels E & F). In terms of morphology, the cells identified were highly diverse and included long (12 μm) and short (8 μm) filaments and single rods (2–4 μm), but mostly rod-shaped cells of approximately 2 μm in diameter which occurred in disordered clusters or as sarcina-like packets (Fig. 5, panels E & F).

Interestingly, both the cell numbers and the rRNA values detected with DSS658 remained rather high in all seasons and depths. Although the rRNA values were highest in July (23%) when sulfate reduction rates were highest (de Beer *et al*., 2005), the *Desulfosarcina/Desulfococcus* group was also abundant at times when only low sulfate reduction rates were recorded in parallel cores. Parallel investigations in Sylt sediments (de Beer *et al*., 2005) have shown that sulfate reductions rates range quite widely from 0.08 to 13.7 mmol m^{-2} d^{-1}, whereas aerobic respiration rates are more stable and higher (105 to 175 mmol m^{-2} d^{-1}). Thus, the maximum of DSS658 rRNA in the upper part of the sediments contributes to the discussion whether and how SRB are able to use sulfate as an electron acceptor in oxic zones (e.g. Cypionka *et al*., 1985; Dannenberg *et al*., 1992; Bühring *et al*., 2005; Mussmann *et al*., 2005). It should be noted that during low tide the complete sediment can turn anoxic, creating temporarily optimal conditions for activity and growth of SRB which would subsequently just survive oxic phases. One might also speculate that alternative electron acceptors like Fe (III) or even oxygen are used by members of the *Desulfosarcina/Desulfococcus* group. However, the alternative of using oxygen as electron acceptor seems highly unlikely since *Desulfosarcina* spp. and *Desulfococcus* spp. are known as strict anaerobes (Rabus *et al*., 2004). Moreover, no member of this group has been reported so far that can use organic compounds in the presence of oxygen. In contrast to *Desulfosarcina* spp. and *Desulfococcus* spp., other SRB such as *Desulfovibrio* spp., which were found generally in low abundances in Sylt sandy sediment and some strains of the genera *Desulfobulbus* and *Desulfobacterium* could respire organic compounds (e.g. formate, acetate, or propionate) at low oxygen concentrations or fully oxic conditions (Dilling & Cypionka, 1990; Dannenberg *et al*., 1992; Cypionka 2000).

Alphaproteobacteria* and *Gammaproteobacteria

Probe ALF968 was used to estimate the abundance of *Alphaproteobacteria* which is an abundant group of marine bacteria. It is important to realize that this probe not only targets members of the *Alphaproteobacteria* but also a variety of *Deltaproteobacteria* including various sulfate-reducing bacteria as well as members of the genera *Pelobacter*, *Geobacter*

and *Desulfuromonas*. Thus, false positives can not be excluded. Cell numbers and rRNA concentrations quantified with ALF968 were comparable to those obtained with CF319a (Table 1). The highest abundance was observed within the upper 6 cm of the sediment, with cell numbers ranging between 2.4 x 10^6–1.4 x 10^8 cells ml^{-1} and rRNA values up to 15% of total rRNA. Slight seasonal differences were observed only in the first 6 cm, while the values below this depth were rather constant at the three time points examined. FISH detected lower cell numbers in March and October than in July while slot blot hybridization showed the lowest values in March. The cell morphotypes detected were short and long rods. Rods with a length of approximately 4 μm were dominant in all samples.

Comparative sequence analysis of the 16S rDNA libraries indicated an important role of members of the subphylum *Gammaproteobacteria*. GAM42a-positive populations represented an important fraction of the microbial community in Sylt sediments based on both slot blot hybridization and FISH (Table 1). The cell numbers ranged between 2.2 $\times 10^6$ – 1.2 $\times$ 10^8 cells ml^{-1} and the specific rRNA concentration was as high as 23% of total rRNA. Seasonal differences were observed in rRNA concentrations, with again, lower values in the March samples. Some of the gammaproteobacterial sequences were related to bacteria potentially able to oxidize sulfide. Five of ten sequences (Sylt6, Sylt29, Sylt39, Sylt45, Sylt49) match probe GAM660 designed to quantify a group affiliated with free-living and endosymbiotic sulfur-oxidizing bacteria (Ravenschlag *et al*., 2001). In addition, GAM660 also targets closely related sequences retrieved from other marine sediments as well as the endosymbionts of *Riftia pachyptila*, other vestimentiferan tubeworms and several bivalves. FISH counts (Table 1) were sometimes quite high (up to 14%), however, highly inconsistent within replicates and between the three seasonal samples. Since slot blot hybridization failed to detect GAM660-positive rRNA this study does not provide conclusive information about the abundance of this group in the Sylt sediments.

Due to the incomplete coverage of the target group and the high number of false-positive out-group hits recorded with the probes ALF968 and GAM42a, a detailed discussion of the quantitative hybridization results obtained with these two probes would be unreliable and therefore is avoided. It is very evident that due to the rapid and continuous extension of the rRNA databases the number of out dated probes will increase in the near future. Only larger sets of probes targeting smaller groups e.g the *Roseobacter* clade within the *Alphaproteobacteria* or specific subgroups within *Gammaproteobacteria* or *Bacteroidetes* will solve this problem and will offer a more complete description of the microbial community.

In conclusion, this study showed that the microbial community structure of Sylt sediments in the top 12 cm was relatively stable over depth, most probably due to deep substrate and oxygen penetration caused by advection and bioturbation. Seasonal

differences of total cell numbers and rRNA concentrations correlated well with the availability of organic carbon and temperature. The high abundance (5–15% of total prokaryotic cells) of the presumably aerobic heterotrophic members of the phylum *Planctomycetes* is in good agreement with the high rates of aerobic degradation recorded by de Beer *et al*. (2005). The relative importance of SRB for organic carbon mineralization in Sylt sediments may be reduced by the high input of oxygen (de Beer *et al*., 2005). However, this needs to be further investigated due to the high abundance of members of the *Desulfosarcina/Desulfococcus* group. In the sandy sediments in Sylt, *Planctomycetes* seem to be even more abundant than members of the *Cytophaga/Flavobacterium* group which might have a similar function. High values for *Planctomycetes* and the CFB group have been reported for two other oxygenated sandy sediments (Bühring *et al*., 2005; Rusch *et al*., 2003) suggesting that this dominance might be a common characteristic feature for this type of ecosystem. Future molecular and cultivation studies should attempt to further characterize these two important groups of marine benthic bacteria.

2. Molecular characterization of chemoautotrophic sulfur-oxidizing symbionts in co-occurring closely related nematode species of the genus *Leptonemella*

Sylt sandy sediments were recently described to harbour a very rich population of symbiont-carrying nematodes belonging to the genera *Leptonemella* (subfamily Stilbonematinae) (Riemann *et al*., 2003). Two closely related species, *L. aphanotecae* and *L. vicina,* were the most abundant inhabitants of these sediments occurring in dense populations (up to 7 individuals ml^{-1} sand) near polychaete's *Arenicola marina* burrows (Riemann *et al*., 2003). These nematodes carry a multilayered coat of coccoid ectosymbiotic bacteria, firmly attached to their body surface (Riemann *et al*., 2003; Ott *et al*., 2004 a, b) (see Introduction, Fig. 3). The bacterial coat is normally removed together with the old cuticle during moulting, a phenomenon that takes place four times during their development into adults. Interestingly, *Leptonemella* adults or juveniles without symbiotic coat were never observed in Sylt sediments (Riemann *et al*., 2003). Although it is not yet clear how the symbionts are acquired it was hypothesized that the worms may gain them from the environment after hatching or moulting through a specific recognition mechanism based on lectine-sugar interactions (Ott *et al*., 2004b; Nussbaumer *et al*., 2004). Considering that the symbionts might be acquired from the environment and that the closely related nematode hosts *Leptonemella aphanothecae* and *L. vicina* co-occur in Sylt sandy sediments, it is of high scientific interest to determine if these 2 species carry identical symbionts or each harbours its own, species-specific symbiont. Such investigation would provide further information

concerning the influence of the shared biogeographical conditions on the establishment and evolution of symbiotic associations.

In order to answer this question numerous *Leptonemella* specimens were collected from Sylt intertidal sandflat ('Hausstrand' 55°5'N/8°26'E) in October 2003 and March 2004. Sediment cores of 5 cm diameter and 15 cm depth were collected from the vicinity of polychaete burrows during low tide. *Leptonemella* specimens were extracted from the sediment by washing, decanting and sieving. They were collected under a dissection microscope, washed in filter-sterilized sea water and preserved in 70% ethanol at 4 °C for FISH and DNA extraction. Total DNA was extracted as previously described (Schizas *et al*., 1997) from eight worms individually and further used for molecular characterization of the nematode hosts and of the ectosymbionts. Fluorescence *in situ* hybridization with general eubacterial (EUB338) and group specific (GAM42a) probes was applied on worm cross-sections in order to confirm that the sequences obtained were from the symbionts.

Molecular characterization of the nematode host

L. vicina and *L. aphanothecae* described in Sylt sediments are very similar morphologically, making their identification and differentiation problematic. Furthermore, these worms tend to coil in compact curls, obscuring anatomical details that are important for species differentiation (Riemann *et al*., 2003). Identification of these two nematodes species based on their morphology was attempted, but the results were not always in accordance with the 18S rRNA sequencing. Nevertheless, the strict identification of the hosts as *L. vicina* or *L. aphanothecae* was not relevant for the purpose of the present work, and therefore they were further differentiated as species A and B based only on 18S rRNA gene sequencing. A 1200 bp fragment of the 18S rRNA gene was amplified with specifically designed primers, cloned and sequenced. For each individual, 5 to 10 sequences were obtained and analysed using the ARB software (Ludwig *et al*., 2004). Comparison of these sequences revealed two different 18S rRNA phylotypes sharing 96–96.3% sequence identity, which corresponded to the host species A (4 individuals) and species B (4 individuals). The sequence identity within each host group was ≥ 99.8% for species A and ≥ 99.6% for species B. Phylogenetic analysis revealed that *Leptonemella* sp. A and *Leptonemella* sp. B were most closely related with each other and with an undescribed *Leptonemella* sp. collected from shallow sediments in Adriatic Sea (95% sequence identity). These nematodes formed, together with other symbiont-carrying nematodes of the subfamily Stilbonematinae (e.g. *Laxus*, *Robbea* or *Catanema* species) a distinct clade within the phylum Nematoda (Fig. 6). These results are in good agreement with previous phylogenetic analyses based on both 18S rRNA gene sequences and morphological characters (e.g. unique glandular sense organs) showing that

all marine nematodes with sulfur-oxidizing chemoautotrophic ectosymbionts belong to a monophyletic group classified as the subfamily Stilbonematinae (Bauer-Nebelsick *et al*., 1995; Kampfer *et al*., 1998; Ott *et al*., 2004b).

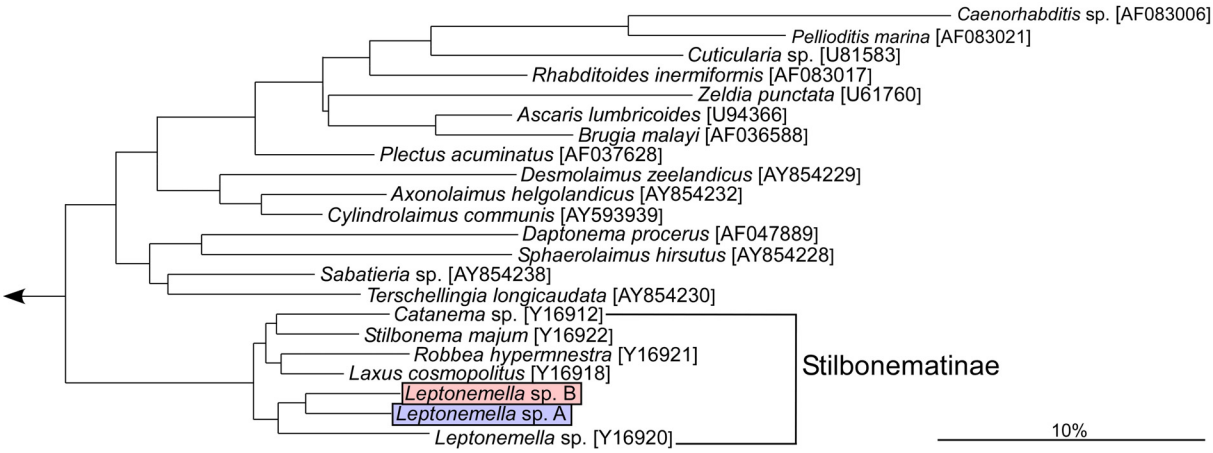

Fig. 6. 18S rRNA-based phylogenetic reconstruction showing the affiliation of the *Leptonemella* sp. A (blue frame) and *Leptonemella* sp. B (pink frame) with selected reference sequences. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Molecular characterization of the bacterial symbionts

A first approach for characterization of the symbiont was amplification, cloning and sequencing of the 16S rRNA gene. Sequences obtained from symbionts of both host species were nearly identical, (99.5% sequence identity) with only minor nucleotide differences within almost complete 16S rRNA sequences (1496 bp) (Table 2). These minor differences were not consistent within each host group and did not form a specific pattern that could differentiate between symbionts of the two host species. Phylogenetic analyses showed that all symbiont sequences of *Leptonemella* sp. were clustering together within the *Gammaproteobacteria* and formed a monophyletic clade with sequences of endosymbionts of gutless oligochaetes (*Olavius* sp., *Inanidrilus* sp.) and ectosymbionts of the gut-bearing nematode *Laxus* sp. (Fig. 7).

18S rRNA	16S rRNA nucleotide position									
	223	432	460	578	1010	1012	1031	1240	1250	1468
A-L33	C	C	C	T	G	G	T	G	A	A
A-L18	C	C	T	T	G	T	T	G	G	G
A-L11	C	T	C	T	G	G	T	G	A	A
A-L42	C	T	C	T	G	G	T	G	A	A
B-L34	T	C	T	T	G	T	T	G	A	G
B-L45	T	C	T	T	G	T	T	G	A	G
B-L6	T	C	T	T	G	G	T	G	A	G
B-L7	T	C	T	C	A	G	A	A	A	A

Table 2. Sequence differences at the level of 16S rRNA gene between symbionts of *Leptonemella* sp. A and B.

Their closest relatives were the Gamma 1 symbiont of *Olavius algarvensis* and the ectosymbiont of *Laxus* sp. (96–98% sequence identity). These sequences of symbiotic bacteria are most closely related to a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family *Chromatiaceae* (91–93% sequence identity). Previous phylogenetic studies on bacterial symbionts of gutless oligochaetes have shown that there is a constant association within the *Gammaproteobacteria* of these symbionts and the ectosymbionts of gut bearing nematodes (Dubilier *et al*., 1999, 2001; Blazejak *et al*., 2005). A plausible explanation for this close evolutionary association is the co-occurrence in similar geographic locations and environments of the two hosts groups which could influence the establishment of these symbioses (Dubilier *et al*., 2005). The affiliation of *Leptonemella* sp. symbionts to the oligochaete-nematode symbionts clade further supports the above hypothesis.

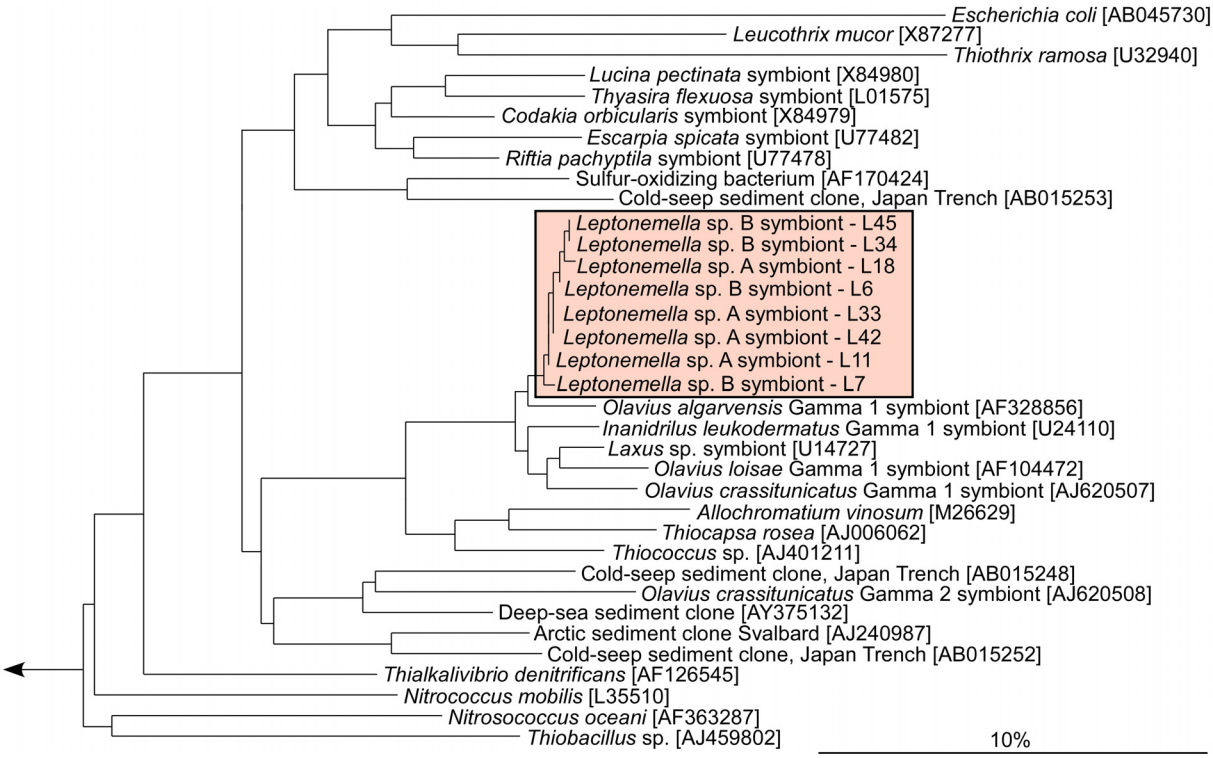

Fig. 7. 16S rRNA-based phylogenetic reconstruction showing the affiliation of the *Leptonemella* species A and B symbionts sequences (pink frame) with selected reference sequences from the *Gammaproteobacteria*. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Comparison of the 16S rRNA sequences showed that the symbionts of the two *Leptonemella* species are very closely related and cannot be distinguished based on analysis of the 16S rRNA gene alone (Table 2). The 16S rRNA gene offers adequate resolution for comparisons from the species level up to kingdom level, but is often not divergent enough to give a good separation in close relationships such as species of the same genus or among conspecific strains (Stackebrandt & Goebel, 1994; Normand *et al*., 1996). For resolution at or below the species level, other more variable sequences, such as

the internal transcribed spacer (ITS) located between the 16S and 23 rRNA genes, are analysed as a supplement to 16S rRNA sequencing (Jensen *et al*., 1993; Kong *et al*., 1999; Lee *et al*., 2002; Osorio *et al*., 2005). The ITS shows a high degree of variability between closely-related species and even between strains of the same species, both in length and as sequence information (Gürtler & Stanisich, 1996). The size of the spacer may vary considerably for different species, and even among the different operons within a single strain (Condon *et al*., 1995). Most bacterial species harbour multiple copies (alleles) of the ribosomal operon in their genome, feature which increases the sequence variation of the spacer regions. The variation in length is typically due to the type and number of the tRNA genes contained.

Since analysis of the 16S rRNA gene was clearly not sufficient to distinguish between symbionts of the two *Leptonemella* species, fragments encompassing, the almost complete 16S rRNA gene, the 16S-23S ITS and a small fragment (189 bp) of the 23S rRNA gene were amplified from the individuals previously investigated. The primers used were 8f (Hicks *et al*., 1992) and L189r (Yu & Mohn, 2001). The 16S rRNA gene was re-amplified together with the ITS to provide a correlation of the nucleotide differences observed in the 16S rRNA with the corresponding ITS sequences. Approximately 10 clones for each worm were sequenced and compared. The sequences from each individual worm were identical. All ITS fragments obtained had approximately the same length (472 to 475 bp) and contained two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. These tRNA genes showed 100% identity in all individuals investigated. Sequence identity between the ITS fragments of species A was 97–99%, while the sequence identities shared between ITS fragments of the species B was 95.5–100%. The sequence identities between the two species were within the same range (95.3–99.6% sequence identity). In total, within 8 individuals investigated, 7 different ITS fragments were obtained. Only one ITS phylotype was shared by two individuals, L45 and L34, belonging to *Leptonemella* sp. B (Fig. 8). These two individuals had also identical 16S rRNA sequences. It seems that there is a strong correlation between the 16S rRNA nucleotide differences and the ITS phylotypes. As an example the 16S rRNA sequence of the individual A-18, which had the greatest similarity with the 16S rRNA sequences of individuals B-45 and B-34 (only one nucleotide different; Table 2), was also highly similar at the level of ITS (only 4 nucleotide different; data not shown). Despite the little variation between ITS phylotypes, a species-specific pattern was not observed (Fig.8).

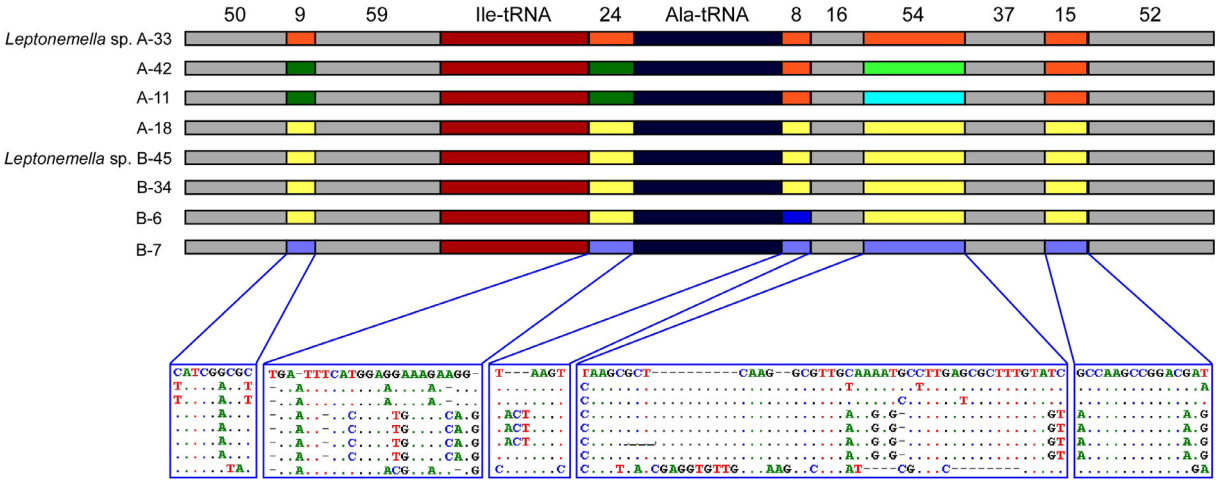

Fig. 8. Mosaic-like structure of ISR sequences of the symbionts of Leptonemella species A and B. The grey colour represents sequence blocks that are identical in ISR of all symbionts investigated. Other colours indicate sequence blocks that varied between different individuals. The sequence information of the variable regions is shown as alignment. Nucleotide positions that are conserved are indicated by dots. Gaps that have been included to obtain optimal sequence alignment are indicated by dashes. Identical fragments between sequences are denoted by the same colour. Two tRNA genes (for Ile and Ala) were found in all symbiont's ISR sequences. The numbers above columns represent sequence length in bp.

