# THE BACTERIAL COMMUNITY OF MARINE COPEPODS

by

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Photos on cover page:
Source: http://planktonnet.awi.de, 10/2010; Photos: Susanne Knotz
From left to right:
*Temora longicornis*, *Monstrilla helgolandica*, *Calanus helgolandicus*, *Acartia clausi*, *Centropages typicus*

# CONTENTS

# ABSTRACT

Marine copepods form one of the most abundant mesozooplankton groups in the oceans and are associated with various numbers of different bacterial species. This has already been shown using culture-dependent microbiological methods or microscopy and more recently by using molecular tools. The detection of human pathogens, such as *Vibrio cholerae* was the main focus of a great number of these studies. However, to date little effort has been made to obtain an overview of the whole bacterial community associated with the copepods.

Therefore, this thesis aims to provide more information about the whole bacterial community of marine copepods. After determining the best suited DNA extraction technique for investigating the copepod-bacteria-consortia for further analysis with denaturing gradient gel electrophoresis (DGGE), four copepod key genera (*Acartia* sp., *Temora* sp., *Centropages* sp. and *Calanus* sp.) were investigated over a sampling period of two years (February 2007 to March 2009). The bacterial community fingerprints of these different copepod taxa were compared for determining differences in the community composition of the different copepod genera and for temporal changes in community structure. Neither differences of the bacterial community patterns among different copepod genera nor seasonal changes or succession were found throughout the survey. To identify the members of the bacterial community associated with marine copepods, bacterial phylotypes of the four copepod key- and three less frequently appearing genera (*Pseudo-/Paracalanus* sp., *Euterpina* sp. and *Candacia* sp.) were identified by 16S rRNA gene sequencing. From the phylogenetic analysis, it could be concluded that the investigated marine copepods were associated with bacteria of four phyla: Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. *Roseovarius* spp., *Sulfitobacter* spp., *Psychrobacter* spp. and *Photobacterium* spp. were the most abundant bacterial genera. Some human and aquatic animal pathogens were identified but no *Vibrio cholerae*. Nonetheless, no copepod- or even copepod genera-specific bacterial communities could be detected.

The investigation of copepod associated bacteria is very important because copepods are the central part of the marine pelagic food web. They contribute to the microbial loop and serve as food for organisms of higher trophic levels.

This study contributed the first detailed insights of the whole bacterial community of different marine copepod genera. Additionally it provides insights whether the bacterial community composition is copepod genera-specific and/or whether it changes seasonally. These findings can provide a new platform for identifying bacterial symbionts and parasites of the copepods by investigating the function and abilities of the detected bacteria.

# CHAPTER I

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## BACTERIA IN THE MARINE PLANKTONIC FOOD WEB

Pomeroy (1974) proposed that microbes are the movers of energy and nutrients in marine food webs. This idea was later formulated as the 'microbial loop' (Azam et al. 1983). In the 'microbial loop' energy and nutrients lost from the planktonic food web in form of dissolved organic matter (DOM) are recovered and repackaged by heterotrophic bacterioplankton before entering the 'classic food web'.

The 'microbial loop' is functionally intertwined with the more familiar food web of plants, herbivores and carnivores (Fig. 1). It channels energy and carbon via bacteria to protozoa, to larger zooplankton such as copepods and krill, and on to jellyfishes, fishes and finally cetaceans (Pomeroy et al. 2007).

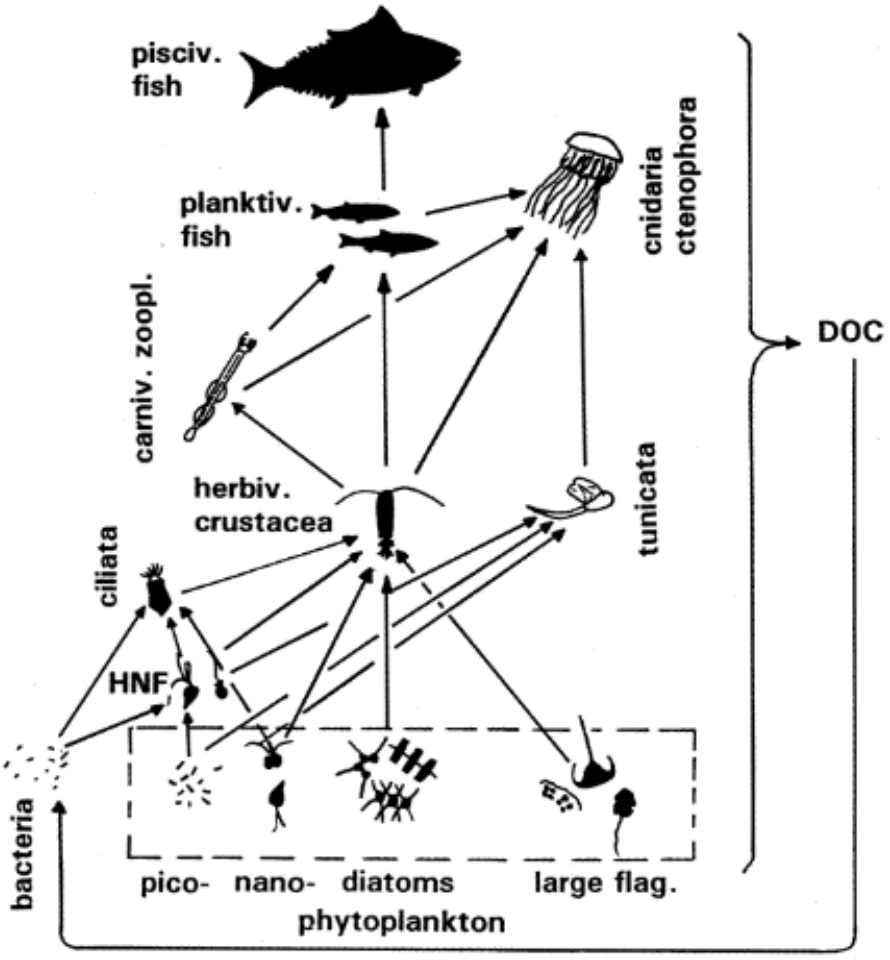



Fig. 1: Schematic overview of the marine food web (after Sommer 2005). DOC = dissolved organic carbon, HNF = heterotrophic nanoflagellates

Phytoplankton and heterotrophic bacteria form the basis of the food web, as primary producers and decomposers (Loreau 2001), but several other trophic levels are organised within the pelagic microbial food web. The main predators of planktonic prokaryotes (picoplankton, 0.2-2 µm) are small heterotrophic and mixotrophic nanoflagellates mainly of 3-5 µm in size (Sherr and Sherr 2002). Ciliates (>20 µm) in turn are important omnivorous grazers of nanoplankton (2-20 µm) (Sherr and Sherr 2002). Mesozooplankton (0.2-2 mm) which prey on phytoplankton as well as on different heterotrophic protists, connect the microbial food web with the classical algae-zooplankton-fish food chain (Kiørboe 1997).

Similar to other trophic levels, bacterioplankton communities are regulated by bottom-up factors, e.g. nutrient limitation, and also by top-down factors, e.g. protist predation and viral infection (Zöllner et al. 2009).

The marine environment offers a wide range of different habitats and niches which are occupied by bacteria. They can occur free-living in the water column or attached to particles, surfaces, such as the chitinous exoskeleton of copepods, and can be part of biofilms.

## PLANKTONIC BACTERIA

Being present in all aquatic ecosystems planktonic bacteria play a key role in the pelagic food web, both in transfers of organic energy and in cycling of nutrients (Azam et al. 1983; Azam 1998; Kan et al. 2006; Mary et al. 2006; Danger et al. 2007a; Danger et al. 2007b).

Traditionally, bacteria have been considered primarily as re-mineralisers of nutrients in dissolved (DOM) and particulate organic matter (POM) (Porter et al. 1988), but these microorganisms affect most biogeochemical processes in the oceans and their activities influence the element cycling on a global scale (Davey and O'Toole 2000; Zöllner et al. 2009). They play a major role in the production and degradation of organic matter, the degradation of many environmental pollutants, and the cycling of nitrogen, phosphorus, sulphur and many metals (Davey and O'Toole 2000).

Bacteria comprise a vast phylogenetic (Venter et al. 2004) as well as metabolic diversity (DeLong et al. 2006). Photoautotrophic bacteria derive energy through phytosynthesis and chemolitotrophs through the oxidation of inorganic

compounds whereas heterotrophic bacteria obtain energy from organic compounds. Furthermore, mixotrophs such as photo-heterotrophic bacteria are common marine organisms.

Generally, Alpha- and Gammaproteobacteria and Bacteroidetes dominate the free-living bacterial communities in sea water (Glöckner et al. 1999; Mary et al. 2006), but there are seasonal changes in abundance of the bacterial groups (Eilers et al. 2001; Gerdts et al. 2004; Fuhrman et al. 2006). These dynamics are regulated by seasonal changes in abiotic and biotic factors, especially phytoplankton dynamics and seasonal cycles in the source and lability of DOM (Crump et al. 2003). A direct relationship between temperature and bacterial production (Pinhassi and Hagström 2000) as well as seasonal succession of the community structure (Gerdts et al. 2004) was shown for marine bacterioplankton.

Perduzzi and Herndl (1992) documented the importance of marine copepods in powering bacterial populations by DOM production. Only the combination of phytoplankton and zooplankton yielded a significant increase in monomeric carbohydrate and a subsequent increase in bacterial numbers (Møller and Nielsen 2001; Danger et al. 2007b). The observed tight coupling between phytoplankton and bacteria on the one hand, and between bacteria and zooplankton on the other supported the idea of a trophically relevant role of bacteria within the food web.

## BACTERIA ASSOCIATED WITH PARTICLES AND SURFACES

In aquatic environments bacteria have a strong affinity for a variety of surfaces (Zobell 1943; Cooksey and Wigglesworth-Cooksey 1995; Costerton et al. 1995; Carman and Dobbs 1997; Gugliandolo et al. 2008). This may involve adherence to surfaces of material easily degraded by the bacteria (e.g. detritus particles, ‘marine snow’) to provide nutrients, or to inert surfaces (e.g. stones, rock, glass) (Brown et al. 1977). For such attachment some bacteria have developed specialised structures (e.g. holdfasts in *Caulobacter* spp.) (Brown et al. 1977). However, it has already been suggested by Zobell and Anderson (1936) that in most species the production of a sticky extracellular polysaccharide facilitates attachment.

The reasons for the preferential growth on surfaces are not fully understood yet, but attached bacteria can have different advantages against the free-living ones. These advantages may be protection from detrimental environments and predation, increasing nutrient availability and metabolic interaction with the host or other bacteria, facilitating genetic exchange, maintaining extracellular enzyme activities and formation of a higher level of microbial organisation (Dang and Lovell 2000; Molin and Tolker-Nielsen 2003; Hall-Stoodley et al. 2004).

In comparison with the surrounding water, aggregates have higher concentrations of nutrients and show elevated microbial activities (Caron et al. 1982) and the bacteria attached to these aggregates also tend to be larger (DeLong et al. 1993). It is assumed that this is due to the fact that more nutrients are available.

Free-floating aggregates with diameters greater than 0.5 mm (Alldredge and Silver 1988) can originate from, e.g., aggregating phytoplankton cells, faecal pellets, moults or mucus from zooplankton (Caron et al. 1982). They are called ‘marine snow’. Researchers have shown that these macroscopic particles are enriched in microbial biomass, nutrients, and trace metals and are involved in biogeochemical transformation of POC in the pelagic environment (Caron et al. 1986).

Direct observation of a wide variety of natural habitats has established that the majority of microbes persist attached to particles and other surfaces within a structured biofilm ecosystem and not as free-floating organisms (Costerton et al. 1995). Hence, although bacteria can have an independent planktonic existence, an interdependent lifestyle in which they function as an integral part of a population or community is also possible and is, in fact, more typical (Davey and O'Toole 2000). Bacteria associated with plankton can survive longer in adverse environmental conditions than free-living forms (Carman and Dobbs 1997). Environmental signals can influence initial attachment of bacteria, such as osmolarity, pH, iron availability, oxygen tension, and temperature (Davey and O'Toole 2000 and references therein). These environmental signals can vary from organism to organism.

Bacteria attached to particles and other surfaces may be a substantial food source for metazoans (Lawrence et al. 1993). An obvious prerequisite for this

trophic link is that the dominant metazoan in the pelagic ocean, such as calanoid copepods, would be able to digest the ingested attached bacteria. The digestibility of bacteria is also of interest from the standpoint of the survival strategies of attached bacteria (Pedrós-Alió and Brock 1983) and the role of bacteria in faecal pellet decomposition (Jacobsen and Azam 1984). Johnson and co-workers (1984) and Gowing and Wishner (1986) showed that copepods do not digest bacteria and therefore prey-attached bacteria would not be an energy source for the copepods. However, Lawrence et al. (1993) showed that copepods can digest bacteria which create a link to the microbial loop. If the ingested attached bacteria survive gut passage and thrive in faecal pellets, then attachment to particles may actually be a survival strategy for bacteria (Hastings and Nealson 1977). Another indication that the attachment is a survival strategy for bacteria: Biofilm-bacteria can be up to 1,000-fold more resistant to antibiotic treatments than the same organism grown planktonically (Gilbert et al. 1997) but the mechanisms by which the biofilm-grown bacteria attain this resistance are still matter of speculation (Davey and O'Toole 2000).

Although researchers such as Zobell (1943) have now recognised and studied surface-attached bacteria for nearly 70 years, we are still only beginning to realise the significance of biofilm-communities (Davey and O'Toole 2000). From an ecological perspective, populations of bacteria arise from individual cells. Metabolically similar populations (e.g. sulfate- and sulphur-reducing bacteria) constitute groupings referred to as guilds (Davey and O'Toole 2000). Sets of guilds (e.g. fermentative, sulfate- and sulfur-reducing, and methanogenic bacteria) conducting interdependent physiological processes form microbial communities (Davey and O'Toole 2000). Biofilms can be composed of a population that developed from a single species or a community derived from multiple microbial species, and they can form on a vast array of abiotic and biotic surfaces (Davey and O'Toole 2000).

Biofilms can be found, e.g., at hydrothermal vents, oil, sea ice, rocks, wood, glass, pipelines, phytoplankton and zooplankton. The surfaces of marine phyto-, zooplankton and other animals are usually associated with bacteria. For example, the chitinous exoskeletons of crustaceaens, such as copepods, are nutrient sources that encourage bacterial attachment and colonisation in the marine environment. Chitin is the most abundant polymer in the marine

environment (Keyhani and Roseman 1999; Bartlett and Azam 2005). Chitinous substrates include arthropods, their moults and their faecal pellets, some diatoms and fungi. These organisms are widespread over wide geographic and spatial ranges in rivers, estuaries and oceans (Bartlett and Azam 2005). Some bacteria are specialised in degrading chitin, e.g., some Gammaproteobacteria, such as *Vibrio* spp., Bacteroidetes and some members of the Alphaproteobacteria.

## BACTERIA ASSOCIATED WITH COPEPODS

Conventional ecological research views mesozooplankton, such as copepods, and bacteria as two weakly and indirectly connected functional groups (e.g. Azam and Malfatti 2007). However, recent studies have shown that their occurrence and ecological functions can be closely linked (e.g. Møller et al. 2007 and Tang et al. 2009a). The bacterial abundance associated with copepods can even be orders of magnitude higher than that in the ambient water, indicating active bacterial colonisation and growth in these microenvironments (Tang 2005).

The investigation of the bacterial communities of marine planktonic copepods is therefore highly relevant. The fact that marine copepods may constitute up to 80 % of the mesozooplankton biomass (Verity and Smetacek 1996) underlines this. They are key components of the food web as grazers of primary production and as food for fish (Fig. 1) (Cushing 1989; Møller and Nielsen 2001). Similarly their moults and carcasses can be populated and decomposed by bacteria (Tang et al. 2006a; Tang et al. 2006b; Tang et al. 2009b). Additionally copepods contribute to the microbial loop (Azam et al. 1983) via 'sloppy feeding' (Møller and Nielsen 2001) and the leakage of nutrients from faecal pellets (Hasegawa et al. 2001; Møller and Nielsen 2001; Møller et al. 2003; Steinberg et al. 2004).

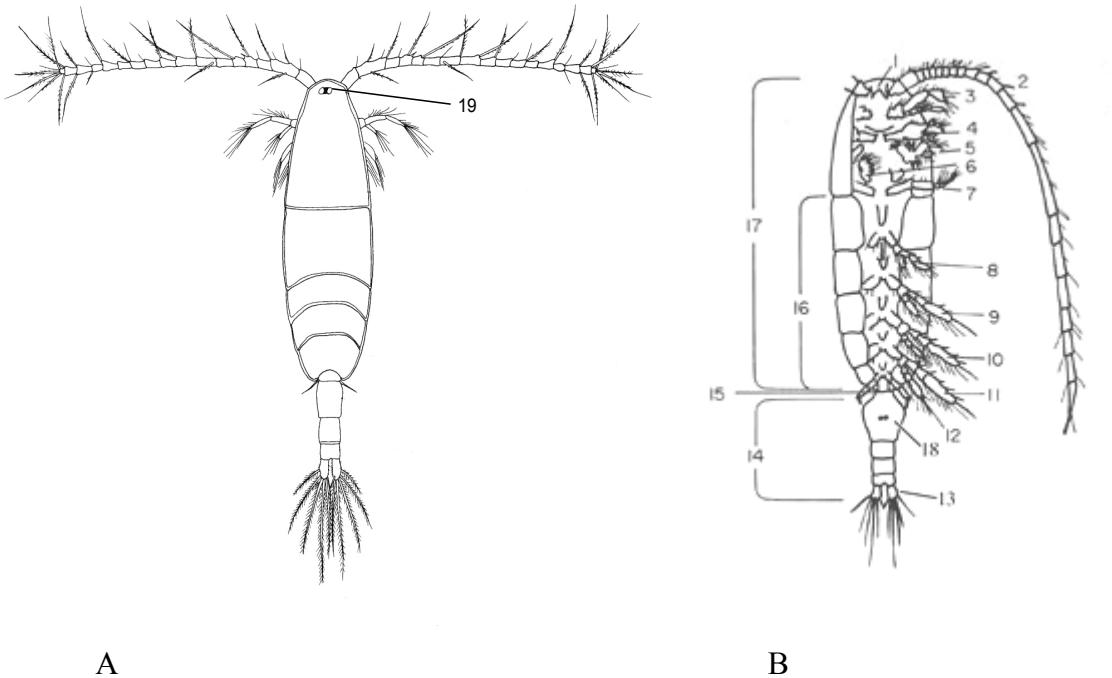
Bacteria located on copepod's exterior, its gut, faecal pellets and carcasses have been investigated with different methods in a number of previous studies (Harding 1973; Sochard et al. 1979; Nagasawa et al. 1985; Nagasawa and Nemoto 1988; Nagasawa 1992; Carman 1994; Delille and Razouls 1994; Kirchner 1995; Hansen and Bech 1996; Carman and Dobbs 1997; Tang 2005; Møller et al. 2007; Tang et al. 2009b; Brandt et al. 2010). With culture-dependent and -independent methods, mostly Gammaproteobacteria, such as

*Vibrio spp*. were detected associated with different copepod genera (e.g. Sochard et al. 1979; Holland and Hergenrader 1981; Huq et al. 1983; Hansen and Bech 1996; Gugliandolo et al. 2008), or *Pseudomonas* spp. (e.g. Sochard et al. 1979; Holland and Hergenrader 1981; Hansen and Bech 1996), *Aeromonas* spp. (e.g. Hansen and Bech 1996; Gugliandolo et al. 2008) and *Cytophaga/Flavobacterium* spp. (e.g. Sochard et al. 1979; Holland and Hergenrader 1981; Hansen and Bech 1996). In recent studies Alphaproteobacteria were also detected (e.g. Møller et al. 2007; Brandt et al. 2010).

However, these studies were only snap shots investigating geographically different marine sites and several different copepod genera with low sample numbers and applying different methods. Nevertheless, the available literature data suggest that the epibiotic bacterial assemblage associated with zooplankton is taxonomically distinct from free-living bacteria (Carman and Dobbs 1997).

## BACTERIA ASSOCIATED WITH COPEPOD SURFACES

Surfaces of many, if not most, marine metazoans are suitable sites for attachment and growth of epibiotic microorganisms (Carman and Dobbs 1997). Animal surfaces, especially the chitinous exoskeletons of crustaceans, e.g. of copepods, appear to act as nutrient source encouraging bacterial attachment and growth and thus undergo extensive colonisation (Sieburth et al. 1976; Carman and Dobbs 1997). For marine copepods, especially the oral region, body appendages (Huq et al. 1983; Nagasawa et al. 1985; Nagasawa 1989), intersegmental regions (Nagasawa et al. 1985; Carman and Dobbs 1997) and the region around the anus are heavily colonised by bacteria (Fig. 2), indicating bacterial exploitation of labile dissolved organic carbon (DOC) released from sloppy feeding and defecation (Hansen and Bech 1996). These bacteria apparently proliferate in areas where excretion or leakage of organic and inorganic nutrients is greatest, presumably exploiting these resources (Carman and Dobbs 1997).




A B

Fig. 2: A) Schematic illustration of the dorsal side of a copepod (*Acartia tonsa*) (Modified after Westheide/Rieger (ed.) 2007). B) Schematic illustration of the ventral side of a copepod, showing the position of appendages (http://www.tafi.org.au/zooplankton/imagekey/copepoda/#image 10/2010). 1) rostrum, 2) first antenna, 3) second antenna, 4) mandible, 5) first maxilla, 6) second maxilla, 7) maxilliped, 8-12) swimming legs, 13) caudal ramus, 14) urosome, 15) principle point of articulation, 16) metasome (thorax), 17) prosome, 18) genital segment (abdominal 1+2), 19) eye

'Sloppy feeding' is the key factor in the large amounts of DOC released during copepod grazing activity (Hasegawa et al. 2001; Møller and Nielsen 2001; Møller et al. 2003). When copepods graze not all of the material grazed is transferred to higher trophic levels (Møller 2005). It is assumed that 49 % of the carbon removed from suspension was already lost by sloppy feeding (Møller et al. 2003). Respiration, excretion, egestion and leakage from faecal pellets all contribute to the loss of carbon and nutrients (see Fig. 3) (Gardner and Paffenhöfer 1982; Båmstedt 1985).

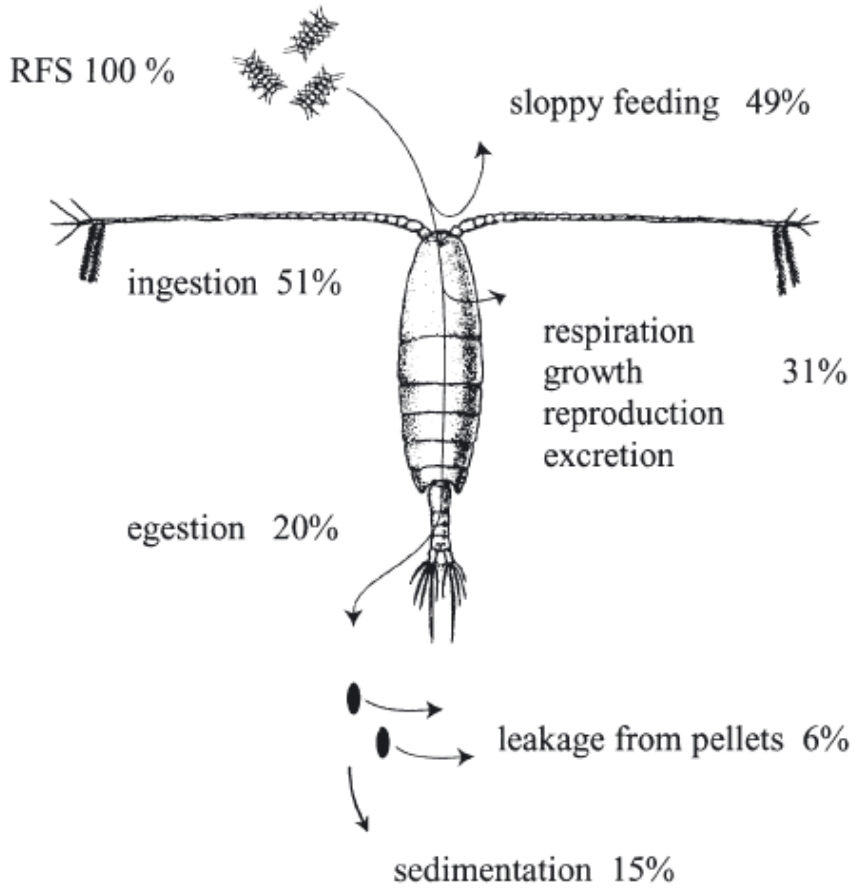



Fig. 3: *Calanus* spp. Schematic illustration of carbon flux of copepods during a spring bloom in Disko Bay, western Greenland, showing percentage of carbon removed from suspension (RFS) that ends up in the different pools (Møller et al. 2003).

These compounds could attract bacteria which prefer the nutrient rich regions i.g. the oral region, the body appendages and intersegmental regions. For carbon from egestion or early leakage of faecal pellets it would be favourable for bacteria to colonise the anal region.

The chitinous carapace itself is also a nutrient source. During the life cycle of copepods from nauplii to the adult stage, bacteria do not accumulate because the animals shed their carapace eleven times (and any associated epibionts) in the transition between each naupliar and copepodite stage (Holland and Hergenrader 1981). However, they do not moult again after adulthood, thus, bacterial (and other) epibionts are more likely to accumulate on adult copepods (Carman and Dobbs 1997). As such, copepod populations consisting primarily of copepodites should contain relatively fewer epibionts than populations consisting primarily of adults (Carman and Dobbs 1997).

Nagasawa (1986) estimated that adult *Acartia* sp. had up to $10^5$ epibiotic bacteria (Carman and Dobbs 1997). Holland & Hergenrader (1981) estimated epibiotic bacterial abundance on freshwater copepods to be approximately $6 \times 10^5$ bacteria per copepod.

Going even further, Nagasawa (1987) observed pits and scars on copepod exoskeletons that were the result of activity by chitinoclastic epibiotic bacteria and thus proposed that some epibiotic bacteria were parasitic on marine calanoid copepods (*Acartia* sp.). However, chitinoclastic activity by epibiotic bacteria on copepods appears to be the exception rather than the rule (Carman and Dobbs 1997).

Sochard et al. (1979) used culture techniques to characterise and quantify the bacteria associated with the calanoid *Acartia tonsa*. They found bacteria primarily associated with the external surfaces of copepods. They were dominated by members of the genera *Vibrio* and *Pseudomonas* of the Gammaproteobacteria.

However, many studies have focused on the relationship between planktonic copepods and human pathogens, especially *Vibrio* spp., due to their potential impact on public health (Kaneko and Colwell 1975; Huq et al. 1983; Heidelberg et al. 2002; Huq et al. 2005). Pathogenic bacteria associated with the surface of copepods could contribute to the transfer of pathogens through the food chain, since copepods are the main dietary constituents of many marine carnivores including planktivorous fish, medusae and chaetognaths (Dumontet et al. 1996).