In other studies, ITS sequence analyses clearly showed significant differences between subspecies of the same species. Thus, from two subspecies of *Photobacterium damselae* (*P. damselae* subsp. *piscida* and *P. damselae* subsp. *damselae*) that have identical 16S rRNA genes but are morphologically distinguishable, a number of 17 different ITS variants have been amplified. These varied in length from 296 to 803 bp and contained different tRNA genes (Osorio *et al*., 2005). The result obtained with symbionts of the two *Leptonemella* species is more close to the situation encountered between strains of the same species (Kong *et al*., 1999). In such a case, comparison of ITS fragments from two strains of *Aeromonas hydrophila* showed sequence identity of 95 to 99% and the presence of identical $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (Kong *et al*., 1999).

In this context, the results obtained in the present study strongly suggest that the symbionts of the two *Leptonemella* species are at most sub-species, if not strains of the same species. Obviously, this mix of symbiont strains co-occurring in Sylt sediments is able to colonize both hosts, indicating very similar surface-specific recognition mechanisms. A similar result was obtained by Won *et al*. (2003) in an investigation by using ITS sequencing to differentiate between SOB endosymbionts of two vent mussels, *Bathymodiolus putioserpentis* and *B. azoricus,* from Mid-Atlantic Ridge. The symbionts of these mussel species shared the same 16S ribotype and species-specific ITS patterns at sites were the host species were not mixed. At sites where the hosts were mixed, this species-specific ITS pattern was not found anymore, and both *Bathymodiolus* species carried a mix of symbiont strains. Another possibility is that the symbionts of the two Leptonemella species are indeed species-specific, but cannot be distinguished by using ITS analysis. To verify this

hypothesis, a larger number of *Leptonemella* individuals from both species, A and B, have to be investigated. The analysis should be expanded by including other genes such as 23S rRNA, protein-coding genes *aprA* or RubisCO or more neutral gene such as housekeeping genes. Also, higher resolution methods such as multi locus sequence analysis (MLSA) or the use of repetitive extragenic palindromes as priming sites to generate diagnostic banding patterns (REP-PCR) could be applied to differentiate between these symbionts (Gilson *et al*., 1987; Versalovic *et al*., 1991).

In situ detection of the symbionts

Catalyzed reporter deposition (CARD)-FISH with general eubacterial (EUB338) and group specific (GAM42a) probes was applied on *Leptonemella*-cross sections (Fig. 9). Ten individuals were fixed (see above), embedded in 3% agar, dehydrated and embedded in paraffin. Cross-sections of 6 μm thickness were re-hydrated, permeabilized and hybridized as previously described (Penthaler *et al*., 2002). As a negative control, NON338 probe was used (Fig. 9). This result confirmed that the symbionts were affiliated with the *Gammaproteobacteria*. However, the confirmation that the 16S rRNA sequences and the corresponding ITS fragments analysed originated from the symbionts would be provided by CARD-FISH with symbiont specific probes.

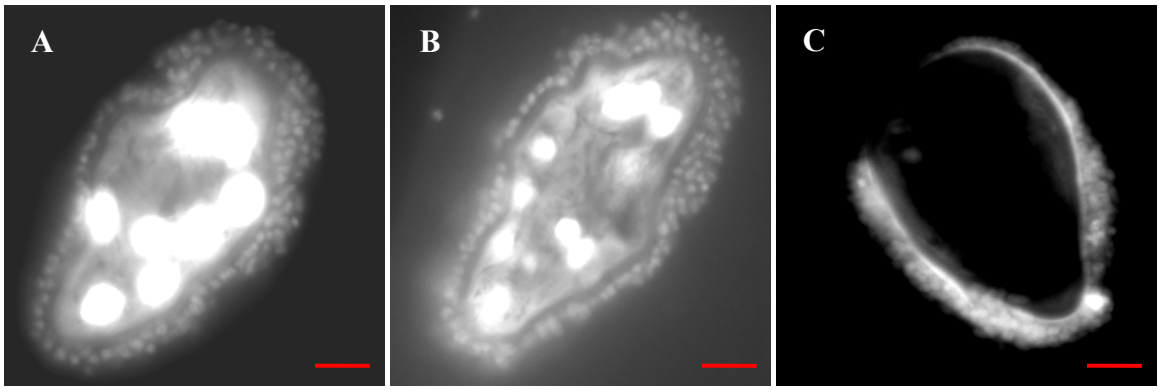

Fig. 9. In situ detection of symbionts of *Leptonemella* sp. FISH images showing cross-sections through the worm with DAPI staining of host nuclei (white-shiny). (A) Hybridization with the general bacterial probe (EUB338); (B) Hybridization with the group-specific probe GAM42a; (C) Hybridization with the negative control probe (NON338). Scale bars are equivalent to 10 μm.

3. Molecular and morphological characterization of the symbiosis between bacterial endosymbionts and the nematode *Astomonema* sp. from the Bahamas

Mouthless nematodes of the genus *Astomonema* were described to harbour endosymbiotic bacteria within their body (Ott *et al*., 2004b). The reduction of the digestive system (e.g. lack of mouth, unfunctional gut) of *Astomonema* species, and the observation of large bacterial cells filing nearly the whole body of the worm, suggested that the bacterial symbionts provide their hosts with nutrition (Ott *et al*., 1982; Giere *et al*., 1995). Also, the preferred occurrence of these nematodes in sulfidic sediments and the presence of numerous vesicles in the bacterial cytoplasm that are assumed to contain sulfur indicate that these symbionts are sulfur-oxidizers. Up to date the symbionts of mouthless nematodes have not been characterized using molecular methods so that nothing is presently known about their identity and relationship with other bacterial symbionts.

The present work had focused on the mouthless nematode *Astomonema* sp., an undescribed species from coral reef sediments in the Bahamas. The worms have been collected from subtidal sediments off Lee Stocking Island during low tide in 1999 and 2004. The sediment at the sampling site was coarse, calcareous, with small amounts of detritus and a yellow-pinkish color. The average water depth was 2 m at low tide. The number of *Astomonema* sp. individuals was found to increase with increasing sediment depth as follows: 1.7×10^3 worms m^{-3} (at 0 to 5 cm depth), 3.4×10^4 m^{-3} (5 to 10 cm), 1.8×10^5 m^{-3} (10 to 15 cm) and 2.9×10^5 m^{-3} at 15 to 20 cm depth. The specimens varied in size from 1 to 4 mm long and 15 to 30 μm wide (Fig. 10) and they had a shiny white to pinkish colour which disappeared during fixation. The *Astomonema* specimens were extracted for the sediment by decantation and sieving, and collected under a dissecting microscope. After collection, the worms were fixed in 70% ethanol and stored at 4°C for DNA analyses and for FISH, and in Trump's fixative (McDowell and Trump, 1976) for TEM.

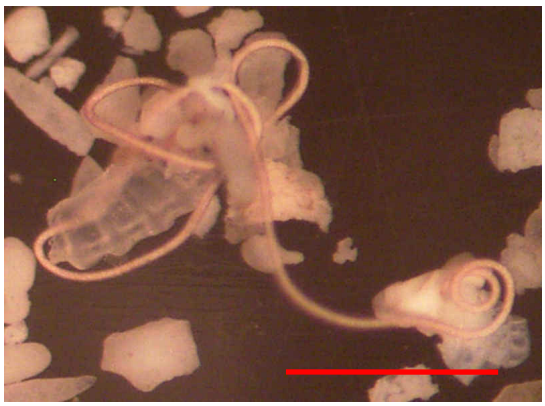

Fig. 10. Photomicrograph of *Astomonema* sp. from Bahamas coral reef sediments. Scale bar = 0.5 mm.

Morphological characterization of the symbionts

The morphology of the endosymbionts was investigated by transmission electron microscopy (TEM). TEM on cross sections through *Astomonema* sp. (Fig. 11A) specimens revealed very large extracellular bacterial cells of a length of about 8-10 μm and a width of 4–5 μm, completely filling the central lumen of the worm (Fig. 11B). The bacteria were surrounded by a layer of long and irregular host cells (intestinal wall cells). This layer was delimited by muscular cells, the epidermis and the cuticle. Thus, the bacteria are extracellular endosymbionts, living in the gut lumen of the worm. A similar extracellular location of the bacterial symbionts was described for *Astomonema southwardorum* from pockmarks in the North Sea (Giere *et al*., 1995).

The bacterial cytoplasm was densely filled with globular greyish and white vacuoles (Fig. 11B). The grey globules appeared membrane-bound, but details of the membrane structure could not be ascertained due to suboptimal fixation. The content of the white vacuoles was presumably dissolved during the fixation procedure. The ultrastucture of the mouthless nematode symbionts was similar to that of the sulfur-oxidizing Gamma 1 symbionts of gutless marine oligochaetes (Dubilier *et al*., 2005 and Bright & Giere 2005). In the oligochaete Gamma 1 symbionts, the bacterial cytoplasm is filled with sulfur and poly-β-hydroxyalkanoate vesicles.

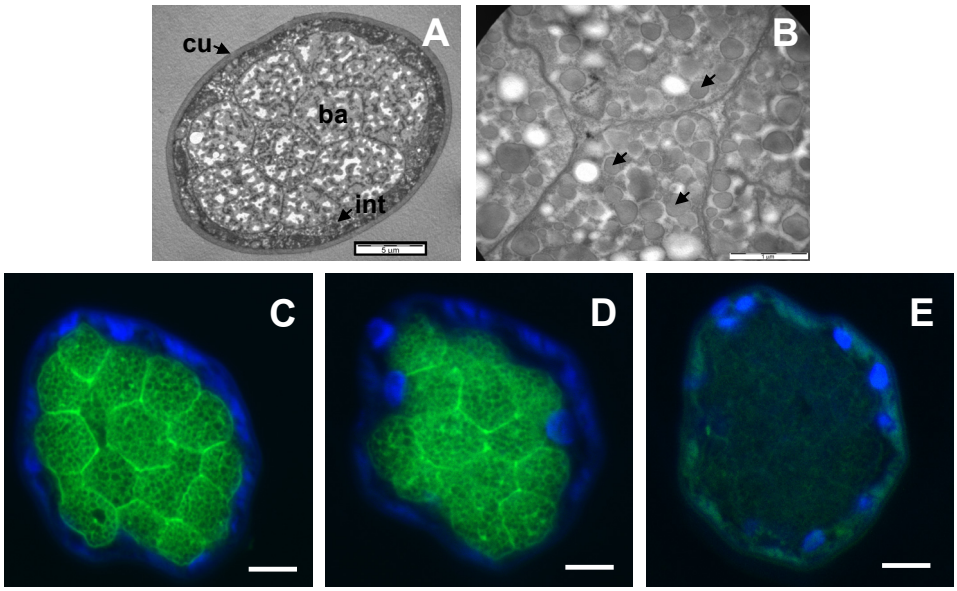

Fig. 11. Transmission electron micrograph (A, B) and FISH identification (C, D, E) of the endosymbionts of *Astomonema* sp. (A) The bacterial symbionts (ba) fill almost the entire body of the worm and are surrounded by intestinal cells (int). The cuticle (cu) is very thick. (B) Higher magnification of the symbionts showing numerous globular vacuoles in the bacterial cytoplasm (arrows). (C, D, E) FISH images showing consecutive cross-sections through the symbiont-containing region of a worm with DAPI staining of host nuclei in the body wall shown in blue. (C) Hybridization with the general bacterial probe (EUB338) with the symbionts in green; (D) Hybridization with the symbiont-specific probe A179 showing the bacterial symbionts in green; (E) Hybridization with the negative control probe (NON338). Scale bars are equivalent to 5 μm (A, C, D, E) and 1 μm (B).

Molecular characterization of the symbionts

16S rRNA gene

In order to identify and characterize the symbionts, several *Astomonema* sp. individuals were investigated by amplification, cloning and sequencing of the 16S rRNA gene (Table 3). For each nematode host a 16S rRNA clone library was constructed using the bacterial-specific primers 8f (Hicks *et al*., 1992) and 1492r (Kane *et al*., 1993). The clone libraries were screened by partial sequencing of 48 to 86 randomly chosen clones. Comparison of the partial sequences showed that each clone library was dominated by a single clone family belonging to the *Gammaproteobacteria* (Table 3). Representatives of each clone family were chosen for complete sequencing of the 16S rRNA gene. Thus, a total of 38 nearly complete 16S rRNA sequences (1496 bp) were obtained. Sequence identity within each clone family was always high, of ≥ 99.6% for partial sequences and 99.8% for full sequences.

Worm individual	1	2	3	4	5	6	7
Gamma symbiont	62 (9)	56 (8)	47 (6)	85 (6)	64 (4)	54 (2)	62 (3)
Others	1	3	1	1	5	0	1
Total no. of clones sequenced	63	59	48	86	69	54	63

Table 3. 16S rRNA clone libraries from 7 *Astomonema* sp. individuals. The numbers of fully sequenced 16S rRNA clones are shown in parentheses.

Parsimony, distance, and maximum-likelihood analyses (Fig. 12) revealed that the dominant gammaproteobacterial 16S rRNA sequence from *Astomonema* sp. consistently grouped with the Gamma 1 sulfur-oxidizing endosymbionts of gutless oligochaetes such as

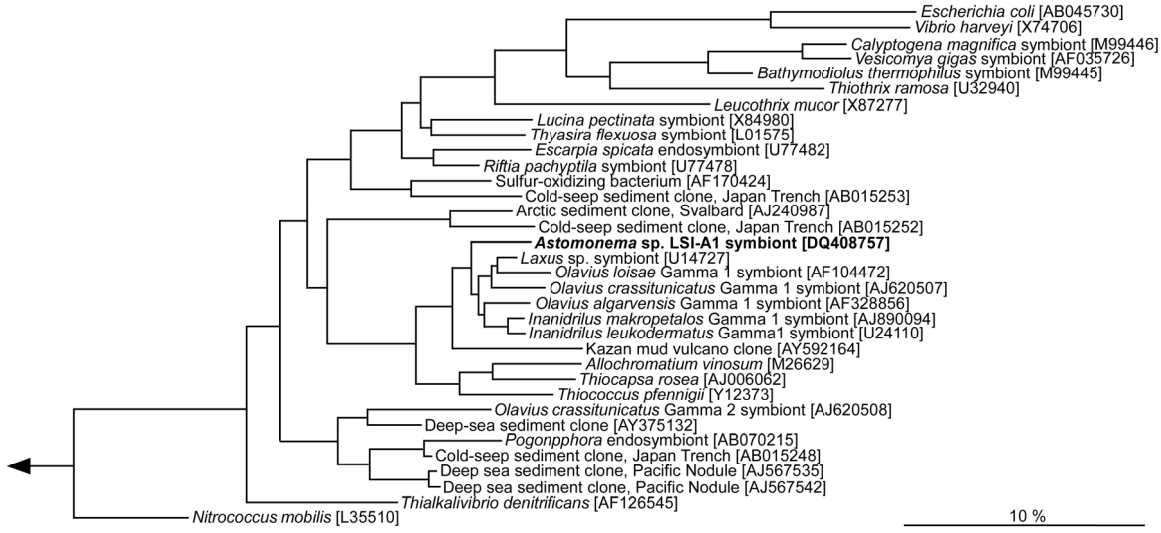

Fig. 12. 16S rRNA-based phylogenetic reconstruction showing the affiliation of the *Astomonema* sp. symbiont sequence (printed in bold) with selected reference sequences from the *Gammaproteobacteria*. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Olavius algarvensis and *Inanidrilus leukodermatus* (95.7–95.9% sequence identity), and the sulfur-oxidizing ectosymbiont of the gut-bearing nematode *Laxus* sp. (96.4% sequence identity). These sequences from symbiotic bacteria together with a clone sequence isolated from the Kazan mud volcano in the eastern Mediterranean have as closest cultivable relatives (90–92% sequence identity) a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family *Chromatiaceae*. The close association, in which the endosymbionts of *Astomonema* sp., endosymbionts of gutless oligochaetes and ectosymbionts of gut bearing nematodes form a monophyletic clade was intriguing considering the different integration levels of the endo- and ectosymbioses, and the significant phylogenetic distance between these host groups. Considering all these aspects it is very clear that these associations were not established through co-speciation but rather independently of each other in convergent evolution. It is highly probable that the co-occurrence of these host groups in shallow reduced sediments exposed them to a similar pool of environmental microbes and led to the uptake of very closely related bacteria as symbionts. The extracellular position of the symbionts in all these host groups is a common characteristic that supports this assumption. Despite the monophyly of the symbionts of nematodes and oligochaetes, their level of integration and location in or on the worm is different within each host group: the *Astomonema* sp. endosymbiots occur in the gut lumen, the oligochaete endosymbiots underneath the cuticle, and the stilbonematinae nematode ectosymbionts on the cuticle. This suggests a remarkable flexibility of the ancestors of these symbionts to establish associations with different invertebrate groups and to develop variable modes of ecto- and endosymbiotic lifestyles.

Fluorescence *in situ* hybridization of the symbiont

In order to establish if the dominant sequences retrieved by clone libraries indeed belong to the endosymbionts, catalyzed reporter deposition (CARD)-FISH with group- (EUB338, GAM42a) and sequence-specific (A179) oligonucleotide probes was applied on cross sections of the worm. The sections, obtained from worms fixed in ethanol and double embedded in agar and paraffin, were permeabilized and hybridized with the above-mentioned probes labeled with horse radish peroxidase (HRP) (for a detailed description see Manuscript 2). The FISH results obtained with the probe A179 confirmed that the dominant sequences originated from endosymbiotic bacteria in the worm (Fig. 11D). The bacterial cells as revealed by hybridization were consistent in shape, size, and distribution with the morphology and structure observed with TEM (Fig. 11A, B). The FISH signals from A179 probe and the general Bacteria probe EUB338 (Fig. 11C) were similar, indicating that no additional bacteria are present in these worms. This corresponds well with the TEM analyses showing only a single bacterial morphotype. The fluorescent signal in all

hybridizations was localized mostly at the edge of the cells suggesting a concentration of ribosomes in this area (Fig. 11C, D). This particular localization of the ribosomes could be caused by the presence of the numerous globular vacuoles which occupy most of the cytoplasm volume, as demonstrated by TEM (Fig. 11 A, B). The apparent angular shape of the cells in FISH images is most likely an artifact created during sample fixation and was observed previously (Giere *et al*., 1995). Positive FISH signals were also obtained with the general gammaproteobacterial probe GAM42a (data not shown), whereas the negative control probe NON338 showed no signal (Fig. 11E).

Adenosine-5'-phosphosulfate (APS) reductase gene

The close phylogenetic association of the *Astomonema* sp. endosymbionts with symbiotic and free-living sulfur-oxidizing bacteria (SOB) and the ultrastructural similarities between these endosymbionts and SOB symbionts of gutless-oligochaetes suggests that the *Astomonema* sp endosymbionts might have similar metabolic abilities (Fig. 12, Dubilier *et al*., 2005; Bright & Giere, 2005). In order to verify this hypothesis, i.e. if the endosymbionts of *Astomonema* sp. have the ability to oxidize reduced sulfur compounds, amplification and sequence analysis of the adenosine-5'-phosphosulfate (APS) reductase gene was attempted. APS reductase is a key enzyme involved in both dissimilatory sulfate reduction and dissimilatory sulfur oxidation pathways of the sulfur metabolism. This enzyme is present in sulfate-reducing bacteria, where it catalyzes the reduction of APS to sulfite, and in sulfur-oxidizing microorganisms where is responsible for the reverse reaction (Rabus *et al*., 2004; Hipp *et al*., 1997; Sanchez *et al*., 2001; Friedrich, 2002). APS reductase has two subunits, alpha and beta, which form a 1:1 $\alpha\beta$ heterodimer. These two subunits are encoded by the *aprA* and *aprB* genes, respectively. The *aprA* gene was proposed as a useful phylogenetic marker for bacteria involved in both oxidative and reductive sulfur metabolism (Hipp *et al*., 1997).

Using specifically designed primers (Kuever, unpublished) the *aprA* gene from two *Astomonema* sp. specimens was amplified, cloned and sequenced. The deduced amino acid sequences, comprising 131 amino acids, were identical between the *Astomonema* sp. individuals and most closely related with the *aprA* of the Gamma 1 SOB symbionts of gutless oligochaetes *Inanidrilus leukodermatus* and *I. makropetalus* (98% sequence identity). The closest cultivated relatives were the sulfur-oxidizers *Thiobacillus denitrificans* (*Betaproteobacteria* 87% sequence identity) and *Chlorobium tepidum* (green sulfur bacteria 71% sequence identity) (Fig. 13). The presence of *aprA* and its close phylogenetic affiliation with *aprA* of free-living and symbiotic SOB further sustain the thiotrophic nature of the *Astomonema* sp. endosymbionts. However, a direct proof that the *aprA* sequences obtained by PCR amplification belong indeed to the symbiont is still lacking. This could be

demonstrated for example by mRNA FISH targeting the transcripts of the *aprA* gene. Alternatively, the sulfur-oxidizing nature of the symbionts could be demonstrated by incubation of living worms in the presence of ^{35}S-sulfide, followed by autoradiography on cross-sections.

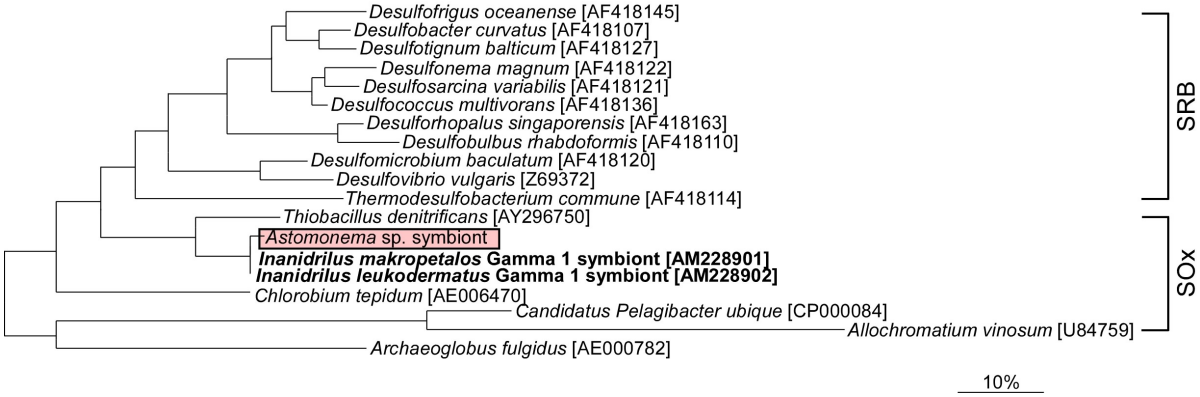

Fig. 13. Phylogenetic placement of APS genes (*aprA*) from *Astomonema* sp. symbiont (pink highlight) based on deduced aminacid sequences. Clone sequences from symbionts are in bold face type. Sulfate-reducing bacteria (SRB), sulfur-oxidizing bacteria (SOx). The phylogenetic tree is based on maximum likelihood analysis. The bar represents 10% estimated sequence divergence.

Molecular characterization of the nematode host

Based exclusively on morphological characteristics, (e.g. length and shape of the body, size of the nerve ring, absence of the somatic setae), genus *Astomonema* was placed within the family Siphonolaimidae of the class Adenophorea (currently Chromadorea), phylum Nematoda (Ott *et al*. 1982). Later descriptions and careful reexaminations of new *Astomonema* species such as *A. otti*, *A. obscura* (Vidakovic & Boucher, 1987), and *A. southwardorum* (Austen *et al*., 1993; Giere *et al*., 1995) emphasized a number of differences between these species, thereby questioning the taxonomic coherence of this group and its present position within the Siphonolaimidae (Ott *et al*., 2004b).

Although 18S rRNA sequence information has been recently applied in combination with morphological characters to refine the phylogeny and taxonomy of the phylum Nematoda (Blaxter *et al*., 1998; De Ley & Blaxter, 2002), such information is currently lacking for members of the genus *Astomonema*. Therefore, the *Astomonema* species investigated in this study was characterized by sequencing of the 18S rRNA gene followed by phylogenetic analysis. Using primers specific for the domain *Eukarya* 1f and 2023r (Pradillon *et al*., 2006) the 18S rRNA gene was amplified, cloned and sequenced. The sequences obtained (1783 bp) were aligned to the ARB database and used for phylogenetic reconstructions, using for comparison available sequences of symbionts-carrying and free-living nematodes. Comparison of the 18S rRNA sequences showed 99.6 and 100% sequence identity between the *Astomonema* sp. individuals, indicating that all specimens

belonged to the same species. Phylogenetic analyses corroborated that *Astomonema* sp. is a member of the class Chromadorea of the phylum Nematoda (Fig. 14). Its closest relatives

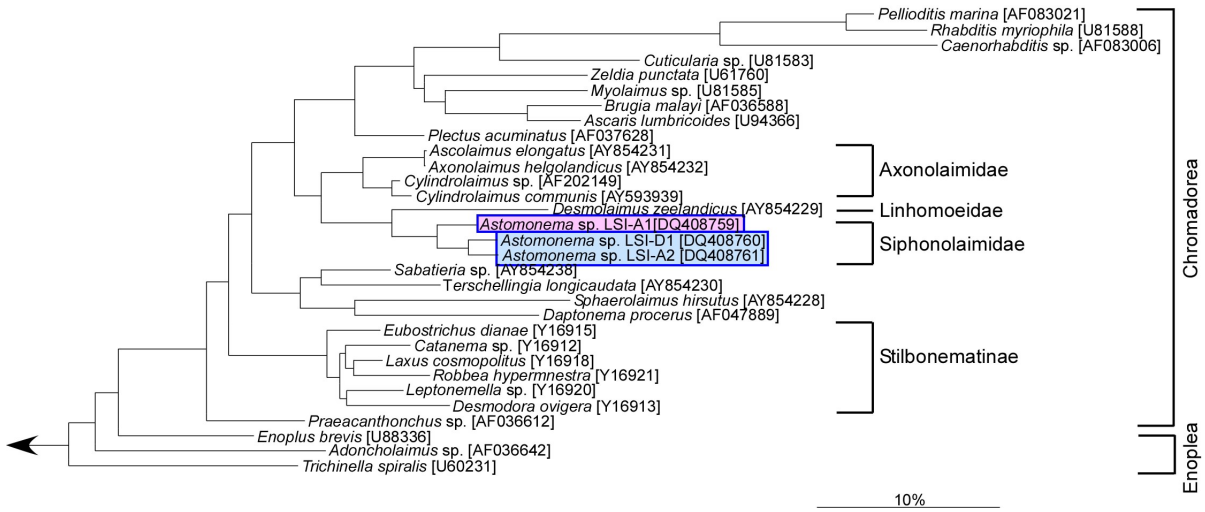

Fig. 14. 18S rRNA-based phylogenetic reconstruction showing the affiliation of the *Astomonema* sp. sequence (pink highlight) with selected reference sequences. Other yet uncharacterized *Astomonema* sp. found in coral reef sediments in the Bahamas are highlighted in blue. The phylogenetic tree is based on maximum likelihood analysis. The scale bar represents 10% estimated sequence divergence.

were free living non-symbiotic marine nematodes of the family Axonolaimidae (*Axonolaimus helgolandicus*, *Cylindrolaimus* spp. and *Ascolaimus elongates*) and Linhomoeidae (*Desmolaimus zeelandicus*) with 89.2–89.5% sequence identity (Fig. 14). Nematodes of the family Stilbonematinae that are characterized by associations with ectosymbiotic bacteria (Ott *et al*., 2004a, b) were not closely related to *Astomonema* sp. (Fig. 14). The close relationship of the *Astomonema* sp. from this study to members of the families Axonolaimidae and Linhomoeidae corresponds well with morphological analysis that describe these families as the closest relatives of the Siphonolaimidae (Lorentzen 1981, 1994; Decraemer *et al*., 2003; De Ley & Blaxter, 2002). This suggests that at least the *Astomonema* sp. from this study belongs to the family Siphonolaimidae. An intriguing question for further studies is if all nematode species with endosymbiotic bacteria such as *A. otti*, *A. obscura* (Vidakovic and Boucher, 1987), and *A. southwardorum* (Austen *et al*., 1993; Giere *et al*., 1995) form a coherent taxonomic group.

Phylogenetic diversity of mouthless nematodes in coral reef sediments

Molecular analysis of a larger number of *Astomonema* sp. individuals from the same sampling area revealed the presence of two other distinct host phylotypes (Fig. 14). Parsimony, distance, and maximum-likelihood analyses showed that these phylotypes were clustered in a monophyletic clade. The sequence identity between the two new phylotypes was 97%, and they showed 94–95% sequence identity with the first described *Astomonema*

sp. phylotype. The closest relatives of this clade of mouthless nematodes were members of the families Axonolaimidae and Linhomoeidae (Fig. 14). This result showed that mixed populations of closely related nematodes of the genus *Astomonema* are present in the coral reef subtidal sediments off Lee Stocking Island. In order to correlate these 18S rRNA gene data with morphological description of the host, numerous other *Astomonema* sp. specimens from the same sampling area are currently investigated at the University of Vienna by Prof. Dr. Jörg Ott. Also, it will be interesting to compare the results regarding the phylogeny and taxonomy of these nematode hosts with 16S rRNA sequences obtained from their bacterial symbionts. This study will expand our knowledge on (a) the phylogeny of free-living nematodes, the most abundant metazoans on Earth, (b) the host-symbiont relationship with respect to the establishment and evolution of these symbiotic associations and (c) the diversity and phylogenetic relationships of SOB symbionts of marine nematodes with symbionts of other marine invertebrates.

4. Cultivation attempts of sulfate-reducing symbionts of *Olavius* spp. from shallow sediments in Elba

Gutless oligochaetes of the genus *Olavius* are known as hosts able to establish associations with multiple symbiont species (Dubilier *et al*., 2001; 2005). The bacterial symbionts occur extracellularly, in a thick layer just below the cuticle of the worms between extensions of the epidermal cells. Molecular investigations have revealed the presence within a single host species of sulfur-oxidizing (SOB) and sulfate-reducing (SRB) symbionts. It has been showed that the SOB and the SRB symbionts are involved in a syntrophic sulfur cycle within the host: the sulfate-reducing symbionts oxidize inorganic or organic compounds, producing sulfide which is in turn oxidized by the sulfur-oxidizing symbiont (Dubilier *et al*., 2001). There is evidence that the SOB symbionts are autotrophic, deriving the carbon needed for synthesis of cell constituents from fixation of CO_2. Nevertheless, the source of carbon and reductant for the SRB partner is presently unknown. Several possibilities can be envisioned concerning the source of substrates for the SRB symbiont: (a) transfer from the autotrophic SOB symbiont; (b) diffusion from the environment, as the worms cuticle has large pores that allow the transport of molecules as large as 70 kDa or (c) transfer of organic compounds resulting from the metabolism of the host. To investigate these possibilities, but also to get an insight into the host-symbiont relationship, isolation of the sulfate-reducing symbionts in pure culture was attempted with *Olavius* spp. (*O. algarvensis* and *O. ilvae*), species that co-occur in sandy sediment in the Mediterranean Sea (Dubilier *et al*., 2005).

The specimens were collected from sediments off the island of Elba in June 2003 and 2004. The worms were extracted from sediment by decanting and sieving. The freshly collected specimens were kept in plastic jars half filled with sediment from the worm's habitat and half with filter-sterilized sea water. In these conditions, hundreds of worms could be maintained alive for several months in the laboratory. To create hot-spots of sulfide production, required by the SOB symbionts, cyanobacterial biomass was autoclaved and buried into the sediment in the jars. Within days black spots formed around the buried organic material, indicating intense production of sulfide. The worms were observed to migrate and settle around these sulfidic spots.

For cultivation of the SRB symbionts 5 to 10 worms were collected from the jars, washed 3–5 times in 1 × PBS until the surface of the worm was free of sand grains or other particles. The worms were homogenised gently in a few drops of anoxic artificial sea water (ASW). The homogenized suspension was further used as inoculum for the preparation of agar dilution series. Preparation of agar dilution series was done as described (Widdel and Bak, 1992). The agar tubes were supplemented with different substrates (Table 4), and with the same substrates in combination with a mixture of yeast extract (4% wt/v final concentration), mussel-oyster extract (10% v/v) and sediment extract (10% v/v). The sediment extract was obtained from North Sea sandy sediment that was vigorously mixed with sea water and centrifuged for 20 min at 4500 rpm; the supernatant was collected and filter-sterilized. The mussel-oyster extract was obtained by homogenizing and autoclaving the tissue collected from 10–20 mussels and oysters. Other factors tested included different reductants, and thiosulfate as alternative electron acceptor to sulfate (Table 4).

Substrate	+/- thiosulfate	Reductant none, ascorbate, sulfide
Acetate + H_2		
Lactate + pyruvate		
Propionate + acetate + succinate		
Butyrate + valerate + caproate		
Ethanol		
Propanol		
Propane		
Butane		
Acetate + H_2 + mixture		
Lactate + pyruvate+ mixture		
Propionate + acetate + succinate+ mixture		
Butyrate + valerate + caproate+ mixture		

Table 4. Combinations of substrate, reductants, alternative electron acceptors and pH used for cultivation of the SRB symbionts of *Olavius* spp.

For each of these combinations a dilution series from 10^{-1} to 10^{-5} was done. All tubes were incubated at room temperature and in the dark. Negative controls, containing the inoculum but no substrates were also prepared.

Following incubation colonies grew only in tubes with sulfide as reductant and with the following substrates: acetate + H_2 (with and without mixture), lactate + pyruvate (with and without mixture), butyrate + valerate + caproate (with and without mixture). Transfer into liquid medium and measurements of sulfide showed that only four of the isolates were sulfate-reducers. These strains were identified by amplification and sequencing of the 16S rRNA gene. The cells from 1 ml of each culture were collected by centrifugation (5 min at 8000 rpm), washed 2 times with sterile water and eluted in 50 μl PCR water. One μl of this

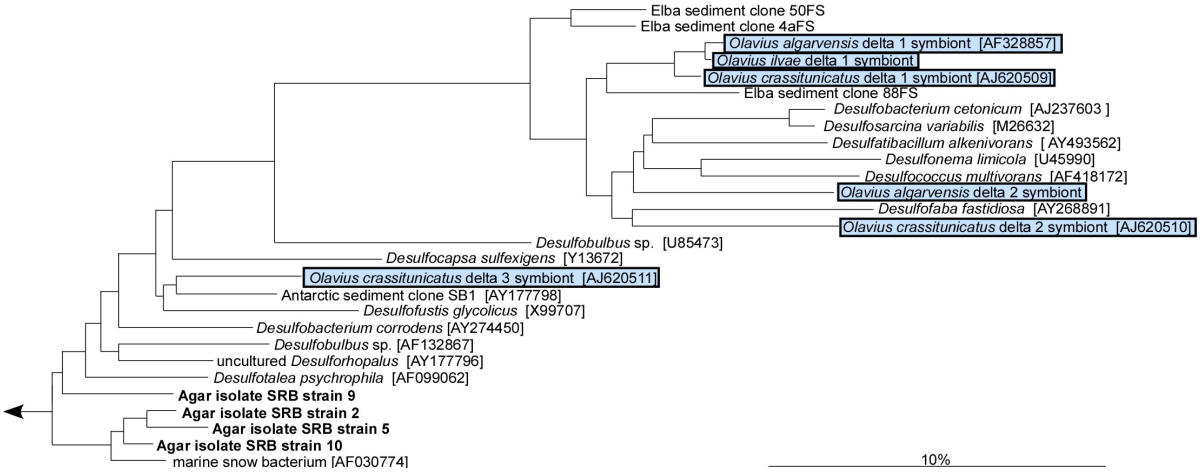

Fig. 15. 16S rRNA phylogenetic reconstruction of the cultivated sulfate-reducing bacteria (bold face type). The phylogenetic tree is based on maximum likelihood analysis. Sequences of sulfate-reducing symbionts are highlighted in blue. The scale bar represents 10% estimated sequence divergence.

volume was used as template in a PCR reaction with bacterial-specific primers (8f and 1492r). The sequences obtained were compared with known sequences of the SRB symbionts of *Olavius* species and with available sequences in the databases. None of the purified strains were similar with the SRB symbionts. Three of the isolates were clustered together (98 % sequence identity) and they were most closely related with marine snow associated bacteria (98% sequence identity) (Fig. 15). This cluster of isolates shared 94% sequence identity with the fourth isolate and their closest cultivated relative was *Desulfotalea psychrophila* (92–94% sequence identity; Fig. 15).

This result, together with the low number of isolates obtained from the high number of substrates combinations used, raised the question whether the symbionts were still present inside of the worms after maintenance under laboratory conditions for more than 2 months. To verify this, 4 worms were randomly sampled from the jars, washed and used for DNA extraction as previously described (Schizas *et al*., 1997). The extracted DNA was used for amplification of the 16S rRNA gene with the primer 8f and the probe DSS658 as a reverse primer, to amplify preferentially the SRB symbionts. The PCR product obtained was purified and sequenced. The retrieved sequences belonged to the SRB symbionts of *Olavius* species indicating their presence inside of the host. Nevertheless, this result did not

provided information regarding the metabolic state of these symbionts. To determine if the SRB symbionts were still active, 6 worms were randomly sampled from different jars, fixed in ethanol, embedded in paraffin and cross-sectioned in 6 μm slices. FISH was performed with the general eubacterial probe (EUB338), the group-specific probe targeting *Desulfosarcina* related species (DSS658) and symbiont-specific probes (C. Rühland, unpublished). The SRB symbionts were not detected with any of these probes. The only symbionts detected were the SOB that hybridized with the general probe EUB338 (data not shown).

Overall, these results suggested that the SRB symbionts were still present inside of the host, but their activity was significantly reduced, leading to a decrease in the number of ribosomes to the level when they were not anymore detectable by FISH. Survival of the worms for extended time periods in the lab (up to 1 year), together with the FISH data, clearly showed that the autotrophic SOB symbionts which supply the host with organic compounds were still active. Considering this, a possible explanation for the reduced activity of SRB symbionts is that they were lacking a substrate which is normally gained from the environment. This substrate was apparently none of those used for preparation of the agar dilution series, since no colonies of the symbiotic SRB were formed. It is also possible that the SRB symbionts are slow growing bacteria and traces of oxygen could have diffused through the stopper inhibiting colony formation. Another option that should be considered is the tight association of the SRB with the SOB symbionts: they are in close physical contact within the host and are involved in a syntrophic sulfur cycle (Dubilier *et al*., 2005). Thus, the lack of growth could also be a direct consequence of a highly-dependent relationship between these two symbionts resulting in the impossibility of cultivating one without the other. Cultivation of the SRB symbionts could therefore be attempted with freshly collected worms that have not been kept under laboratory conditions. Assuming that the SRB symbionts need growth factors which are supplied by the SOB symbiont, simultaneous cultivation of both symbionts, for example by creating artificial sulfide and oxygen gradients in agar tubes for the SOB symbiont and supplying organic substrates for the SRB symbiont, could be an alternative strategy.

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Publications

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1. **Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea**
   Niculina Musat, Ursula Werner, Katrin Knittel, Steffen Kolb, Tanja Dodenhof, Justus E.E. van Beusekom, Nicole Dubilier, and Rudolf Amann

2. **Molecular and morphological characterization of the symbiosis between bacterial endosymbionts and the nematode *Astomonema* sp. from the Bahamas**
   Niculina Musat, Olav Giere, Armin Gieseke, Frank Thiermann, Rudolf Amann, and Nicole Dubilier

3. **Molecular characterization of chemoautotrophic sulfur-oxidizing symbionts in co-occurring closely related nematode species of the genus *Leptonemella***
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4. **Transport and mineralization rates in North Sea sandy intertidal sediments, Sylt-Rømø Basin, Wadden Sea**
   Dirk de Beer, Frank Wenzhöfer, Timothy G. Ferdelman, Susan E. Boehme, Markus Huettel, Justus E.E. van Beusekom, Michael E. Böttcher, Niculina Musat and Nicole Dubilier

I

Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea

Niculina Musat[1], Ursula Werner[1], Katrin Knittel[1], Steffen Kolb[1], Tanja Dodenhof[1], Justus E.E. van Beusekom[2], Nicole Dubilier[1], and Rudolf Amann[1]*

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Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea

Niculina Musat[a], Ursula Werner[a], Katrin Knittel[a], Steffen Kolb[a], Tanja Dodenhof[a], Justus E.E. van Beusekom[b], Dirk de Beer[a], Nicole Dubilier[a], Rudolf Amann[a,*←]

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Abstract

Molecular biological methods were used to investigate the microbial diversity and community structure in intertidal sandy sediments near the island of Sylt (Wadden Sea) at a site which was characterized for transport and mineralization rates in a parallel study (D. de Beer, F. Wenzhöfer, T. Ferdelman, S.E. Boehme, M. Huettel, J.E.E. van Beusekom, M.E. Böttcher, N. Musat, N. Dubilier, Transport and mineralization rates in North Sea sandy intertidal sediments, Sylt-Romo Basin, Wadden Sea, Limnol. Oceanogr. 50 (2005) 113 127). Comparative 16S rRNA sequence analysis revealed a high bacterial diversity. Most sequences retrieved by PCR with a general bacterial primer set were affiliated with *Bacteroidetes*, *Gammaproteobacteria*, *Deltaproteobacteria* and the *Pirellula* cluster of *Planctomycetales*. Fluorescence in situ hybridization (FISH) and slot-blot hybridization with group-specific rRNA-targeted oligonucleotide probes were used to characterize the microbial community structure over depth (0 12 cm) and seasons (March, July, October). We found high abundances of bacteria with total cell numbers up to 3×10^9 cells ml^{-1} and a clear seasonal variation, with higher values in July and October versus March. The microbial community was dominated by members of the *Planctomycetes*, the *Cytophaga/Flavobacterium* group, *Gammaproteobacteria*, and bacteria of the *Desulfosarcina/Desulfococcus* group. The high abundance (1.5×10^7 1.8×10^8 cells ml^{-1} accounting for 3 19% of all cells) of presumably aerobic heterotrophic polymer-degrading planctomycetes is in line with the high permeability, deep oxygen penetration, and the high rates of aerobic mineralization of algal biomass measured in the sandy sediments by de Beer et al. (2005). The high and stable abundance of members of the *Desulfosarcina/Desulfococcus* group, both over depth and season, suggests that these bacteria may play a more important role than previously assumed based on low sulfate reduction rates in parallel cores (de Beer et al., 2005).

Keywords: 16S rRNA diversity; Community structure; FISH; Slot-blot hybridization; Marine sediment; Sand; *Planctomycetes*; *Pirellula*; *Desulfosarcina/Desulfococcus* group

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Introduction

Although sandy marine sediments cover 70% of coastal zones, they have only recently received more attention by microbiologists and biogeochemists [6,11,13,25,26,48 50,60]. The first wave of studies on molecular microbial ecology of marine sediments focused on muddy sediments [31,32,39,44,47,51]. In our opinion, there are two main reasons why sandy sediments have been understudied in the past.

First, they have organic contents that are 1 2 orders of magnitude lower than those of muddy sediments [7,50,54]. This low organic content has led to the erroneous assumption that sandy sediments have lower microbial activity. Furthermore, the large grain size of sand results in less surface area for bacterial attachment compared to muddy sediments [27,29]. Recent biogeochemical studies on sandy sediments show that, in contrast to the above assumption sandy sediments are microbially highly active. Organic matter remineralization rates and oxygen uptake measured in sandy sediments were comparable with those of organic rich muddy sediments [7,11,13,48,50,54] suggesting comparable microbial activities. Element cycling in the upper layers of sandy sediments is enhanced mainly by pore water circulation that effectively transports organic material, nutrients and oxygen into the sediment. Sandy sediments act as filter systems, trapping and concentrating suspended particles, algae, and bacteria [11,13,24], and thereby stimulating microbial activity.

The second reason is that sandy sediments are more difficult to study than muddy sediments. They are quite heterogeneous, consisting of an immobilized microbial community on the sand grains in the form of more or less tightly attached biofilms and a mobile pore water community that is easily lost during sampling. It might, therefore, be partly due to differences in the cell detachment protocols that even basic microbiological data like total cell concentrations show significant discrepancies of 1 2 orders of magnitude between related samples (e.g., [26,32,50]). This makes it difficult to compare different studies on the microbial community structure of sandy sediments. Furthermore, coastal sediments generally have steep gradients, e.g., of oxygen, which in the case of coarse, permeable sands oscillate with the tidal cycle.