## BACTERIA ASSOCIATED WITH COPEPOD GUT AND FAECAL PELLETS

As already mentioned above it is likely that not only the exoskeleton of copepods but also their intestine is populated by bacteria. Many researchers have investigated copepods' intestines and their faecal pellets (e.g. Honjo and Roman 1978; Sochard et al. 1979; Gowing and Silver 1983; Jacobsen and Azam 1984; Nagasawa and Nemoto 1988; Nagasawa 1992) mostly by cultivation-dependent approaches. Nevertheless, it is still questionable whether copepods actually harbour a distinct permanent bacterial gut flora (Honjo and Roman 1978; Gowing and Silver 1983; Gowing and Wishner 1986). Nott et al. (1985) for instance found any enteric flora, since the epithelium of the gut wall

contributes the peritrophic membrane to the faecal pellet and therefore no bacteria were found in the empty guts of starving copepods.

Copepods produce membrane-covered faecal pellets (Gauld 1957; Yoshikoshi and Ko 1988; Frangoulis et al. 2004). This peritrophic membrane appears to consist of chitinous microfibrils and a basic substance containing acid mucopolysaccharides and proteins (Yoshikoshi and Ko 1988) although Honjo and Roman (1978) doubted its chitinous nature.

Gowing and Silver (1983) found most bacteria within these pellets. They assumed these had either passed through the gut undigested or were enteric. Lawrence et al. (1993) found no bacteria in faecal pellets when copepods were fed with axenic food. That leads to the assumption that bacteria egested into the faecal pellets derived from the food (Lawrence et al. 1993).

# FOCUS OF THE STUDY

## STUDY SITE AND OBJECTS

The study site for all investigations presented in this thesis was Helgoland Roads (54°11.3' N and 7°54.0' E), German Bight, North Sea. The long-term sampling station Helgoland Roads, where all samples were taken, is located between the main island and the sandy dune (Fig. 4). At Helgoland Roads continuous work-daily measurements of several abiotic parameters and phytoplankton counts are carried out since 1962 and result in one of the richest temporal marine data sets available - the Helgoland Roads time series (Wiltshire et al. 2010).

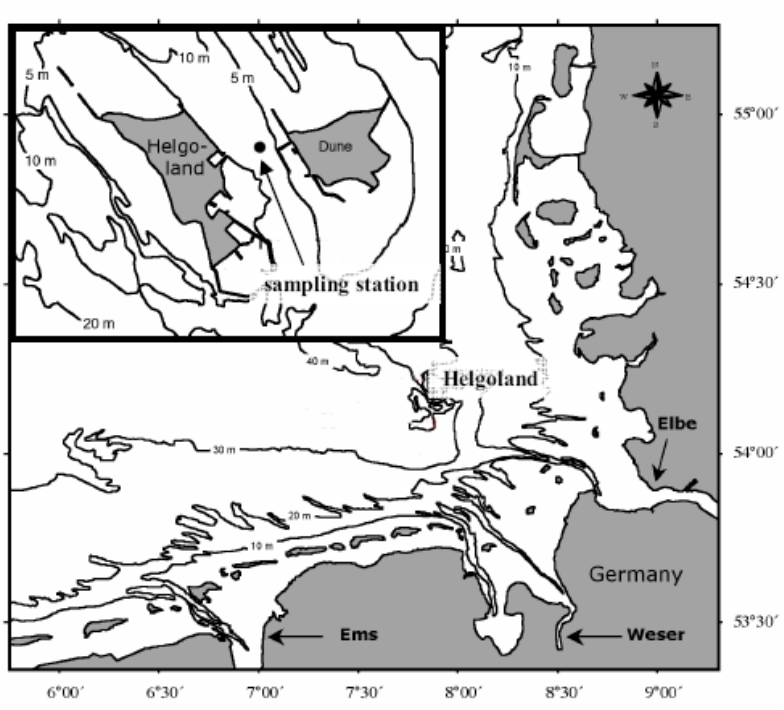



Fig 4: Map of the German Bight and Helgoland Island with the location of the long-term sampling station Helgoland Roads (54° 11.3`N, 7° 54.0`E). The contour lines show the water depth (Wesche et al. 2007).

The island of Helgoland is situated in the central part of the German Bight about 60 km off the German coast. It is subject to both coastal influences from the shallow Wadden Sea as well as marine influences from the open North Sea. The water column around the island is permanently mixed (Radach et al. 1990). The German Bight is influenced by the North Atlantic Ocean and coastal waters with plumes of the rivers Elbe and Weser and has a salinity of approximately 32

(Wesche et al. 2007). A strong seasonality can be observed concerning temperature and nutrient conditions. In late winter temperature can drop to 2°C whereas in summer temperature can rise until 20°C (Wiltshire and Manly 2004). In winter months inorganic nutrient concentrations are high but decrease during spring reaching lowest concentrations in early summer. After the spring bloom of phytoplankton phosphorus limitation can occur. Bacterioplankton and phytoplankton of the German Bight also show seasonality (Gerdts et al. 2004; Sapp et al. 2007). The bacterioplankton of the German Bight has been described for example by Eilers et al. (2001) as mainly consisting of Alpha-, Gammaproteobacteria and Bacteroidetes.

Near the island of Helgoland the most abundant calanoid copepod species are the small herbivorous to omnivorous copepods *Acartia* spp. (770-1,100 µm) (Hickel 1975; Halsband and Hirche 2001), *Temora longicornis* (875-1,220 µm) (Halsband and Hirche 2001), *Centropages spp.* (880-1,330 µm) (Halsband and Hirche 2001)*, Pseudocalanus elongatus* (790-1,100 µm) (Halsband and Hirche 2001) and *Paracalanus* spp. (750-1,000 µm). These were frequently detected until 1974 and 2004 (Greve et al. 2004) as well as the bigger calanoid copepod *Calanus helgolandicus* (Jonasdottir and Koski 2010). Cyclopoida represent carnivorous as well as omnivorous zooplankton such as *Oithona* spp., *Cyclopina* spp. and *Corycaeus* spp., whereas the Harpacticoida represent detritivorous and omnivorous zooplankton such as *Euterpina acutifrons*, *Tisbe* spp. and *Microsetella* spp. (Greve et al. 2004). *Candacia armata* is caught only sporadically in the Southern North Sea (Krause et al. 1995). The main predators of these copepods are fish larvae and adult planktivorous fish as well as ctenophores, e.g., *Pleurobrachia pileus* (Fig. 6). The ctenophore population usually increases around June and feeds on mesozooplankton, mainly copepods (Greve et al. 2004).

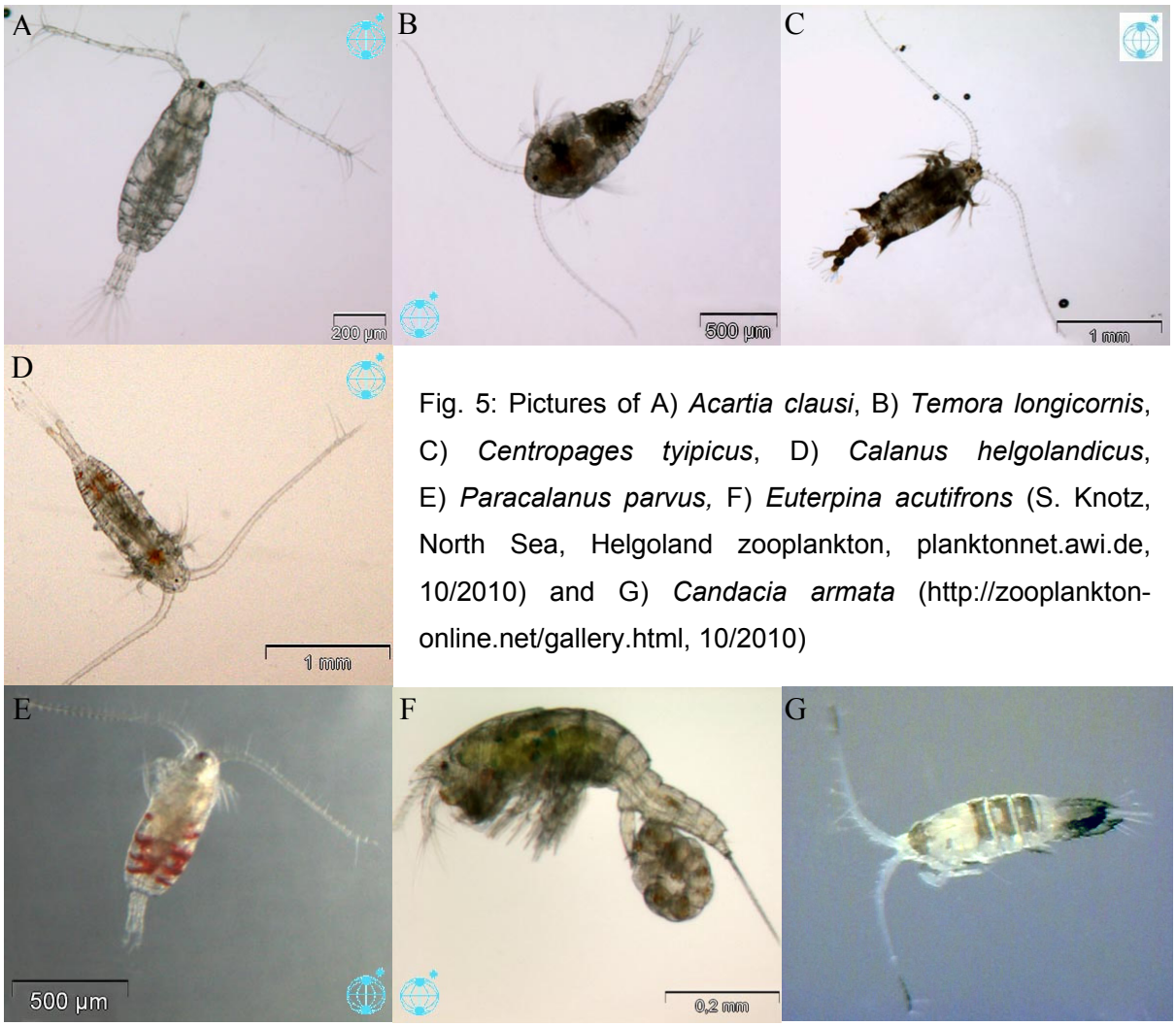



Fig. 5: Pictures of A) *Acartia clausi*, B) *Temora longicornis*, C) *Centropages tyipicus*, D) *Calanus helgolandicus*, E) *Paracalanus parvus,* F) *Euterpina acutifrons* (S. Knotz, North Sea, Helgoland zooplankton, planktonnet.awi.de, 10/2010) and G) *Candacia armata* (http://zooplankton-online.net/gallery.html, 10/2010)

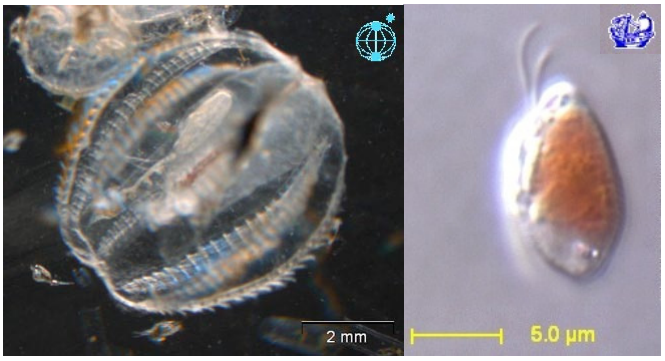



Fig 6: *Pleubrachia pileus* (S. Knotz, North Sea, Helgoland zooplankton, plantonnet.awi.de, 10/2010) (left) and *Rhodomonas salina* (D. Vaulot, Roscoff Culture Collection, planktonnet.awi.de, 10/2010) (right)

## METHODS

Our lack of knowledge of the real conditions under which most of the bacteria grow in their natural environment (Muyzer and Smalla 1998) makes it impossible to culture all bacteria. With the methods currently available the successful cultivation of approximately 20 % has been achieved (Selje et al. 2005). Thus, for a better understanding of microbial diversity in ecosystems, other approaches, which complement the traditional microbiological approaches, are required (Muyzer and Smalla 1998).

The analysis of the genes encoding for the small subunit of ribosomes (rDNA) has an enormous advantage: these genes have highly conserved as well as very variable regions. Thus, the phylogenetic diversity can be detected on phylum to even species level.

Using molecular tools, many novel microorganisms from various environments have been discovered (Kan et al. 2006). Most results have been obtained by cloning 16S rDNA fragments after amplification of DNA extracted from these different habitats (Muyzer and Smalla 1998). On the basis of 16s rRNA genes further molecular tools have been established to identify bacteria, to monitor and compare community diversity over time or from different habitats, e.g., fluorescence *in situ* hybridisation (FISH), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), single-stranded-conformation polymorphism (SSCP), denaturing high performance liquid chromatography (dHPLC), temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE).

Molecular community fingerprinting techniques, such as DGGE, allow a rapid analysis of the phylogenetic structure of bacterial communities (Mary et al. 2006) and the identification of unknown populations by subsequent sequencing bands. DGGE of PCR amplified 16S rRNA genes was first introduced in microbial ecology as a quick fingerprinting method to study bacterial dynamics at the community level (Muyzer et al. 1993). DGGE has now been successfully used to elucidate complex microbial assemblages in a range of very different environments, including lakes and rivers, coastal waters, polar regions and extreme environments (examples can be found in Kan et al. 2008).

Therefore, DGGE with subsequent sequencing of DGGE bands was also chosen as the technique of choice for this study.

However, it is known that many factors (DNA extraction, primer selection, PCR cycles, gene copy number etc.) can influence the outcome of PCR, particularly when applied to environmental samples (Kan et al. 2006). Therefore, not only a substantial effort in culture-independent investigations of the bacteria associated with copepods is needed, but also a proper assessment of those DNA-extraction techniques that are suited for these consortia.

For this study the above technique was applied to four of the most abundant and three of the rarer but important genera of marine copepods occurring at Helgoland: *Acartia* sp., *Temora longicornis*, *Centropages* sp. (Fig. 5), *Calanus helgolandicus*, *Pseudo-/Paracalanus* sp., *Euterpina* sp. and *Candacia* sp. (see Fig. 5).

To establish the DNA-extraction, PCR and DGGE and for comparison of exterior and interior associated bacterial community patterns as well as for a tri-trophic laboratory feeding experiment, laboratory-grown *Acartia tonsa* were used.

In the tri-trophic feeding experiment the ctenophore *Pleurobrachia pileus* (Fig. 6) served as secondary consumer after the copepod *Acartia tonsa.* As primary producer for the feeding experiment, *Rhodomonas salina* (Fig. 6) was chosen due to the fact that this cryptophyte is easy to culture in great densities.

# AIM OF THE STUDY

Although the bacteria associated with copepods have already been investigated with different methods for about 30 years, nearly nothing is known about the whole associated bacterial community. Regarding copepod-attached bacteria, understanding which bacterial communities dominate and what they respond to, is ecologically important (see above).

For this reason this study aimed to further our knowledge of the bacteria associated with marine copepods.

Therefore with this study four main questions should be answered:

**1. Which bacteria are associated with marine copepods?**

For this study of North Sea copepods, the abundant copepod genera *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Pseudo-/Paracalanus* sp. and the rarer genera *Calanus helgolandicus*, *Euterpina* sp. and *Candacia* sp. were investigated concerning their bacterial communities by PCR-DGGE Furthermore, to complement the PCR-DGGE analyses, clone libraries of two different time points were generated for the three most abundant copepod species. Subsequent sequencing of DGGE bands and clone types led to the identification of the associated bacterial phylotypes (**Chapter III**).

**2. Do different copepod genera harbour distinct bacterial communities?**

The bacterial community fingerprints of the copepod genera *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus* obtained by PCR-DGGE were compared to each other to identify differences of the bacterial communities of different copepod genera with the help of statistical methods (**Chapter III**).

**3. Does the bacterial community of marine copepods change during the year?**

The bacterial communites of the copepods genera *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus* were monitored throughout two consecutive years (from February 2007 until March 2009) to understand whether a seasonal succession of the bacterial communities occurred. Furthermore, to complement the PCR-DGGE analyses, clone libraries of two different time points were generated to compare the bacterial communities from June 2007 with June 2008. For detailed methods and results see **Chapter III**.

**4. Are the copepod associated bacteria transferred by food?**

The question whether the bacteria can be transferred from one host to another via feeding was investigated with a tri-trophic feeding experiment with the cryptophyte *Rhodomonas salina* as primary producer, the calanoid copepod *Acartia tonsa* and the ctenophore *Pleurobrachia pileus* as primary and secondary consumers, repectively (**Chapter IV**).

However, first of all it was necessary to **develop a suitable DNA-extraction technique** for investigating the bacterial communities of different copepods genera to get the best start (**Chapter II**).

# LIST OF MANUSCRIPTS

This thesis is based on the following manuscripts, partly submitted or already published.

I. Brandt P, Gerdts G, Boersma B, Wiltshire KH, Wichels A (2010) **Comparison of different DNA-extraction techniques to investigate the bacterial community of marine copepods**. Helgol Mar Res (available online) DOI 10.1007/s10152-010-0188-1

   Sampling, analyses, the text writing and graphical presentation were done by P. Brandt under supervision of Dr. A. Wichels, Dr. G. Gerdts, Prof. Dr. M. Boersma and Prof. Dr. K.H. Wiltshire.

II. Brandt P, Gerdts G, Kreisel K, Boersma M, Wiltshire KH, Wichels A (to be submitted to ISME J) **The microbiome of North Sea copepods**.

   Sampling, DGGE-analyses, the text writing and graphical presentation were done by P. Brandt under supervision of Dr. A. Wichels, Dr. G. Gerdts, Prof. Dr. M. Boersma and Prof. Dr. K.H. Wiltshire. Analysis of cloning was done by K. Kreisel under supervision of Dr. A. Wichels and P. Brandt (Diploma-Thesis 2009). Statistical and phylogenetic analyses were done by P. Brandt with help of Dr. G. Gerdts and Dr. A. Wichels, respectively.

III. Brandt P, Gerdts G, Schoo KL, Wichels A, Wiltshire KH, Boersma M (proposed journals: Aquat Ecol or Biol Let) **Transfer of bacteria through trophic levels? Does P-limitation make any difference?**

   Set up of the experiment was conducted by K. L. Schoo and P. Brandt. Sampling for and the DGGE-analyses, the text writing and graphical presentation were done by P. Brandt under supervision of Dr. A. Wichels, Dr. G. Gerdts, Prof. Dr. M. Boersma and Prof. Dr. K.H. Wiltshire. Statistical analyses were done by P. Brandt with help of Dr. G. Gerdts.

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# Chapter II

## COMPARISON OF DIFFERENT DNA-EXTRACTION TECHNIQUES

# COMPARISON OF DIFFERENT DNA-EXTRACTION TECHNIQUES TO INVESTIGATE THE BACTERIAL COMMUNITY OF MARINE COPEPODS

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## ABSTRACT

Marine zooplanktic organisms, such as copepods, are usually associated with large numbers of bacteria. Some of these bacteria live attached to copepods' exoskeleton, while others prevail in their intestine and faecal pellets. Until now, general conclusions concerning the identity of these bacteria are problematic since the majority of previous studies focused on cultivable bacteria only. Hence, to date little is known on whether copepod genera or species harbour distinct bacterial populations and about the nature of this association. To shed more light on these copepod/bacteria consortia, the focus of this study was the development and evaluation of a suitable approach to extract bacterial DNA from different North Sea copepod genera. Furthermore, the bacterial DNA was analysed by PCR-DGGE and subsequent sequencing of excised bands. The result of this work was an appropriate extraction method for batches of ten to one copepod specimens and offered first insights as to which bacteria are attached to the copepods *Acartia* sp. and *Temora* sp. from Helgoland Roads (German Bight) and a laboratory-grown *Acartia tonsa* culture. It revealed the prevalence of Alphaproteobacteria.

## INTRODUCTION

Copepods are important components of the marine food web. Their significance as a link between primary producers and higher trophic levels has long been recognised (Cushing 1989; Møller and Nielsen 2001; Møller 2005; Olsen et al. 2005). Copepods also contribute to the microbial loop (Azam et al. 1983; Møller and Nielsen 2001) by releasing dissolved organic carbon (DOC) through sloppy feeding, excretion or more indirectly by leakage of DOC from their faecal pellets (Hasegawa et al. 2001; Møller and Nielsen 2001; Møller et al. 2003; Steinberg et al. 2004) or via the decomposition of copepod carcasses by bacteria (Tang et al. 2006a, b).

Bacteria typically have a strong affinity to both biotic and abiotic surfaces (Zobell 1943; Costerton et al. 1978; Cooksey and Wigglesworth-Cooksey 1995; Carman and Dobbs 1997) and in most cases benefit from this attachment. Hence, also animal surfaces such as the chitinous exoskeleton of crustaceans can undergo extensive colonisation by bacteria (Sieburth et al. 1976; Carman and Dobbs 1997) depending on the life stage or fitness of the animal (Carman and Dobbs 1997). Using scanning electron microscopy, Nagasawa et al. (1985) found that between 0 and 100% of the members of a copepod population were colonised by bacteria, and that the epibiotic load of adult *Acartia* sp. can be anywhere between 10 and $10^5$ per specimen (Nagasawa et al. 1985; Nagasawa 1989).

From previous studies, it is known that different bacterial species show different distribution or colonisation patterns on copepods (Carman and Dobbs 1997). Especially the oral region, body appendages (Huq et al. 1983), intersegmental regions and the region around the anus (Carman and Dobbs 1997) are colonised by bacteria, reflecting the bacterial exploitation of DOC released by sloppy feeding or defecation (Hansen and Bech 1996).

The study of microbial epibionts on crustaceans in aquatic environments was in its infancy in 1997 (Carman and Dobbs 1997), and this situation did not change until Tang et al. (2006a, b) first investigated bacterial communities on decomposing copepod carcasses using molecular techniques such as PCR-DGGE. These studies showed that the bacterial community of freshly dispatched copepods was different from that of copepods which were incubated for some days. Møller et al. (2007) first investigated the bacterial community

associated with *Calanus* sp. using PCR-DGGE and compared it with the community of the surrounding water. They observed that the copepod samples consisted of less than five discernable DGGE bands, whereas much higher numbers of bands could be found in the surrounding water samples. However, part of the free-living bacteria could also be found attached to the copepods. The authors also reported that the first extraction technique they applied, an enzyme/SDS extraction protocol without use of phenol-chloroform, led to the release of PCR-inhibiting substances which required an altered DNA-extraction protocol.

Therefore, not only a substantial effort in culture-independent investigations of the bacteria associated with copepods is needed, but also a proper assessment of those DNA-extraction techniques that are suited for these consortia. Only then do we stand a chance to substantially further our knowledge on the community structure and the identity of the bacteria associated with marine copepods.

Hence, the present study focuses on the evaluation of different DNA-extraction techniques of copepod-bacteria consortia. To this end, we used commercial lysis reagents and extraction kits as well as a common method such as phenol-chloroform DNA extraction. Furthermore, the bacterial community-DGGE-pattern of laboratory-grown *Acartia tonsa* and field-caught *Acartia* sp. and *Temora* sp. is described, and selected DGGE bands are further analysed by DNA sequencing.

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND PREPARATION

Zooplankton samples from Helgoland Roads in the North Sea (54°11.3’ N, 7°54.0’ E) were collected in June 2007 using a 150- and 500-µm net aboard the research vessel ‘‘Aade’’. With a stereo microscope, the animals were sorted by genus with sterile tweezers and washed twice with sterile sea water. A culture of *Acartia tonsa* was grown from egg to adult stage at 18°C and LD 16:8 in filtered sea water with a salinity of 31. These copepods were fed with *Rhodomonas* sp. Adult specimens from laboratory-reared cultures of *Acartia tonsa*, field-caught *Acartia* sp. and *Temora* sp. were frozen in sterile tubes with one, five or ten individuals per tube.

## DNA EXTRACTION

A number of cell lysis and DNA-extraction techniques were applied to the copepod samples (Fig. 1) covering a range of chemical and physical treatments. Additionally, protocols for differentiation of the attached bacterial community on copepod surfaces from the entire bacterial community were tested and are described later. Obviously, it would be of interest to differentiate between individual copepods, as no information is available on the variation of the bacterial community among specimens of copepods. On the other hand, given the very low densities of bacteria on copepods reported in previous studies, it was first of all necessary to determine the quantity of copepod specimens needed to obtain an adequate amount of bacterial DNA for further analysis. Due to the fact that the numbers of bacteria associated with copepods could be very low, the extraction techniques were first tested on batches of ten and five copepods per sample.

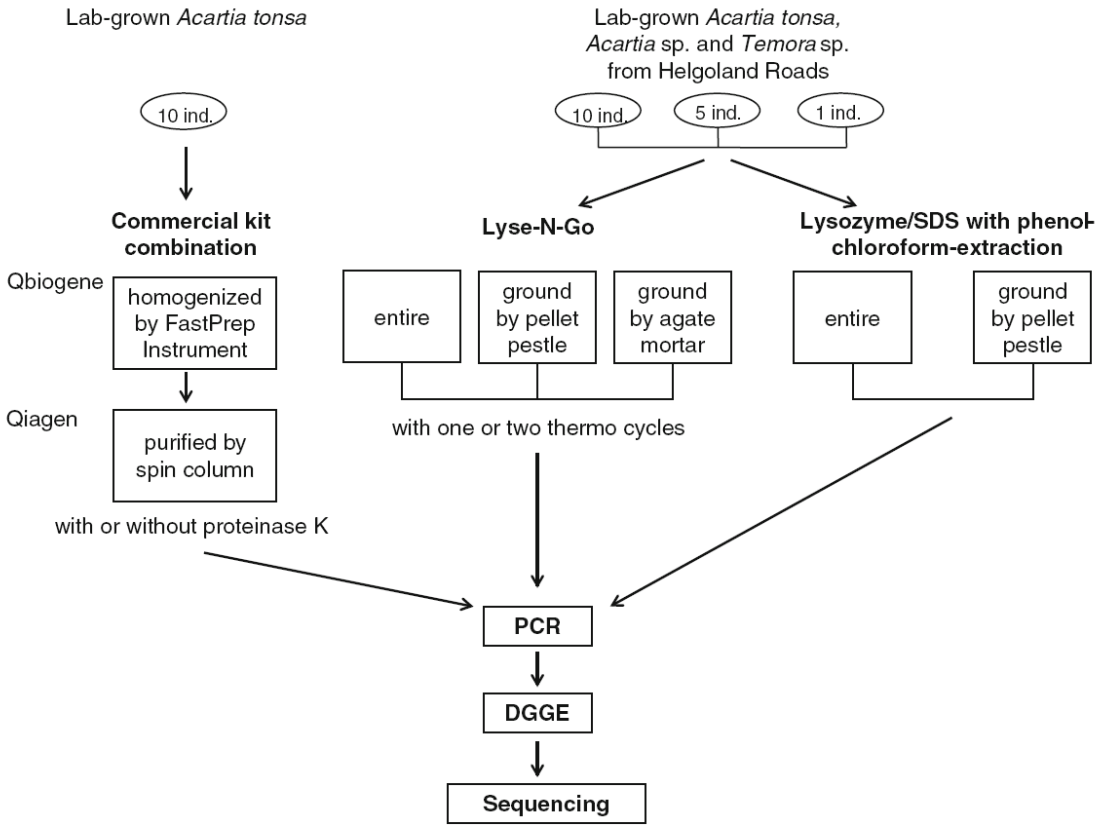


Fig. 1: Schematic overview of sample preparation

## EPIBIOTIC BACTERIAL COMMUNITY

### COPEPOD SURFACE-ATTACHED BACTERIA WERE INVESTIGATED USING TWO PROTOCOLS

One used the Lyse-N-Go reagent (Pierce, Rockford, IL, USA) and the other employed lysozyme and SDS for cell lysis followed by phenol-chloroform DNA extraction and DNA precipitation with isopropanol, leaving the copepods intact.