Marine coastal sediments are known to contain a rich diversity of microorganisms from different physiological and phylogenetic groups. Therefore, previous molecular studies focused on the general microbial community structure [4,6,26,32,50] and the abundance and depth distribution of specific functional groups, e.g., polymer degrading, sulfur-oxidizing or sulfate-reducing bacteria (SRB) [19,47,60]. In temperate zones, microbial communities in coastal sediments should also be influenced by the strong seasonality in temperature and the availability of organic matter. This also applies to our study site, a typical intertidal sand flat, near the island of Sylt (Wadden Sea, south-eastern North Sea). Over the 2-year period examined in this study (1999/2000) the tidal water had typical strong algal blooms beginning in the spring until early summer and temperatures ranged between 1 and 20 °C (Fig. 1). To our knowledge, seasonal changes in the microbial communities of sandy sediments have only been examined in a single study which reported higher cell numbers and activity in the summer months versus fall and winter [50].

This study provides the first extensive description of the microbial community structure of coastal marine sandy sediment which is based on two quantitative rRNA-based methods, fluorescence in situ hybridization (FISH) and slot-blot hybridization. Additional information on bacterial phylogenetic diversity was obtained by comparative sequencing of 16S rRNA genes. Considering the heterogeneity of sandy sediment we argue that a comparison of two different molecular methods one based on single cell detection (FISH), the other on extraction of total RNA should yield a more complete description of microbial community composition over depth and over different seasons. Inconsistencies in the results obtained with the two methods would facilitate the identification of experimental bias and would thereby prevent an over-interpretation of the results.

This study represents the second part of a large integrated study on the Sylt intertidal sand flat. The first part focusing on the biogeochemistry of this site was recently published [13]. The samples were taken in close collaboration with de Beer et al. [13] at the same collection sites and times so that the microbiological data from this study can be directly compared to a large set of biogeochemical information.

Materials and methods

Study site and sampling procedure

The Wadden Sea is a coastal area along the Dutch, German, and Danish North Sea coast. Our investigations took place in the northern Wadden Sea on an intertidal sand flat (55°02′N, 8°26′E) directly south of the harbor in List on the island Sylt. The sand flat is approximately 80 100 m wide (from the high to low water line). The sediment is mainly composed of coarse silicate sand (grain size of ca. 200 400 μm) which is highly permeable ($k = 1.5\ 7 \times 10^{-11}\ m^2$). The sediment color was yellow from surface to 4 cm depth, gray below 4 cm, and changed to black below 10 cm indicating high sulfide concentrations in this region. For a detailed site description, see DeBeer et al. [13]. Tidal range is 2 m.

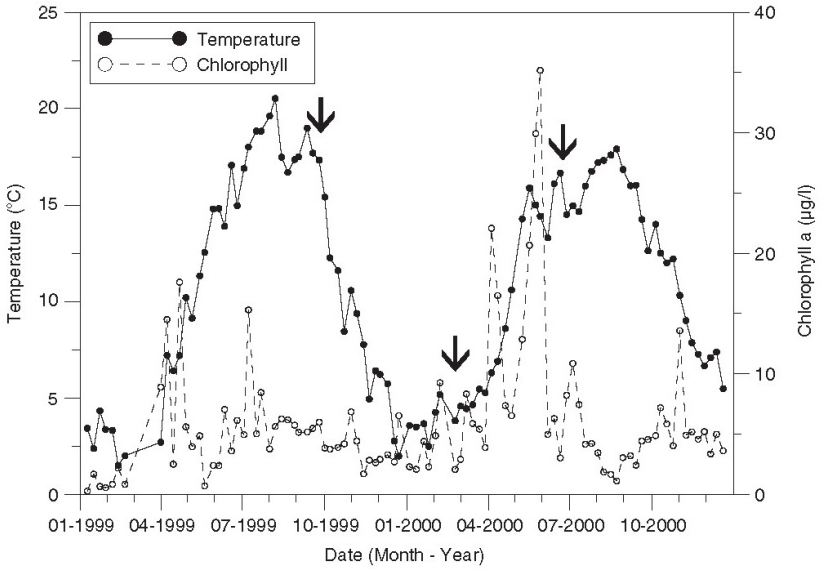

Fig. 1. Seasonal variation of chlorophyll *a* concentrations and temperature in the water column in the main tidal channel near the sampling site. The values are weekly means. Sampling times are indicated by arrows.

The inundation time also depends on the prevailing weather conditions. The environmental conditions in the water column at two nearby stations during the period of examination are shown in Fig. 1. These data are part of an ongoing monitoring program. Temperature shows a clear seasonal cycle with maximum water temperatures of about 18 20 °C in August and minimum temperatures of about 1 °C in winter. Phytoplankton biomass (measured as chlorophyll *a*) shows a clear diatom-dominated spring bloom in April of 10 20 µg chlorophyll/l sometimes followed by a second bloom of the Haptophycea *Phaeocystis globosa* (e.g., in May 2000).

The sampling was performed during low tide in the middle of the flat, approximately 40 m in the offshore direction from the high water line on June 22, 1999, October 6, 1999, March 7, 2000, and July 5, 2000. The water salinity was about 29‰ independent of season, temperature or tides. The temperature was measured before, and during sampling. The in situ sediment temperature ranged from 20 to 22 °C at the surface and decreased to 16.4 17.0 °C at 10 cm depth in June 1999, October 1999 and July 2000. In March 2000, the temperature was 6.2 7.3 °C at the sediment surface, and 5 °C at 10 cm depth. Three parallel cores were collected from each season for molecular analyses. Within 2 h after sampling the sediment cores were sub-sampled and stored at −80 °C for nucleic acids extraction and fixed in formaldehyde for FISH analysis.

Fluorescence in situ hybridization (FISH)

Two parallel sediment cores from each season were used for FISH and total cell counts analysis. Cores were sectioned in 1 cm layers and the sediment was fixed for 2 3 h with 3% (final concentration) formaldehyde at 4 °C, washed twice with 1 × phosphate-buffered saline (PBS: 10 mM sodium phosphate [pH 7.2], 130 mM NaCl) and then stored in 1 × PBS:ethanol (1:1) at −20 °C. Subsamples were diluted with 1 × PBS buffer (final dilution 1:40) and treated by sonication (probe MS73, Sonopuls UW70, Bandelin, Berlin, Germany) at an amplitude of 105 µm and 35 kW for 80 s without break (October 1999 sediment cores). For March 2000 and July 2000 cores, the protocol was changed-in an attempt to increase the number of bacteria detached from the sand grains without cell lysis. A mild sonication at an amplitude of 42 µm and less than 10 kW for 7 times at 30 s each, with a 30 s break between each sonication was used. For FISH, sonicated samples were vortexed and a 50 70 µl aliquot of the supernatant was filtered on a 0.2 µm pore-size type GTTP polycarbonate filter (Milipore, Eschborn, Germany). The cells were hybridized, stained with 4′,6′-diamidino-2-phenylindole (DAPI) and microscopically counted as described previously [55]. Competitor probes and formamide concentrations used are shown in Table 1. Counts are reported as means calculated from 10 15 randomly chosen microscopic fields corresponding to 700 1000 DAPI-stained cells. Values were corrected for

Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′→3′)	Target RNA	Position[a]	Slot-blot (T_d) (°C)[b]	FISH (FA)[c]	Source or reference
UNI1390	Universal – all organisms	GACGGGCGGTGTGTACAA	16S, 18S	1390–407	44	NU	[61]
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	16S	915–935	56	NU	[41]
EUK1379	Eukarya	TACAAAGGGCAGGGAC	18S	1379–1394	42	NU	[21]
EUB338-I	Most bacteria	GCTGCCTCCCGTAGGAGT	16S	338–355	54	10	[1]
EUB338-II	Supplement to EUB 338: *Planctomycetales*	GCAGCCACCCGTAGGTGT	16S	338–355	64	NU	[10]
EUB338-III	Supplement to EUB 338: *Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	16S	338–355	63	NU	[10]
NON338	Antisense of EUB338	ACTCCTACGGGAGGCAGC	16S	338–355	NU	10	[57]
ALF968	*Alphaproteobacteria*, wide variety of *Deltaproteobacteria*	GGTAAGGTTCTGCGCGTT	16S	968–985	50	20	[36]
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	23S	1027–1043	58[b]	35	[36]
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	23S	1027–1043	60[b]	35	[36]
PLA886	*Planctomycetales*, many Eukarya	GCCTTGCGACCATACTCCC	16S	886–904	62	35	[40]
CF319a	*Cytophaga/Flavobacterium*	TGGTCCGTGTCTCAGTAC	16S	319–336	56[b]	35	[35]
DSR651	*Desulforhopalus* spp.	CCCCCTCCAGTACTCAAG	16S	651–668	62[b]	35	[37]
DSS658	*Desulfosarcina* spp./*Desulfofaba* spp.	TCCCACTTCCCTCTCCCAT	16S	658–685	58[b]	60	[37]
DSV698	*Desulfovibrio* spp.	GTTCCTCCAGATATCTACGG	16S	698–717	58[b]	35	[37]
GAM660	16SrDNA clone sequences affiliated with endosymbionts and some other species in the *Gammaproteobacteria*	TCCACTTCCCTCTAC	16S	660–674	52[b]	35	[43]

NU, not used.

Competitor probes were used for FISH as follows: for BET42a the competitor was GAM42a, for GAM42a the competitor was BET42a and for PLA886 the competitor was cPLA886.

[a]Position in the 16S or 23S rRNA of *E.coli*.

[b]Dissociation temperatures (T_d) were determined with the washing buffer containing 1 × SSC and 0.1% SDS.

[c]FA, formamide concentrations in the hybridization buffer calculated as percent (vol/vol).

the signals counted with the probe NON338. Oligonucleotides labeled with Cy3 were purchased from Thermo Electron (Ulm, Germany).

RNA extraction and slot-blot hybridization

RNA was extracted from 2 8 ml of wet sediment (per layer) from October 1999, March 2000 and July 2000 cores by bead beating, phenol extraction, and isopropanol precipitation as described previously [34,56]. The quality and purity of RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Ambion). Up to 50 ng RNA was blotted onto nylon membranes (Magna Charge; Micron Separations, Westborough, MA) in triplicate and hybridized with ^{33}P labeled oligonucleotide probes as described by Stahl et al. [56] specific activities were in the range of $0.24-0.26 \times 10^8$ cpm/μg probe. Membranes were washed at different temperatures depending on the dissociation temperature (T_d) of the probe. The probes and dissociation temperatures used in this study are given in Table 1. For probes EUB338-II, EUB338-III and ALF968, dissociation temperatures of 64, 63 and 50 °C, respectively, were determined as described by Raskin et al. [41].

Hybridization signal intensity was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantified as described previously [51]. Reference rRNA isolated from pure cultures of *Cytophaga lytica* (DSM 7489), *Rhodopirellula baltica* strain SH1 (DSM 10527), *Methanolobus tindarius* (DSM 2278), *Zoogloea* strain Cadagno, *Planctomyces maris* (DSM 8797), *Verrucomicrobium spinosum* (DSM 4136), *Rhizobium rubri* (DSM 6772), *Lactococcus lactis* (DSM 20481), *Desulfovibrio salexigenes* (DSM 2638), strain LSv22 (DSM 13039) [30] and strain "Milos OAII2" as well as the rRNA of *Saccharomyces cerevisiae* and *Escherichia coli* (purchased from Roche, Mannheim, Germany) served as standards for hybridization with the probes given in Table 1.

DNA extraction and PCR

Sediments from two different seasons (June 1999 and July 2000) were used for clone library construction. Total DNA was extracted as described previously by Zhou et al. [62]. Nearly complete 16S rRNA genes were amplified by PCR using bacterial-specific primers 8f [21] and 1492r [28]. The PCR reactions contained 50 pmol (1 μl) of each primer, 1 mM (10 μl) total dNTPs, 1 × PCR buffer (10 μl), 0.3 mg ml^{-1} BSA (10 μl), and 1 U (1 μl) of either *Pfu* polymerase (Promega, Madison, USA) or TaKaRa-*Taq* polymerase (BioWhittaker UK, Ltd.). The amplification was done with a Thermocycler Mastercycler (Eppendorf, Hamburg, Germany) as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 42 °C, and 3 min elongation at 72 °C, with a final extension step of 10 min at 72 °C. Blunt-end PCR products generated by the *Pfu* polymerase were 3′-A tailed by an incubation for 30 min at 70 °C with 5 U of Super*Taq*-Polymerase (HT Biotechnology LTD, Cambridge, UK) and 10 mM ATP.

Clone library construction and amplified rDNA restriction analysis (ARDRA)

The PCR products were purified with the QIAquick Purification Kit (Qiagen, Hilden, Germany) and cloned either into the pGEM-T Easy vector (Promega, Madison, WI) or pCR4 TOPO vector (Invitrogen, Groningen, Netherlands) according to the manufacturer's recommendations. The recombinant vectors were transformed into chemically competent *E. coli* cells following the manufacturer's instructions or into electro-competent *E. coli* cells as previously described [45]. The 16S rDNA (rRNA gene) libraries were screened by ARDRA. The clones were categorized into different phylotypes based on the patterns obtained by dual digestion (3 h, 37 °C) with two restriction enzymes, *Hae*III and *Rsa*I (8.3 U [μg DNA]$^{-1}$).

Sequencing and phylogenetic analysis

Representatives of major ARDRA-pattern groups were chosen for sequencing. Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen). Sequencing with vector primers and universal rRNA gene-specific primers, was done by GAG BioScience (Bremen, Germany) or AGOWA (Berlin, Germany). Sequence data were analyzed with the ARB software package [33]. Phylogenetic trees were calculated by performing neighbor-joining, maximum likelihood and maximum parsimony analysis using different sets of filters exclude the influence of highly variable positions and without any filter. Topologies derived by these different approaches were compared with each other to construct a consensus tree. For tree reconstruction, only nearly full-length sequences (>1300 bp) were considered. Partial sequences were then inserted into the reconstructed tree by applying parsimony criteria, without allowing changes in the overall topology. The nucleotide sequence accession data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession no. AM040097 AM040147.

Results

Clone library

In order to obtain a first insight in the bacterial diversity of the Sylt sandy sediments, a 16S rDNA library was constructed from a 0 12 cm core in June 1999 and a 0 9 cm core taken in July 2000. A total of 48 clones from June and 58 clones from July cores were screened by ARDRA and divided into similarity groups. Representatives of each group were sequenced and used for phylogenetic tree reconstruction. Thus, 20 clones were fully sequenced from June 1999 and 23 clones were fully and 7 partially sequenced for July 2000. Sequences were phylogenetically highly diverse and included numerous cultivated as well as uncultivated bacterial lineages. Sequence similarity to cultivated species was in all cases lower than 97%. In contrast, sequence similarity to other environmental 16S rDNA libraries was often high with up to 99%.

Comparative 16S rRNA sequence analysis (Fig. 2) revealed that most sequences belonged to four bacterial groups: *Bacteroidetes* (formerly *Cytophaga/Flavobacterium/Bacteroides* phylum), *Planctomycetes*, *Deltaproteobacteria*, and *Gammaproteobacteria*. A high number of phylotypes (11) were detected within the *Bacteroidetes*. They were widely distributed throughout the phylum, did not form specific clusters, and the similarity to known species was lower than 95%. The *Planctomycetes* group was represented by 9 different phylotypes affiliated with *Pirellula* spp. A total number of 12 phylotypes were distributed within the *Deltaproteobacteria* group and formed separate clusters: 5 phylotypes were related with *Desulfosarcina/Desulfococus* species, 4 phylotypes were closely affiliated with the genera *Desulfocapsa* and *Desulfobulbus*, and 3 phylotypes were not affiliated with any known deltaproteobacterial species but rather with uncultivated species. Within the *Gammaproteobacteria* the 10 phylotypes were affiliated with symbiotic and free-living sulfur-oxidizing bacteria. A few other sequences were affiliated with the *Holophaga-Acidobacteria* (2), *Verrucomicrobiales* (2), *Deinococcales* (2), *Actinobacteria* (1), *Deferribacterales* (1) and Candidate division OP3 (1). The library analysis was not intended to yield an in-depth description of the presumably quite high bacterial diversity but rather to assist in the selection of group-specific hybridization probes.

Total cell counts

The total number of microbial cells was determined in two parallel cores for each sampling season (March, July and October), using DAPI staining (Fig. 3A). For all seasonal comparisons it should be considered that the cell detachment protocol for the October 1999 sample was different to that of March and July 2000. Cell numbers were relatively high in all samples, ranging from 0.4 to 3.3×10^9 cells ml^{-1} sediment. Cell numbers were two-fold lower in March than in July and October, with up to 1.1×10^9 cells ml^{-1} sediment in March, and 2.0 3.3×10^9 cells ml^{-1} sediment in July and October. Single peaks in cell numbers always occurred in the first 6 cm, but overall there was no obvious decrease of total cell numbers with increasing depth. Little variation was observed between the two parallel cores from each season (Table 2), despite the fact that the distance between each core was as large as 10 cm.

Domain-specific probing

Bacterial, archaeal, and eukaryotic rRNA were quantified using slot-blot hybridization with probes specific for these three domains. Bacterial rRNA (using the EUB338-I probe) accounted for up to 77% of total rRNA in March, and 58% and 83% of total rRNA in July and October, respectively (Table 2). These percentages did not increase significantly after probing with EUB338-II (fully complementary to the 16S rRNA of some *Planctomycetes* species which are missed by probe EUB338-I) and EUB338-III (designed for *Verrucomicrobiales* species), as their rRNA accounted for only 0.5 1.8% of total rRNA (data not shown). Archaeal and eukaryotic rRNA amounts were also very low, with highest values at only 3% archaeal and 1% eukaryotic rRNA (Table 2). Total bacterial, archaeal, and eukaryotic rRNA (EUB338-I + EUB338-II + EUB338-III, ARCH915, and EUK1379) never summed up to the total amount of rRNA as determined with the UNI1390 probe, despite the fact that the measurements were repeated several times.

The seasonal and vertical distribution of "prokaryotic rRNA", defined as the sum of slot-blot values obtained with probes EUB338-I + EUB338-II + EUB338-III + ARCH915, corresponded well with total prokaryotic cell numbers as determined by DAPI staining for the top 6 cm (Fig. 3, panels A and B). Total prokaryotic rRNA in March was at most half of the amount measured in July and October in the upper 6 cm. As with total prokaryotic cell numbers, the highest rRNA amounts were always in the top layer. Below 6 cm, there were no obvious seasonal differences in total prokaryotic rRNA.

Average cellular rRNA contents were calculated by dividing the amount of "prokaryotic rRNA" by the DAPI counts. The winter values (March 2000) were not lower than the summer and fall values (Fig. 3, panel C). The values ranged quite strongly from 0.1 to 2.2 fg rRNA $cell^{-1}$, with peaks occurring at 2 and 8 cm sediment depth.

Fig. 2. 16S rRNA-based phylogenetic reconstruction showing the affiliation of Sylt sequences (printed in bold) with selected reference sequences. Branching orders that were not supported by all phylogenetic methods used are shown as multifurcations. Partial sequences were added to the existing tree, without allowing changes in the overall tree topology. The scale bar represents 10% estimated sequence divergence.

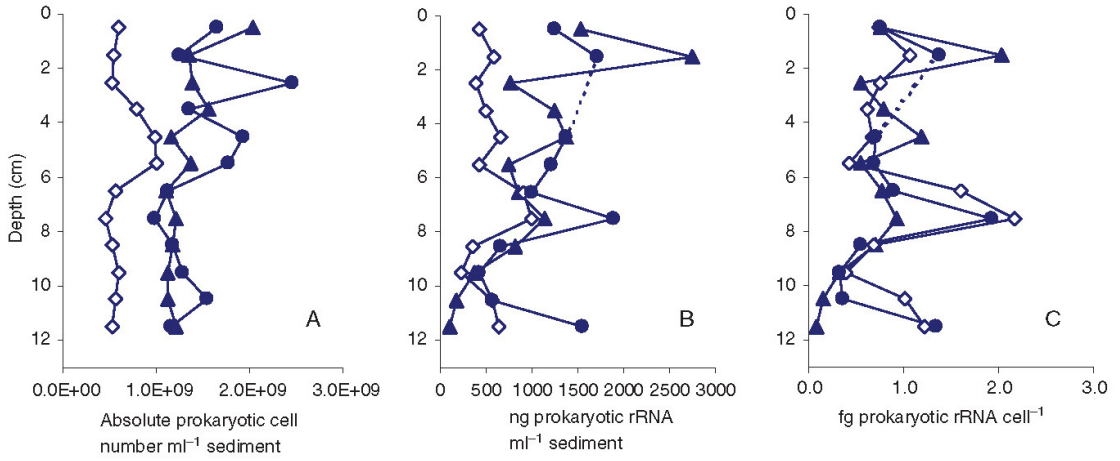

Fig. 3. Vertical profiles of total cell counts (panel A, mean of two parallel cores), prokaryotic rRNA recovery (B), and prokaryotic cellular rRNA content (C) in sandy Sylt sediments. The samples were collected in October 1999 (▲), March 2000 (◇) and July 2000 (●).

Group-specific probing

Planctomycetes was the most abundant bacterial group based on slot-blot hybridization (Table 2 and Fig. 4, panel A). Specific cell counts with probe PLA886 ranged between 1.5×10^7 and 1.8×10^8 cells ml^{-1} (3 19% of total cells). They were highest in the upper 8 cm. A summer peak of *Planctomycetes* was observed with FISH over the full length of the core, whereas slot-blot hybridization values of probe PLA886 were higher only in the upper 6 cm of the sediment in summer and fall. Particularly at the sediment surface very high slot-blot values were measured with PLA886 (up to 70% of total rRNA). This was most likely caused by cross-hybridization of PLA886 to the 18S rRNA of ciliates and unicellular algae (see Discussion). FISH counts are less biased since the morphology of *Planctomycetes* is quite specific. The cells detected by PLA886 were usually large cocci of different sizes (up to 1 μm diameter), occurring as single cells or in disordered clusters (Fig. 5, panels A and B). This morphology is significantly different from that of ciliates and unicellular algae so that we can exclude the possibility of false-positive FISH signals from these members of the Wadden Sea ecosystem.

Probe CF319a [35] was used to quantify members of the *Cytophaga/Flavobacterium* group (CF) of the phylum *Bacteroidetes*. These were abundant in the Sylt sand flat, but only at the sediment surface, based on both slot-blot and FISH (Table 2 and Fig. 4, panels C and D). The CF were most abundant in the top 2 cm, with cell numbers ranging between 1.2×10^6 and 1.8×10^8 cells ml^{-1} (1 11% of total cells). Up to 8% of total rRNA was detected by slot-blot hybridization with probe CF319a. Seasonal differences were observed in the upper 2 cm. Corresponding to the general trend, cell numbers and rRNA values were lower in March than in July and October. Most of the cells identified within this cluster were morphologically diverse and included long and short rods (0.5 1.5 μm in length) and short filaments (Fig. 5, panels C and D).

Probe ALF968 was used to estimate the abundance of *Alphaproteobacteria* which are an abundant group of marine bacteria. It is important to realize that this probe not only targets members of the *Alphaproteobacteria* but also a variety of *Deltaproteobacteria* including various SRB as well as members of the genera *Pelobacter*, *Geobacter* and *Desulfuromonas*. Thus, false positives cannot be excluded. Cell numbers and rRNA concentrations quantified with ALF968 were comparable to those obtained with CF319a data not shown.

Comparative sequence analysis of the 16S rDNA libraries indicated an important role of members of the *Gammaproteobacteria*. GAM42a-positive populations represented an important fraction of the microbial community in Sylt sediments based on both slot-blot hybridization and FISH (Table 2). The cell numbers ranged between 2.2×10^6 and 1.2×10^8 cells ml^{-1} and the specific rRNA concentration was as high as 23% of total rRNA. Seasonal differences were observed in rRNA concentrations, with again, lower values in the March samples.

Some of the gammaproteobacterial sequences were related to bacteria potentially able to oxidize sulfide. Five of ten sequences (Sylt6, Sylt29, Sylt39, Sylt45, Sylt49) match probe GAM660 designed to quantify a group affiliated with free-living and endosymbiotic sulfur-oxidizing bacteria [43]. In addition, GAM660 also targets closely related sequences retrieved from other marine sediments as well as the endosymbionts of

Table 2. Quantification of different microbial groups using FISH (core A/B) (% of DAPI) and rRNA slot blot hybridization (core C) (% of total RNA)

Depth [cm]	Absolute prok. cell counts			Total RNA (probe UNI1390)*←			Bacteria (probe EUB338-I)						*Gammaproteobacteria* (probe GAM42a)					
	Oct.	Mar.	Jul.	Oct.	Mar.	Jul.	Oct.		Mar.		Jul.		Oct.		Mar.		Jul.	
		[10^9 ml^{-1}]			[ng RNA ml^{-1}]		% DAPI	% RNA*←	% DAPI	% RNA*←	% DAPI	% RNA*←	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA
0.5	2.0/2.0[a]	0.6/0.6	2.0/1.3	1814	880	3288	35/34	83	63/56	47	83/79	36	2/4	18	10/12	8	7	13
1.5	1.3/1.4	0.5/0.6	1.4/1.1	3474	1478	4420	49/47	77	51/52	38	85/87	36	2/4	17	15/9	8	5	16
2.5	1.3/1.4	0.5/0.6	3.3/1.6	1594	1460	ND	42/38	48	65/45	26	65/65	ND	2/4	13	10/9	6	3	ND
3.5	1.6/1.6	0.6/1.0	1.6/1.1	1906	1318	ND	ND/33	63	55/57	35	81/72	ND	ND/3	15	5/10	8	4	ND
4.5	1.1/1.2	1.0/1.0	2.0/1.9	1898	1124	2264	36/33	71	51/46	56	70/76	57	1/2	13	5/8	8	4	16
5.5	1.4/1.4	1.1/0.9	1.8/1.7	1365	1145	2252	30/31	53	42/51	34	63/69	53	0/2	11	4/10	9	7	21
6.5	1.1/1.1	0.7/0.5	ND/1.1	1654	1125	1924	31/33	50	61/56	77	ND/80	50	1/2	23	11/7	9	6	17
7.5	1.2/1.2	0.4/0.5	1.0/0.9	2087	1401	3291	30/24	53	63/49	66	62/58	55	0/2	13	10/6	10	9	18
8.5	1.2/1.2	0.4/0.6	1.2/1.2	1779	828	1847	ND/27	45	24/58	41	65/76	32	ND/0	11	ND/9	9	5	17
9.5	1.1/1.2	0.6/0.5	1.2/1.3	832	620	1034	32/22	43	14/57	37	74/66	38	0/ND	10	ND/6	8	4	15
10.5	1.1/1.1	0.6/0.5	1.6/1.5	320	1211	1380	26/17	55	14/45	43	76/72	38	ND	11	ND/8	10	2	18
11.5	1.2/1.2	0.6/0.5	0.7/1.6	165	1193	2561	16/25	63	11/56	50	73/78	58	ND	9	ND/ND	12	3	11

Depth [cm]	*Planctomycetes* probe (PLA886)						*Cytophaga–Flavobacterium* (probe CF319a)						*Desulfosarcinales* (probe DSS658)					
	Oct.		Mar.		Jul.		Oct.		Mar.		Jul.		Oct.		Mar.		Jul.	
	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA	% DAPI	% RNA
0.5	7/4	70	7/19	33	13	29	4/7	3	11/7	5	6	7	3/3	20	4/5	16	1	23
1.5	7/6	48	10/13	17	15	31	8/8	5	9/9	5	9	8	2/2	15	5/7	14	1	19
2.5	6/6	42	5/13	6	9	ND	9/11	5	9/2	3	2	ND	2/2	20	1/5	14	1	ND
3.5	ND/6	39	4/6	12	11	ND	ND/7	4	6/5	3	2	ND	ND/3	17	1/5	16	0	ND
4.5	6/5	27	9/6	24	10	31	4/5	4	5/4	2	2	6	3/4	17	2/6	17	0	20
5.5	5/5	20	17/4	16	9	27	4/6	2	8/3	2	2	5	2/5	15	1/4	14	2	17
6.5	7/4	20	19/6	26	9	28	2/4	2	7/3	2	2	5	2/3	16	2/5	17	1	19
7.5	5/5	27	18/4	30	13	18	3/2	2	3/3	2	1	6	3/3	11	3/6	14	2	17
8.5	ND/7	31	6/7	16	9	8	ND/2	2	4/3	2	1	4	ND/4	18	1/10	20	4	15
9.5	3/5	18	7/5	10	5	26	2/6	2	2/1	2	1	4	3/6	16	1/8	21	5	19
10.5	3/3	37	8/5	18	7	30	6/0	2	6/1	2	1	5	2/3	20	1/7	19	5	19
11.5	4/5	46	8/4	23	8	43	4/0	2	2/2	2	1	4	2/3	15	3/6	17	6	19

ND: not determined.

*From de Beer et al. [11].

[a]Core A value/core B value.

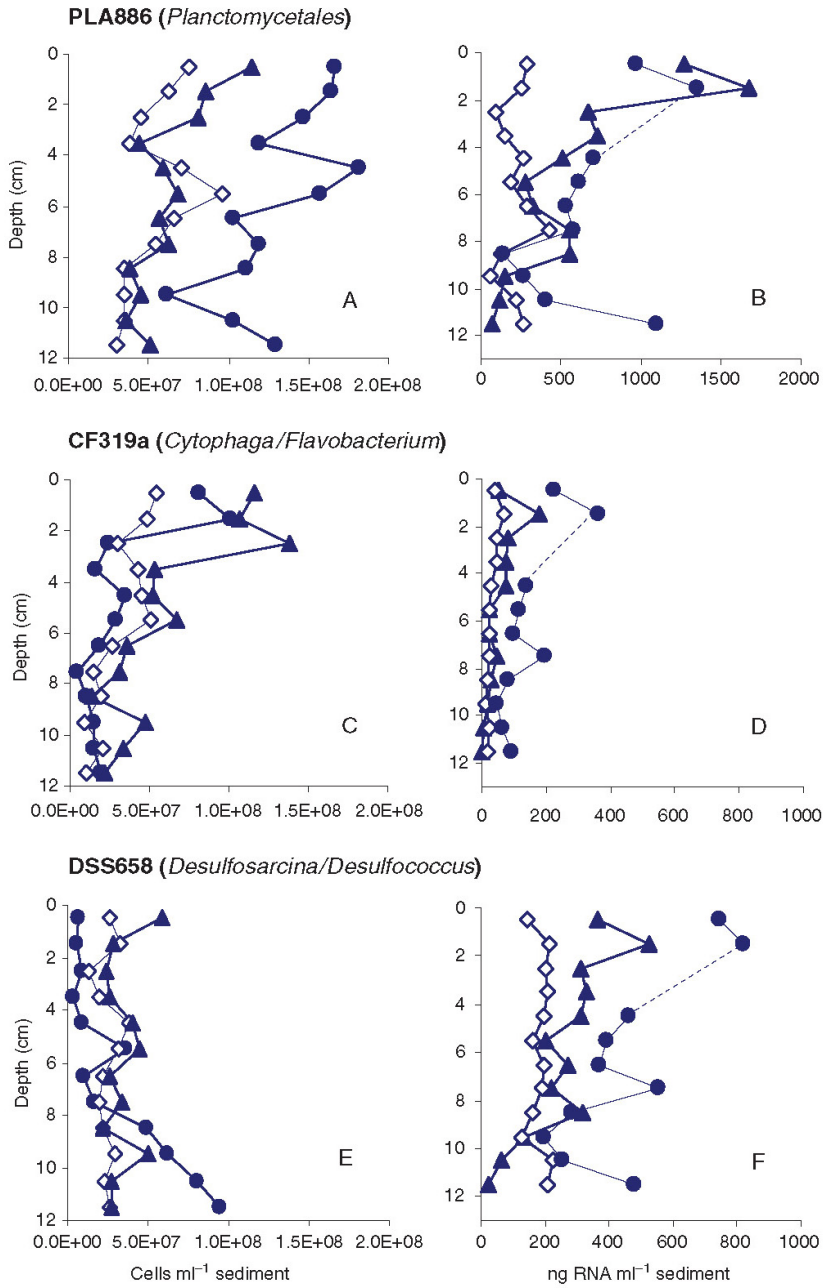

Fig. 4. Vertical profiles of bacterial groups in Sylt sediments as detected by FISH (A, C, E) and rRNA slot-blot hybridization (B, D, F) in October 1999 (▲), March 2000 (◇) and July 2000 (●). Note the different horizontal scales.

Riftia pachyptila, other vestimentiferan tubeworms and several bivalves. FISH counts (Table 2) were sometimes quite high (up to 14%), however, highly inconsistent within replicates and between the three seasonal samples. Since slot-blot hybridization failed to detect GAM660-positive rRNA this study does not provide

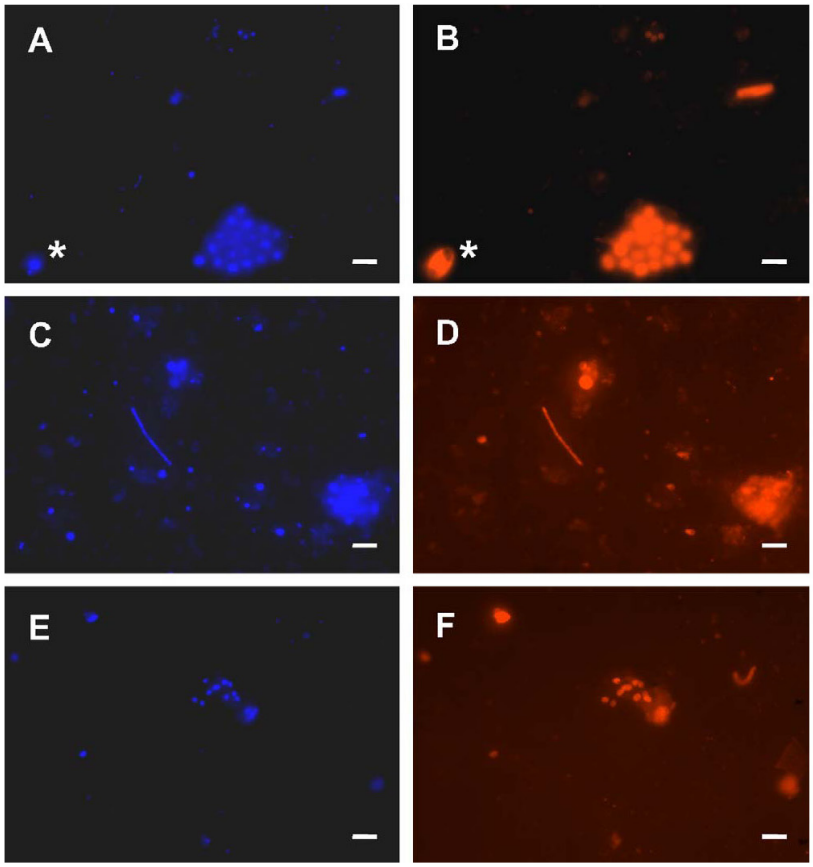

Fig. 5. Epifluorescence micrographs of bacteria in sediment samples from Sylt. Left panels show DAPI staining (blue) and right panels the corresponding FISH signal with the different probes (red). (A–B) Probe PLA886*, (C–D) probe CF319a and (E–F) probe DSS658. The scale bar is equivalent to 5 μm. *Diatom cell, not counted.

conclusive information about the abundance of this group in the Sylt sediments.

FISH and slot-blot hybridization results were also inconsistent for SRB from the *Deltaproteobacteria*. In this case the rRNA fractions detected were generally higher than relative cell abundance (Table 2 and Fig. 4, panels E and F). Of the three probes used to detect deltaproteobacterial SRB, the probe for the *Desulfosarcina/Desulfococcus* group (DSS658) detected the highest cell numbers (3.2×10^6 9.5×10^7 cells ml^{-1}; 0.4 10% of DAPI) and rRNA concentrations (14 23% of total rRNA). This corresponds well with our phylogenetic analyses which showed a cluster of *Desulfosarcina*-related sequences in the Sylt clone library. *Desulfosarcina/Desulfococcus* was abundant in all sediment layers examined. As with all other bacterial groups, the rRNA concentrations were lower in March than in July and October, but this trend was not observed in the FISH data. In terms of morphology, the cells identified were highly diverse and included long (12 μm) and short (8 μm) filaments and single rods (2 4 μm), but mostly rod-shaped cells of approximately 2 μm in diameter which occurred in disordered clusters or as sarcina-like packets (Fig. 5, panels E and F).

Other SRB detected in the North Sea sediments were members of the frequently cultivated genus *Desulfovibrio* and members of the genus *Desulforhopalus*. Members of both groups were present at all seasons and depths, with highest values in March at the sediment surface (up to 13% and 9% of total cells, respectively). In July and October, members of the *Desulfovibrio* and *Desulforhopalus* groups showed similar values of 1 2% of total cells (data not shown). Slot-blot hybridization revealed 1 2% abundance of *Desulfovibrio* spp. for all seasons and depths, while rRNA recovery of *Desulforhopalus* spp. was constantly below

the detection limit. Probes for other SRB (e.g., *Desulfomicrobium* spp., *Desulfotalea* spp., *Desulfobacter* spp., *Desulfobacterium* spp., *Desulfobulbus* spp.) were also used for FISH in October sediments but signals were always below the detection limit set at 1% of total cells.

Discussion

Microbial cell numbers and seasonal activity

Total microbial cell numbers determined for Sylt sandy sediments were in the range of 0.5 2 × 10^9 cells ml^{-1}. These counts are significantly higher than microbial cell numbers previously reported for sandy sediments which were often around 10^8 cells ml^{-1} [48,50,60] and rather comparable with those reported for muddy sediments (1 5 × 10^9 cells ml^{-1}; [31,32,39,44,51]. Initial experiments on Sylt sandy sediments clearly showed that total cell numbers strongly depend on the cell detachment protocols. Recently, elevated cell numbers, comparable with those obtained in the present study were also reported from another sandy intertidal flat in the Wadden Sea [26]. In muddy sediments cell numbers were reported to decrease in the top 5 10 cm from 3 5 × 10^9 cells ml^{-1} to about 1 2 × 10^9 cells ml^{-1}. The sandy sediments investigated in this study did not have such a significant decrease with depth in total cell number. This might be the result of the higher permeability and the resulting high availability of both substrates and electron acceptors in deeper layers [13]. An alternative explanation would be a continuous mixing of the upper sediment layer which is, however, not supported by other data from this study and that of de Beer [13].

Cell numbers and rRNA concentrations were generally higher in July and October than in March. This is expected and likely caused by the higher availability of degradable organic carbon in summer and fall (Fig. 1, also see estimates in [13]). During summer the main source of organic carbon was from phytoplankton. Just before the July 2000 sampling there was a strong phytoplankton bloom as indicated by an elevated chlorophyll *a* concentration (Fig. 1). In October 1999 the temperature was still high but the phytoplankton abundance was much lower (Fig. 1). At that time, the abundant benthic diatoms were more likely the main source of organic carbon. The March 2000 sampling is characteristic for an end-of-winter situation. It should be noted that, although both the abundance of prokaryotic cells as well as rRNA concentrations were lower in winter, the average cellular rRNA content calculated from these two parameters remained similar. It seems that over the winter months the overall size of the microbial community is reduced rather than the average cellular ribosome content. However, the strong variability of average cellular ribosome contents (0.1 2.2 fg rRNA $cell^{-1}$) over depth suggests that only limited information is provided by averaging for total prokaryotic rRNA. It is possible that different bacterial populations with different cellular rRNA contents dominate during different seasons.

Contribution of *Bacteria*, *Archaea* and *Eukarya* to the overall microbial community

Bacterial, archaeal, and eukaryotic rRNA were quantified using slot-blot hybridization. The sum of rRNA amounts measured with the domain probes did not add up to the rRNA amount determined with the universal probe. This might, at least in part, be a consequence of eukaryotic rRNA underestimation. We checked both probes EUK1379 and UNI1390 against the 18S rRNA sequences of several species of planktonic and benthic ciliates and flagellates which are known to be abundant in the North Sea [3,38,58]. Some of these species (e.g., *Myrionecta rubra*, *Chlamydodon excocellatus*, *Masodinium pulex*, *Cyclidium plouneouri*, *C. porcatum*) are targeted only by the universal probe but not by the eukaryotic probe. Since the detection of *Archaea* was also consistently low, we focused in more detail on the domain that clearly dominates the microbial community of the Sylt sandy marine sediment, *Bacteria*.

Phylogenetic diversity of bacteria in sandy sediments

The 16S rRNA diversity of bacteria in Sylt sandy sediments was high and comparable with that previously described in more detail for organic rich muddy sediments (e.g., [4,5,45]). With our limited sequencing effort we identified a high abundance of diverse representatives of four major bacterial groups, *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, and *Planctomycetes*. These have also been described from muddy sediments. Such a high number of phylotypes within the *Bacteroidetes* and *Planctomycetes* clusters has not been observed previously in sandy sediments. In a study of Puget Sound (USA), sequences related to *Bacteroidetes* were not found and the number of phylotypes within the *Planctomycetes* was limited to two [20]. The contamination of the Puget Sound site with creosote may have contributed to the limited representation of the *Bacteroidetes* and *Planctomycetes* groups. The sandy sediments in Sylt are not strongly influenced by contaminants, and the high diversity observed in this study could be a general characteristic of such environments. The affiliation of all *Planctomycetes* sequences with those of *Pirellula* spp. suggests an

aerobic heterotrophic metabolism and involvement in the degradation of algal polymers [18,53].

Quantification of abundant bacterial groups

Although individual results obtained with the two hybridization methods used for quantification sometimes differed significantly, the overall data indicated a high abundance of *Planctomycetes*, *Bacteroidetes*, *Alphaproteobacteria* (mainly in the upper layers of the sediment), *Gammaproteobacteria* and of deltaproteobacterial sulfate reducers of the *Desulfosarcina/Desulfococcus* group. Members of the *Betaproteobacteria*, *Desulfovibrio* spp. and *Desulforhopalus* spp. were not significantly represented in the microbial community.

Planctomycestes is an abundant group in marine systems, inhabiting the water column and the sediment. In marine sediments members of the *Planctomycetes* are present in the upper layers [26,50] as well as in the anoxic regions [43] often found attached to the sand grains or in association with phytodetrital macroaggregates ("marine snow", [14,42]).

The extremely high values obtained in our study particularly at the sediment surface by slot-blot hybridization and the inconsistence with the seasonal variations of the *Planctomycetes* cells determined by FISH was assumed to be due to a cross-hybridization of the PLA886 probe with the 18S rRNA of eukaryotes that also occur in coastal marine sediments. It was already known at the time of its design that PLA886 could also bind to the 18S rRNA of diatoms, in addition to that of some fungi and protists [26,40]. We checked the probes PLA886 and EUK1379 against the 18S rRNA sequences of abundant species of benthic and planktonic diatoms, ciliates, and flagellates in the North Sea [3,58]. Some of them (e.g., *Cyclidium porcatum, Peridinium* sp., *Chlamydodon excocellatus*, *Zoothamnium* sp., *Uronema* sp.) were found to be targeted only by probe PLA886 but not by probe EUK1379, leading us to the assumption that the *Planctomycetes* rRNA might be overestimated. The slot-blot values measured with PLA886 should therefore not be used to quantify their abundance. Reliable FISH quantification with PLA886 (which was originally designed for this purpose) is, however, possible because of clear microscopic differentiation between prokaryotes and eukaryotes based on cell size and organization. FISH data show that *Planctomycetes* account for 3 19% of all prokaryotic cells. The highest abundance occurred in the top 8 cm in the March and July 2000 samples, the highest absolute number of 1.8×10^8 cells ml^{-1} in July 2000 at 5 cm depth. In all but one depth layer of this summer core *Planctomycetes* made up more than 1.0×10^8 cells ml^{-1}. We consider this a direct consequence of the higher abundance of phytoplankton in July and postulate a dominant role of *Planctomycetes*, especially of the *Pirellula/Blastopirellula/Rhodopirellula* group [53], in the main microbial process occurring in the upper layers of sandy sediments, the aerobic heterotrophic mineralization of algal biomass [13]. Genome analysis of *Rhodopirellula baltica*, a coastal isolate of *Planctomycetes*, has revealed a high number of sulfatases involved in the degradation of polysaccharidic algal cell wall components [18].

High abundance of members of the CF of the *Bacteroidetes* was first reported from marine sediments by Llobet-Brossa et al. [32]. Since then, CF have often been found to be abundant in oxic and anoxic regions of marine sediments (e.g., [6,26,43,50]), but also in coastal marine bacterioplankton (e.g., [8,15,16]). Similar to *Planctomycetes*, many members of this group are specialized in the degradation of complex macromolecules. Even though they occur in enormous numbers in habitats rich in organic material, especially those involved in the aerobic or anaerobic degradation of algae blooms [17,46,47,52], some species can adapt to low nutrient levels as well [22,23].