### LYSE-N-GO

Entire copepods in batches of either ten, five or one individuals were treated with 10 µl Lyse-N-Go reagent, followed by either one or two cycles of the thermocycle program recommended by the manufacturer. Lyse-N-Go isolates were directly subjected to PCR using either 2 µl of pure extract or 2 µl of a 1:10 dilution thereof, or were stored at -20°C until further analysis.

### LYSOZYME/SDS

Entire copepods in batches of either ten, five or one individuals were treated with 370 µl of STE-buffer (6.7% saccharose, 50 mM Tris, 1 mM EDTA, pH 8). Cell lysis was accomplished by incubating the samples with 100 µl lysozyme (10 mg/ml) for 30 min at 37°C with gentle shaking at 400 rpm. Next, 50 µl EDTA-Tris (250, 50 mM) and 30 ll SDS-Tris-EDTA (20%, 50, 20 mM) were added and samples were incubated for 60 min at 50°C with gentle shaking. The salt concentration was increased by adding a 1/10 volume of NaCl (5 M), and proteins were removed using 1 volume of phenol-chloroform (1:1). DNA was then precipitated by overnight incubation with isopropanol at -20°C. Precipitates were washed with 75% ethanol and dried in a laminar flow cabinet for 15 min prior to resuspension with 10 µl sterile water. DNA extracts were stored at -20°C until further analysis.

## ENTIRE BACTERIAL COMMUNITY

DNA of bacteria internally and externally associated with sampled copepods was extracted using one of three protocols after mechanical processing of each sample: first, treatment with Lyse-N-Go reagent; second, treatment with lysozyme/SDS followed by phenol-chloroform DNA extraction and precipitation

with isopropanol; and third, a combination of two commercial DNA extraction kits.

#### LYSE-N-GO

Copepods in batches of either ten, five or one individuals were suspended in 10 µl of Lyse-N-Go reagent and transferred to a sterile agate mortar using sterile cut pipet tips. An additional 10 µl of Lyse-N-Go reagent was added to the copepod suspension prior to grinding the sample with agate mortar and pestle by hand for 30 s in a laminar flow cabinet. The homogenised sample was pipetted into a sterile micro reaction tube. The agate mortar and pestle were then rinsed with an additional 10 µl of Lyse-N-Go, which was added to the homogenate in the tube. Alternatively, another grinding procedure was conducted with a pellet pestle and pellet pestle motor (Kontes, Vineland, NJ, USA) for 30 s with 10 µl Lyse-N-Go reagent inside a sterile micro reaction tube before carrying out the recommended thermal cycle program one or two times.

#### COMMERCIAL KIT COMBINATION

BIO101 FastDNA Kit (Qbiogene, Carlsbad, CA, USA) and Blood and Tissue Kit (Qiagen, Hilden, Germany) were combined as follows: 1,000 µl of CLS-TC buffer (chaotropic lysis buffer, Qbiogene) was added to a sample of copepods (10 ind.) and pipetted with a sterile cut pipet tip into a matrix tube (Qbiogene). The sample was homogenised with FastPrep Instrument for 30 s at a speed setting of 4.0. After a 5-min centrifugation step at 16 000 x g, the entire supernatant was transferred into a new micro reaction tube. A 1/10 volume of proteinase K (Roche, Mannheim, Germany) was added to one of the supernatant samples and another sample was left without proteinase to test whether a difference in the released amount of DNA or in the amount of PCR product exists. Both samples were then first incubated for 30 min at 37°C followed by 60 min incubation at 56°C in a thermomixer with gentle shaking. A 1/2 volume of buffer AL (Qiagen) and a 1/2 volume of ethanol (96%) were added, and the solution was transferred to a spin column tube containing a silica matrix for DNA purification (Qiagen). DNA binding and elution steps were performed as recommended by the manufacturer, with one exception: The final

elution step was conducted twice using 30 µl of elution buffer AE (Qiagen) for each step.

#### LYSOZYME/SDS

Copepods (10, 5, 1 ind.) were ground in 10 µl STE-buffer for 30 s with pellet pestle, using pellet pestle motor followed by addition of 360 µl of STE and 100 µl lysozyme (10 mg/ml), respectively. The next steps were the same as described previously.

### PCR CONDITIONS

PCR amplification of 16S rRNA gene fragments was performed using the bacteria specific primers P3-clamp (Muyzer et al. 1993) with a 40-bp GC-rich sequence at the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3'). PCR mixtures with a volume of 50 µl contained 5 µl of 10 x Taq buffer (Eppendorf, Hamburg, Germany), 8 µl of Master Enhancer (Eppendorf) for initial PCRs and no enhancer for reamplification after DGGE, 300 µM dNTPs (Promega, Mannheim, Germany), 0.2 µM of each primer, 2 U of Taq DNA polymerase (Eppendorf) and either 2 µl of pure or 1:10 diluted DNA prior to or 1 µl after DGGE.

'Touchdown' PCR was performed as described by Sapp et al. (2007). PCR fragments on 1.2% agarose gels were visualised by ethidium bromide (0.5 mg $l^{-1}$) and images were captured with a ChemiDoc XRS System (BioRad, München, Germany). The thickness and intensity of each band visualised was used to gauge the relative volume of the corresponding product used for DGGE.

## DGGE

All 16S rRNA gene amplicons were resolved on 6% (w/v) polyacrylamide gels in 0.5 x TAE buffer (20 mM TrisHCl, 10 mM acetic acid, 0.5 mM EDTA) with denaturing gradients of 15–55% urea/formamide (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 60°C and 150 V for 10 h (Sigler et al. 2004) using a DCode mutation detection system (BioRad). DGGE gels were stained with SYBRGold (Invitrogen, Karlsruhe, Germany). Imaging was performed with a ChemiDoc XRS System (BioRad).

Prominent DGGE bands were excised, eluted (Sambrook et al. 1989), reamplified and confirmed by an additional DGGE.

As a standard for all DGGE gels, the combined PCR-amplicons (P3-clamp/907r) of five bacteria (*Polaribacter filamentus* DSM 13964, *Sulfitobacter mediterraneus* DSM 12244, *Arthrobacter agilis* DSM 20550, *Microbacterium maritypicum* DSM 12512, *Leifsonia aquatica* DSM 20146) with different GC-contents were used.

## SEQUENCING AND PHYLOGENETIC ANALYSIS

Selected DGGE bands were excised, the DNA was eluted and reamplified using the primers 341f (without GC-clamp) (Muyzer et al. 1993) and 907r. PCR products were checked on 1.2% (w/v) agarose gels prior to sequencing. PCR products with the right size (~ 566 bp) were excised from the agarose gels and used for sequencing.

DNA sequencing of PCR products was performed by Qiagen GmbH using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing primers were 341f and 907r. Nearest relatives were searched using BLAST (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997). Sequence data were checked for the presence of PCR-amplified chimeric sequences by the CHECK_CHIMERA program (Cole et al. 2003). The ARB software package (http://www.arb-home.de) was used for phylogenetic analysis (Ludwig et al. 2004). After addition of sequences to the ARB 16S rDNA sequences database (release May 2005), alignment was carried out with the Fast Aligner integrated in the program and refined by comparison of closest relatives retrieved by BLAST. Sequences with more than 1,300 nucleotides were used to calculate phylogenetic trees. The ARB ‘‘parsimony interactive’’ tool was used to add partial sequences to respective trees. Phylogenetic relationships were deduced by the neighbour joining method including the correction algorithm of Felsenstein (1993).

## NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The sequences obtained in this study are available from GenBank under accession numbers FJ226496–FJ226515.

# RESULTS

## DNA EXTRACTION AND PCR

DNA extracts yielded from the different extraction techniques were not quantified before the use as PCR template, because it was very plausible that especially with the ground copepods also copepod DNA was extracted. Nevertheless, PCR amplification of the targeted 16S rRNA gene region was successful. The differences observed between the extraction methods tested here are described in the following paragraphs.

### EPIBIOTIC BACTERIAL COMMUNITY

#### LYSE-N-GO

Applying the Lyse-N-Go treatment using one thermocycle on ten isolated copepods yielded sufficient bacterial DNA for successful bacterial 16S rRNA gene amplification by PCR, regardless of the copepod group (laboratory-grown *Acartia tonsa*, field-caught *Acartia* sp. or field-caught *Temora* sp.) selected. Employing an additional thermocycle during DNA extraction yielded a higher quantity of PCR product. Replicate extractions using Lyse-N-Go reagent with one or two thermocycles on one or five copepods per sample yielded DNA which, when used as a PCR template, led to only inconsistent results regarding the amplification of PCR product.

#### LYSOZYME/SDS

Enzymatic/detergent cell lysis followed by phenol–chloroform DNA extraction performed on ten, five or one copepod(s) in suspension yielded PCR amplifiable bacterial DNA in all cases. Quality checks of the amplification products revealed consistently higher band intensity than products derived from Lyse-N-Go DNA extracts. No variation among the three investigated copepod groups and replicates were observed.

### ENTIRE BACTERIAL COMMUNITY

#### LYSE-N-GO

Grinding ten, five or one copepod individual(s) in Lyse-N-Go reagent with an agate mortar resulted in inconsistent success with PCR amplification. PCR products generated using DNA extracts from pellet-pestle-homogenised

copepod suspensions as template yielded PCR products with a higher concentration than those generated from agate mortar DNA extracts. Amplification was inconsistent when the pellet pestle technique was applied to five or one copepod(s). The procedure with two thermocycles yielded higher amounts of detectable PCR product than that with one thermocycle, irrespective of which extraction steps were taken before.

#### COMMERCIAL KIT COMBINATION

Combining the BIO101 FastDNA Kit to extract DNA from copepod tissue and the silica-based DNA purification in spin-columns from the Blood and Tissue Kit successfully retrieved PCR-amplifiable DNA. The amounts of DNA and PCR product detected were comparable to Lyse-N-Go treatment with pellet pestle. Incubation with proteinase K yielded lower quantities of PCR product.

#### LYSOZYME/SDS

The highest amount of PCR product for all investigated copepod groups was achieved using the lysozyme/SDS treatment in combination with phenol–chloroform DNA extraction together with pellet pestle homogenisation. PCR amplification was consistent for copepod suspensions with ten, five or one individual(s). PCR product quality checks revealed that amplification with DNA from phenol–chloroform DNA extracts of laboratory-grown and field-caught *Acartia* sp. resulted in notably less amount of PCR product compared to those of field-caught *Temora* sp. (Fig. 2).

## DGGE

Bacteria-specific PCR products obtained from DNA extracted with different methods generated notably different DGGE banding patterns. A lower number of DGGE bands were observed with PCR products received from the Lyse-N-Go procedure compared to banding patterns obtained with PCR products achieved from phenol–chloroform DNA extraction. Up to 20 distinct DGGE bands were generated using PCR products from *Temora* sp. and approximately 10 for both groups of *Acartia* sp. irrespective of the number of copepods used for extraction. Clear differences are notable between the laboratory-grown and field-caught *Acartia* sp. groups (Fig. 3). The highest number of DGGE bands

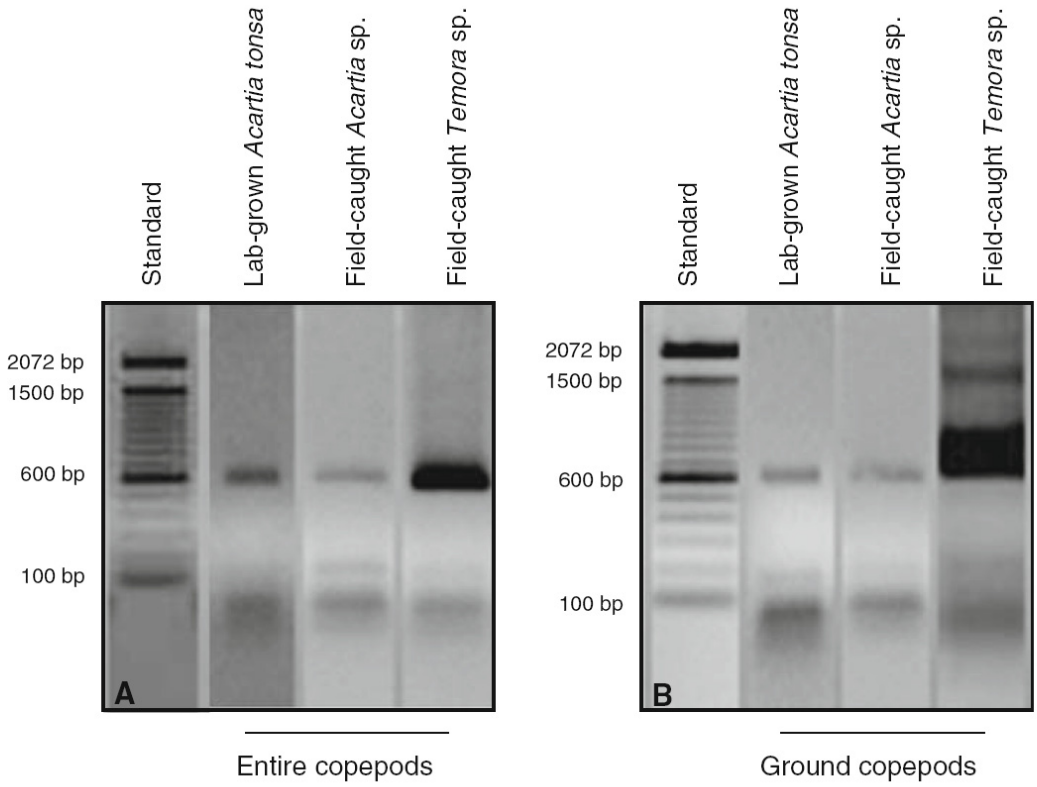


Fig. 2: Comparison of PCR product amounts of the three investigated copepod groups. A Using intact copepods for phenol–chloroform DNA extraction and B using pellet pestle-ground copepods

and a very stable and reproducible pattern could be observed using PCR products of phenol–chloroform extracted DNA regardless of whether the copepods were left intact or ground by pellet pestle prior to analysis. DGGE finger print patterns showed higher numbers of bands when using the ground copepods than the intact ones (Fig. 4). Unexpectedly, some bands are only visible in the intact copepods (Fig. 4, lower white box).

## SEQUENCING AND PHYLOGENETIC ANALYSIS

Prominent DGGE bands were sequenced from excised agarose gel bands. Sequence data of 20 excised bands from ground copepods could be retrieved representing nine different bacterial phylotypes (Table 1). Closest relatives of the sequenced bands derived from BLAST analyses are also listed in Table 1. The results revealed many close matches with 98–100% similarity to bacterial 16S rRNA gene sequences in the GenBank. Seventeen sequences of DGGE

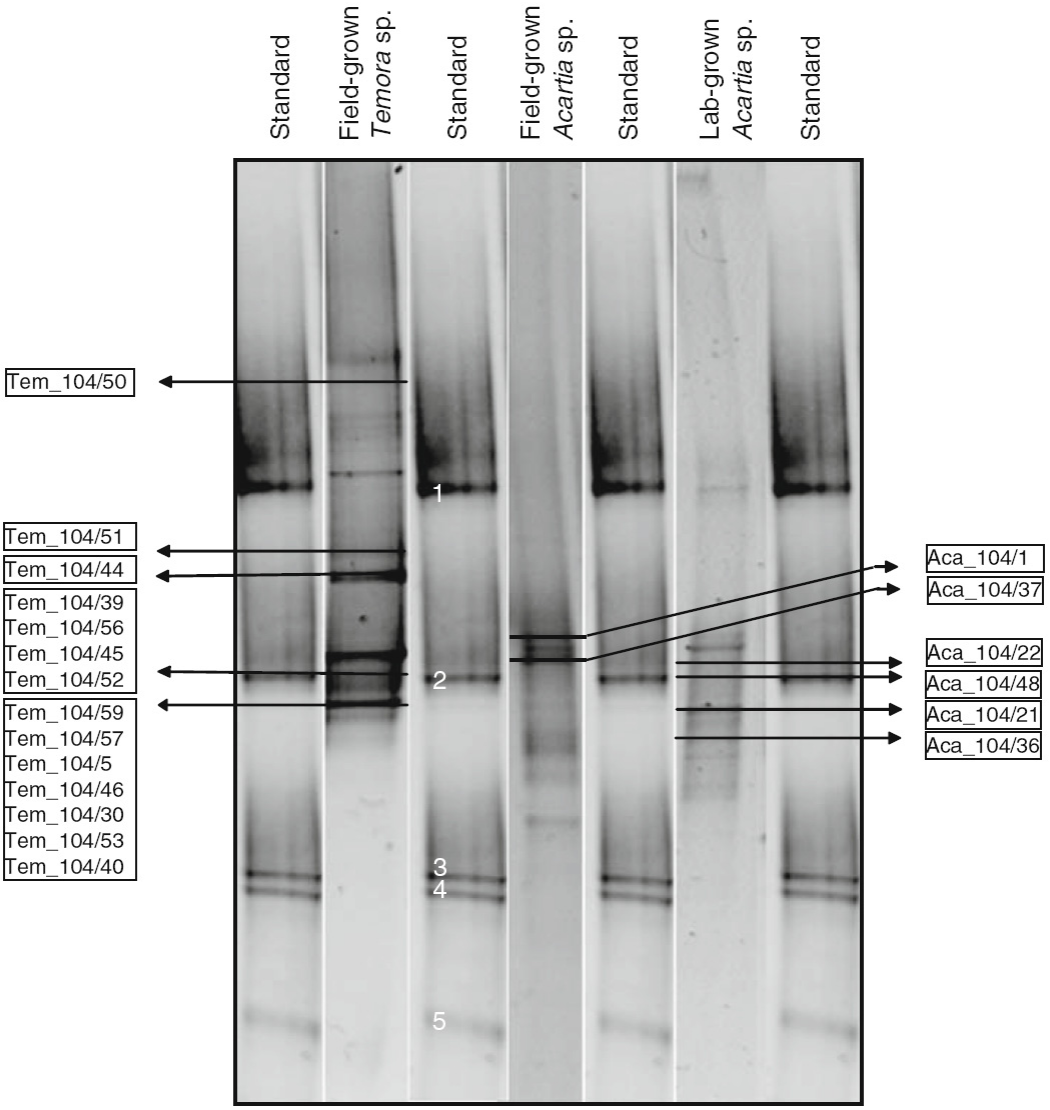


Fig. 3: DGGE banding patterns of the three investigated copepod groups. DNA was extracted using pellet pestle and phenol–chloroform DNA extraction. Arrows with numbers show cut and sequenced DGGE bands. Numbers on standard-bands mark the gel positions of the PCR-amplicons of five bacteria: 1 *Polaribacter filamentus* DSM 13964, 2 *Sulfitobacter mediterraneus* DSM 12244, 3 *Arthrobacter agilis* DSM 20550, 4 *Microbacterium maritypicum* DSM 12512, 5 *Leifsonia aquatica* DSM 20146

bands showed high similarity with sequences of Alphaproteobacteria, only one with Deltaproteobacteria, and two with Bacteroidetes. No Gammaproteobacteria were observed.

Comparison of sequence data of excised bands appearing at the same position in DGGE gels revealed identical closest relatives in most cases (e.g. Tem_104/59, Tem_104/57, Tem_104/5, Tem_104/46, Tem_104/30,

Tem_104/53 and Tem_104/40). Moreover, bands Aca_104/21, Aca_104/22 and Aca_104/48 resulted in the same sequence although they were excised at different gel positions (Table 1). A neighbour-joining tree (Fig. 5) of selected members of the Alphaproteobacteria, Deltaproteobacteria and Bacteroidetes shows that the excised bands sequences Tem_104/59 (Tem_104/57, Tem_104/5, Tem_104/46, Tem_104/30, Tem_104/53 and Tem_104/40 are not shown in the tree) cluster with a sequence of a surface-attached marine bacterium of the *Roseobacter* clade (EU005307) (Dang et al. 2008). Aca_104/21, Aca_104/22 and Aca_104/48 are strongly related to an uncultured marine bacterium (DQ372849) (Morris et al. 2006), while the sequences Tem_104/44, Tem_104/52 and Tem_104/45 (Tem_104/39 and Tem_104/56 are not shown in the tree) are grouped with the sequences of copepod-associated bacteria (DQ839253 and DQ839261) found by Møller et al. (2007).

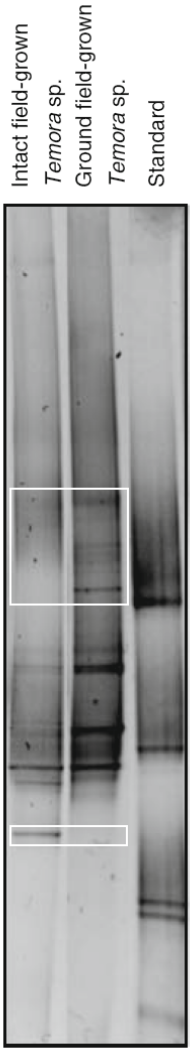


Fig. 4: Differences in the DGGE-banding pattern of intact or ground copepods. White boxes highlight the differences

Table 1: Closest relatives to the sequenced DGGE bands

| Band | Host | Similarity (%) | Alignment length | Accession number | Closest relative | Taxonomic affiliation as reported in GenBank |
|---|---|---|---|---|---|---|
| Tem_104/59 | *Temora* sp. field | 99 | 510/511 | EU005307 | Clone G3-45 | Uncultured Alphaproteobacterium |
| Tem_104/57 | | 99 | 510/511 | | | |
| Tem_104/5 | | 99 | 473/474 | | | |
| Tem_104/46 | | 99 | 460/461 | | | |
| Tem_104/30 | | 99 | 476/477 | | | |
| Tem_104/53 | | 100 | 435/435 | | | |
| Tem_104/40 | | 100 | 463/463 | | | |
| Tem_104/52 | *Temora* sp. field | 100 | 465/465 | DQ839261[a] | DGGE band NS16 | Uncultured Alphaproteobacterium |
| Tem_104/39 | | 99 | 459/462 | | | |
| Tem_104/45 | | 99 | 460/464 | | | |
| Tem_104/56 | | 98 | 484/490 | | | |
| Tem_104/44 | *Temora* sp. field | 97 | 439/451 | DQ839261[a] | DGGE band NS16 | Uncultured Alphaproteobacterium |
| Tem_104/50 | *Temora* sp. field | 97 | 510/522 | EF215784 | Clone PV2-37 | Uncultured Bacteroidetes |
| Tem_104/51 | *Temora* sp. field | 91 | 496/540 | DQ831068 | Isolate LP-OTU63 | Uncultured Bacteroidetes |
| Aca_104/21 | *Acartia* sp. lab | 98 | 452/459 | DQ372849 | Clone NH10_29 | Uncultured Alphaproteobacterium |
| Aca_104/22 | *Acartia* sp. lab | 97 | 442/453 | DQ372849 | Clone NH10_29 | Uncultured Alphaproteobacterium |
| Aca_104/48 | *Acartia* sp. lab | 96 | 412/426 | DQ372849 | Clone NH10_29 | Uncultured Alphaproteobacterium |
| Aca_104/36 | *Acartia* sp. lab | 95 | 402/420 | DQ395758 | Clone ctg_CGOF389 | Uncultured Alphaproteobacterium |
| Aca_104/1 | *Acartia* sp. field | 96 | 440/454 | DQ234217 | Clone DS134 | Uncultured Alphaproteobacterium |
| Aca_104/37 | *Acartia* sp. field | 93 | 443/472 | DQ911789 | DGGE band GB03-a10-p | Uncultured Deltaproteobacterium |

[a] Sequence accession number of copepod-associated bacteria found by Møller et al. (2007)

Phylotype Aca_104/1 clusters with a bacterium detected in a Taiwanese mangrove ecosystem (DQ234217) (Liao et al. 2007), while sequence Tem_104/37 is related to a surface water bacterium from the German Bight (DQ911789). Sequence Tem_104/50 is grouped with an uncultured surface-attached bacterium (EF215784) (Dang et al. 2008), whereas Aca_104/36 is related to a deep sea octacoral bacterium (DQ395758).

## DISCUSSION

Marine bacteria have a strong affinity to surfaces (Zobell 1943; Costerton et al. 1978; Cooksey and Wigglesworth-Cooksey 1995; Carman and Dobbs 1997). In many cases, such as with marine snow, this can be explained by the greater availability of resources in these aggregates, or in case of surface layers and biofilms, by some sort of protective features of these structures (Matz et al. 2005). In the case of the bacterial communities associated in- and outside of the marine copepods, little is known about their function and even less about their identity. Obviously, bacteria located around the mouth and anus could well be consuming waste and excretory products of the animals, but to our knowledge,

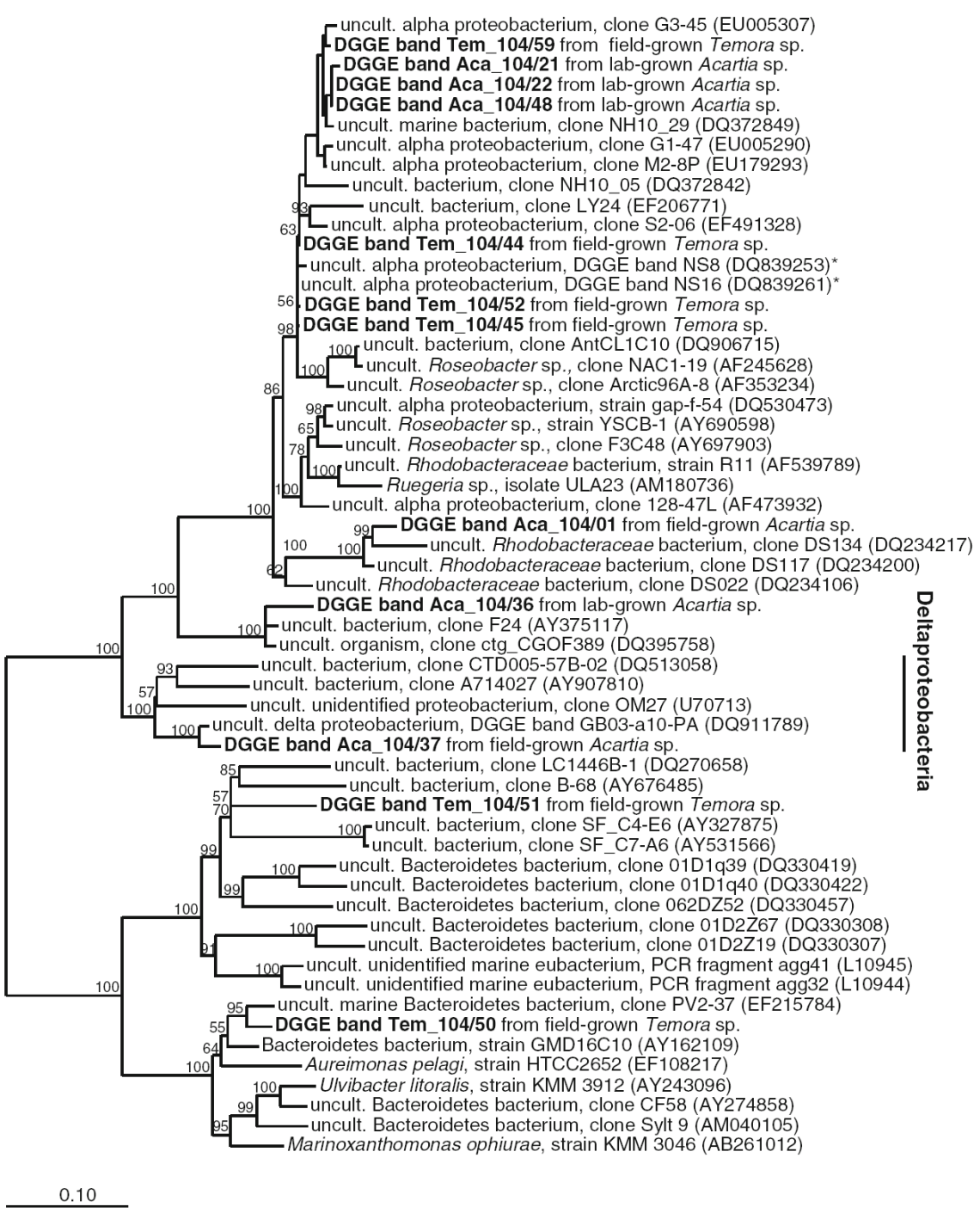


Fig. 5: Phylogenetic tree of Alphaproteobacteria, Deltaproteobacteria and members of the Bacteroidetes. GenBank accession numbers are given in parentheses. Bootstrap values above 50% are displayed. Marked with stars: Accession numbers of copepod-associated bacteria found by Møller et al. (2007)

no information as to their functional role exists. In fact, it is not even known precisely whether these bacteria are simply epibionts, or possibly harmful or pathogenic for the copepods themselves, for other aquatic animals or even for

humans. In earlier studies, on the identification of the copepod-associated bacteria Sochard et al. (1979) used culture techniques to characterise and quantify the bacteria with the well-known disadvantages of cultivation (Muyzer et al. 1993). So the use of culture-independent techniques or a combination of culture-dependent and -independent techniques (Peter and Sommaruga 2008) could possibly shed more light on the identity of the copepod-associated bacteria. For example, Tang and co-workers (Tang 2005; Tang et al. 2006a, b) investigated the bacteria on copepod carcasses and faecal pellet colonisation using molecular techniques. Their DGGE results showed that the bacterial community of copepod carcasses alter over the time of incubation. But from the results of this study, however, it remains unclear whether the bacteria were already associated with the living copepods or whether they colonised the animals after their death. In the case of the faecal pellets, it is unclear whether they were colonised by the bacteria in the gut or after defecation. Additionally, the bacteria found in these studies were not identified.