In this study a clear seasonal difference in CF abundance was observed only at the sediment surface, with higher cell numbers and rRNA concentrations in July and October (Fig. 2A, B). The maxima of cell numbers (up to 1.8×10^8 cells ml^{-1}) and rRNA concentrations were in the upper 3 cm. For this probe, the two quantification methods agreed well, supporting a relative contribution of CF319a-positive cells to the microbial community of sandy sediments of 1 10%. Abundance was higher in surface layers, as also reported in previous studies on sandy sediments [26,32]. It seems that certain populations of the CF dwell in this mostly fully aerobic zone receiving relatively high amounts of organic matter from the overlaying water column [13]. Probing with CF319a showed also a relatively constant depth distribution down to 12 cm, supporting a recent study on 40-cm cores [26]. The relatively constant CF abundance below the maximum might be due to the presence of aerobic species sustained by occasional oxygen penetration to depths up to 8 cm based on water flow or to even higher depth based on bioturbation [13]. Alternatively, the presence of anaerobic, fermentative members of the CF in deeper sediment layers is also a possible explanation [43,47]. The *Bacteroidetes* 16S rRNA sequences have a much lower similarity to each other than, e.g., to those of the *Planctomycetes*. This supports the assumption that specific CF populations have different metabolic functions in sandy sediments.

Parallel investigations in the Sylt sediments [13] have shown that sulfate reduction rates range quite widely from 0.08 to 13.7 mmol m^{-2} d^{-1}, whereas aerobic respiration rates are more stable and higher (105 to 175 mmol m^{-2} d^{-1}). Previous studies on marine sediments have shown that SRB constitute an important part of the microbial community [4,31,44,51,60]. Within

the polyphyletic group of SRB, the deltaproteobacterial *Desulfosarcina/Desulfococcus* group was described as the most abundant in muddy (e.g., [31,39,44]) and sandy sediments [6,26]. Our study confirms these findings. Using FISH, probe DSS658 detected up to 81% of all cells hybridizing with a set of deltaproteobacterial probes and using slot-blot hybridization, up to 96% of the rRNA was detected with the same probe set. *Desulfosarcina* spp. and *Desulfococcus* spp. are known to be nutritionally versatile in their use of electron donors and are capable of complete oxidation of organic carbon to CO_2 [59].

Interestingly, both the cell numbers (up to 10%) and the rRNA values (11 23%) detected with DSS658 remained rather high in all seasons and depths. Although the rRNA values were highest in July (23%) when sulfate reduction rates were highest [13], the *Desulfosarcina/Desulfococcus* group is also abundant at times when only low sulfate reduction rates were recorded in parallel cores. The maximum of DSS658 rRNA in the upper part of the sediments contributes to the discussion whether and how SRB are able to use sulfate as an electron acceptor in oxic zones (e.g., [6,9,12,39]). It should be noted that during low tide the complete sediment can turn anoxic, creating temporarily optimal conditions for activity and growth of SRB which would subsequently just survive oxic phases. One might also speculate that alternative electron acceptors like Fe (III) or even oxygen are used by members of the *Desulfosarcina/Desulfococcus* group.

We refrain from an in-depth discussion of the quantitative hybridization results obtained with probes ALF968 (up to 15%) and GAM42a (up to 23%) since these group-specific probes are known to be compromised by both incomplete coverage of the target group and an ever increasing number of false-positive outgroup hits. The hybridization results obtained here by FISH and slot-blot hybridization agree with the high number of gammaproteobacterial 16S rRNA sequences in the clone library. Group-specific probing is an intermediate step in a complete description of community structure that requires in addition a larger set of more specific probes [2] targeting smaller groups, e.g., the *Roseobacter* clade within the *Alphaproteobacteria* or specific subgroups within the *Bacteroidetes*. It should also be examined whether the *Planctomycetes* are indeed dominated by species related to the *Pirellula* cluster.

In conclusion, the microbial community structure of Sylt sediments in the top 12 cm was relatively stable over depth, most probably due to deep substrate and oxygen penetration caused by advection and bioturbation. Seasonal differences of total cell numbers and rRNA concentrations correlated well with the availability of organic carbon and temperature. The relative importance of SRB for organic carbon mineralization in Sylt sediments may be reduced by the high input of oxygen [13]. However, this needs to be further investigated due to the high abundance of members of the *Desulfosarcina/Desulfococcus* group. The high abundance of presumably aerobic heterotrophic members of the phylum *Planctomycetes* accounting roughly for 5 15% of total prokaryotic cells is in good agreement with the high rates of aerobic degradation shown by de Beer et al. [13]. In the sandy sediments in Sylt, *Planctomycetes* seem to be even more abundant than members of the CF which might have a similar function. High values for *Planctomycetes* and the CF group have been reported for two other oxygenated sandy sediments [6,50], suggesting that this dominance might be a common characteristic feature for this type of ecosystem. Future molecular and cultivation studies should attempt to further characterize these two important groups of marine benthic bacteria.

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II

Molecular and morphological characterization of the symbiosis between bacterial endosymbionts and the nematode *Astomonema* sp. from the Bahamas

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Summary

Marine nematode worms of the genus *Astomonema* have been described from intertidal sandflats, deep-sea muds, and methane-rich pock marks. All *Astomonema* species lack a mouth and morphological studies show that they are associated with endosymbiotic bacteria, but to date nothing is known about the identity and phylogeny of symbionts from this host group. In this study, we characterized the symbiosis in an undescribed *Astomonema* species from coral reef sediments in the Bahamas. Phylogenetic analysis of the host based on its 18S rRNA gene showed that *Astomonema* sp. belongs to the class Chromadorea with non-symbiotic nematodes of the families Linhomoeidae and Axonolaimidae as its closest relatives. Comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization revealed that the *Astomonema* sp. symbionts belong to the *Gammaproteobacteria* and are most closely related (95–96% sequence similarity) to gammaproteobacterial sulfur-oxidizing symbionts from 2 other host groups: the Gamma 1 endosymbionts of gutless oligochaetes and the ectosymbionts of gut-bearing nematodes. The close phylogenetic relationship of the bacteria from *Astomonema* sp, gutless oligochaetes, and gut-bearing nematodes can not be explained by co-speciation given the distant relationships between these three host groups. Instead, the shared biogeographical distribution of these hosts is assumed to have played a key role in the formation of these symbiotic associations.

Introduction

Symbiotic associations between marine invertebrates and chemoautotrophic sulfur-oxidizing bacteria are widely found in nature, in habitats ranging from deep sea hydrothermal vents and cold seeps to shallow water, sheltered intertidal and subtidal sediments (Cavanaugh, 1985; Giere, 1992; Fisher, 1990; Nelson and Fisher, 1995). A wide variety of marine invertebrates from different phyla such as the Annelida, Bivalvia, or Nematoda are known to host such symbiotic associations (Fisher, 1990; Cavanaugh, 1994; Ott *et al*., 2004a, b; Bright and Giere, 2005; Dubilier *et al*., 2005). The bacterial partner can occur as an intra- or extracellular endosymbiont inside the animal, or as an ectosymbiont attached to the host's body. The symbionts use the energy obtained through oxidation of reduced sulfur compounds, such as sulfide, to fix CO_2 into organic compounds. These organic compounds are passed on to the host or the symbionts are digested (Southward, 1987; Ott *et al*., 1991).

Within the Nematoda both endo- and ectosymbiotic associations have been described. In the subfamily Stilbonematinae*,* ectosymbiotic bacteria are attached to the worm's cuticle and cover most of its body surface (Ott *et al*., 2004a, b). These worms have a fully functional gut and appear to graze on their symbionts (Polz *et al*., 1992, Ott *et al*., 1991, 2004b). In contrast, nematodes with endosymbiotic bacteria have reduced their digestive system. The mouth is absent and the gut, if present, is non-functional (Giere *et al*., 1995, Ott *et al*., 2004b). These nematodes belong to the genera *Astomonema* (Ott *et al*., 1982; Vidacovic and Boucher, 1987; Austen *et al*., 1993; Giere *et al*., 1995), *Parastomonema* (Kito, 1989) and *Rhabdothyreus* (Hope and Murphy, 1969; Riemann, 1993). Although *Astomonema* and *Parastomonema* species prefer shallow intertidal or subtidal sediments while *Rhaptothyreus* species live in deep sea sediments, all these nematodes have morphologically similar, oval-shaped endosymbiotic bacteria (Ott *et al*., 1982; Kito, 1989; Austen *et al*., 1993; Giere *et al*., 1995; Riemann, 1993).

The genus *Astomonema* contains four described species: *A. jenneri* (Ott *et al*., 1982), *A. otti*, *A. obscura* (Vidacovic and Boucher, 1987) and *A. southwardorum* (Austen *et al*., 1993; Giere *et al*., 1995). The reduction of the digestive system in all *Astomonema* species and the observation of large bacterial cells filling nearly the entire body of the worm suggests that the bacterial symbionts provide their hosts with nutrition (Ott *et al*., 1982; Giere *et al*., 1995). Moreover, the preferential occurrence of the nematodes in sulfidic sediments and the presence of large vesicles in the bacteria that are assumed to contain sulfur indicate that these symbionts are thiotrophic (Ott *et al*., 1982, 2004b; Giere *et al*., 1995). However, endosymbionts from marine nematodes have not yet been characterized using molecular methods so that nothing is currently known about their phylogenetic relationship to bacterial symbionts of other marine invertebrates.

In this study, we investigated the symbiotic association of *Astomonema* sp. from shallow intertidal sediments in the Bahamas. The ultrastructure of the symbiosis was examined by transmission electron microscopy (TEM). The phylogeny of the bacterial symbiont and the nematode host was investigated by comparative 16S rRNA and 18S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH) was used to confirm the symbiotic origin of the 16S rRNA sequence obtained from the worm.

Results and Discussion

Transmission electron microscopy. The *Astomonema* specimens have been collected at low tide from subtidal sediments off Lee Stocking Island. The sediment at the sampling site was coarse, calcareous, with small amounts of detritus and a yellow-pinkish color. The number of *Astomonema* sp. individuals was found to increase with increasing sediment depth as follows: 1.7×10^3 worms m^{-3} (at 0 to 5 cm depth), 3.4×10^4 m^{-3} (5 to 10 cm), 1.8×10^5 m^{-3} (10 to 15 cm) and 2.9×10^5 m^{-3} at 15 to 20 cm depth. The specimens varied in size from 1 to 4 mm long and 15 to 30 μm wide (Fig. 1) and they had a shiny white to pinkish colour which disappeared during fixation.

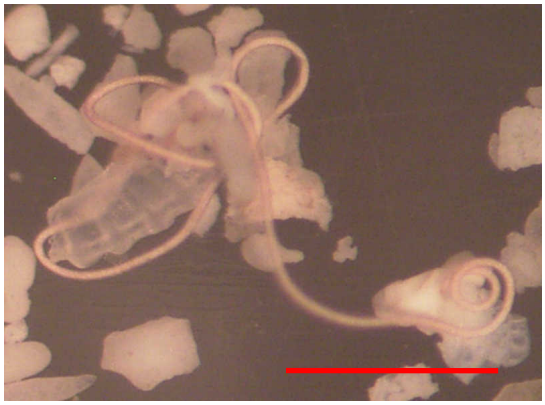

Fig. 1. Photomicrograph of *Astomonema* sp. from Bahamas coral reef sediments. Scale bar = 0.5 mm.

Cross sections through specimens of *Astomonema* sp. (Fig. 2A) showed that a central lumen was nearly completely filled with very large, extracellular bacterial cells, about 8–10 μm long and 4–5 μm wide (Fig. 2B). The bacteria were surrounded by a layer of long and irregular intestinal wall cells. This layer was distally bordered by muscular cells, the epidermis and the cuticle. Thus, the bacteria are extracellular endosymbionts, living in the gut lumen of the worms. A similar extracellular gut symbiosis was described in *A. southwardorum* from pockmarks in the North Sea (Giere *et al*. 1995).

The bacterial cytoplasm was densely filled with globular greyish and white vacuoles (Fig. 2B). The grey globules appeared membrane-bound, but details of the membrane structure could not be ascertained due to suboptimal fixation. The contents of the white vacuoles were presumably dissolved during the fixation procedure. The ultrastructure of the nematode bacteria was very similar to that of the sulfur-oxidizing Gamma 1 symbionts of gutless marine oligochaetes (reviewed in Dubilier *et al*. 2005 and Bright and Giere 2005). In

the oligochaete Gamma 1 symbionts, the bacterial cytoplasm is filled with sulfur and poly-hydroxy-beta-alkanoate vesicles.

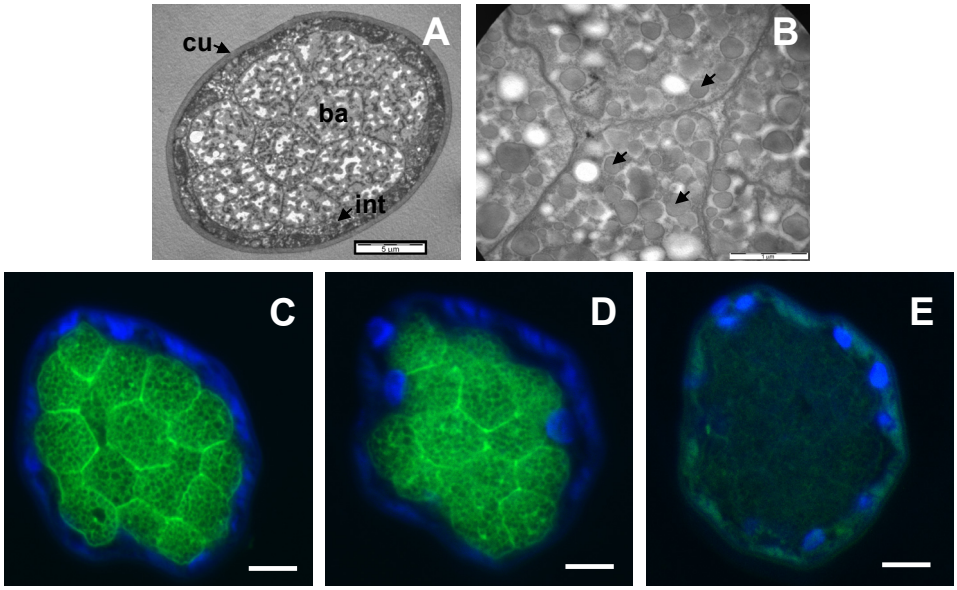

Fig. 2. Transmission electron micrograph (A, B) and FISH identification (C, D, E) of the endosymbionts of *Astomonema* sp. (A) The bacterial symbionts (ba) fill almost the entire body of the worm and are surrounded by intestinal cells (int), and the cuticle (cu). (B) Higher magnification of the symbionts showing numerous globular vacuoles in the bacterial cytoplasm. (C, D, E) FISH images showing consecutive cross-sections through the symbiont-containing region of a worm with DAPI staining of host nuclei in the body wall shown in blue. (C) Hybridization with the general bacterial probe (EUB338) with the symbionts in green; (D) Hybridization with the symbiont-specific probe A179 showing the bacterial symbionts in green; (E) Hybridization with the negative control probe (NON338). Scale bars are equivalent to 5 μm (A, C, D, E) and 1 μm (B).

Phylogeny of the host. The 18S rRNA sequences of the *Astomonema* sp. individuals shared 99.6–100% identity, indicating that they all belonged to the same species. In all phylogenetic analyses, the *Astomonema* sp. 18S rRNA sequence falls within the class Chromadorea of the phylum Nematoda (Fig. 3). The closest relatives are other marine nematodes from the families Axonolaimidae (*Axonolaimus helgolandicus*, *Cylindrolaimus* spp. and *Ascolaimus elongates*), and Linhomoeidae (*Desmolaimus zeelandicus*) with 89.2–89.5% sequence identity. Nematodes from the subfamily Stilbonematinae that are characterized by associations with ectosymbiotic bacteria are not closely related to *Astomonema* sp. (Ott *et al*., 2004a, b; Fig. 3).

Using morphological characteristics, Ott *et al*. (1982) placed the first *Astomonema* species described, *A. jeneri*, in the family Siphonolaimidae of the class Adenophorea (currently Chromadorea). There are currently no 18S rRNA sequences available for known members of the family Siphonolaimidae (such as *Siphonolaimus* spp.). Our molecular analysis therefore dose not confirm the placement of *Astomonema* in the family Siphonolaimidae. However, the close relationship of the *Astomonema* sp. from this study to members of the families Axonolaimidae and Linhomoeidae corresponds well with morphological analyses that describe these families as the closest relatives of the

Siphonolaimidae (Lorentzen 1981, 1994; Decraemer *et al*., 2003; De Ley and Blaxter, 2002). This suggests that at least the *Astomonema* sp. from this study belongs to the family Siphonolaimidae. An intriguing question for further studies is the taxonomic coherence of nematodes with endosymbiotic bacteria. Are nematode species such as *A. otti*, *A. obscura* (Vidakovic and Boucher, 1987), and *A. southwardorum* (Austen *et al*., 1993; Giere *et al*., 1995) really closely related? Significant morphological differences between these species suggest that their placement within the single genus *Astomonema* may not be correct (Ott *et al*., 2004b).

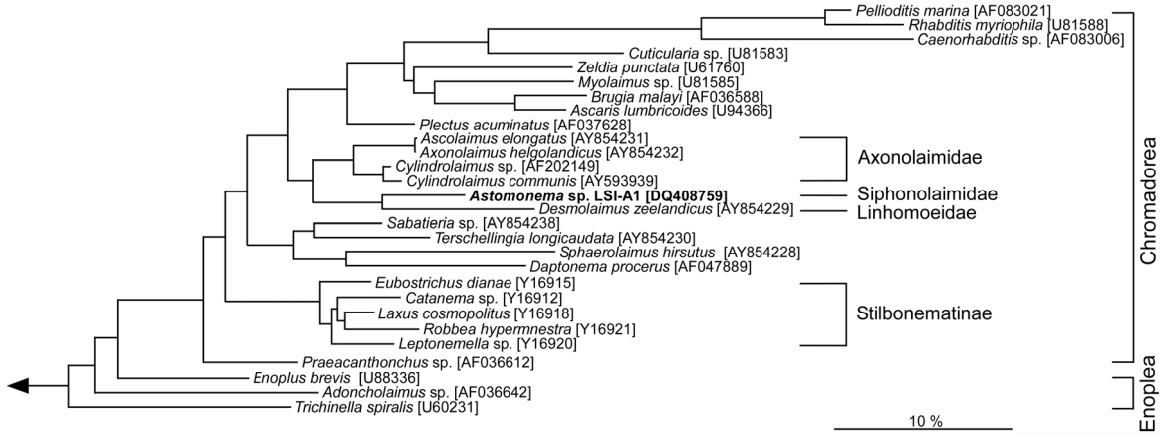

Fig. 3. 18S rRNA-based phylogenetic reconstruction showing the affiliation of the *Astomonema* sp. sequence (printed in bold) with selected reference sequences. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence. The nematode families indicated are in accordance with the classification system proposed by Lorenzen, 1994.

Phylogeny of the symbiont. 16S rRNA clone libraries were constructed for *Astomonema* sp. individuals collected in 1999 and 2004, and characterized by partial sequencing of randomly chosen clones. Comparison of the partial sequences showed that a single clone family belonging to the *Gammaproteobacteria* was dominant in all clone libraries (Table 1). Sequence identity within this clone family was always high (≥ 99.6% for partial sequences and ≥ 99.8% for full sequences).

Table 1. 16S rRNA clone libraries from 7 *Astomonema* sp. individuals. The numbers of fully sequenced 16S rRNA clones are shown in parentheses

Worm individual	1	2	3	4	5	6	7
Gamma symbiont	62 (9)	56 (8)	47 (6)	85 (6)	64 (4)	54 (2)	62 (3)
Others	1	3	1	1	5	0	1
Total no. of clones sequenced	63	59	48	86	69	54	63

Parsimony, distance, and maximum-likelihood analyses (Fig. 4) revealed that the dominant gammaproteobacterial 16S rRNA sequence from *Astomonema* sp. consistently grouped with the Gamma 1 sulfur-oxidizing endosymbionts of gutless oligochaetes such as *Olavius algarvensis* and *Inanidrilus leukodermatus* (95.7–95.9% sequence identity), and the sulfur-oxidizing ectosymbiont of the gut-bearing nematode, *Laxus* sp. (96.4% sequence identity). These sequences from symbiotic bacteria together with a clone sequence isolated from the Kazan mud volcano in the eastern Mediterranean have as closest cultivable relatives (90–92% sequence identity) a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family *Chromatiaceae* (Fig. 4).

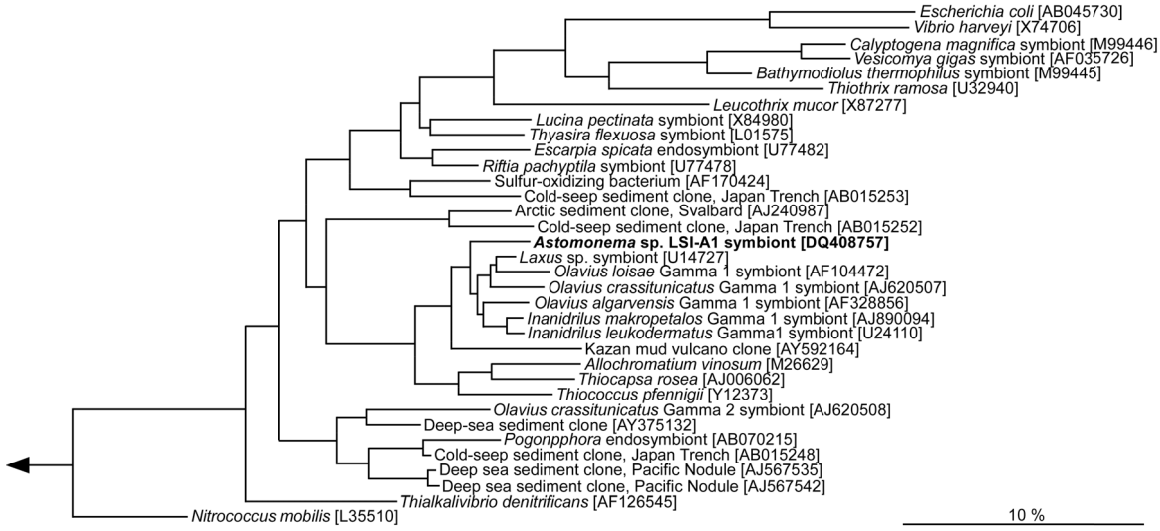

Fig. 4. 16S rRNA-based phylogenetic reconstruction showing the affiliation of the *Astomonema* sp. symbiont sequence (printed in bold) with selected reference sequences from the *Gammaproteobacteria*. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Fluorescence *in situ* hybridization (FISH) of the symbiont. FISH with the oligonucleotide probe A179 specific to the dominant 16S rRNA sequence from *Astomonema* sp. confirmed that this sequence originated from endosymbiotic bacteria in the worm (Fig. 2 D). The hybridization signal from the A179 probe was consistent in shape, size, and distribution with the morphology and structure of the large, oval-shaped bacteria observed with TEM in the body cavity of *Astomonema* sp. (Fig. 2A, B). The FISH signals from the A179 probe and the general Bacteria probe EUB338 (Fig. 2C) were similar, indicating that no additional bacteria are present in these worms. This corresponds well with the TEM analyses showing only a single bacterial morphotype in these worms. Positive FISH signals were also obtained with the general gammaproteobacterial probe GAM42a (data not shown), whereas the negative control probe NON338 showed no signal (Fig. 2E).

Protein-coding genes. The close phylogenetic association of the *Astomonema* sp. endosymbionts with symbiotic and free-living sulfur-oxidizing bacteria (SOB) and the ultrastructural similarities between these endosymbionts and SOB symbionts of gutless-oligochaetes suggests that the *Astomonema* sp endosymbionts might have similar metabolic abilities (Fig. 4, Dubilier *et al*., 2005; Bright and Giere, 2005). In order to verify this hypothesis, i.e. if the endosymbionts of *Astomonema* sp. have the ability to oxidize reduced sulfur compounds, amplification and sequence analysis of the adenosine-5'-phosphosulfate (APS) reductase gene was attempted. APS reductase is a key enzyme involved in both dissimilatory sulfate reduction and dissimilatory sulfur oxidation pathways of the sulfur metabolism. This enzyme is present in sulfate-reducing bacteria, where it catalyzes the reduction of APS to sulfite, and in sulfur-oxidizing microorganisms where is responsible for the reverse reaction (Rabus *et al*., 2004; Hipp *et al*., 1997; Sanchez *et al*., 2001; Friedrich, 2002). APS reductase has two subunits, alpha and beta, which form a 1:1 $\alpha\beta$ heterodimer. These two subunits are encoded by the *aprA* and *aprB* genes, respectively. The *aprA* gene was proposed as a useful phylogenetic marker for bacteria involved in both oxidative and reductive sulfur metabolism (Hipp *et al*., 1997).

Using specifically designed primers (Kuever, unpublished) the *aprA* gene from two *Astomonema* sp. specimens was amplified, cloned and sequenced. The deduced amino acid sequences, comprising 131 amino acids, were identical between the *Astomonema* sp. individuals and most closely related with the *aprA* of the Gamma 1 SOB symbionts of gutless oligochaetes *Inanidrilus leukodermatus* and *I. makropetalus* (98% sequence identity). The closest cultivated relatives were the sulfur-oxidizers *Thiobacillus denitrificans* (*Betaproteobacteria* 87% sequence identity) and *Chlorobium tepidum* (green sulfur bacteria 71% sequence identity) (Fig. 5). The presence of *aprA* and its close phylogenetic affiliation with *aprA* of free-living and symbiotic SOB further sustain the thiotrophic nature of the *Astomonema* sp. endosymbionts.

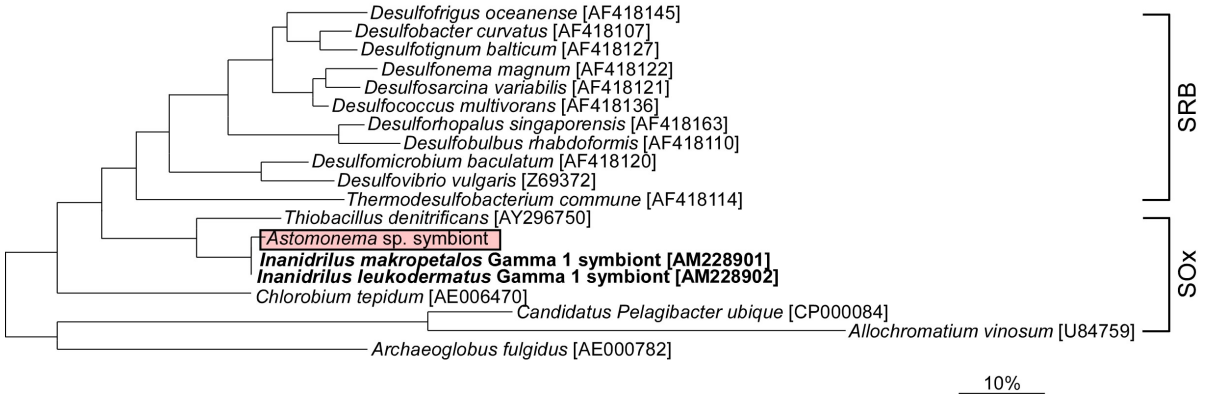

Fig. 5. Phylogenetic placement of APS genes (*aprA*) from *Astomonema* sp. symbiont (highlighted) based on deduced aminacid sequences. Clone sequences from symbionts are in bold face type. Sulfate-reducing bacteria (SRB), sulfur-oxidizing bacteria (SOx). The phylogenetic tree is based on maximum likelihood analysis. The bar represents 10% estimated sequence divergence.

Conclusions. Marine nematode worms lacking a mouth and gut were first described in 1969 (Hope and Murphy, 1969). It was not until the discovery of chemoautotrophic sulfur-oxidizing bacteria in the hydrothermal vent tube worm *Riftia pachyptila* in 1981 (Cavanaugh *et al*., 1981) that a similar type of symbiosis was hypothesized to occur in moutless nematodes (Ott *et al*., 1982; Giere *et al*., 1995). The analysis of the gene coding for enzyme diagnostic of sulfur metabolism, together with the presence of vesicles assumed to contain sulfur in the *Astomonema* sp. endosymbionts and their close phylogenetic relationship to the Gamma 1 sulfur-oxidizing symbionts of gutless oligochaetes indicates that the *Astomonema* sp. bacteria are also chemoautotrophic sulfur oxidizers. The complete reduction of the mouth in these worms suggests that the endosymbionts are their main source of nutrition.

The close phylogenetic relationship between the endosymbionts of *Astomonema* sp., those of gutless oligochaetes, and the ectosymbionts of gut-bearing nematodes is intriguing. These three host groups are not closely related to each other. It is therefore clear that these associations were not established through co-speciation but rather independently of each other in convergent evolution. Given the regular co-occurrence of these three host groups in reduced sediments of coral reefs, it is likely that their exposure to a similar pool of environmental microbes led to the uptake of very closely related bacteria as symbionts. The extracellular position of the symbionts in all these host groups is a common characteristic that supports this assumption. Despite the monophyly of these nematode and oligochaete symbionts, their level of integration and location in or on the worm is very different within each host group: the *Astomonema* sp. endosymbionts occur in the gut lumen, the oligochaete endosymbionts underneath the cuticle, and the stilbonematinae nematode ectosymbionts on the cuticle. This suggests a remarkable flexibility of the ancestors of these bacterial symbionts, in their ability to establish associations with different hosts developing variable ecto- and endosymbioses.

Experimental procedures

Specimen collection. The collection site was located near Lee Stocking Island, Exuma Cays, Bahamas (23°46'N, 076°06'W). Worms were collected during low tide from shallow subtidal sediments in 1999 and 2004. The average water depth at the sampling site at low tide was 2 m. In order to determine the optimum sediment depth for sampling with the maximum abundance of individuals, six different sediment cores were collected from the same area (approx. 0.5–1 m distance between cores) by using a plexiglas corer of 5 cm diameter and 25 cm length. Each core was sliced in sub-cores as follows: 0–5 cm, 5–10 cm, 10–15 cm and 15–20 cm. The sub-cores corresponding to the same depth were pooled

together. The worms were extracted by decantation and sieving through a sieve with a mesh size of 63 μm, counted and collected under a dissecting microscope. We found the highest number of individuals between 15–20 cm sediment depth (see Results). Multiple other cores were then sampled from this depth only, proceeding with the specimen collection as described above. After collection, the worms were fixed in 70% ethanol and stored at 4°C for DNA analyses and for FISH, and in Trump's fixative (McDowell and Trump, 1976) for TEM.

DNA extraction and PCR amplification. DNA was extracted individually from *Astomonema* specimens as described (Schizas *et al*., 1997), with slight modifications. Proteinase K treatment was followed by three cycles of freezing (3 min in liquid nitrogen) and thawing (3 min 65°C). The DNA was purified with the Gene Releaser reagent (BioVentures, Murfreesboro, Tenn.) according to the manufacturer's specifications. The 16S rRNA and the 18S rRNA genes were amplified by PCR using the universal bacterial primers 8f (Hicks, 1992) and 1492r (Kane, 1993) and the general eukaryotic primers 1F (Winnepenninckx, 1995) and 2023r (Pradillon *et al*., 2006), respectively. In addition to the rRNA genes, the gene coding for the APS reductase (*aprA*) from two *Astomonema* specimens was amplified. For the specific amplification of the *aprA* gene the primers aps1F and aps4R (Kuever, unpublished data) were used.

Cloning and sequencing. The PCR products from each specimen were purified (PCR purification Kit, Qiagen, Hilden, Germany) and cloned separately using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. For screening of 16S rRNA genes, 48–86 clones per individual were randomly picked and controlled for the correct insert size by PCR with the M13F and M13R primers (Yanisch-Perron *et al*., 1985). For screening of the 18S rRNA and *aprA* genes, 2–3 clones per individual were checked for the correct insert size by PCR with the same primers (M13F and M13R). PCR products of the correct size (~ 1500 bp) for the 16S rRNA gene were further screened by partial sequencing using the GM1F primer (Muyzer *et al*., 1993). The partial sequences were aligned and compared with the BioEdit software (Hall, 1999), and grouped based on sequence similarity (more than 99.6%). Representative clones from each similarity group were fully sequenced. PCR products of the right size for the 18S rRNA gene (~ 2000 bp) and aprA gene (400 bp) were fully sequenced. All sequencing reactions were performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, Calif., USA). The 16S rRNA sequence of the symbiont and the 18S rRNA sequence of the *Astomonema* sp. were deposited in the GenBank database under accession numbers DQ408757 (symbiont) and DQ408759 (host).

Phylogenetic analysis. The 16S rRNA sequences of the symbiont and the 18S rRNA sequences of the host were compared with sequences in the database using BLAST for similarity searches (Altschul *et al*., 1990). The 16S rRNA and 18S rRNA sequences were analyzed with the ARB software package (Ludwig *et al*., 2004). Phylogenetic trees were calculated by performing parsimony, distance, and maximum likelihood analyses with different filter sets. For tree reconstruction, only sequences with more than 1250 bp for 16S rRNA sequences and more than 1400 bp for 18S rRNA sequences were used. For the 16S rRNA gene phylogenetic tree, sequences of *Rhizobiaceae*, *Alphaproteobacteria*, were used as out-group. Sequences of *Priapulus* spp. and *Halicryptus* spp. (phylum *Priapula*) were used as out-group for the 18S rRNA phylogenetic tree.

FISH. Twenty *Astomonema* sp. specimens were prepared for FISH analyses as follows: the ethanol fixed specimens were embedded in 3% agar (BD Diagnostic Systems, Heidelberg, Germany) as previously described (Winsor, 1994), dehydrated in an increasing ethanol series, and embedded in paraffin. Sectioning of the samples and prehybridization treatments were performed as described (Dubilier *et al*, 1995), with slight modifications: tissue sections were incubated for 5 min in xylene, ethanol and proteinase K. The proteinase K concentration in the reaction buffer (0.01 M Tris-HCl [pH 7.8], 0.05 M EDTA [pH 8], 0.5% SDS) was increased from 5 to 50 μg ml^{-1}. For *in situ* detection of the bacterial symbionts, catalyzed reporter deposition (CARD)-FISH with horseradish peroxidase (HRP)-labeled probes and tyramide signal amplification was applied as described (Pernthaler *et al*., 2002). Following prehybridization treatment (see above), tissue sections were hybridized with the HRP-labeled probes for 3 h at 46°C. After hybridization the sections were washed for 15 min at 48°C in washing buffer (Pernthaler *et al*., 2002), and equilibrated in 1 x phosphate-buffered saline (PBS, pH 7.6) for 30 min at room temperature (RT). The moist tissue sections were incubated with the amplification solution (1 × PBS, 0.0015% [vol/vol] H_2O_2, 1% Alexa Flour 488 dye [Molecular Probes, Leiden, The Netherlands]) for 20 min at 37°C in the dark and rinsed in 1× PBS for 15 min at RT. After air drying, sections were stained with 3 μg ml^{-1} 4',6'-diamidino-2-phenylindol (DAPI) for 5 min at RT in the dark. After DAPI staining the tissue sections were washed in 80% ethanol, air dried, and embedded in a mixture of low fluorescence glycerol mountant (Citifluor AF1, Citifluor Ldt, London) and mounting fluid Vecta Shield (Vecta Laboratories, Burlingame, CA) in a 3:1 v/v ratio, and stored for microscopic evaluation at −20°C for 1–2 days. The oligonucleotide probe A179 targeting the 16S rRNA sequence of the *Astomonema* sp. symbiont (Table 2) was designed with the PROBE_DESIGN tool of the ARB program. Probe specificity was checked against the ARB dataset and the Ribosomal Database Project (Maidak *et al*., 2000). Probe specificity was tested with a pure culture of *Thauera linaloolentis*, strain 47Lol

(DSM 12138) that has a 16S rRNA sequence with a region containing 1 central mismatch to the probe A179. Two recently published environmental clones from groundwater samples (Miyoshi *et al*., 2005) have 16S rRNA sequences with a region that is 100% identical to the specific probe A179. Since these sequences were retrieved from a terrestrial environment and affiliated with the *Betaproteobacteria*, it is highly unlikely that they occur in *Astomonema* sp.. Nevertheless, for a direct confirmation, cross-sections of the worms were hybridized with the general betaproteobacterial probe (BET42a). The hybridized cross-sections showed no signal (data not shown). The general Bacteria probe (EUB338) (Amann *et al*., 1990) and the general gammaproteobacterial probe (GAM42a) (Manz *et al*., 1992) were used as positive controls, and the nonsense probe (NON338) was used as a negative control. All hybridizations were performed with formamide concentrations that ensured high specificity (Table 2).

Table 2. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5'-3')	Target RNA	Position[a]	CARD-FISH (FA)[b]	Source or Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S	338-355	35	Amann *et al*., 1990
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	23S	1027-1043	35	Manz *et al*., 1992
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	23S	1027-1043	35	Manz *et al*., 1992
A179	Gamma symbiont	TTTCTCCCACAGGACGTA	16S	179-197	30-35	This study
NON338	Negative control, antisense of EUB338	ACTCCTACGGGAGGCAGC	16S	338-355	35	Wallner *et al*., 1993

[a] Position in the 16S or 23S rRNA of *E.coli.*
[b] FA, formamide concentrations in the hybridization buffer calculated as percent (vol/vol).
BET42a was used as a competitor probe for GAM42a.

Transmission electron microscopy

The TEM fixed *Astomonema* sp. specimens were washed in a 0.05 M solution of Na-cacodylate and post-fixed in 0.5% osmiumtetroxide. Alcohol dehydration was followed by embedding in LR-White resin and further conventional processing and sectioning for TEM preparations.

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III

Molecular characterization of chemoautotrophic sulfur-oxidizing symbionts in co-occurring closely related nematode species of the genus *Leptonemella*

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Summary

Sylt sandy sediments were recently described to harbour a very rich population of symbiont-carrying nematodes belonging to the genera *Leptonemella* (subfamily Stilbonematinae). Two species, *L. aphanothecae* and *L. vicina*, carrying morphologically similar ectosymbiotic bacteria, were described as the most abundant nematodes in these sediments. The two host species were differentiated and characterized by analysis of the 18S rRNA gene. The 18S rRNA sequence analysis showed that the two nematode species were closely related, sharing 96–96.3% sequence identity, and were phylogenetically affiliated with the subfamily Stilbonematinae, family Desmodoridae (94–95% sequence identity). 16S rRNA sequences obtained from symbionts of both host species were nearly identical, (99.5% sequence identity) with only minor nucleotide differences. These differences were not consistent within the symbionts of each host group and did not form a specific pattern that could differentiate between symbionts of the two hosts. Phylogenetic analyses showed that symbiont sequences of *Leptonemella* sp. were clustering together within the *Gammaproteobacteria* and formed a monophyletic clade with sequences of endosymbionts of gutless oligochaetes (*Olavius* sp., *Inanidrilus* sp.) and ectosymbionts of the gut-bearing nematode *Laxus* sp. Internal transcribed spacer (ITS) fragments of symbionts from both hosts had approximately the same length and contained two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. The ITS sequences were highly similar and a species-specific pattern was not observed. In this context, the results obtained in this study strongly suggest that the symbionts of the two *Leptonemella* species are at most sub-species, if not strains of the same species. Alternatively, the possibility that the symbionts of the two *Leptonemella* species are indeed species-specific, but cannot be distinguished by using ITS analysis cannot be ruled out.

Introduction

Free living marine nematodes harbouring sulfur-oxidizing chemoautotrophic ectosymbiotic bacteria constitute an important part of the metazoan community in sulfidic benthic habitats. These symbiont-bearing nematodes are members of the subfamily Stilbonematinae within the family Desmodoridae of the class Chromadorea (Ott *et al*., 2004). Up to now, 30 symbiont-bearing nematode species belonging to 8 genera have been described from different areas ranging from tropical and subtropical (Caribbean Sea) to temperate zones (Mediterranean Sea, North Sea) (Ott and Novak 1989; Ott *et al*., 1991; 2004; Ott 1995; Riemann *et al*., 2003). They live in sheltered intertidal and subtidal sediments mainly at the interface between oxidized surface layer and the deeper anoxic sediment layer (Ott and Novak 1989; Ott *et al*., 1991).

Intensively studied in the past 15 years, these symbioses revealed a remarkable specific association between partners. Thus, the symbionts which cover the greatest part of the host body in a highly ordered and species-specific pattern require reduced sulfur compounds as well as electron acceptors (e.g. oxygen) for their oxidation (Ott *et al*., 1991, 2004). According with previous observations, the nematode hosts expose their symbionts alternately to reducing or oxidizing conditions by migrating between oxidized surface sediment layers and reduced sulfidic layers (Ott *et al*., 1991). The nematodes appear to take advantage from this association by grazing their own symbionts (Polz *et al*., 1992; Hoschitz *et al*., 1999; Riemann *et al*., 2003), and it has been suggested that the bacterial coat protect the host against toxic hydrogen sulfide (Ott, 1995; Hentschel *et al*., 1999). Sheltered intertidal sandy sediments off the Island of Sylt of the North Sea are known to harbour a very rich population of Stilbonematinae represented exclusively by members of the genus *Leptonemella* Cobb, 1920 (Riemann *et al*., 2003). Three species of *Leptonemella* co-occur in these sediments, and two of them, *L. aphanotecae* and *L. vicina*, are the most abundant inhabitants (Riemann *et al*., 2003). All *Leptonemella* species and even the juveniles from Sylt sediments were shown to carry a multilayered coat of coccoid bacteria on the body surface excepting the head (Riemann *et al*., 2003). The *Leptonemella* species as all Stilbonematinae species are moulting four times during their developments into adults removing their symbiotic bacterial coat together with the old cuticle (Ott *et al*., 2004). Although it is not yet known, how the symbionts are acquired, it was hypothesized that the worms may gain their symbionts from the environment after hatching or moulting through a specific recognition mechanism based on lectine-sugar interactions (Ott *et al*., 2004; Nussbaumer *et al*., 2004). Considering the very close relationship between *L. aphanotecae* and *L.vicina* (only slight species differences, Riemann *et al*., 2003) and their co-existence, it

is of a great scientific interest to establish whether the symbionts of *L. aphanotecae* and *L. vicina* are identical or whether each host species carry its own species-specific symbiont.

In order to answer to this question the whole 16S rRNA gene together with the 16S-23S rRNA internal transcribed spacer region (ITS or ISR) from the symbionts were amplified and compared. Fluorescence *in situ* hybridization was applied to confirm that the sequences obtained correspond to the symbiotic bacteria. Due to morphological similarities between *L. aphanotecae* and *L. vicina,* the two hosts were identified based on 18S rRNA gene sequencing.

Material and Methods

Study site and sampling procedure. The Wadden Sea is a coastal area along the Dutch, German, and Danish North Sea coast. The site of investigation is located in the northern Wadden Sea on an intertidal sand flat situated adjacent to the Wattenmeer Research Station in List on the Island Sylt ("Hausstrand", 55° 5' N/8° 26' E). For a detailed site description, see Riemann *et al*., 2003.The sampling was performed at low tide, in the middle part of the flat, near *Arenicola marina* burrows in October 2003 and March 2004. All samples were taken using a plexiglas corer of 5 cm diameter and 25 cm length which was usually pushed up to 20 cm depth into the sand. The worms were extracted by decantation and sieving through a sieve with a mesh size of 63 μm, and collected under a dissecting microscope. After collection, the worms were fixed in 70% ethanol and stored at 4 °C for DNA analyses and for fluorescence *in situ* hybridization (FISH).

DNA extraction and PCR amplification. The total DNA was extracted from 8 worms individually as described (Schizas *et al*., 1997), with slight modifications. The Proteinase K treatment was followed by three cycles of freezing (3 min in liquid nitrogen) and thawing (3 min 65 °C). The DNA was purified with the Gene Releaser reagent (BioVentures, Murfreesboro, Tenn.) according with the manufacturer's specifications. The whole 16S rRNA gene together with the ITS region were amplified using bacterial primers 8f (position 8 of the E. coli16S rRNA gene; Hicks, 1992) and L189r (position 189 of the E.coli 23S rRNA gene; Yu and Mohn, 2001). The specific designed primers Nem01F (5'-GCCGAATTATGGTGA-3') and Nem01R (5'-CAGACAAATCGCTCC-3') were used to amplify a 1200 bp fragment of the 18S rRNA gene of the nematode hosts. All primers were purchased from Interactiva (Ulm, Germany). PCR for the 16S rRNA-ITS fragment was performed as follows: 50 pmol of each primer, 2.5 μmol of each dNTP, 1 x Super-*Taq*-buffer (HT Biotehnology Ltd, Cambridge, UK) and 1 U of *Taq* DNA polymerase (Promega, Mannheim, Germany) were adjusted to a total volume of 50 μl with sterile water. After an initial denaturation step of 1 min at 96 °C, 28 cycles at 94 °C for 1 min, 48 °C for 2 min, and

72 °C for 3 min were performed followed by a final elongation at 72 °C for 10 min. The 18S rRNA fragment was amplified in the same conditions but with an annealing temperature of 42 °C.

Cloning and sequencing. The PCR products of each specimen were purified (PCR purification Kit, Qiagen, Hilden, Germany) and separately cloned by using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. For screening of 16S rRNA-ITS and the 18S rRNA genes, 5-10 clones were randomly picked and controlled for the correct insert size (~ 2400 bp) and ~1400 bp, respectively by PCR with M13F and M13R primers (Yanisch-Perron *et al*., 1985). PCR products of the correct size were purified and sequenced. All sequencing reactions were performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, Calif., USA).