To our knowledge, Møller et al. (2007) were the first to investigate the bacterial community of the marine copepod *Calanus* sp. with PCR-DGGE followed by sequencing of DGGE bands. They reported difficulties with bacterial DNA-extraction from these copepods. These authors initially used a modified enzyme/SDS-extraction technique without the use of phenol–chloroform, but it seemed that the complete lysis of the copepods with this method resulted in inhibition of the following PCR reaction. The extraction method was changed accordingly and they continued with a commercial DNA extraction kit where the copepods were not visibly lysed.

Therefore, one of the aims of our study was to develop an extraction protocol suited for the analysis of copepod associated bacterial DNA. This optimised DNA extraction technique is absolutely essential for molecular biology, since a modification of the extraction method is required for each different sample and each tissue (Roose-Amsaleg et al. 2001). Two previous studies on the extraction of DNA from copepod-associated bacteria for PCR-DGGE analysis have been published so far (Tang et al. 2006b; Møller et al. 2007). Tang and co-workers used a treatment with zirconium beads and hot phenol–chloroform–isoamylalcohol, whereas Møller et al. used two other different methods. This shows that so far no standard DNA-extraction technique has been established.

In the present study, a modified lysozyme/SDS phenol–chloroform DNA extraction protocol was compared with a combination of two commercially available DNA-isolation kits and a commercial cell lysis reagent. These techniques differ in sample processing time and also in their efficiency. In addition, three different grinding procedures were tested to have the possibility to compare the surface-attached as well as the entire internal and external bacterial communities of the copepods. Due to the fact that, especially when using grinding techniques, also copepod DNA was extracted, the extracted DNA amounts were not quantified in this study. When the DNA extracts were used as PCR template, however, bacterial PCR product could be obtained in most cases.

Generally, the Lyse-N-Go-procedure was the fastest of the investigated techniques. It took only a few minutes to prepare the samples without grinding the copepods. With this procedure, there was no visible lysis of the copepods. Grinding with the pellet pestle took another 30 s, while grinding with the agate mortar required more time for transferring the copepods into the mortar and back to the test tube. Additionally, during this transfer, a lot of material was lost due to the large surface area of pestle and mortar, resulting in very faint PCR bands. For PCR reaction of the Lyse-N-Go processed samples, the mixture had to be diluted 10 fold, to get an adequate PCR product. This might be due to the presence of some inhibitory substances, as already described by Møller et al. (2007), when using two or more microlitres of the original mixture.

The combination of two commercial DNA extraction kits was even more time consuming. A combination of two kits was required because of the grinding procedure of the copepods. The lysing-matrix tube and the CLS-TC buffer (Bio1O1) were used with the corresponding FastPrep Instrument (Bio1O1) to homogenise the copepods, while the spin column of the Qiagen Blood and Tissue Kit was used to purify the DNA.

The lysozyme/SDS phenol–chloroform DNA extraction was the most time consuming procedure, because the precipitation step took place over night. However, the PCR product amount yield was best of all investigated procedures, regardless of whether the copepods were intact or ground. In contrast, Møller et al. (2007) reported that their modified enzyme/SDS extraction protocol led to a release of PCR-inhibiting substances which was not observed in our procedure,

as we used a phenol–chloroform step after enzyme/SDS-extraction to get rid of these substances. Therefore, from our investigations, we conclude that the lysozyme/SDS phenol–chloroform DNA extraction method is the one best suited, allowing even for the analysis of single copepod individuals.

The band intensities of PCR-products on ethidium bromide–stained agarose gels of phenol–chloroform extracts retrieved from laboratory-grown and field-caught *Acartia* sp. and field-caught *Temora* sp. were compared. Based on strong PCR product signals on an agarose gel, it can be assumed that field-caught *Temora* sp. may harbour more bacteria than field-caught and laboratory-grown *Acartia* sp. (Fig. 2). Since the copepods were not measured or weighed in this study, nor were the attached bacteria counted, we only presume that *Temora* sp. harbour more bacteria than *Acartia* sp. because of their larger size. However, this will need further investigations.

The DGGE finger printing analysis revealed clear differences among the investigated DNA extraction techniques. The banding patterns varied both in band numbers and band intensities. This might be due to low DNA concentrations, resulting in too low template concentrations for production of an adequate amount of PCR product. If the quantities of PCR product are too low, no DGGE-band can be formed. Therefore, it has to be considered that DGGE bands of a single copepod species can vary in number, although they harbour the identical bacterial community. These results underline the importance of a highly efficient DNA-extraction technique, which is essential for investigating the bacterial communities not only of copepods.

Distinct banding patterns are evident for each of the three copepod groups subjected to phenol–chloroform DNA extraction treatment and PCR-DGGE (Fig. 3). Hence, it is probable that specific copepod genera harbour different bacterial communities. Additionally, there were differences in the DGGE-banding patterns of intact and ground copepods extracted with phenol–chloroform, clearly suggesting that there are differences between the bacteria populations in- and outside of the copepods. To gain more information about this topic, it would be necessary to investigate, e.g., the gut of the copepods separately, because this should be the body part inside the copepods which harbours the most bacteria. That work would also contribute to answer the

question of whether the gut microflora of these important links of the marine food web is transient or permanent (Carman and Dobbs 1997).

For sequencing, the PCR products of the appropriate size were excised from the agarose gel to exclude unspecific by-products (approx. 100 bp) (Fig. 2). After sequencing the most prominent DGGE bands of the investigated copepod groups two bacterial phyla were found by sequence analysis. The identified phylotypes in this study belonged to the Alphaproteobacteria, Deltaproteobacteria and the Bacteroidetes. The phylotypes were related to marine surface-water bacteria as well as to surface-attached bacteria. One DGGE band (Tem_104/51) was related with a bacterium associated with larval stages of the tropical rock lobster (*Panulirus ornatus*) (Payne et al. 2007).

Four identified sequences of *Temora* sp.-associated bacteria (Tem_104/52, Tem_104/39, Tem_104/45 and Tem_104/56) (Table 1) were strongly related to two sequences of attached bacteria of the copepod *Calanus* sp. found by Møller et al. (2007). These bacteria were members of the *Roseobacter* lineage of the Alphaproteobacteria.

The finding that copepod-attached bacterial phylotypes were related to the *Roseobacter* lineage is not surprising, considering that members of the *Roseobacter* lineage are among the most predominant bacterial phylotypes recovered from marine plankton clone libraries and have been found to be dominant in the North Sea (Eilers et al. 2001).

Some excised bands (Aca_104/21, Aca_104/22 and Aca_104/48) result in the same sequence although they were excised from different gel positions (Tab. 1). This is probably due to microheterogeneity of different rRNA-operons present in this bacterial species, as was reported previously (Nübel et al. 1996; v. Wintzingerode et al. 1997). Although several other studies observed members of the genus *Vibrio* among the attached bacteria on copepods (Sochard et al. 1979; Huq et al. 1983; Tamplin et al. 1990), no members of this genus were found in our study. Members of the bacterial genus *Vibrio* seem to have a particularly strong affinity for all marine surfaces, especially under nutrient-limiting conditions (Dawson et al. 1981; Tamplin et al. 1990; Carman and Dobbs 1997). Nalin and co-workers (Nalin et al. 1979) found a connection between chitinous surfaces and *Vibrios*. Huq and coworkers (Huq et al. 1983. 1984), using culture techniques, detected both *V. cholerae* and *V. parahaemolyticus*

preferentially attached to planktonic copepods relative to other bacteria (e.g. *Escherichia coli* and *Pseudomonas* sp.). The absence of *Vibrio* sp. in the present study cannot be explained yet. However, maybe the DGGE-bands which represented *Vibrio* sp. were not excised because they were too faint or the sequencing of the excised band failed. Muyzer et al. (1993) and Murray et al. (1996) reported that PCR-DGGE is sensitive enough to detect bacteria which make up only 1–2% of bacterial populations in the mixed assemblage of selected bacterial strains. Thus, the PCR-DGGE method tends to bias towards the predominant groups within a community (v. Wintzingerode et al. 1997; Casamayor et al. 2000). Moreover, the detection limit of the PCR-DGGE method is affected by the relative abundance of a population as well as by ribosomal RNA (rrn) operon copy numbers (Kan et al. 2006). Nonetheless, substantial information about the species composition can be obtained from complex microbial communities by DGGE analysis (Muyzer and Smalla 1998).
The present and other similar studies allow only a small insight into the identity of the bacteria colonising the exoskeleton and the intestine of marine copepods. Hence, for further insights into the bacterial communities of different copepod genera, or even species, as well as into the copepod gut microflora and faecal pellet-associated bacteria, more studies have to be conducted. The outcomes of these studies strongly depend on highly efficient molecular biological techniques. A suitable bacterial DNA extraction technique for this research was developed and could be evaluated in this study.

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# CHAPTER III

# THE MICROBIOME OF NORTH SEA COPEPODS

# THE MICROBIOME OF NORTH SEA COPEPODS

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## ABSTRACT

Copepods can be associated with different kinds and numbers of bacteria. This has already been shown in the past with culture-dependent microbiological methods or microscopy and more recently by using molecular tools. Here, we investigated for the first time the bacterial community of four frequently occurring copepod genera, *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus* from the North Sea over a period of two years using denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing. Exemplarily three other copepod species *Pseudo-/Paracalanus sp*., *Candacia sp*. and *Euterpina* sp. were included to obtain a comprehensive insight into the structure of the whole bacterial community associated with North Sea copepods. To complement the DGGE analyses, clone libraries of two different time points of the sampling period were generated.

Based on the DGGE banding patterns of the two years survey, we could find neither any seasonality in the bacterial communities nor any significant differences among distinct copepod species. Overall, we identified 44 different bacterial genera falling into the four bacterial phyla of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. The most abundant phylotypes were affiliated to the bacterial classes of Alpha- and Gammaproteobacteria. In comparison of DGGE and clone libraries, gammaproteobacterial phylotypes dominated the clone libraries, whereas alphaproteobacterial were most abundant in the DGGE analyses.

## INTRODUCTION

Marine copepods may constitute up to 80 % of the mesozooplankton biomass (Verity and Smetacek 1996). They are key components of the food web as grazers of primary production and as food for organisms of higher trophic levels (Cushing 1989; Møller and Nielsen 2001). Copepods supply material and energy to the microbial loop (Azam et al. 1983) by to 'sloppy feeding' (Møller and Nielsen 2001), excretion and defecation (Hasegawa et al. 2001; Møller et al. 2003; Steinberg et al. 2004). Their moults and carcasses can also be populated and decomposed by bacteria (Tang et al. 2006a; Tang et al. 2006b; Tang et al. 2009b).

Copepods can be colonised by bacteria, especially in the oral and anal regions as well as on body appendages (Huq et al. 1983), the intersegmental parts (Carman and Dobbs 1997) and the intestine (Sochard et al. 1979; Nagasawa and Nemoto 1988). The nature of this association, whether the bacteria are parasites, commensals or symbionts, and also the origin of these bacteria are not yet understood. To date it is also not known whether copepods exhibit a distinct bacterial community related to their lifestyle or metabolism or whether they represent just polymeric chitinous surfaces in the marine environment. Bacteria located on the copepod's exterior, in the gut, on faecal pellets and on carcasses have been investigated with different methods in a number of previous studies (e.g. Harding 1973; Sochard et al. 1979; Nagasawa et al. 1985; Nagasawa and Nemoto 1988; Nagasawa 1992; Carman 1994; Delille and Razouls 1994; Kirchner 1995; Hansen and Bech 1996; Carman and Dobbs 1997; Tang 2005; Møller et al. 2007; Tang et al. 2009b; Brandt et al. 2010). However, these studies were only snap shots investigating geographically different marine sites and several copepod genera with only few samples and applying a vast variety of methods.

Historically, classical microbiological cultivation methods were used to obtain an insight into the bacterial community of some copepod genera (e.g. Sochard et al. 1979). Culture-dependent methods impart a bias towards microorganisms that can be easily cultured (DeLong et al. 1993; Heidelberg et al. 2002a). Molecular techniques that do not require cultivation recently opened new perspectives (Heidelberg et al. 2002a) in the analysis of the copepod associated bacterial community (Møller et al. 2007). Nevertheless, since the majority of former

studies focussed solely on the identification of pathogens, e.g. *Vibrio cholerae* (e.g. Kaneko and Colwell 1975; Nalin et al. 1979; Belas and Colwell 1982; Huq et al. 1983; Huq et al. 1984; Tamplin et al. 1990; Heidelberg et al. 2002b), not much is known about the whole bacterial community associated with copepods or seasonal dynamics of the associated bacteria to date.

The present study investigated the bacterial communities of four different marine calanoid copepod genera, *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus* during two seasonal cycles. Furthermore, the analysis of the bacterial community using DGGE and subsequent sequencing of dominant bands was analysed using clone libraries at two distinct time points of the sampling period. The main focus of this study was not only to get an overview of the microbiome of North Sea copepods in general, but also to examine species specificity or seasonality of certain bacterial populations.

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND PREPARATION

Zooplankton samples from Helgoland Roads in the North Sea (54°11.3' N and 7°54.0' E) were collected between February 2007 and March 2009 using a 150 µm and 500 µm net aboard the research vessels "Aade" and "Diker". The sampling intervals were weekly (first six months), bi-weekly (second six months) and monthly (second year), respectively. Specimens from *Acartia* sp. (ACA), *Temora longicornis* (TEM), *Centropages* sp. (CEN) and *Calanus helgolandicus* (CAL) were sampled during the two years, whereas specimens of *Euterpina* sp. (EUT), *Pseudo-/Paracalanus* sp. (PSE), and *Candacia* sp. (CAN) were sampled exemplarily during the first year when they occurred. Using a stereo microscope, the animals were sorted by genus with sterile tweezers and washed twice in sterile sea water. Until further analysis, individual copepods were frozen in sterile reaction tubes at -20 °C.

### DNA EXTRACTION

DNA extraction was carried out using a pellet pestle with pellet pestle motor (Kontes, Vineland, NJ, USA). Bundles of three (CAL) or five (all other copepod genera) copepod individuals were ground for 30 s. This was followed by a phenol-chloroform-DNA extraction with SDS and lysozyme (Brandt et al. 2010).

## PCR

PCR-amplification of 16S rRNA gene fragments for DGGE was performed using the bacteria specific primers 341f with GC-clamp (P3) (Muyzer et al. 1993) with a 40-bp GC-rich sequence at the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3') (Muyzer et al. 1995).

PCR mixtures with a volume of 50 µl contained 5 µl of 10 x Taq buffer (5 Prime, Hamburg, Germany), 8 µl of Master Enhancer (5 Prime) for initial PCRs and no enhancer for reamplification after DGGE, 200 µM dNTPs (Promega, Mannheim, Germany), 0.2 µM of each primer, 2 U of Taq DNA polymerase (5 Prime) and 2 µl of DNA prior and after DGGE. 'Touchdown'-PCR was performed as described by Sapp et al. (2007b). PCRs were conducted in a Mastercycler (Eppendorf, Hamburg, Germany) and PCR products were separated on 1.2% (w/v) agarose gels (50 min at 100 V in 0.5 x TBE).

For the cloning approach the primers 63f and 1387r (Marchesi et al. 1998) were used for PCR (composition of PCR mixtures see above) to get a 16S rDNA-fragment as long as possible. The temperature profile was as follows: 5 min initial denaturing at 94 °C, 30 cycles with denaturing at 94 °C for one min, annealing at 55 °C for one min and elongation at 68 °C for two min was followed by a final elongation step at 68 °C for 6 min.

Separated PCR products were visualised by ethidium bromide (0.5 mg $l^{-1}$) and images were captured with a ChemiDoc XRS System (BioRad, München, Germany). The thickness and intensity of each band visualised were used to gauge the relative volume of the corresponding product used for DGGE (see below).

## DGGE

All 16S rRNA gene amplicons were resolved on 6% (w/v) polyacrylamide gels in 0.5 x TAE buffer (20 mM TrisHCl, 10 mM acetic acid, 0.5 mM EDTA) with denaturing gradient of 15–55% urea/formamide (100% denaturant contains 7M urea and 40% formamide). Electrophoresis was performed at 60 °C and 150 V for 10 h (Sigler et al. 2004) using a DCode mutation detection system (BioRad). DGGE gels were stained with SYBRGold (Invitrogen, Karlsruhe, Germany). Imaging was performed with a ChemiDoc XRS System (BioRad). Prominent

DGGE bands were excised, eluted in 50 µl PCR-water (Eppendorf) by gentle shaking at 37 °C for 60 min, reamplified and confirmed by an additional DGGE. As a marker for comparative analyses of all DGGE gels the combined PCR-amplicons (GC-341f/907r) of four bacteria (*Polaribacter filamentus* DSM 13964, *Sulfitobacter mediterraneus* DSM 12244, *Arthrobacter agilis* DSM 20550, *Leifsonia aquatica* DSM 20146) were used.

## CLONING

The 1324 bp 16S rDNA-PCR fragment of selected copepod samples of TEM, ACA and CEN of two dates, June 2007 (05.06.2007) and 2008 (03.06.2008) were cloned into the cloning vector pCR® 4-TOPO® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The clones were differentiated into different clone types via single base sequencing (Schmidt et al. 1991). Sequencing was conducted with DNA Sequencer LONG READER IR4200 (LI-COR, Lincoln, NE, USA) and SequiTherm EXCEL™ II DNA Sequencing Kit LC (Epicentre, Madison, WI, USA) after manufacturer's protocol. Identical banding patterns with a similarity of more than 99% (less than 5 bp differences) were combined to one clone type.

## SEQUENCING AND PHYLOGENETIC ANALYSIS

For sequencing, eluted DGGE-bands were reamplified using the primers 341f (without GC-clamp) and 907r. PCR-products were checked on 1.2% (w/v) agarose gels prior to sequencing. PCR-products with the correct size (~566 bp) were excised from the agarose gels and used for sequencing.

The PCR-products of different clone types were reamplified with the primer pair 63f and 1387r and purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

DNA sequencing of PCR-products was performed by Qiagen GmbH using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as used for reamplification. Sequence data were checked for the presence of PCR amplified chimeric sequences by the CHECK_CHIMERA program (Cole et al. 2003). Nearest relatives for all sequences were searched using BLAST (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997). The ARB software package (http://www.arb-home.de) was used for

phylogenetic analysis (Ludwig et al. 2004). After addition of sequences to the ARB 16S rDNA sequences database (release May 2005), alignment was carried out with the 'Fast Aligner' integrated in the program and refined by comparison of closest relatives retrieved by BLAST. Sequences with more than 1300 nucleotides were used to calculate phylogenetic trees. The ARB 'parsimony interactive' tool was used to add partial sequences to respective trees. Phylogenetic relationships were deduced by the neighbour joining method including the correction algorithm of Felsenstein (1993).

After the cloning approach and phylogenetic analysis rarefaction method (Sanders 1968; Hurlbert 1971) was used to estimate whether the bacterial diversity of each clone sample was detected. The rarefaction curve was calculated with the online software FastGroupII (http://biome.sdsu.edu/fastgroup/cal_tools.html).

### STATISTICAL ANALYSIS

Analyses of DGGE fingerprints were carried out with Bionumerics 5.0 software package (Applied Maths NV, Sint-Martens-Latem, Belgium). Normalisation of DGGE gels was performed using a marker consisting of combined PCR-amplicons (GC-341f / 907r) of four bacteria with different GC-contents (see above).

Band matching analysis was performed with Bionumerics 5.0 software (Applied Maths NV) for sample comparison. Bands were assigned to classes of common bands within all profiles. Ordination techniques based on DGGE fingerprints were used to analyse the bacterial community at the phylotype level and the factors affecting specific bacterial phylotypes. Multivariate analysis of fingerprints was performed using the subroutines of non-metric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM) of the PRIMER 6.1.6 software suite (PRIMER-E Ltd., Plymouth, UK). ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA). Analysis of similarity (ANOSIM; Primer; Jaccard distance measure, 999 permutations) was used to test differences in bacterial community composition of different copepod genera, of the different months, seasons (March-May =

spring; June-August = summer; September-November = autumn; December-February = winter) and years.

### NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

(not yet available)

## RESULTS

### COMPARISON OF DGGE BANDING PATTERNS

Overall more than 2000 copepod individuals were sampled over the two years. For DGGE analyses we pooled three (CAL) to five copepod individuals (all others) from the same genus of one sampling day and obtained DGGE patterns from 118 different copepod pools (CAL = 20; CEN = 21; ACA = 30; TEM = 29; PSE = 5; CAN = 6; EUT = 6) and 16 pools from laboratory-reared ACA, field-caught ACA and TEM from a previous study (Brandt et al. 2010).

For all investigated copepod genera the DGGE band numbers ranged between two and 20 per sample.

For statistical analysis a total of 37 band classes were assigned. A few bands could not be included in the statistical analysis because they appeared outside the marker band positions of the DGGE gels. These bands were, however, used for further sequencing and phylogenetic analysis.

Analysis of similarities (ANOSIM) showed neither significant differences among the DGGE patterns of different copepod genera (Global R = 0.021; p = 20.6%) nor among different months (Global R = 0.083; p = 0.5%), seasons (Global R = 0.017; p = 21.1%) or years (Global R = 0.059; p = 4.7%). In Fig. 1 and 2 the two-dimensional nMDS plots of seasons and genera are shown. The stress value is 0.27 which is quite high. A three-dimensional plot based of the same data set (not shown), however, also displays a high stress value (0.19). Values of stress in the range 0.2-0.3 should be treated with a great deal of scepticism (Clarke and Warwick 2001). Concerning alpha-diversity, a slight dependency (not significant) between OTU (band) richness and months was observed. The number of band classes increased from January to March and decreased until December (data not shown).

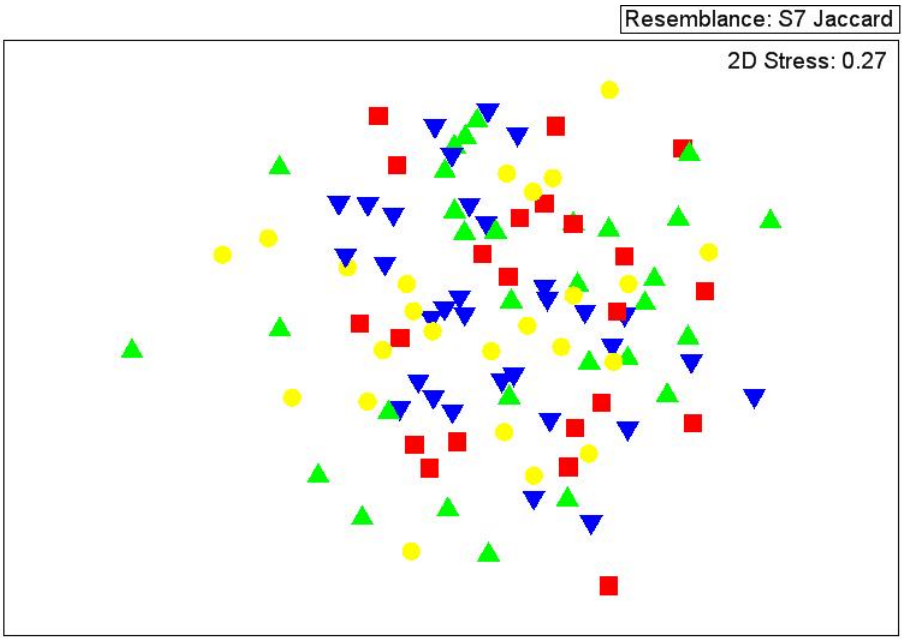



Fig. 1: nMDS plot based on Jaccard similarities of DGGE fingerprints of bacterial communities of four different copepod genera (▲ *Acartia* sp., ▼ *Temora longocornis*, ■ *Centropages* sp. and ● *Calanus helgolandicus*)

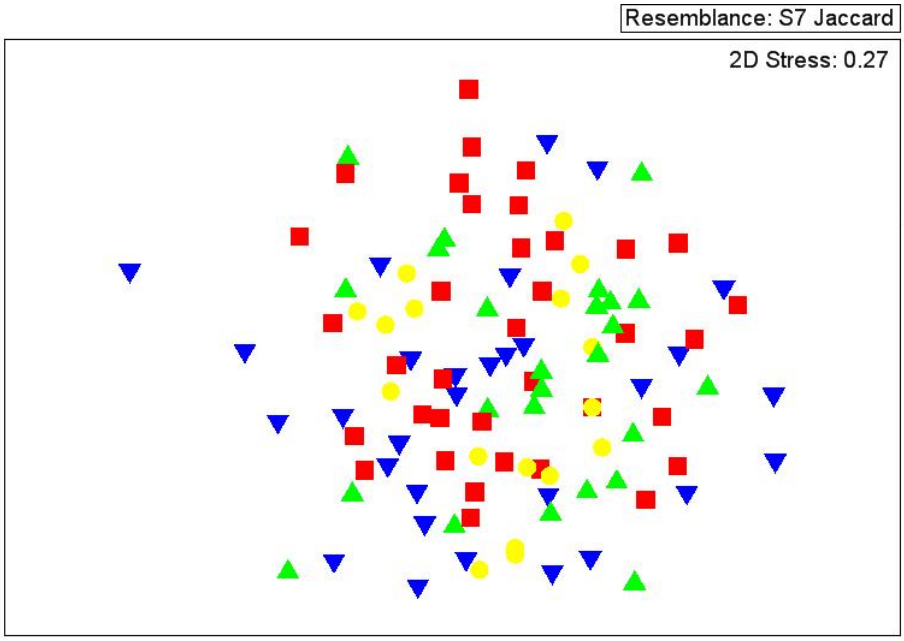



Fig. 2: nMDS plot based on Jaccard similarities of DGGE fingerprints of bacterial communities of four different copepod genera (*Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) during different seasons. ▲ winter, ▼ spring, ■ summer, ● autumn

## ANALYSIS OF EXCISED 16S RDNA DGGE BANDS

Overall 223 DGGE bands were successfully sequenced (see Tab.1a). Several of these bands occurred at similar positions in DGGE gels, representing identical band classes. Those sequences which exhibited almost identical BLAST results and positions in phylogenetic trees were combined to distinct phylotypes (in trees and tables) when they originated from the same copepod genus, including different pools or sampling dates. By this procedure we identified 36, 64, 16, 23, seven, eight and two different bacterial phylotypes for ACA, TEM, CEN, CAL, PSE, EUT and CAN, respectively (Tab. 1a). Overall, for all seven copepod genera, the obtained bacterial phylotypes fell into four different bacterial phyla: Actinobacteria (7%), Bacteroidetes (10%), Firmicutes (3%), and Proteobacteria (80%) (data not shown). For PSE, EUT and CAN only very few bacterial sequences were obtained and hence were excluded from further analysis. Beside the bacterial sequences, two chloroplast sequences were obtained (from TEM and CEN). Additionally, one DNA sequence matched with 18S rDNA of *Calanus* sp. (from CAL).

For the four copepod genera we found bacterial sequences of three and four different bacterial phyla, respectively: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Fig. 3). For *Centropages* sp. the phylum Firmicutes was absent. Overall the Proteobacteria were the dominating phylum (Fig.3).

Tab. 1a: Obtained sequences and phylotypes from DGGE-bands for the seven investigated copepod genera. Sequences were combined to one phylotype when cut DGGE bands were at the same gel position and sequences appeared at the same position in phylogenetic trees. Additionally only sequences with differences not more than 1% were combined to one phylotype.

| Copepod genus | Label | Number of sequences | Number of phylotypes |
|---|---|---|---|
| *Acartia* sp. | ACA | 53 | 36 |
| *T. longicornis* | TEM | 94 | 64 |
| *Centropages* sp. | CEN | 26 | 16 |
| *C. helgolandicus* | CAL | 28 | 23 |
| *Pseudo-/Paracalanus* sp. | PSE | 8 | 7 |
| *Euterpina* sp. | EUT | 12 | 8 |
| *Candacia* sp. | CAN | 2 | 2 |

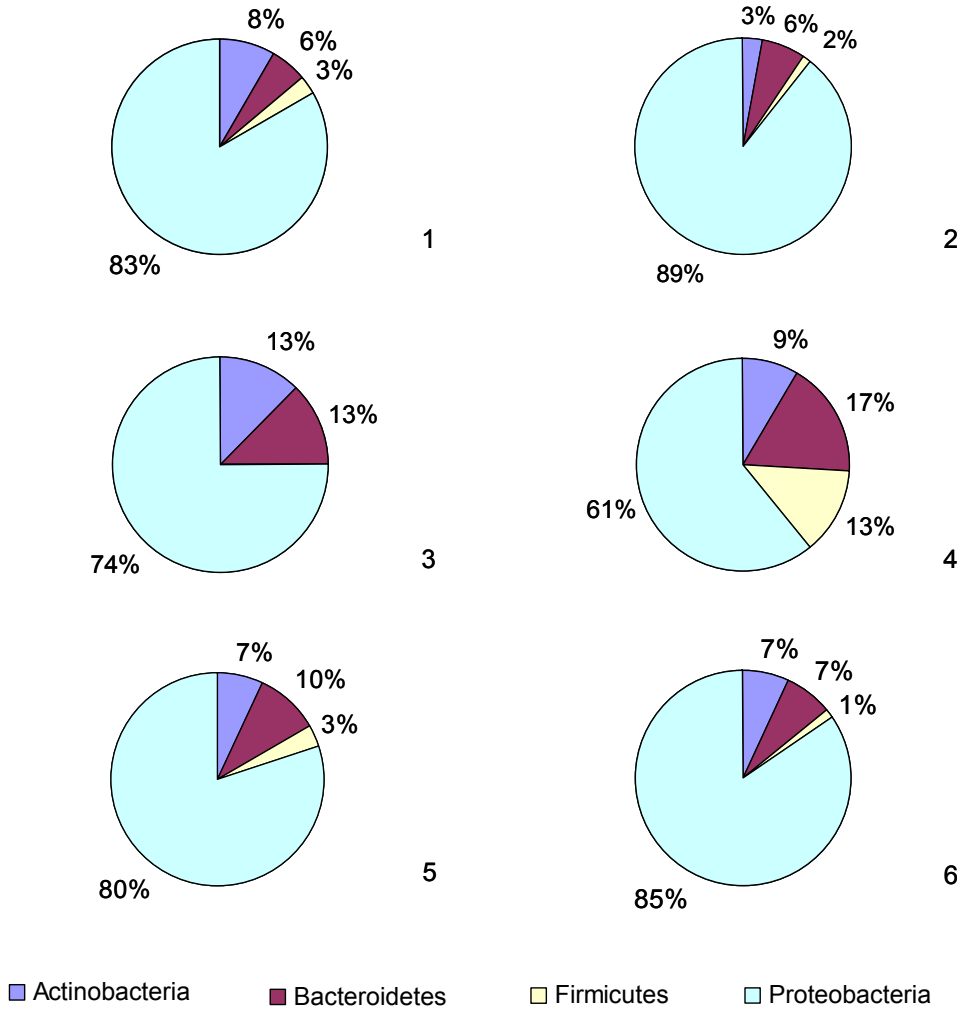



Fig. 3: Bacterial phyla associated with 1) *Acartia* sp., 2) *Temora longicornis*, 3) *Centropages* sp., 4) *Calanus helgolandicus* using PCR-DGGE; and comparison of bacterial phyla found with 5) DGGE (pooled: *Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) and 6) cloning (pooled: *Acartia* sp., *Temora longocornis* and *Centropages* sp.). Percentages <1% are not displayed.

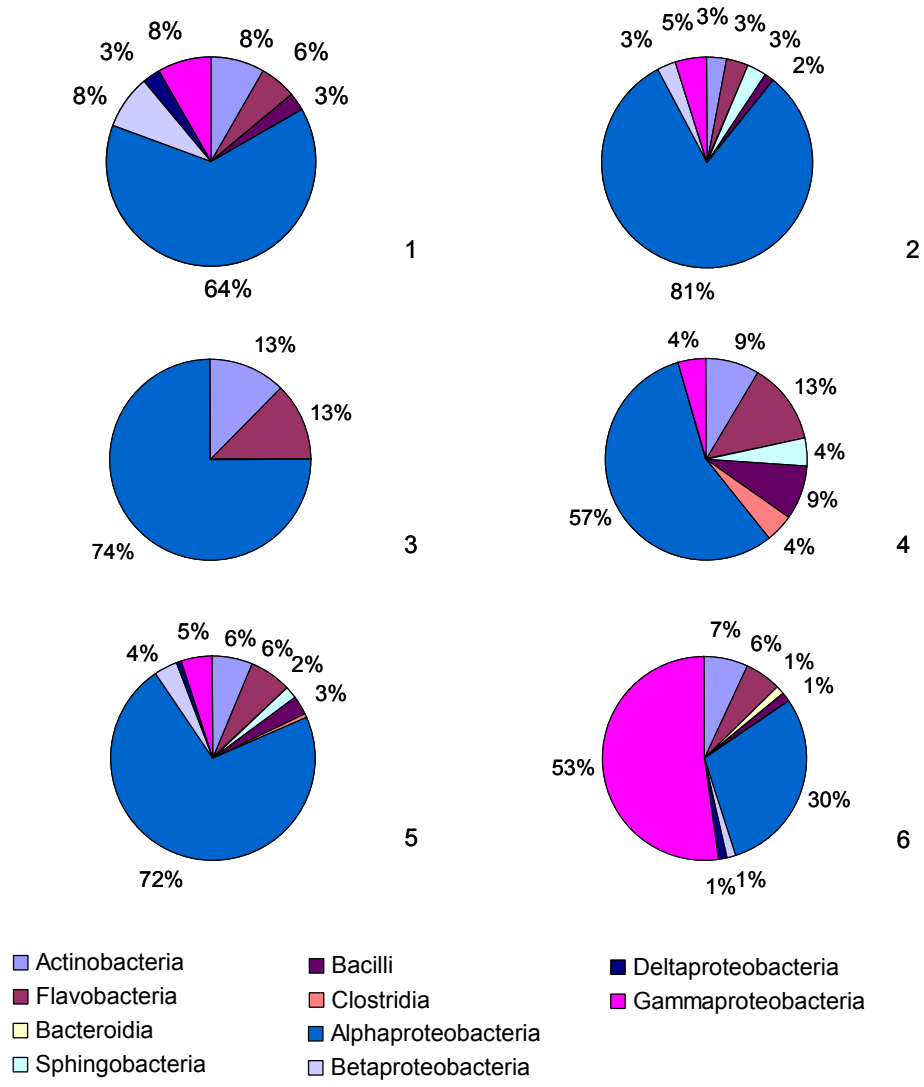



Fig. 4: Bacterial classes associated with 1) *Acartia* sp., 2) *Temora longicornis*, 3) *Centropages* sp., 4) *Calanus helgolandicus* using PCR-DGGE; and comparison of bacterial phyla found with 5) DGGE (pooled: *Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) and 6) cloning (pooled: *Acartia* sp., *Temora longocornis* and *Centropages* sp.). Percentages <1% are not displayed.

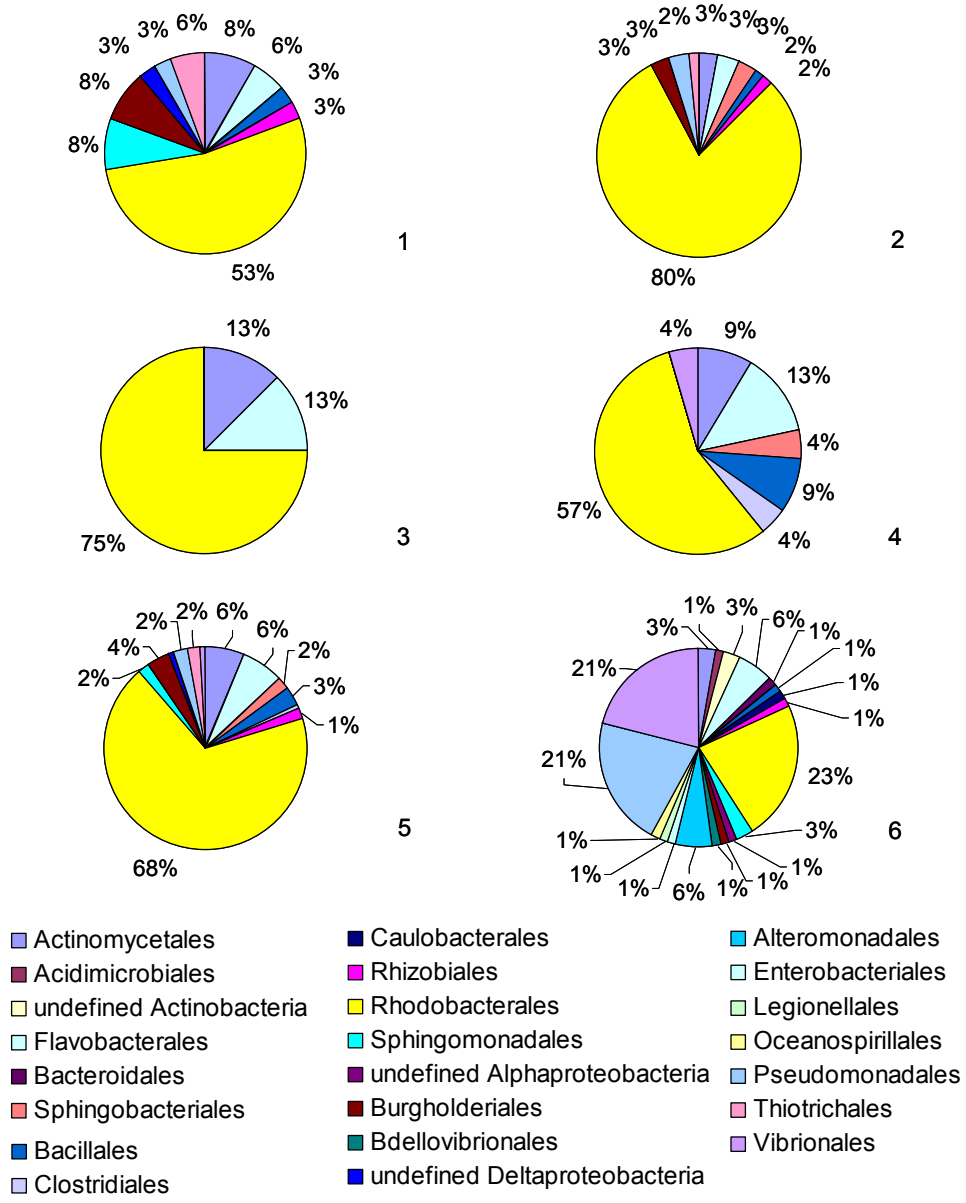



Fig. 5: Bacterial orders associated with 1) *Acartia* sp., 2) *Temora longicornis*, 3) *Centropages* sp., 4) *Calanus helgolandicus* using PCR-DGGE; and comparison of bacterial phyla found with 5) DGGE (pooled: *Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) and 6) cloning (pooled: *Acartia* sp., *Temora longocornis* and *Centropages* sp.). Percentages <1% are not displayed.

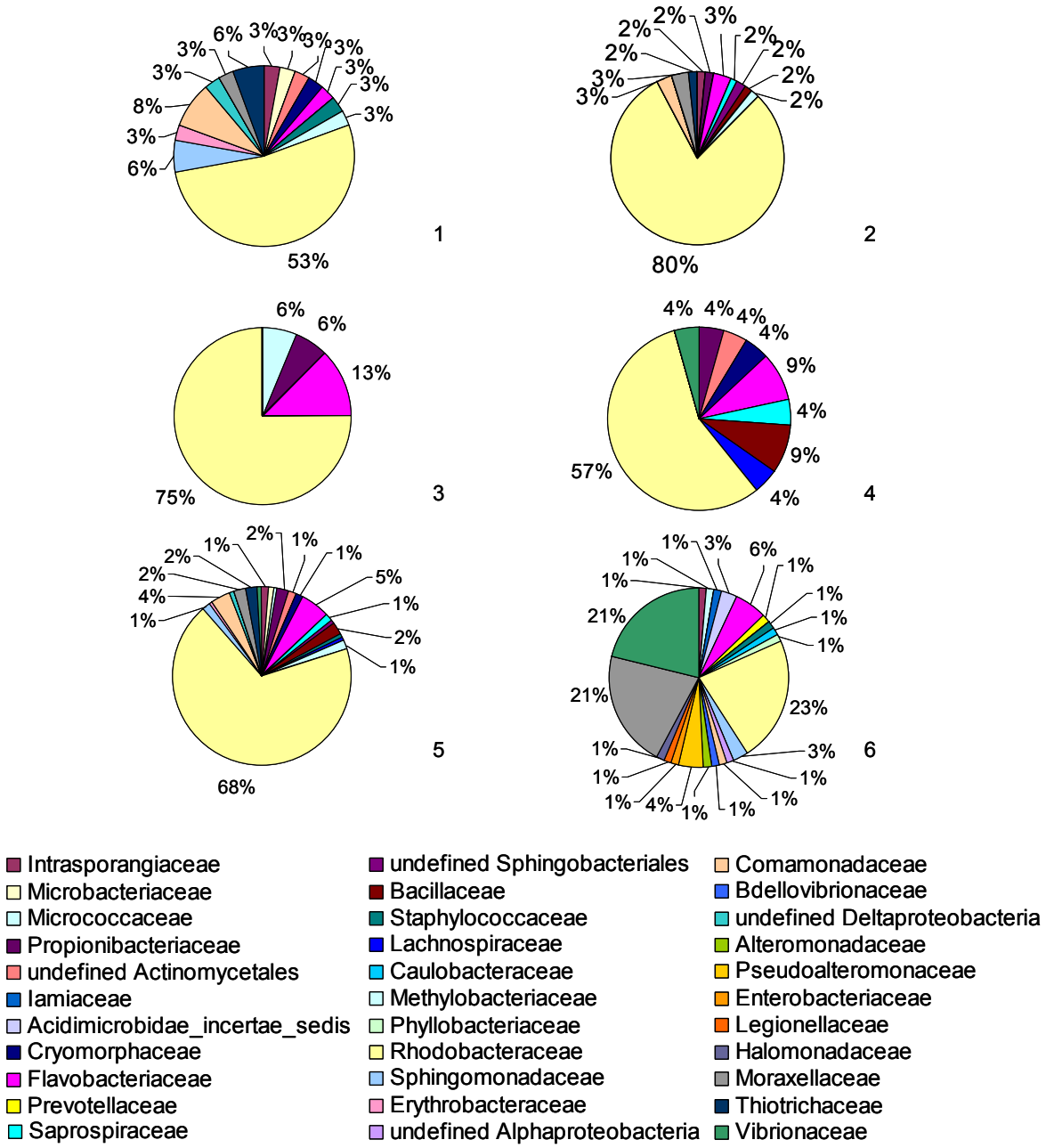



Fig. 6: Bacterial families associated with 1) *Acartia* sp., 2) *Temora longicornis*, 3) *Centropages* sp., 4) *Calanus helgolandicus* using PCR-DGGE; and comparison of bacterial phyla found with 5) DGGE (pooled: *Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) and 6) cloning (pooled: *Acartia* sp., *Temora longocornis* and *Centropages* sp.). Percentages <1% are not displayed.

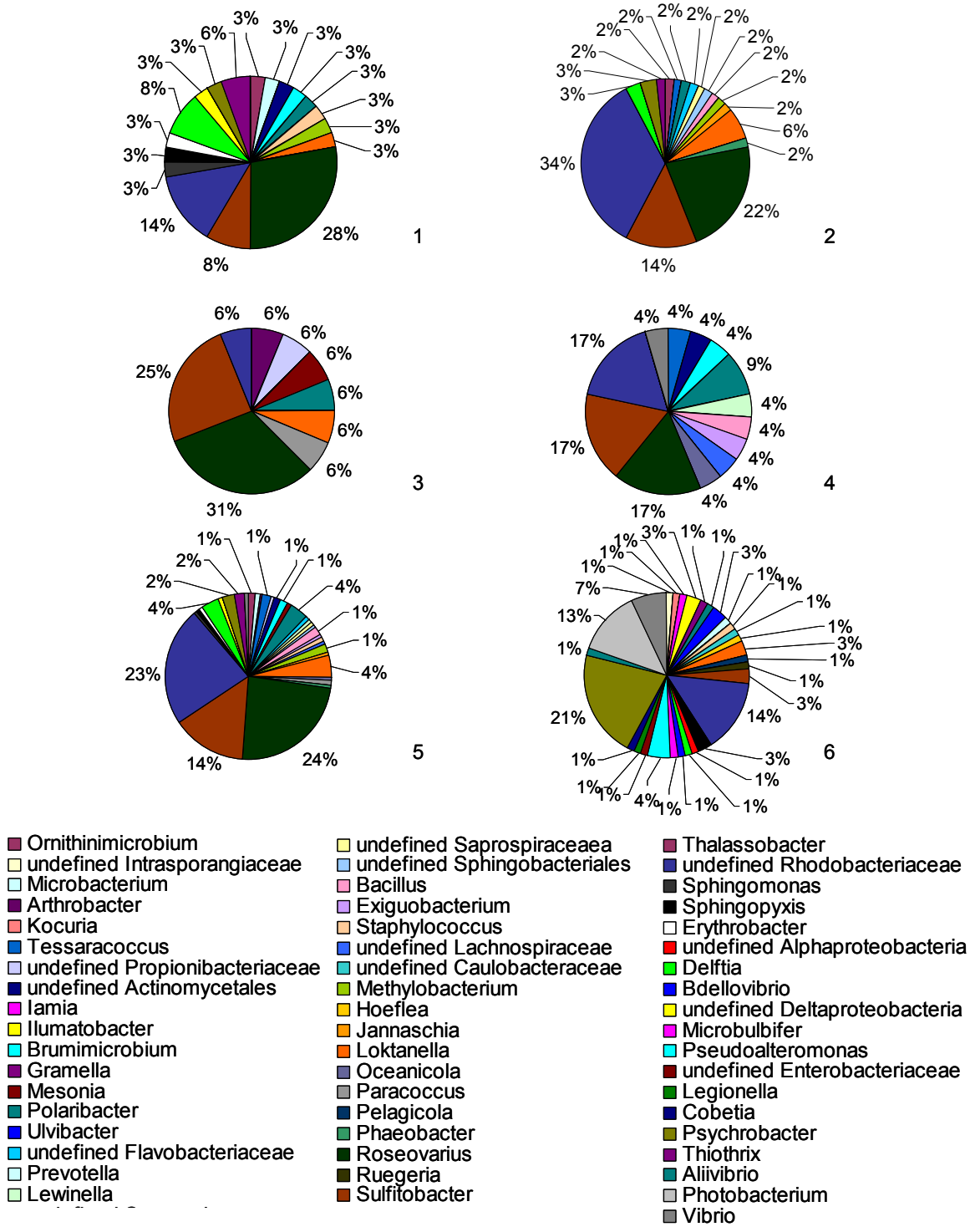



Fig. 7: Bacterial genera associated with 1) *Acartia* sp., 2) *Temora longicornis*, 3) *Centropages* sp., 4) *Calanus helgolandicus* using PCR-DGGE; and comparison of bacterial phyla found with 5) DGGE (pooled: *Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) and 6) cloning (pooled: *Acartia* sp., *Temora longocornis* and *Centropages* sp.). Percentages <1% are not displayed.

Overall, for the four investigated key copepod genera, we identified nine different bacterial classes (Fig. 4). Comparing the four different copepod genera Alphaproteobacteria were identified as the most abundant class. Three copepod genera harboured seven different bacterial classes each. For CEN only four different classes were identified.

We detected twelve different bacterial orders altogether (Fig. 5). Comparing the four different copepod genera, it became obvious that Rhodobacterales was the dominating order. Sphingobacteriales were only found associated with TEM and CAL. Within the class of Alphaproteobacteria the order Rhizobiales was found only associated with ACA and TEM, whereas the Sphingomonadales were discovered only associated with ACA. Burgholderiales were not detected in CAL-samples whereas only in these samples Vibrionales could be observed. CEN-samples only contained Actinomycetales, Flavobacterales and Rhodobacterales.

The twelve orders could be separated into 18 different bacterial families (Fig. 6). ACA harboured the highest number of different bacterial families (twelve). Ten families each were associated with TEM and CAL and only four with CEN. The Rhodobacteraceae dominated in all four copepod genera.

Overall we identified 26 different bacterial genera associated with the four copepods (Fig. 7, see also Tab. 2-6 in the supplementary material for the phylogenetic classification of the detected phylotypes): *Ornithinimicrobium* was the only genus within the Intrasporangiaceae, whereas *Microbacterium* was the only one within the Microbacteriaceae. *Arthrobacter* fell into the family of Micrococcaceae and *Tessaracoccus* into the Propionibacteriaceae. *Brumimicrobium* was the only Cryomorphaceae-genus, whereas the Flavobacteriaceae included two genera, *Mesonia* and *Polaribacter*. The genus *Lewinella* belonged to the Saprospiraceaea and *Bacillus* as well as *Exiguobacterium* fell into the Bacillaceae. *Staphylococcus* was the only member of the Staphylococcaceae. Furthermore, *Methylobacterium* belonged to the Methylobacteriaceae, and the Rhodobacteraceae were represented by *Jannaschia*, *Loktanella*, *Oceanicola*, *Paracoccus*, *Phaeobacter*, *Roseovarius* and *Sulfitobacter*. *Sphingomonas* and *Sphingopyxis* fell into the Sphingomonadaceae. *Erythrobacter* belonged to the Erythrobacteraceae, *Delftia* to the Comamondaceae. *Psychrobacter* fell into the Moraxellaceae,

*Thiothrix* belonged to the Thiothrichaceae and *Vibrio* represented the Vibrionaceae.

Undefined sequences made up to 1% of all sequences on genus-level each, Propionibacteriaceae, Flavobacteriaceae, Saprospiraceaea and Lachnospiraceae. 23% were undefined Rhodobacteriaceae. The most abundant genus was *Roseovarius* with 24%, followed by *Sulfitobacter* with 14% of all sequences on genus-level (Fig. 7). CEN harboured seven different bacterial genera whereas CAL harboured nine. 15 different bacterial genera were associated with ACA and 13 with TEM. *Vibrio* was only found associated with CAL (Fig. 7).

## ANALYSIS OF CLONE LIBRARIES

For cloning we used bundles of five copepod individuals of three copepod genera from two sampling dates in June 2007 and 2008. We collected 108, 126, and 34 clones for ACA, TEM and CEN in 2007, and 25, 89 and 50 clones in 2008 (CAL was absent on these sampling days), respectively (for details see Tab. 1b). The number of clone types was low for CEN in June 2007 (two) and highest for TEM sampled in June 2007 (26). Altogether 72 clone types could be assigned.

Tab. 1b: Obtained clones and clone types from the three investigated copepod genera. Clones were combined to one clone type when single base sequencing showed a similarity of at least 99 %.

| Sampling day | Copepod genus | Label | Number of clones | Number of clone types |
|---|---|---|---|---|
| 05.06.2007 | *Acartia* sp. | KK_C | 108 | 11 |
| | *T. longicornis* | KK_D | 126 | 26 |
| | *Centropages* sp. | KK_E | 34 | 2 |
| 03.06.2008 | *Acartia* sp. | KK_F | 25 | 11 |
| | *T. longicornis* | KK_A | 89 | 13 |
| | *Centropages* sp. | KK_B | 50 | 9 |

As compared to DGGE we found four different phyla in the clone libraries: Actinobacteria (7%), Bacteroidetes (7%), Firmicutes (1%) and Proteobacteria (85%) (Fig. 3). The low bacterial diversity of CEN-samples detected by DGGE is reflected in the respective clone library.

In the following section we avoid listing all identified bacterial classes, orders, families and genera detected in the clone libraries, but indicate main differences to the DGGE approach.