Sequence analysis. The 16S rRNA-ITS sequences of the symbionts and the 18S rRNA sequences of the hosts were compared with sequences in the GenBank database by using BLAST for similarity searches (Altschul *et al*., 1990). Also, these sequences were routinely aligned and compared with the BioEdit software (Hall, 1999). The 16S rRNA and 18S rRNA sequences data were further analyzed with the ARB software package (Ludwig *et al*., 2004) and used for phylogenetic trees reconstruction. Phylogenetic trees were calculated by performing parsimony, distance, and maximum likelihood analyses by using different sets of filters. For tree reconstruction, only sequences with more than 1250 bp were used. ITS sequences were screened for the presence of tRNA genes using the tRNA gene search tool at http://bioweb.pasteur.fr/seqanal/interfaces/fastrna.html.

Fluorescence *in situ* hybridization. Ten *Leptonemella* sp. specimens were prepared for FISH analyses as follows: the ethanol fixed specimens were embedded in 3% agar (BD Diagnostic Systems, Heidelberg, Germany) as previously described (Winsor, 1994), dehydrated in an increasing ethanol series, and embedded in paraffin. Sectioning of the samples and prehybridization treatments were performed as described (Dubilier *et al*, 1995), with slight modifications: tissue sections were incubated for 5 min in xylene, ethanol and proteinase K. The proteinase K concentration in the reaction buffer (0.01 M Tris-HCl [pH 7.8], 0.05 M EDTA [pH 8], 0.5% SDS) was increased from 5 to 50 μg ml^{-1}. For *in situ* detection of the bacterial symbionts, catalyzed reporter deposition (CARD)-FISH with horseradish peroxidase (HRP)-labeled probes and tyramide signal amplification was applied as described (Pernthaler *et al*, 2002). Following prehybridization treatment (see above), tissue sections were hybridized with the HRP-labeled probe for 3 h at 46 °C. After hybridization the sections were washed for 15 min at 48 °C in washing buffer (Pernthaler *et*

al., 2002), and equilibrated in 1 x phosphate-buffered saline (PBS, pH 7.6) for 30 min at room temperature. The moist tissue sections were incubated with the amplification solution (1 x PBS, 0.0015% [vol/vol] H_2O_2, 1% Alexa Flour 488 dye [Molecular Probes, Leiden, The Netherlands]) for 20 min at 37 °C in the dark and rinsed in 1 x PBS for 15 min at room temperature. After air drying, sections were stained with 3 µg ml^{-1} 4',6'-diamidino-2-phenylindol (DAPI) for 5 min at room temperature in dark. After DAPI staining the tissue sections were washed in 80% ethanol, air dried and embedded in a mixture of low fluorescence glycerol mountant (Citifluor AF1, Citifluor Ldt, London) and mounting fluid Vecta Shield (Vecta Laboratories, Burlingame, CA) in a 3:1 v/v ratio, and stored for microscopic evaluation at −20 °C for 1–2 days. The general *Bacteria* probe (EUB338) (Amann *et al*., 1990) and the general *Gammaproteobacteria* probe (GAM42a) (Manz *et al*., 1992) were used to detect the bacterial symbionts, and the nonsense probe (NON338) was used as a negative control. All hybridizations were performed with formamide concentrations that ensured high specificity or with competitor (Table1).

Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5'-3')	Target RNA	Position[a]	CARD-FISH (FA)[b]	Source or Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S	338-355	35	Amann *et al*., 1990
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	23S	1027-1043	35	Manz *et al*., 1992
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	23S	1027-1043	35	Manz *et al*., 1992
NON338	Negative control, antisense of EUB338	ACTCCTACGGGAGGCAGC	16S	338-355	35	Wallner *et al*., 1993

[a] Position in the 16S or 23S rRNA of *E.coli.*
[b] FA, formamide concentrations in the hybridization buffer calculated as percent (vol/vol).
BET42a was used as a competitor probe for GAM42a.

Results and Discussions

Molecular characterization of the nematode host

L. vicina and *L. aphanothecae* described in Sylt sediments are very similar morphologically, making their identification and differentiation problematic. Furthermore, these worms tend to coil in compact curls, obscuring anatomical details that are important for species differentiation (Riemann *et al*., 2003). Identification of these two nematodes species based on their morphology was attempted, but the results were not always in accordance with the 18S rRNA sequencing. Nevertheless, the strict identification of the hosts as *L. vicina* or *L. aphanothecae* was not relevant for the purpose of the present work, and therefore they were further differentiated as species A and B based only on 18S rRNA gene sequencing.

A 1200 bp fragment of the 18S rRNA gene was amplified with specifically designed primers, cloned and sequenced. For each individual, 5 to 10 sequences were obtained and analysed using the ARB software (Ludwig *et al*., 2004). Comparison of these sequences revealed two different 18S rRNA phylotypes sharing 96–96.3% sequence identity, which corresponded to the host species A (4 individuals) and species B (4 individuals). The sequence identity within each host group was ≥ 99.8% for species A and ≥ 99.6% for species B. Phylogenetic analysis revealed that *Leptonemella* sp. A and *Leptonemella* sp. B were most closely related with each other and with an undescribed *Leptonemella* sp. collected from shallow sediments in Adriatic Sea (95% sequence identity). These nematodes formed, together with other symbiont-carrying nematodes of the subfamily Stilbonematinae (e.g. *Laxus*, *Robbea* or *Catanema* species) a distinct clade within the phylum Nematoda (Fig. 1). These results are in good agreement with previous phylogenetic analyses based on both 18S rRNA gene sequences and morphological characters (e.g. unique glandular sense organs) showing that all marine nematodes with sulfur-oxidizing chemoautotrophic ectosymbionts belong to a monophyletic group classified as the subfamily Stilbonematinae (Bauer-Nebelsick *et al*., 1995; Kampfer *et al*., 1998; Ott *et al*., 2004).

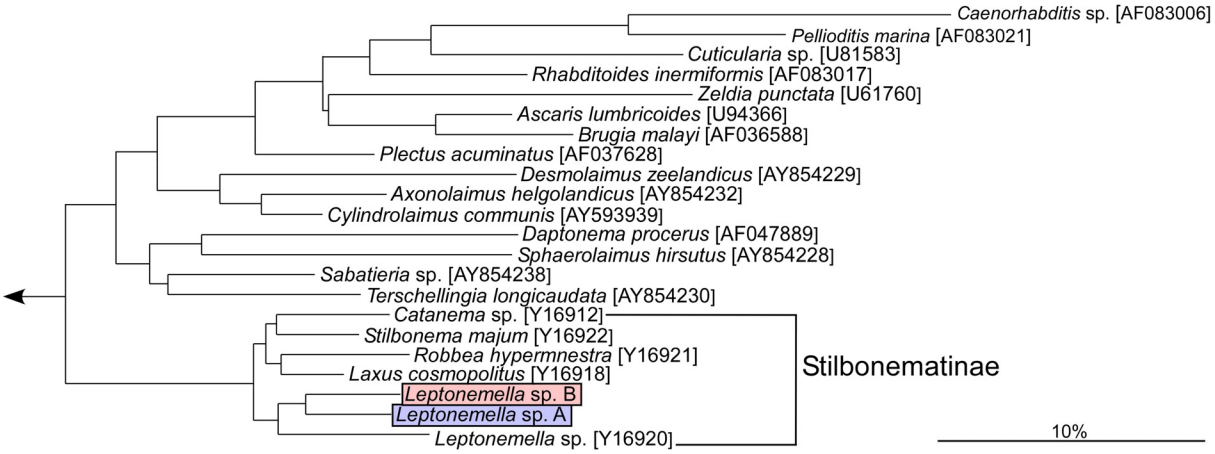

Fig. 1. 18S rRNA-based phylogenetic reconstruction showing the affiliation of the *Leptonemella* sp. A (blue frame) and *Leptonemella* sp. B (pink frame) with selected reference sequences. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Molecular characterization of the bacterial symbionts

A first approach for characterization of the symbiont was by sequencing of the 16S rRNA gene. Sequences obtained from symbionts of both host species were nearly identical, (99.5% sequence identity) with only minor nucleotide differences within almost complete 16S rRNA sequences (1496 bp) (Table 2). These minor differences were not consistent within each host group and did not form a specific pattern that could differentiate between symbionts of the two host species. Phylogenetic analyses showed that all symbiont sequences of *Leptonemella* sp. were clustering together within the *Gammaproteobacteria*

and formed a monophyletic clade with sequences of endosymbionts of gutless oligochaetes (*Olavius* sp., *Inanidrilus* sp.) and ectosymbionts of the gut-bearing nematode *Laxus* sp. (Fig. 2). Their closest relatives were the Gamma 1 symbiont of *Olavius algarvensis* and the ectosymbiont of *Laxus* sp. (96–98% sequence identity). These sequences of symbiotic bacteria are most closely related to a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family *Chromatiaceae* (91–93% sequence identity).

18S rRNA	16S rRNA nucleotide position									
	223	432	460	578	1010	1012	1031	1240	1250	1468
A-L33	C	C	C	T	G	G	T	G	A	A
A-L18	C	C	T	T	G	T	T	G	G	G
A-L11	C	T	C	T	G	G	T	G	A	A
A-L42	C	T	C	T	G	G	T	G	A	A
B-L34	T	C	T	T	G	T	T	G	A	G
B-L45	T	C	T	T	G	T	T	G	A	G
B-L6	T	C	T	T	G	G	T	G	A	G
B-L7	T	C	T	C	A	G	A	A	A	A

Table 2. Sequence differences at the level of 16S rRNA gene between symbionts of *Leptonemella* sp. A and B.

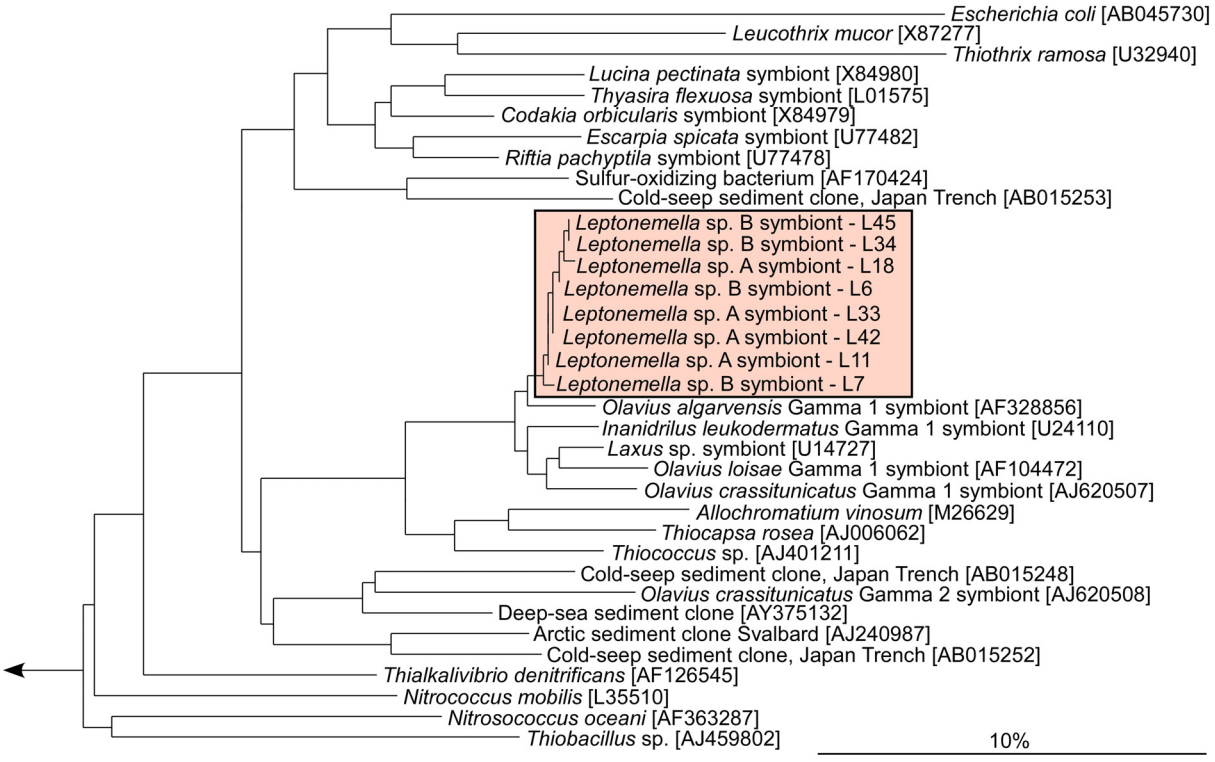

Fig. 2. 16S rRNA-based phylogenetic reconstruction showing the affiliation of the *Leptonemella* species A and B symbionts sequences (pink frame) with selected reference sequences from the *Gammaproteobacteria*. The phylogenetic tree is based on maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Previous phylogenetic studies on bacterial symbionts of gutless oligochaetes have shown that there is a constant association within the *Gammaproteobacteria* of these symbionts and the ectosymbionts of gut bearing nematodes (Dubilier *et al*., 1999, 2001; Blazejak *et al*., 2005).A plausible explanation for this close evolutionary association is the co-occurrence in similar geographic locations and environments of the two hosts groups which could influence the establishment of these symbioses (Dubilier *et al*., 2005). The affiliation of *Leptonemella* sp. symbionts to the oligochaete-nematode symbionts clade further supports the above hypothesis.

Comparison of the 16S rRNA sequences showed that the symbionts of the two *Leptonemella* species are very closely related and cannot be distinguished based on analysis of the 16S rRNA gene alone (Table 2). The 16S rRNA gene offers adequate resolution for comparisons from the species level up to kingdom level, but is often not divergent enough to give a good separation in close relationships such as species of the same genus or among conspecific strains (Stackebrandt & Goebel, 1994; Normand *et al*., 1996). For resolution at or below the species level, other more variable sequences, such as the internal transcribed spacer (ITS) located between the 16S and 23 rRNA genes, are analysed as a supplement to 16S rRNA sequencing (Jensen *et al*., 1993; Kong *et al*., 1999; Lee *et al*., 2002; Osorio *et al*., 2005). The ITS shows a high degree of variability between closely-related species and even between strains of the same species, both in length and as sequence information (Gürtler & Stanisich, 1996). The size of the spacer may vary considerably for different species, and even among the different operons within a single strain (Condon *et al*., 1995). Most bacterial species harbour multiple copies (alleles) of the ribosomal operon in their genome, feature which increases the sequence variation of the spacer regions. The variation in length is typically due to the type and number of the tRNA genes contained.

Since analysis of the 16S rRNA gene was clearly not sufficient to distinguish between symbionts of the two *Leptonemella* species, fragments encompassing, the almost complete 16S rRNA gene, the 16S-23S ITS and a small fragment (189 bp) of the 23S rRNA gene were amplified from the individuals previously investigated. The 16S rRNA gene was re-amplified together with the ITS to provide a correlation of the nucleotide differences observed in the 16S rRNA with the corresponding ITS sequences. Approximately 10 clones for each worm were sequenced and compared. The sequences from each individual worm were identical. All ITS fragments obtained had approximately the same length (472 to 475 bp) and contained two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. These tRNA genes showed 100% identity in all individuals investigated. Sequence identity between the ITS fragments of species A was 97–99%, while the sequence identities shared between ITS fragments of the species B was 95.5–100%. The sequence identities between the two species were within

the same range (95.3–99.6% sequence identity). In total, within 8 individuals investigated, 7 different ITS fragments were obtained. Only one ITS phylotype was shared by two individuals, L45 and L34, belonging to *Leptonemella* sp. B (Fig. 3). These two individuals had also identical 16S rRNA sequences. It seems that there is a strong correlation between the 16S rRNA nucleotide differences and the ITS phylotypes. As an example the 16S rRNA sequence of the individual A-18, which had the greatest similarity with the 16S rRNA sequences of individuals B-45 and B-34 (only one nucleotide different; Table 2), was also highly similar at the level of ITS (only 4 nucleotide different; data not shown). Despite the little variation between ITS phylotypes, a species-specific pattern was not observed (Fig. 3).

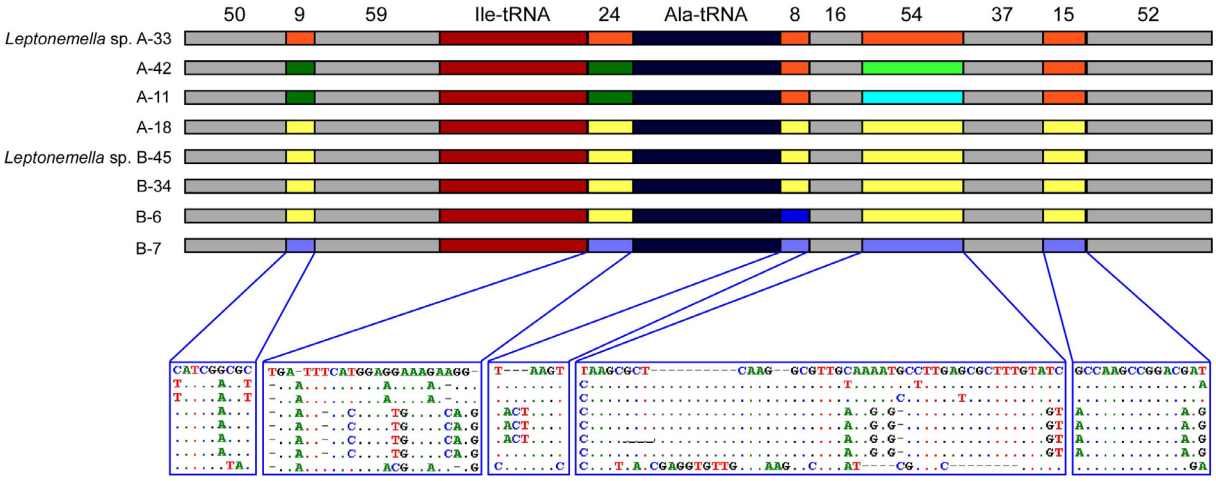

Fig. 3. Mosaic-like structure of ISR sequences of the symbionts of Leptonemella species A and B. The grey colour represents sequence blocks that are identical in ISR of all symbionts investigated. Other colours indicate sequence blocks that varied between different individuals. The sequence information of the variable regions is shown as alignment. Nucleotide positions that are conserved are indicated by dots. Gaps that have been included to obtain optimal sequence alignment are indicated by dashes. Identical fragments between sequences are denoted by the same colour. Two tRNA genes (for Ile and Ala) were found in all symbiont's ISR sequences. The numbers above columns represent sequence length in bp.

In other studies, ITS sequence analyses clearly showed significant differences between subspecies of the same species. Thus, from two subspecies of *Photobacterium damselae* (*P. damselae* subsp. *piscida* and *P. damselae* subsp. *damselae*) that have identical 16S rRNA genes but are morphologically distinguishable, a number of 17 different ITS variants have been amplified. These varied in length from 296 to 803 bp and contained different tRNA genes (Osorio *et al*., 2005). The result obtained with symbionts of the two *Leptonemella* species is more close to the situation encountered between strains of the same species (Kong *et al*., 1999). In such a case, comparison of ITS fragments from two strains of *Aeromonas hydrophila* showed sequence identity of 95 to 99% and the presence of identical $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (Kong *et al*., 1999).

In this context, the results obtained in the present study strongly suggest that the symbionts of the two *Leptonemella* species are at most sub-species, if not strains of the same species. Obviously, this mix of symbiont strains co-occurring in Sylt sediments is able

to colonize both hosts, indicating very similar surface-specific recognition mechanisms. A similar result was obtained by Won *et al*. (2003) in an investigation by using ITS sequencing to differentiate between SOB endosymbionts of two vent mussels, *Bathymodiolus putioserpentis* and *B. azoricus,* from Mid-Atlantic Ridge. The symbionts of these mussel species shared the same 16S ribotype and species-specific ITS patterns at sites were the host species were not mixed. At sites where the hosts were mixed, the species-specific ITS patterns were not found anymore, and both *Bathymodiolus* species carried a mix of symbiont strains. Another possibility is that the symbionts of the two Leptonemella species are indeed species-specific, but cannot be distinguished by using ITS analysis. To verify this hypothesis, a larger number of *Leptonemella* individuals from both species, A and B, have to be investigated. The analysis should be expanded by including other genes such as 23S rRNA, protein-coding genes *aprA* or RubisCO or more neutral gene such as housekeeping genes. Also, higher resolution methods such as multi locus sequence analysis (MLSA) or the use of repetitive extragenic palindromes as priming sites to generate diagnostic banding patterns (REP-PCR) could be applied to differentiate between these symbionts (Gilson *et al*., 1987; Versalovic *et al*., 1991).

In situ detection of the symbionts

Catalyzed reporter deposition (CARD)-FISH with general eubacterial (EUB338) and group specific (GAM42a) probes was applied on *Leptonemella*-cross sections (Fig. 4). The hybridization result confirmed that the symbionts were affiliated with the *Gammaproteobacteria*. A further confirmation that the 16S rRNA sequences and the corresponding ITS fragments analysed originate from the symbionts would be provided by hybridization with specifically designed probes.

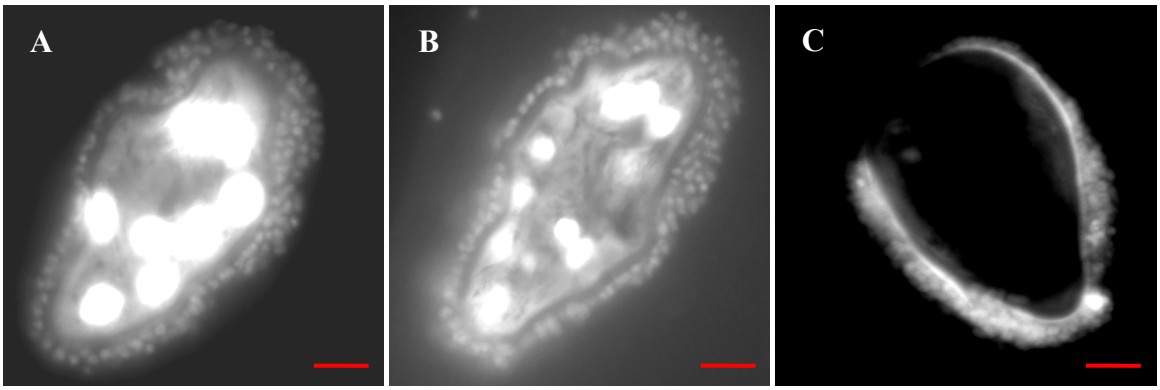

Fig. 4. In situ detection of symbionts of *Leptonemella* sp. FISH images showing cross-sections through the worm with DAPI staining of host nuclei (white-shiny). (A) Hybridization with the general bacterial probe (EUB338); (B) Hybridization with the group-specific probe GAM42a; (C) Hybridization with the negative control probe (NON338). Scale bars are equivalent to 10 μm.

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