On the level of bacterial classes we identified eight classes (Fig. 4). Sphingobacteria and Clostridia were not discovered by cloning but Bacteroidia occurred, although not discovered by DGGE (Fig. 4). In contrast to DGGE-band-sequencing the Gammaproteobacteria were the most dominant class with 53% of all sequences on class-level in all clone libraries. The second most abundant were Alphaproteobacteria with 30%.

Within the eight bacterial classes 17 orders of bacteria were identified. In addition to those detected by DGGE-band-sequencing, Acidimicrobiales within the Actinobacteria and Bacteroidales (Bacteroidia) were discovered. Furthermore, we detected Caulobacterales affiliated to the Alphaproteobacteria, Bdellvibrionales which belong to the Deltaproteobacteria and Alteromonadales, Enterobacteriales, Legionellales as well as Oceanospirillales which represented the Gammaproteobacteria. Both, the Pseudomonadales and Vibrionales dominated with 21% with cloning. Some Actinobacteria and Alphaproteobacteria could not be defined on this level (Fig. 5). We could not detect Sphingobacteriales, Clostridiales, or Thiothrichales, which we found by DGGE-band-sequencing (Fig. 5).

On bacterial family level 19 different families could be detected (Fig. 6). We found Iamiaceae as members of the Acidimicrobiales, Prevotellaceae which belong to the Bacteroidales, and Caulobacteraceae which fell into the Caulobacterales. Also the Alteromonadales-member Pseudoalteromonadaceae and Enterobacteriaceae as representative of Enterobacterales could be detected in the clone libraries. Halomonadaceae which belong to the Oceanospirillales as well as Phyllobacteriaceae which fell into the Rhizobiales could be found. We also detected the Bdellvibrionales-members Bdellovibrionaceae and Alteromonadaceae which fell into the Alteromonadales, in addition to the families detected by DGGE-band-sequencing. Microbacteriaceae, Propionibacteriaceae, Cryomorphaceae, Saprospiraceae, Bacillaceae, Lachnospiraceae, Methylobacteriaceae, Erythrobacteraceae and Thiotrichaceae were missing in the clone-library analysis (Fig. 6).

Dominating families as determined by cloning were Moraxellaceae (21%) and Vibrionaceae (21%) (Fig. 6).

23 different bacterial genera were distinguished (Fig. 7). In addition to the genera identified by DGGE-band-sequencing we detected Micrococcacea-genera *Kocuria*, *Iamia* which belongs to Iamiaceae, the Actinobacterium *Ilumatobacter,* as well as *Gramella* and *Ulvibacter* as members of the Flavobacteriaceae. We found *Prevotella* of the family Prevotellaceae and *Hoeflea* of the family Phyllobacteriaceae. Also *Pelagicola* and *Ruegeria* as members of the Rhodobacteriaceae were additionally detected by clone library analysis. *Bdellovibrio*, *Microbulbifer*, *Pseudoalteromonas, Legionella, Cobetia* as members of the families Bdellvibrionaceae, Alteromonadaceae, Pseudo-alteromonadaceae, Legionellaceae and Halomonadaceae, respectively, were found as well as the Vibrionaceae-genera *Aliivibrio* and *Photobacterium*. Some Intrasprangiaceae, Saprospiraceae, Caulobacteraceae, Rhodobacteraceae and Enterobacteriaceae could not be defined on this level (Fig. 7).

Not detected in the clone-libraries but with DGGE were the genera *Ornithimicrobium, Microbacterium, Arthrobacter, Tessaracoccus, Brumi-microbium, Mesonia, Lewinella, Bacillus, Exiguobacterium, Staphylococcus, Methylobacterium, Jannaschia, Oceanicola, Paracossus, Phaeobacter, Roseovarius,* which was dominating by DGGE-band-sequencing, *Sphingomonas, Erythrobacter and Thiothrix* (Fig. 7).

## DISCUSSION

For the first time the analysis of bacterial assemblages of four copepod key genera (*Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus*) was done over a period of two years using culture-independent techniques. We used DGGE and molecular cloning for the assessment of the phylogenetic diversity of the complex microbiome of these four frequently occurring and three less abundant North Sea copepod genera (*Candacia* sp., *Euterpina* sp. and *Pseudo-/Paracalanus* sp.).

Due to methodological limitations in DGGE only a relatively small part of the 16S rRNA gene can be analysed and used as a phylogenetic discriminator. Hence, it is likely that several ‘species’ are combined in some of the OTUs (bands) (Ferrari and Hollibaugh 1999). The number of 16S rRNA genes per

genome could be a reason for overestimating or underestimating some bacterial groups (Cottrell and Kirchman 2000b). With DGGE only a few Gammaproteobacteria were found (Fig. 4, 10), as was already observed by Alónso-Sáez and co-workers (2007), who could not detect any Gammaproteobacteria using DGGE analysis. In our study, Bacteroidetes, an important group of the coastal marine bacterial community (Eilers et al. 2001), were found by DGGE-band-sequencing as well as in the clone libraries (Fig. 3, 11). Cottrell and Kirchman (2000b) stated that Alphaproteobacteria are generally overestimated and Bacteroidetes are underestimated in clone libraries. Hence, care must be taken when attempting to deduce the structure of a bacterial community based on just one analysis method. We think that in our study the combination of the two molecular techniques provided a comprehensive analysis of the bacterial assemblages of the investigated marine copepod genera.

Although the analysis of the 16S rDNA inheres a number of limitations which are well known, the use of this gene as a phylogenetic marker helps to determine the phylogenetic position of bacteria in the evolutionary tree of life independent of cultivability and complexity of the ecosystem (v. Wintzingerode et al. 1997; Hugenholtz et al. 1998).

In total 44 different bacterial genera which belong to four bacterial phyla were found to be associated with the four investigated key copepod genera (Fig. 7).

Referring to the DGGE analyses, sequences affiliated with the Alphaproteobacteria were predominant (Fig. 4, 8, 9a, 9b). This group was also the most common one among the phylotypes in previous studies on copepods by molecular techniques (Møller et al. 2007; Peter and Sommaruga 2008; Grossart et al. 2009; Tang et al. 2009a; Tang et al. 2009b). Older studies mostly based on culture-dependent methods found Gammaproteobacteria to be the dominating bacterial class (e.g. Sochard et al. 1979). This finding is in accordance with our clone libraries (Fig. 4).

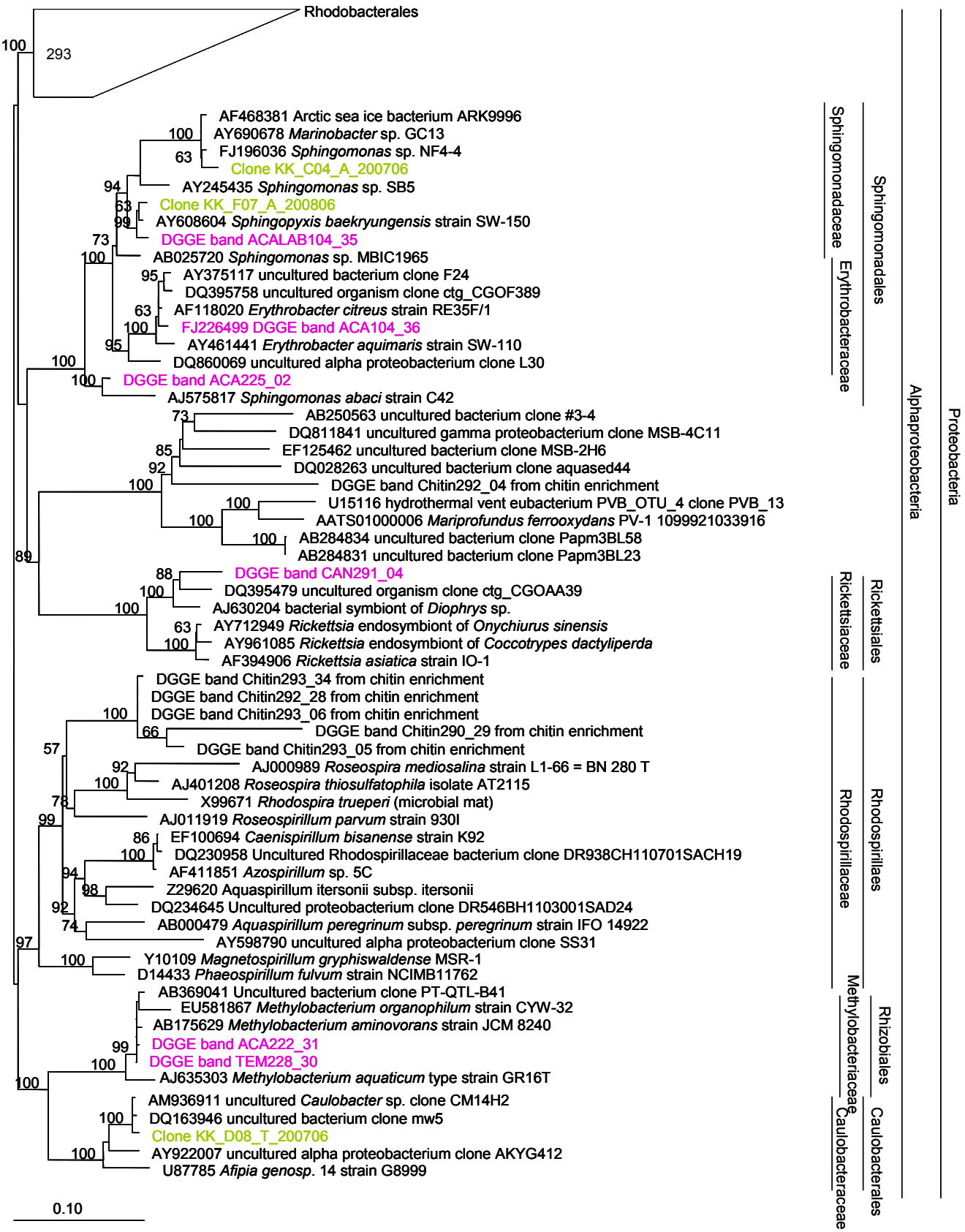



Fig. 8: Phylogenetic tree of Alphaproteobacteria without Rhodobacterales. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.

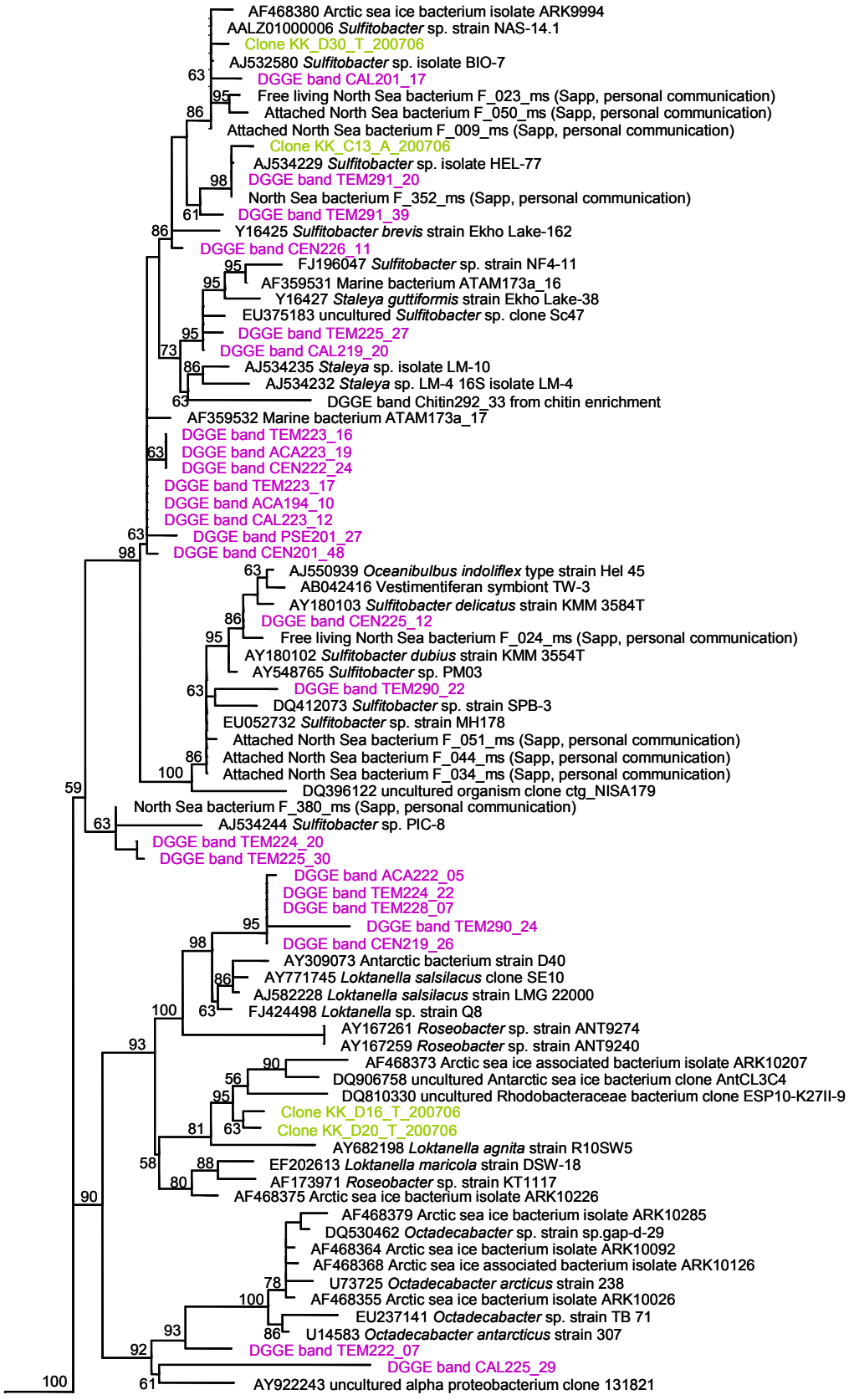



Fig. 9a: Phylogenetic tree of Rhodobacterales. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed (continued on next page).

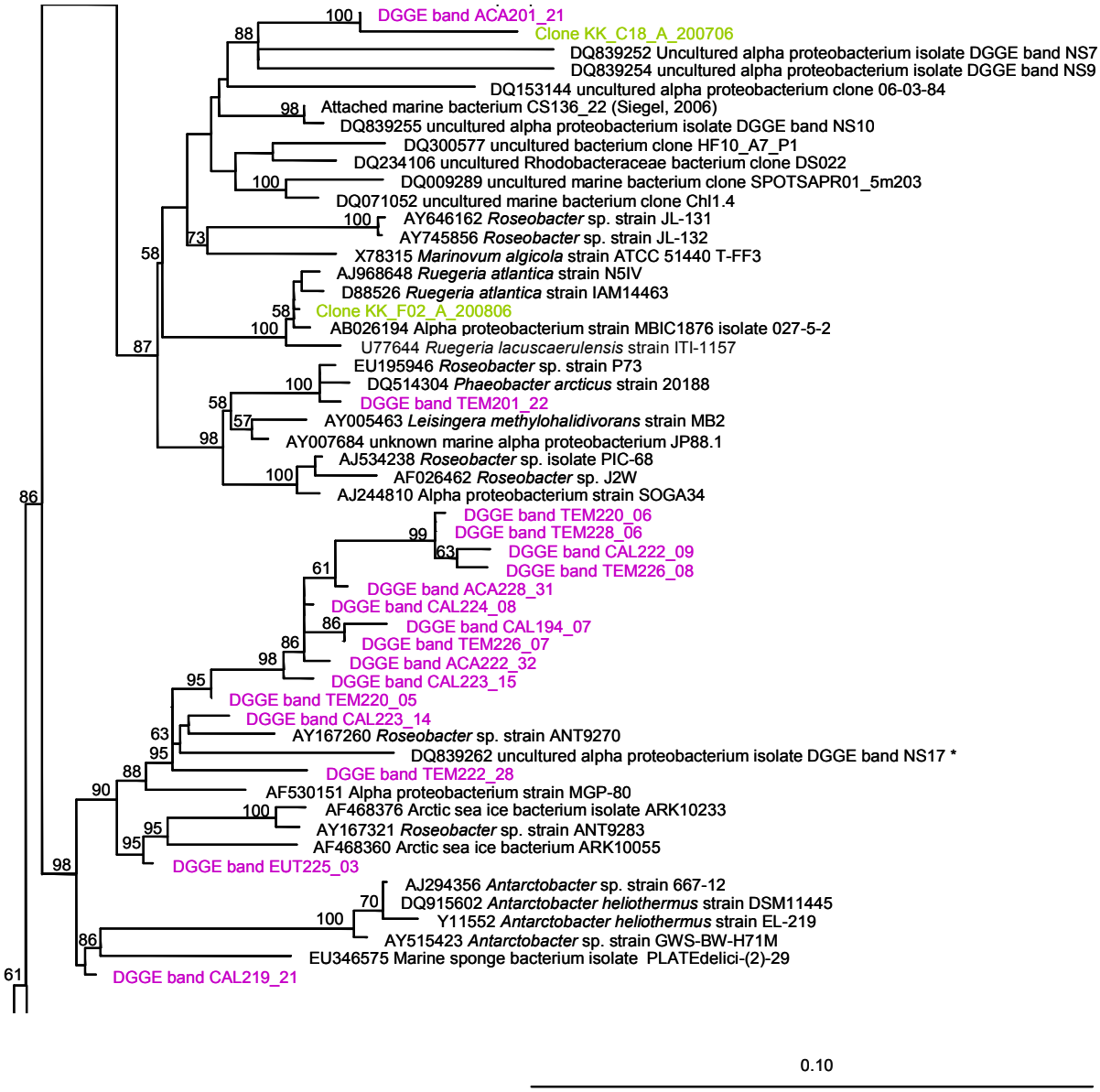
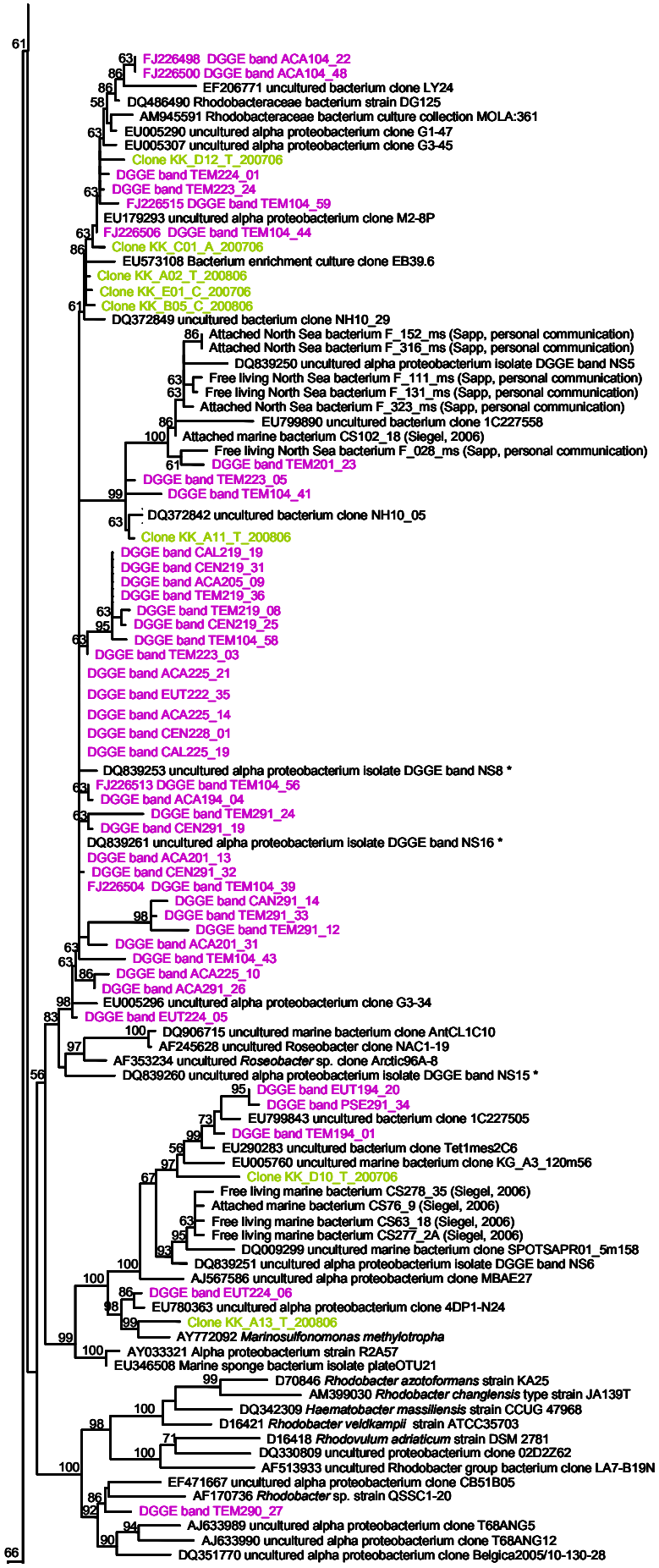



Fig. 9a (continued): Phylogenetic tree of Rhodobacterales. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.

Fig. 9b (next page): Phylogenetic tree of Rhodobacterales (part 2). In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.


FJ226498 DGGE band ACA104_22
FJ226500 DGGE band ACA104_48
EF206771 uncultured bacterium clone LY24
DQ486490 Rhodobacteraceae bacterium strain DG125
AM945591 Rhodobacteraceae bacterium culture collection MOLA:361
EU005290 uncultured alpha proteobacterium clone G1-47
EU005307 uncultured alpha proteobacterium clone G3-45
Clone KK_D12_T_200706
DGGE band TEM224_01
DGGE band TEM223_24
FJ226515 DGGE band TEM104_59
EU179293 uncultured alpha proteobacterium clone M2-8P
FJ226506 DGGE band TEM104_44
Clone KK_C01_A_200706
EU573108 Bacterium enrichment culture clone EB39.6
Clone KK_A02_T_200806
Clone KK_E01_C_200706
Clone KK_B05_C_200806
DQ372849 uncultured bacterium clone NH10_29
Attached North Sea bacterium F_152_ms (Sapp, personal communication)
Attached North Sea bacterium F_316_ms (Sapp, personal communication)
DQ839250 uncultured alpha proteobacterium isolate DGGE band NS5
Free living North Sea bacterium F_111_ms (Sapp, personal communication)
Free living North Sea bacterium F_131_ms (Sapp, personal communication)
Attached North Sea bacterium F_323_ms (Sapp, personal communication)
EU799890 uncultured bacterium clone 1C227558
Attached marine bacterium CS102_18 (Siegel, 2006)
Free living North Sea bacterium F_028_ms (Sapp, personal communication)
DGGE band TEM201_23
DGGE band TEM223_05
DGGE band TEM104_41
DQ372842 uncultured bacterium clone NH10_05
Clone KK_A11_T_200806
DGGE band CAL219_19
DGGE band CEN219_31
DGGE band ACA205_09
DGGE band TEM219_36
DGGE band TEM219_08
DGGE band CEN219_25
DGGE band TEM104_58
DGGE band TEM223_03
DGGE band ACA225_21
DGGE band EUT222_35
DGGE band ACA225_14
DGGE band CEN228_01
DGGE band CAL225_19
DQ839253 uncultured alpha proteobacterium isolate DGGE band NS8 *
FJ226513 DGGE band TEM104_56
DGGE band ACA194_04
DGGE band TEM291_24
DGGE band CEN291_19
DQ839261 uncultured alpha proteobacterium isolate DGGE band NS16 *
DGGE band ACA201_13
DGGE band CEN291_32
FJ226504 DGGE band TEM104_39
DGGE band CAN291_14
DGGE band TEM291_33
DGGE band TEM291_12
DGGE band ACA201_31
DGGE band TEM104_43
DGGE band ACA225_10
DGGE band ACA291_26
EU005296 uncultured alpha proteobacterium clone G3-34
DGGE band EUT224_05
DQ906715 uncultured marine bacterium clone AntCL1C10
AF245628 uncultured Roseobacter clone NAC1-19
AF353234 uncultured *Roseobacter* sp. clone Arctic96A-8
DQ839260 uncultured alpha proteobacterium isolate DGGE band NS15 *
DGGE band EUT194_20
DGGE band PSE291_34
EU799843 uncultured bacterium clone 1C227505
DGGE band TEM194_01
EU290283 uncultured bacterium clone Tet1mes2C6
EU005760 uncultured marine bacterium clone KG_A3_120m56
Clone KK_D10_T_200706
Free living marine bacterium CS278_35 (Siegel, 2006)
Attached marine bacterium CS76_9 (Siegel, 2006)
Free living marine bacterium CS63_18 (Siegel, 2006)
Free living marine bacterium CS277_2A (Siegel, 2006)
DQ009299 uncultured marine bacterium clone SPOTSAPR01_5m158
DQ839251 uncultured alpha proteobacterium isolate DGGE band NS6
AJ567586 uncultured alpha proteobacterium clone MBAE27
DGGE band EUT224_06
EU780363 uncultured alpha proteobacterium clone 4DP1-N24
Clone KK_A13_T_200806
AY772092 *Marinosulfonomonas methylotropha*
AY033321 Alpha proteobacterium strain R2A57
EU346508 Marine sponge bacterium isolate plateOTU21
D70846 *Rhodobacter azotoformans* strain KA25
AM399030 *Rhodobacter changlensis* type strain JA139T
DQ342309 *Haematobacter massiliensis* strain CCUG 47968
D16421 *Rhodobacter veldkampii* strain ATCC35703
D16418 *Rhodovulum adriaticum* strain DSM 2781
DQ330809 uncultured proteobacterium clone 02D2Z62
AF513933 uncultured Rhodobacter group bacterium clone LA7-B19N
EF471667 uncultured alpha proteobacterium clone CB51B05
AF170736 *Rhodobacter* sp. strain QSSC1-20
DGGE band TEM290_27
AJ633989 uncultured alpha proteobacterium clone T68ANG5
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DQ351770 uncultured alpha proteobacterium clone Belgica2005/10-130-28

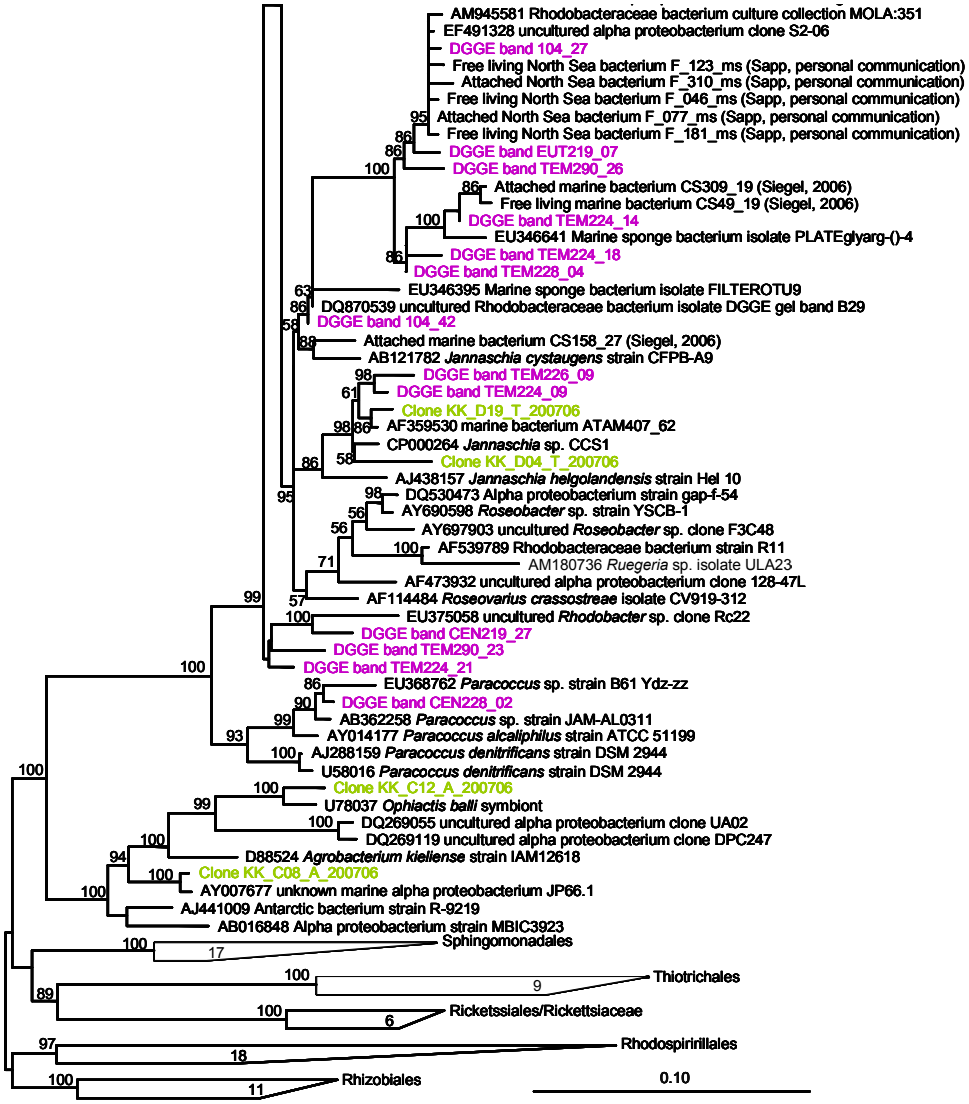



Fig. 9b (continued): Phylogenetic tree of Rhodobacterales (part 2). In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.

Phylogenetic analysis of DGGE-band-sequences and clone libraries revealed that most of the alphaproteobacterial phylotypes belong to the Rhodobacterales-group (Fig. 3, 9a, 9b), which is widely distributed and frequently observed in marine environments (Wagner-Döbler and Biebl 2006). Hence, it is not surprising that in our study, as well as in other recent studies (Møller 2007; Møller et al. 2007; Peter and Sommaruga 2008; Grossart et al. 2009; Tang et al. 2009a; Tang et al. 2009b), Rhodobacterales were found to be associated also with copepods. Rhodobacterales are known to be rapid and

successful primary surface colonisers (Jones et al. 2007; Dang et al. 2008; Porsby et al. 2008).

The Rhodobacterales-phylotypes found in this study (Fig. 5, 9a, 9b) clustered with sequences of surface attached (biotic and abiotic), but also of free-living bacteria. Most of these Rhodobacterales-phylotypes belong to the *Roseobacter*-lineage. As ecological generalists, Roseobacters harbour large gene inventories and a remarkable suite of mechanisms permitting them to obtain carbon and energy (Newton et al. 2010). Members of the *Roseobacter*-lineage play for example an important role for the global carbon and sulphur cycle and are able to produce dimethylsulfide (DMS) (Wagner-Döbler and Biebl 2006).

Dimethylsulfoniumpropionate (DMSP)-consuming bacteria were found to be associated with the marine calanoid copepod *Acartia tonsa* by Tang and co-workers (2001). Møller et al. (2007), who first investigated the bacterial community associated with two bundles of ten *Calanus helgolandicus* (North Sea) individuals using (DGGE and subsequent sequencing, found that all three of the obtained bacterial sequences belonged to the Roseobacter-lineage. Their phylotype NS17 (DQ839262) clusters with 13 sequences of the present study (Fig. 9a). It can therefore be assumed that this phylotype is typical for copepods from the North Sea. Overall 28 phylotypes of the present study are positioned near the sequences NS16 and NS8 (DQ839261 and DQ839253) (Møller et al. 2007) (Fig. 9a) which again indicates the general association of the Roseobacter-lineage with North Sea copepods.

Besides the Rhodobacterales we also identified phylotypes related to facultative methylotrophic Methylobacteriaceae (Rhizobiales) (Fig. 8). It is known that copepods can produce methane during grazing which could indicate the presence of methanogens in micro-niches probably in the copepods' guts (Oremland 1979; de Angelis and Lee 1994).

We also identified phylotypes related to Sphingomonadales (Fig. 8), *Sphingomonas* sp., *Sphingopyxis* sp. and *Erythrobacter* sp. The latter were previously found associated with sea water (Yoon et al. 2004), but also sediment (Zeng et al. 2005), tunicates (Martínez-García et al. 2007) and corals (Penn et al. 2006, unpublished). Some *Sphingomonas* sp. are known to express chitinases for usage of chitin as carbon source (Zhu et al. 2007). More

phylotypes related to chitin-degrading bacteria were affiliated to the classes of Gammaproteobacteria (Fig. 10) and Bacteroidetes (Fig. 11).

Bacteroidetes (Fig. 11) have already been found to be associated with copepods (Sochard et al. 1979; Hansen and Bech 1996; Peter and Sommaruga 2008). They are known to be chemoorganotrophic particle colonisers and especially proficient in degrading various polymers such as cellulose, chitin and pectin but also high molecular DOM (DeLong et al. 1993; Cottrell and Kirchman 2000a; Riemann et al. 2000; Kirchman 2002; Riemann et al. 2006). In our study the majority of bacteroidetes phylotypes belong to the Flavobacteraceae with eight phylotypes related to *Polaribacter* spp. from polar sea ice and sea water (Bowman et al. 1997; Gosink et al. 1998; Brinkmeyer et al. 2003; Riemann et al. 2008). Three Flavobacteraceae phylotypes clustered with sequences obtained from Sargasso sea bacterioplankton (Zengler et al. 2002) and from artificial surfaces submerged in sea water (Dang et al. 2008). Besides these main phylotypes single phylotypes were identified within the Bacteroidetes (Saprospiraceae) which were described mainly to derive from living surfaces (Goffredi et al. 2004) and sediments (Lee 2007, unpublished).

Besides the Alphaproteobacteria (Fig. 2, 8, 9a, 9b) dominating the DGGE analyses, phylotypes of the Gammaproteobacteria (Fig. 2, 10) were the most abundant in the clone libraries (53%) (Fig. 2). In many studies Gammaproteobacteria were identified as the major group associated with marine copepods (e.g. Sochard et al. 1979; Hansen and Bech 1996; Heidelberg et al. 2002b).

*Vibrio*-populations, for example, associated with zooplankton play an important role in the mineralisation of chitin (Kaneko and Colwell 1975; Belas and Colwell 1982; Huq et al. 1983; Tamplin et al. 1990; Bassler et al. 1991; Yu et al. 1991; Heidelberg et al. 2002b). *Vibrio* spp. comprise a significant portion of the natural bacterial flora of zooplankton with a chitinous exoskeleton, such as copepods (Sochard et al. 1979; Huq et al. 1983; Tamplin et al. 1990; Heidelberg et al. 2002b). Pseudoalteromonads, however, are also known to possess chitinases. In our study we found three phylotypes related to *Pseudoalteromonas* spp. (Fig. 7, 10) which were related to sequences from arctic sea water (Harder 2003, unpublished), sea ice (Yu et al. 2009), sponges (Dieckmann et al. 2005), sediment (Azuma 2003, unpublished), chitin enrichments (Brandt 2010, unpubl.)

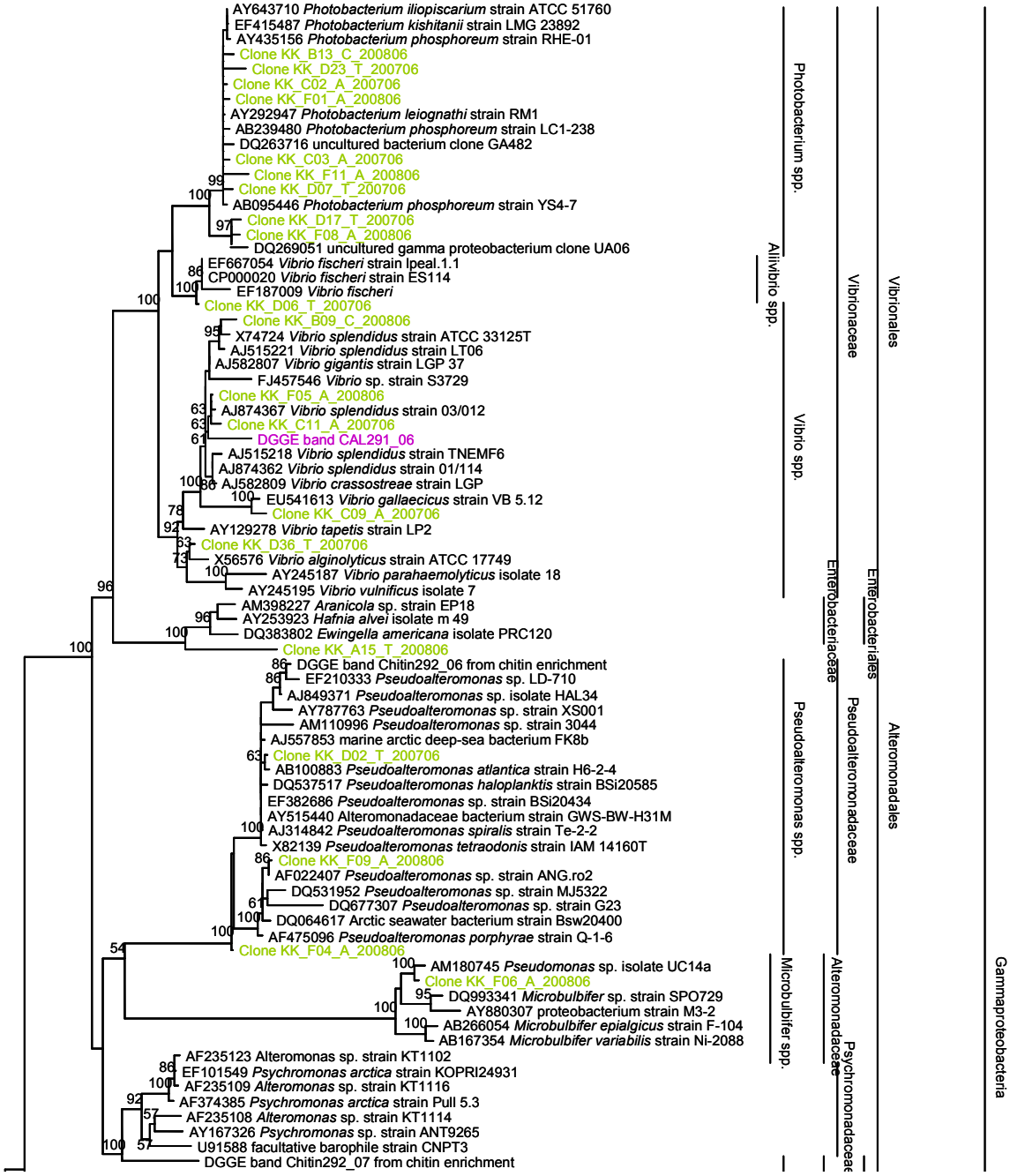



Fig. 10: Phylogenetic tree of Gamma- and Betaproteobacteria. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed (continued on next page).

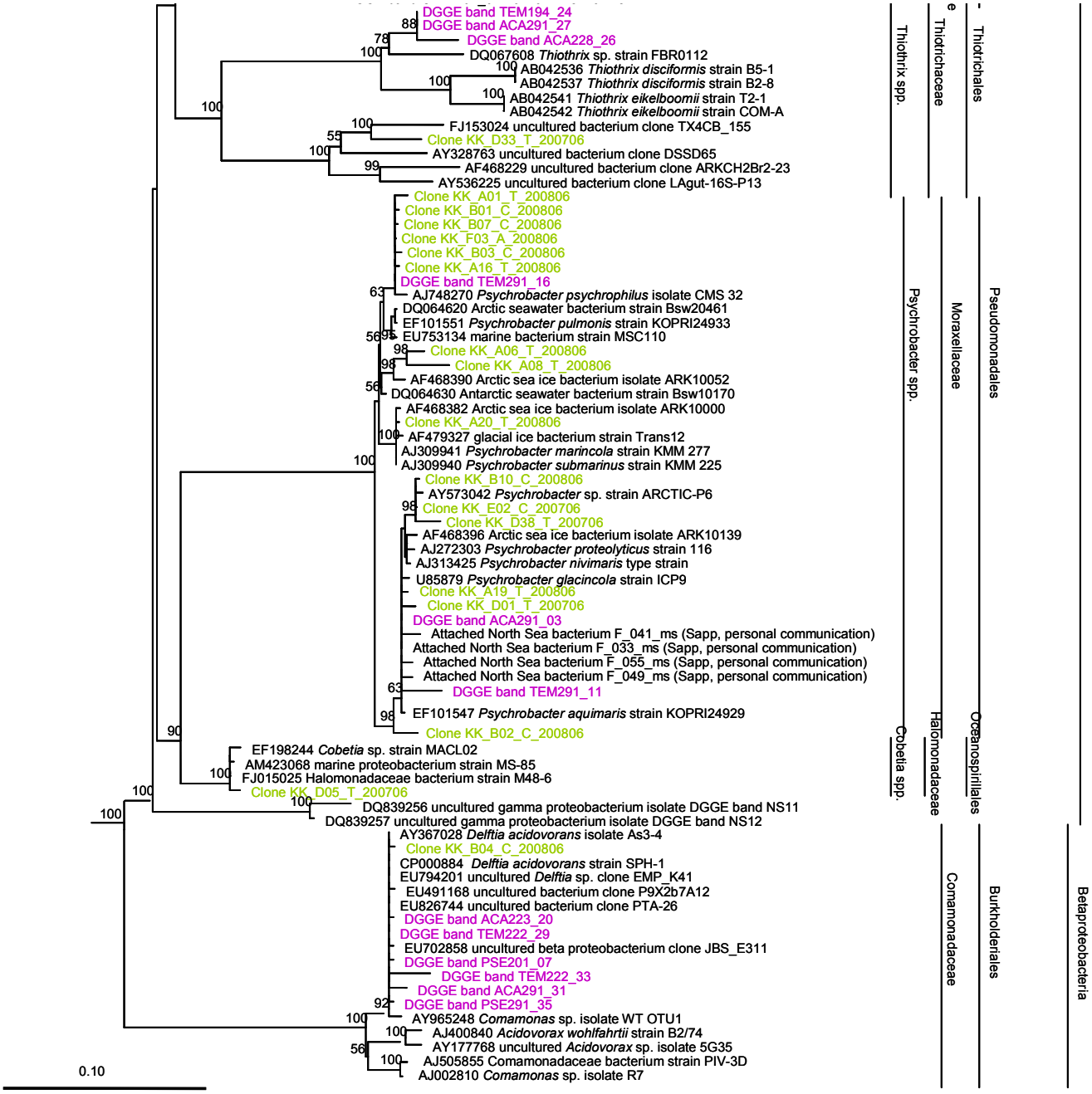



Fig. 10 (continued): Phylogenetic tree of Gamma- and Betaproteobacteria. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.

and the nidamental gland and egg capsules of a squid (Barbieri et al. 2001). Hansen and Bech (1996) found *Pseudoalteromonas* spp. associated with copepod intestines and faecal pellets. Within the Vibrionaceae (Fig. 10) we detected *Aliivibrio* sp. and *Vibrio* spp. *Aliivibrio* spp. were found in the marine

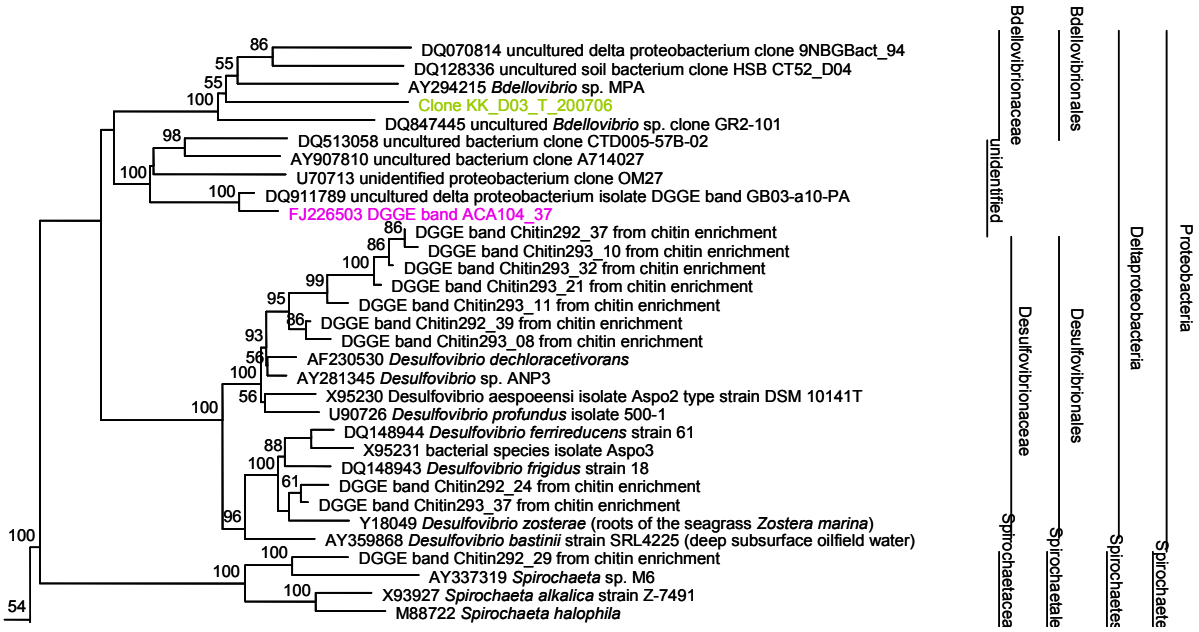



Fig. 11: Phylogenetic tree of Deltaproteobacteria, Bacteroidetes and Spirochaetes. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed (continued on next page).

environment, often associated with animals; some species are mutualistic symbionts or pathogens of marine animals (Urbanczyk et al. 2007). Six phylotypes in this study cluster with *Vibrio*-genera known as pathogens. *Vibrio alginolyticus* (Fig. 10) cause soft tissue infections and is listed as human pathogen, whereas *Vibrio splendidus* (Fig.10) is known to be a fish pathogen (Farmer III and Hickman-Brenner 2006). Some of these species have been associated with mortality in a wide range of marine animals, such as molluscs, fish, shrimps, and octopus (Beaz-Hidalgo et al. 2009).

Nine clone sequences clustered with *Photobacterium* spp. (Fig. 10). *Photobacterium phosphoreum* is a bioluminescent psychrophilic bacterium which is usually found in the intestine, gills and skin of fishes (Farmer III and Hickman-Brenner 2006). *Photobacterium* spp. was also found by Sochard and co-workers (1979) associated with the intestine und surface of copepods.

Members of Moraxellaceae (Fig. 10) formed a large group with 18 phylotypes, which fell more precisely into the genus *Psychrobacter*. They cluster with sequences obtained from macroalgae (Lee et al. 2006), arctic/antarctic sea ice and sea water (Bowman et al. 1997; Brinkmeyer et al. 2003; Zeng et al. 2007), krill (Denner et al. 2001) and tidal flat sand (Nichols et al. 2008). *Psychrobacter* species in general do not metabolise complex substrates such as

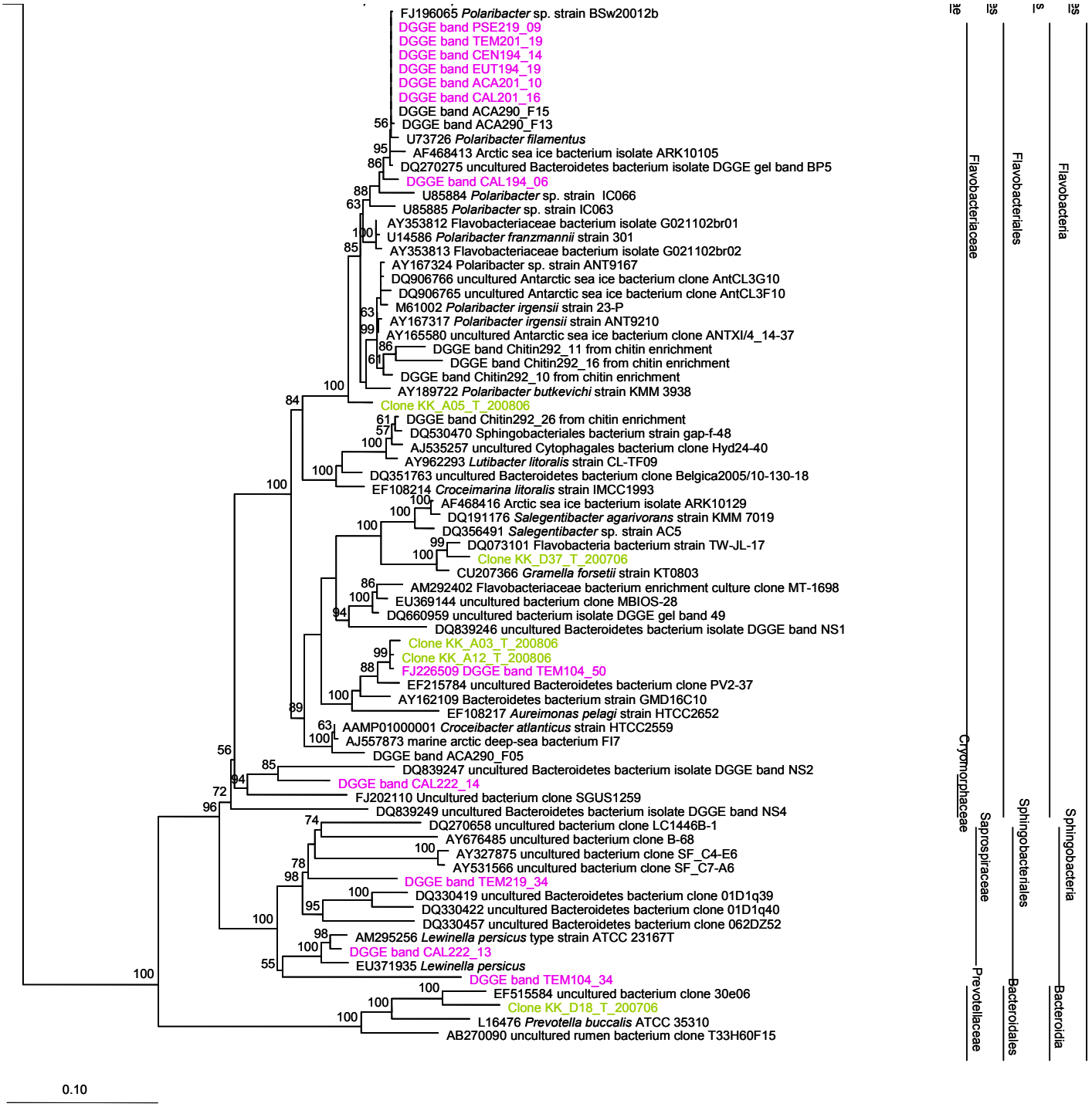



Fig. 11 (continued): Phylogenetic tree of Deltaproteobacteria, Bacteroidetes and Spirochaetes. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.

polysaccharides (Bowman 2006). Various *Psychrobacter* species have been found to occasionally cause infections in humans, animals and fish (Bowman 2006). *Psychrobacter* is considered to be an opportunistic pathogen and generally causes secondary, albeit very serious infections (Bowman 2006).

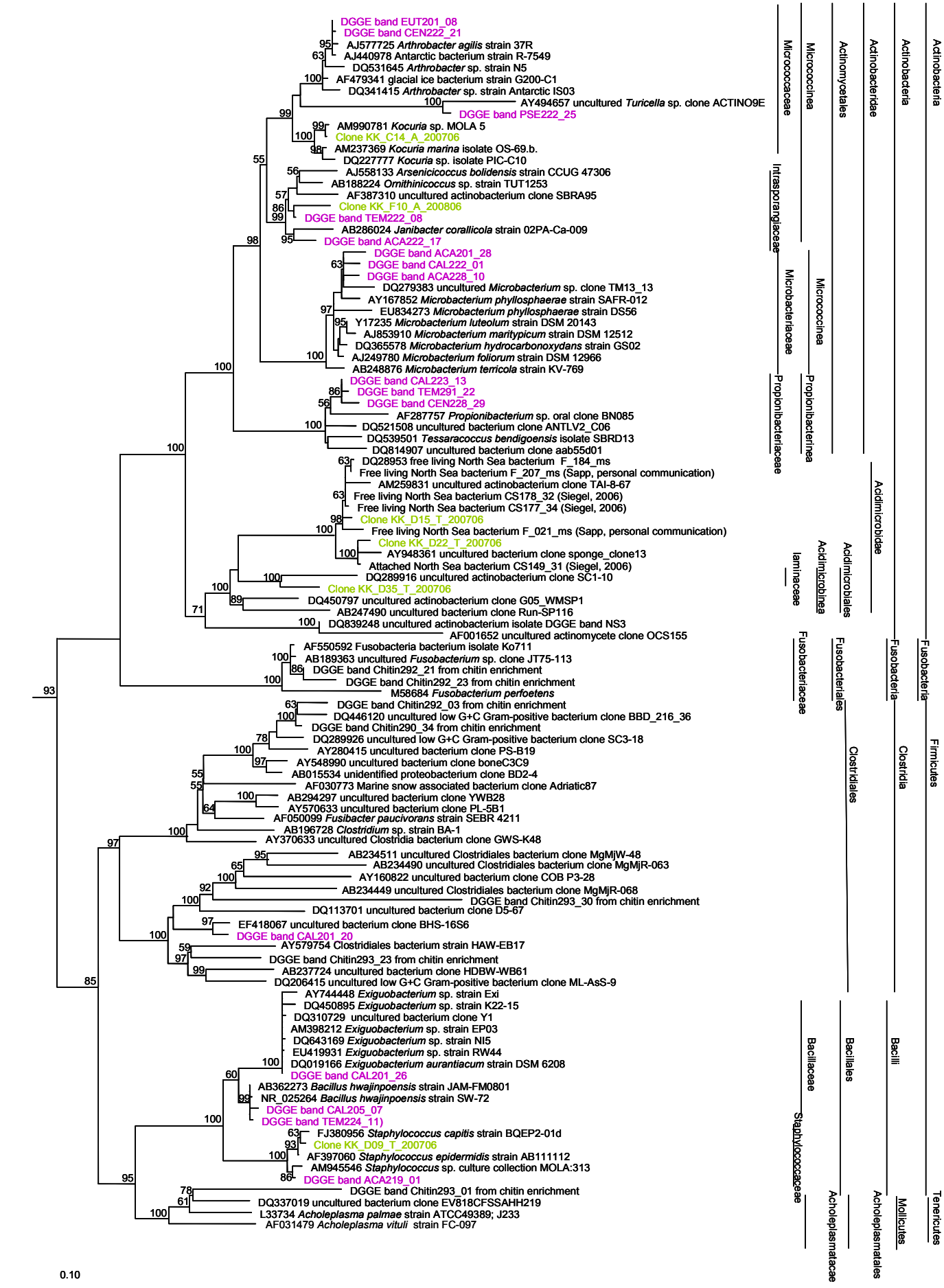
Four sequences fell within the Thiothrichaceae, which were previously found associated with sludge (Kanagawa et al. 2000), soil (Valenzuela-Encinas et al. 2009), polar pack ice (Brinkmeyer and Helmke, 2003 unpublished), and the gut of bivalves (Duplessis et al. 2004). A novel uncultured *Thiothrix* strain was also found endogenous to the marine crustacean *Urothoe poseidonis* (Gillan and Dubilier 2004).

Within the Firmicutes (Fig. 12), five phylotypes were related to the Bacillales, a single pyhlotype fell into the Clostridiales. Both groups contain members capable to degrade chitin (Gooday 1990; Vogan et al. 2008). Interestingly, related *Bacillus* spp. phylotypes have already been identified by Hansen and Bech (1996) associated with the faecal pellets and the gut of copepods.

Within the Actinobacteria (Fig.12), 16 phylotypes fell into several different families. These bacteria mostly cluster with surface attached bacteria or seem to originate from sediment, others are mostly free-living bacteria. It seems that some were only found by coincidence. Seven of these phylotypes fell into the suborder Micrococcinea. Thereof, four sequences belong to the Micrococcaceae. They cluster with sequences from Antarctic ice (Ma et al. 2006, unpublished) and soil samples (Reddy et al. 2004, unpublished) and with a sequence from fish gills (Bowman and Nowak 2004).

Three phylotypes were related with Propionibacteriaceae, other three sequences fell into the Intersporangiaceae which were found in sediments (Collins et al. 2004) and associated with corals (Kageyama et al. 2007b). Three phylotypes were related to *Microbacterium* spp. occurring attached to various biotic and abiotic surfaces (Venkateswaran et al. 2001; Barbieri et al. 2007; Kageyama et al. 2007a). Two sequences fell into the Acidimicrobidae and clustered with sponge (Wichels et al. 2006; Thiel et al. 2007) and North Sea water bacteria (Sapp, personal communication), whereas another sequence fell into the Iaminaceae and clustered with a bacterium from shelf sediment (Hunter et al. 2006).

Fig. 12 (next page): Phylogenetic tree of Actinobacteria and Firmicutes. In green: sequences obtained from cloning approach; in purple: sequences obtained from PCR-DGGE analysis. Bootstrap values over 50 % are displayed.


DGGE band EUT201_08
DGGE band CEN222_21
AJ577725 Arthrobacter agilis strain 37R
AJ440978 Antarctic bacterium strain R-7549
DQ531645 Arthrobacter sp. strain N5
AF479341 glacial ice bacterium strain G200-C1
DQ341415 Arthrobacter sp. strain Antarctic IS03
AY494657 uncultured Turicella sp. clone ACTINO9E
DGGE band PSE222_25
AM990781 Kocuria sp. MOLA 5
Clone KK_C14_A_200706
AM237369 Kocuria marina isolate OS-69.b.
DQ227777 Kocuria sp. isolate PIC-C10
AJ558133 Arsenicicoccus bolidensis strain CCUG 47306
AB188224 Ornithinicoccus sp. strain TUT1253
AF387310 uncultured actinobacterium clone SBRA95
Clone KK_F10_A_200806
DGGE band TEM222_08
AB286024 Janibacter corallicola strain 02PA-Ca-009
DGGE band ACA222_17
DGGE band ACA201_28
DGGE band CAL222_01
DGGE band ACA228_10
DQ279383 uncultured Microbacterium sp. clone TM13_13
AY167852 Microbacterium phyllosphaerae strain SAFR-012
EU834273 Microbacterium phyllosphaerae strain DS56
Y17235 Microbacterium luteolum strain DSM 20143
AJ853910 Microbacterium maritypicum strain DSM 12512
DQ365578 Microbacterium hydrocarbonoxydans strain GS02
AJ249780 Microbacterium foliorum strain DSM 12966
AB248876 Microbacterium terricola strain KV-769
DGGE band CAL223_13
DGGE band TEM291_22
DGGE band CEN228_29
AF287757 Propionibacterium sp. oral clone BN085
DQ521508 uncultured bacterium clone ANTLV2_C06
DQ539501 Tessaracoccus bendigoensis isolate SBRD13
DQ814907 uncultured bacterium clone aab55d01
DQ28953 free living North Sea bacterium F_184_ms
Free living North Sea bacterium F_207_ms (Sapp, personal communication)
AM259831 uncultured actinobacterium clone TAI-8-67
Free living North Sea bacterium CS178_32 (Siegel, 2006)
Free living North Sea bacterium CS177_34 (Siegel, 2006)
Clone KK_D15_T_200706
Free living North Sea bacterium F_021_ms (Sapp, personal communication)
Clone KK_D22_T_200706
AY948361 uncultured bacterium clone sponge_clone13
Attached North Sea bacterium CS149_31 (Siegel, 2006)
DQ289916 uncultured actinobacterium clone SC1-10
Clone KK_D35_T_200706
DQ450797 uncultured actinobacterium clone G05_WMSP1
AB247490 uncultured bacterium clone Run-SP116
DQ839248 uncultured actinobacterium isolate DGGE band NS3
AF001652 uncultured actinomycete clone OCS155
AF550592 Fusobacteria bacterium isolate Ko711
AB189363 uncultured Fusobacterium sp. clone JT75-113
DGGE band Chitin292_21 from chitin enrichment
DGGE band Chitin292_23 from chitin enrichment
M58684 Fusobacterium perfoetens
DGGE band Chitin292_03 from chitin enrichment
DQ446120 uncultured low G+C Gram-positive bacterium clone BBD_216_36
DGGE band Chitin290_34 from chitin enrichment
DQ289926 uncultured low G+C Gram-positive bacterium clone SC3-18
AY280415 uncultured bacterium clone PS-B19
AY548990 uncultured bacterium clone boneC3C9
AB015534 unidentified proteobacterium clone BD2-4
AF030773 Marine snow associated bacterium clone Adriatic87
AB294297 uncultured bacterium clone YWB28
AY570633 uncultured bacterium clone PL-5B1
AF050099 Fusibacter paucivorans strain SEBR 4211
AB196728 Clostridium sp. strain BA-1
AY370633 uncultured Clostridia bacterium clone GWS-K48
AB234511 uncultured Clostridiales bacterium clone MgMjW-48
AB234490 uncultured Clostridiales bacterium clone MgMjR-063
AY160822 uncultured bacterium clone COB P3-28
AB234449 uncultured Clostridiales bacterium clone MgMjR-068
DGGE band Chitin293_30 from chitin enrichment
DQ113701 uncultured bacterium clone D5-67
EF418067 uncultured bacterium clone BHS-16S6
DGGE band CAL201_20
AY579754 Clostridiales bacterium strain HAW-EB17
DGGE band Chitin293_23 from chitin enrichment
AB237724 uncultured bacterium clone HDBW-WB61
DQ206415 uncultured low G+C Gram-positive bacterium clone ML-AsS-9
AY744448 Exiguobacterium sp. strain Exi
DQ450895 Exiguobacterium sp. strain K22-15
DQ310729 uncultured bacterium clone Y1
AM398212 Exiguobacterium sp. strain EP03
DQ643169 Exiguobacterium sp. strain NI5
EU419931 Exiguobacterium sp. strain RW44
DQ019166 Exiguobacterium aurantiacum strain DSM 6208
DGGE band CAL201_26
AB362273 Bacillus hwajinpoensis strain JAM-FM0801
NR_025264 Bacillus hwajinpoensis strain SW-72
DGGE band CAL205_07
DGGE band TEM224_11)
FJ380956 Staphylococcus capitis strain BQEP2-01d
Clone KK_D09_T_200706
AF397060 Staphylococcus epidermidis strain AB111112
AM945546 Staphylococcus sp. culture collection MOLA:313
DGGE band ACA219_01
DGGE band Chitin293_01 from chitin enrichment
DQ337019 uncultured bacterium clone EV818CFSSAHH219
L33734 Acholeplasma palmae strain ATCC49389; J233
AF031479 Acholeplasma vituli strain FC-097
0.10
Micrococcaceae
Micrococcinea
Actinomycetales
Actinobacteridae
Actinobacteria
Actinobacteria
Intrasporangiaceae
Micrococcaceae
Micrococcinea
Actinomycetales
Actinobacteridae
Actinobacteria
Actinobacteria
Microbacteriaceae
Micrococcinea
Actinomycetales
Actinobacteridae
Actinobacteria
Actinobacteria
Propionibacteriaceae
Propionibacterinea
Actinomycetales
Actinobacteridae
Actinobacteria
Actinobacteria
Acidimicrobiales
Acidimicrobidae
Actinobacteria
Actinobacteria
Iamiaceae
Acidimicrobinea
Acidimicrobiales
Acidimicrobidae
Actinobacteria
Actinobacteria
Fusobacteriaceae
Fusobacteriales
Fusobacteria
Fusobacteria
Clostridiales
Clostridia
Firmicutes
Bacillaceae
Bacillales
Bacilli
Firmicutes
Staphylococcaceae
Bacillales
Bacilli
Firmicutes
Acholeplasmataceae
Acholeplasmatales
Mollicutes
Tenericutes

Seven Betaproteobacteria (Fig. 10) phylotypes were found. They all were exclusively related to the Comamonadaceae. The phylotypes cluster with others obtained from sediment (Li et al. 2008, unpublished, Kwon and Finneran 2009) and seafloor lavas (Santelli et al. 2008), but also faecal samples (Patton et al. 2009).
Within the Deltaproteobacteria (Fig. 11) we found a single sequence belonging to the genus *Bdellovibrio*. These bacteria have been isolated from a wide range of water systems: estuaries, oceans, rivers, sewage, fish ponds, and on biofims on surfaces (Jurkevitch 2006). Bdellovibrios attack Gram negative bacteria, such as *Vibrio* spp., they can be regarded as intracellular parasites (Jurkevitch 2006).

An overall view of the phylogenetic analysis can be summarised by the following findings:
i) Most phylotypes clustered with surface-attached or organism-associated bacteria (see phylogenetic trees Fig. 8-12).
ii) The bacterial taxonomic groups associated with marine copepods of the North Sea analysed in this study are similar to those found generally attached or associated in the marine pelagic environment on the phylum/class level (see phylogenetic trees Fig. 8-12).
iii) The commonly found free-living bacteria of coastal North Sea bacterioplankton are generally not the ones which are associated with copepods. Eilers and co-workers (Eilers et al. 2001) found Alpha- (Roseobacter) and Gammproteobacteria (*Pseudoalteromonas, Alteromonas, Colwellia, Photobacterium*), and members of the CF cluster (*Cytophaga, Polaribacter, Flavobacterium*) in the water column of the North Sea near Helgoland. This seems to be comparable to our results but on the higher taxonomic levels clear differences show up (see phylogenetic trees Fig. 8-12).
iv) In our study we found a lot of cold adapted bacteria which were already found associated with polar ice samples. This is not so surprising when we consider that most of the isolates from Arctic sea ice were psychrotolerant and grew optimally between 20 and 25 °C. Only a few strains were psychrophilic with an optimal growth at 10 – 15 °C. (Groudieva et al. 2004). Commonly isolated Gammaproteobacteria phylotypes from both poles that fall amongst

others into the genera *Psychrobacter, Colwellia* and Alphaproteobacteria into the genera *Octadecabacter* and *Sulfitobacter*, and Bacteroidetes into the genera *Polaribacter, Flavobacterium* and *Psychroserpens* (Hollibaugh et al. 2007) were also detected in this study. These genera find the optimal condition to live and accrete in temperate waters.

v) Since many of the associated phylotypes were related with groups capable of degrading chitin or possessing chitinases it might be presumed that the supply of chitin is an important factor for the development of the bacterial community on the copepods' surface. On the other hand, the copepods' chitinous surface may also serve as living surface only, with the advantage that the copepods produce DOM and DMSP by 'sloppy feeding' or defecation and methane during grazing. Nutrient supply to the bacteria will not end until the copepod dies and the carcass is decomposed. Living surfaces are typically nutrient-rich environments where inorganic molecules and metabolic by-products accumulate, often exude different chemical deterrents or cues and are generally complex morphologically (e.g. tissue differentiation). Thus living surfaces are likely to provide very different and more highly differentiated habitats compared to pelagic environments.

Tang and co-workers (2009a) postulated that the life strategies of the different copepod genera with regard to feeding strategies play an important role for the composition of the associated bacterial communities. Interestingly, with our results we could not support this although the different copepod genera investigated had different life styles. Some were herbivorous and some rather omnivorous or detritivorous. This did not seem to make a difference regarding their bacterial assemblages.

Adult copepods do not moult again (Carman and Dobbs 1997), but until adulthood copepods shed their carapace after each naupliar and copepodite stage. In this phase of life, the bacterial community is disturbed after each life stage and has to re-colonise the nauplius or copepodite. Presumably, the community formerly attached to the surface- is lost with the carapace and colonisation begins anew. These different life stages could be the reason why so many primary surface colonisers were present associated with copepods. Factors affecting diversity include the level of disturbance or the harshness of

the environment. For example, disturbed communities may have higher diversity than undisturbed communities in which competition may result in one or a few individual species dominating (Begon et al. 1996). In contrast, harsh environments tend to be species poor and are typically occupied by species that can withstand the extreme conditions (Longford et al. 2007).

Although we identified specific bacterial clusters containing only copepod related bacterial phylotypes in some cases (e.g. within the Rhodobacteraceae, or within the *Polaribacter* group), our results suggest that copepods do not harbour a distinct bacterial flora. Some bacteria that were associated with North Sea copepods seemed to be only coincidentally attached to that surface. Some mostly clustered with surface attached bacteria or seemed to originate from sediment, but some seem to be very typical for copepods or at least for marine surfaces. In some bacteria-host connections, the host-driven evolution plays an important role leading to a distinct or specific bacterial community (e.g.Oh et al. 2010; Thakuria et al. 2010). A distinct bacterial flora was proposed for example for marine sponges (Webster et al. 2001; Hentschel et al. 2002) or marine diatoms (Grossart et al. 2005), but also for these habitats, studies are available presenting contradictory findings (Wichels et al. 2006; Sapp et al. 2007a). We assume that the copepod-associated bacteria are mainly opportunistic ones because clusters of copepod-specific bacteria or crustacean-specific bacteria are most likely rare. Presumably the copepods' chitinous surfaces providing a nutrient-rich environment represent a highly differentiated habitat compared to pelagic environments. As a result, one might expect different assemblages of bacteria between pelagic and host-associated communities (Longford et al. 2007), which was in fact the case.

Heidelberg et al. (2002b) postulated that bacteria associated with zooplankton would most likely display seasonal trends, with larger populations associated with the zooplankton during spring and autumn (Heidelberg et al. 2002b). This hypothesis is in coherence with the study of Huq and Colwell (1996) related to the *Vibrio cholera* epidemics in Bangladesh, which occur biannually, during the spring and fall. This seasonal cycle of cholera outbreaks is closely correlated with copepod abundance (Huq and Colwell 1996).

In this context we searched for seasonality in the bacterial community but were not able to detect it. However, a seasonality of pelagic bacteria, free-living and

attached, is well documented in the North Sea (Eilers et al. 2001; Gerdts et al. 2004; Alónso-Sáez et al. 2007).

It is therefore surprising that we found neither significance for the specificity of associated bacteria nor for a seasonal influence supporting the hypothesis of unspecific bacteria occupying the copepods during the season. In both cases we only found weak trends: i) some specific bacterial clusters supporting the first hypothesis, and ii) a weak indication for higher diversity during spring and summer in the fingerprints.

We are aware that we were not able to identify every single bacterial phylotype associated with marine copepods. Nevertheless, this study allows a very detailed look into which bacteria were associated with North Sea copepods in the years 2007 to 2009, with Alpha- and Gammaproteobacteria dominating the assemblages. To achieve further insights, it will be necessary to differentiate between interior an exterior associated bacteria and physiological approaches could shed more light on the physiology of the bacteria-copepod association.

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## SUPPLEMENTAL MATERIAL

Tab. 2: Bacterial phylotypes associated with *Acartia* sp. The number of sequences means the number of combined identical sequences (difference ≤ 1%) of *Acartia* sp. samples which appeared either at the same position in DGGE gels and in the phylogenetic tree or were identified as the same clone type by single base sequencing.

Tab. 3: Bacterial phylotypes associated with *Temora longicornis*. The number of sequences means the number of combined identical sequences (difference ≤ 1%) of *Temora longicornis* samples which appeared either at the same position in DGGE gels and in the phylogenetic tree or were identified as the same clone type by single base sequencing.

Tab. 4: Bacterial phylotypes associated with *Centropages* sp. The number of sequences means the number of combined identical sequences (difference ≤ 1%) of *Centropages* sp. samples which appeared either at the same position in DGGE gels and in the phylogenetic tree or were identified as the same clone type by single base sequencing.

Tab. 5: Bacterial phylotypes associated with *Calanus helgolandicus*. The number of sequences means the number of combined identical sequences (difference ≤ 1%) of *Calanus helgolandicus* samples which appeared either at the same position in DGGE gels and in the phylogenetic tree or were identified as the same clone type by single base sequencing.

Tab. 6: Bacteria associated with other copepod genera. The number of sequences means the number of combined identical sequences (difference ≤ 1%) of the different copepod genera samples which appeared at the same position in DGGE gels and in the phylogenetic tree.

**Tab. 2**

| DGGE band / clone | Number of Sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| ACA222_17 | 1 | 570 | AB286024 | 97% | *Janibacter corallicola* strain 02PA-Ca-009 | Actinobacteria | Actinobacteria | Actinomycetales | Intrasporangiaceae | *Ornithinimicrobium* |
| ACA228_10 | 1 | 525 | GU933614 | 95% | *Microbacterium* sp. MJ432 | Actinobacteria | Actinobacteria | Actinomycetales | Microbacteriaceae | *Microbacterium* |
| ACA201_28 | 1 | 519 | EU834273 | 91% | *Microbacterium phyllosphaerae* strain DS56 | Actinobacteria | Actinobacteria | Actinomycetales | not defined | not defined |
| KK_C14_A_200706 | 1 | 948 | AM990781 | 99% | *Kocuria* sp. MOLA 5 | Actinobacteria | Actinobacteria | Actinomycetales | Micrococcaceae | *Kocuria* |
| KK_F10_A_200806 | 7 | 1212 | AJ558133 | 95% | *Arsenicicoccus bolidensis* strain CCUG 47306 | Actinobacteria | Actinobacteria | Actinomycetales | Intrasporangiaceae | not defined |
| ACA194_02 | 1 | 547 | FJ202110 | 97% | Uncultured bacterium clone SGUS1259 | Bacteroidetes | Flavobacteria | Flavobacteriales | Cryomorphaceae | *Brumimicrobium* |
| ACA201_10 | 6 | 525 - 526 | FJ196065 | 99 - 100% | *Polaribacter* sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| ACA219_01 | 2 | 544 - 589 | AM945546 | 99 - 100% | *Staphylococcus* sp. MOLA 313 | Firmicutes | Bacilli | Bacillales | Staphylococcaceae | *Staphylococcus* |
| ACA201_21 | 8 | 391 - 512 | DQ421680 | 96 - 97% | Uncultured alpha proteobacterium clone SIMO-4315 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| ACA225_14 | 3 | 560 - 561 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA194_04 | 1 | 525 | DQ839253 | 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA201_13 | 1 | 501 | DQ839253 | 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA205_09 | 1 | 562 | DQ839253 | 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA225_21 | 1 | 561 | DQ839253 | 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA291_26 | 1 | 502 | DQ839253 | 97% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA225_10 | 1 | 493 | DQ839253 | 96% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA201_31 | 1 | 512 | DQ839253 | 96% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACALAB104_22 | 1 | 453 | EF206771 | 97% | Uncultured bacterium clone LY24 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACALAB104_48 | 1 | 428 | EF206771 | 96% | Uncultured bacterium clone LY25 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| ACA291_30 | 1 | 496 | AJ534234 | 96% | *Staleya* sp. LM-9 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| KK_C13_A_200706 | 5 | 811 | AJ534229 | 98% | *Sulfitobacter* sp. isolate HEL-77 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| ACA223_19 | 1 | 562 | DQ683726 | 100% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| ACA194_10 | 1 | 516 | DQ683726 | 99% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| ACA222_05 | 1 | 563 | AJ534223 | 99% | *Methylarcula* sp. DT-12 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| ACA222_32 | 1 | 454 | AJ505788 | 94% | Alpha proteobacterium GRP21 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| ACA228_31 | 1 | 461 | AJ505788 | 94% | Alpha proteobacterium GRP21 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_C18_A_200706 | 2 | 1254 | FJ664792 | 99% | Uncultured alpha proteobacterium clone plankton_B01 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_F02_A_200806 | 1 | 1196 | AB255399 | 100% | *Ruegeria atlantica* | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Ruegeria* |
| ACA291_25 | 1 | 499 | FJ664792 | 99% | Uncultured alpha proteobacterium clone plankton_B01 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_C01_A_200706 | 73 | 1186 | EU573108 | 96% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| ACA225_02 | 1 | 563 | EF494197 | 99% | *Sphingomonas* sp. DS18 | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingomonas* |
| KK_F07_A_200806 | 2 | 1238 | AY608604 | 99% | *Sphingomonas baekryungensis* | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingopyxis* |
| KK_C04_A_200706 | 7 | 1221 | FJ196036 | 98% | *Sphingomonas* sp. NF4-4 | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingopyxis* |
| ACALAB104_35 | 1 | 457 | AY608604 | 96% | *Sphingomonas baekryungensis* strain SW-150 | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingopyxis* |
| ACALAB104_36 | 1 | 419 | EU253576 | 95% | *Erythrobacter* sp. strain MED365 | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Erythrobacteraceae | *Erythrobacter* |
| ACA222_31 | 1 | 562 | AB369041 | 99% | Uncultured bacterium clone PT-QTL-B41 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylobacteriaceae | *Methylobacterium* |
| KK_C08_A_200706 | 3 | 1202 | AY007677 | 99% | Unknown marine alpha proteobacterium JP66.1 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | *Hoeflea* |
| KK_C12_A_200706 | 3 | 1094 | U78037 | 96% | *Ophiactis balli* symbiont | Proteobacteria | Alphaproteobacteria | not defined | not defined | not defined |
| ACA223_20 | 2 | 534 - 587 | EU826744 | 99 - 100% | Uncultured bacterium clone PTA-26 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| ACA223_22 | 1 | 486 | EU702858 | 96% | Uncultured beta proteobacterium clone JBS_E311 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| ACA291_31 | 1 | 497 | EU641637 | 99% | Uncultured Comamonadaceae bacterium clone LW9m-3-69 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| ACA104_37 | 1 | 475 | DQ911789 | 93% | Uncultured delta proteobacterium isolate DGGE band GB03-a10-PA | Proteobacteria | Deltaproteobacteria | not defined | not defined | not defined |
| ACA228_26 | 1 | 468 | AY426613 | 93% | Uncultured *Thiothrix* sp. clone UP23b | Proteobacteria | Gammaproteobacteria | Thiotrichales | Thiotrichaceae | *Thiothrix* |
| ACA291_27 | 1 | 499 | AY426613 | 97% | Uncultured *Thiothrix* sp. clone UP23b | Proteobacteria | Gammaproteobacteria | Thiotrichales | Thiotrichaceae | *Thiothrix* |
| ACA291_03 | 1 | 489 | AY165598 | 95% | Uncultured Arctic sea ice bacterium clone ARKXVI/2-136 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_F03_A_200806 | 2 | 1261 | EU753134 | 98% | Marine bacterium MSC110 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_C02_A_200706 | 6 | 1269 | EF415487 | 99% | *Photobacterium kishitanii* strain LMG 23892 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_C03_A_200706 | 3 | 1285 | FJ456709 | 99% | Uncultured bacterium clone A266_NCI | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_F08_A_200806 | 1 | 1284 | DQ269051 | 98% | Uncultured gamma proteobacterium clone UA06 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_F11_A_200806 | 3 | 1273 | DQ263716 | 98% | Uncultured bacterium clone GA482 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_F01_A_200806 | 2 | 1293 | AY643710 | 99% | *Photobacterium iliopiscarium* strain ATCC 51760 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_C09_A_200706 | 4 | 1297 | EU541613 | 99% | *Vibrio* sp. VB 5.12 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| KK_C11_A_200706 | 1 | 1024 | FJ457546 | 96% | *Vibrio* sp. S3729 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| KK_F05_A_200806 | 2 | 1305 | AJ874367 | 99% | *Vibrio splendidus* strain 03/012 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| KK_F06_A_200806 | 3 | 1286 | AM180745 | 99% | *Pseudomonas* sp. isolate UC14a | Proteobacteria | Gammaproteobacteria | Alteromonadales | Alteromonadaceae | *Microbulbifer* |
| KK_F04_A_200806 | 1 | 1098 | AY515440 | 100% | *Alteromonadaceae* bacterium GWS-BW-H31M | Proteobacteria | Gammaproteobacteria | Alteromonadales | Pseudoalteromonadaceae | *Pseudoalteromonas* |
| KK_F09_A_200806 | 1 | 1292 | AF022407 | 99% | *Pseudoalteromonas* sp. ANG.ro2 | Proteobacteria | Gammaproteobacteria | Alteromonadales | Pseudoalteromonadaceae | *Pseudoalteromonas* |

ACA = field-caught *Acartia* sp. ; ACALAB = laboratory-grown *Acartia tonsa*

TAB. 3

| DGGE band | Number of Sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| TEM222_08 | 1 | 570 | AB286024 | 97% | *Janibacter corallicola* strain 02PA-Ca-009 | Actinobacteria | Actinobacteria | Actinomycetales | Intrasporangiaceae | *Ornithinimicrobium* |
| TEM291_22 | 1 | 482 | DQ521508 | 98% | Uncultured bacterium clone ANTLV2_C06 | Actinobacteria | Actinobacteria | Actinomycetales | Propionibacteriaceae | *Tessaracoccus* |
| KK_D15_T_200706 | 1 | 884 | AM259831 | 98% | Uncultured actinobacterium clone TAI-8-67 | Actinobacteria | Actinobacteria | | Acidimicrobidae_incertae_sedis | *Ilumatobacter* |
| KK_D22_T_200706 | 4 | 918 | AY948361 | 98% | Uncultured bacterium clone sponge_clone13 | Actinobacteria | Actinobacteria | | Acidimicrobidae_incertae_sedis | *Ilumatobacter* |
| KK_D35_T_200706 | 1 | 1260 | DQ289916 | 95% | Uncultured actinobacterium clone SC1-10 | Actinobacteria | Actinobacteria | Acidimicrobiales | Iamiaceae | *Iamia* |
| TEM201_19 | 7 | 517 - 533 | FJ196065 | 97 - 100% | Polaribacter sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| KK_A05_T_200806 | 2 | 1279 | AY285943 | 96% | Flavobacteriaceae bacterium G1B2 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| KK_D37_T_200706 | 1 | 924 | DQ073101 | 97% | Flavobacteria bacterium TW-JL-17 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Gramella* |
| TEM104_50 | 2 | 485 - 530 | EF215784 | 96 - 97% | Uncultured Bacteroidetes bacterium clone PV2-37 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | not defined |
| KK_A12_T_200806 | 2 | 981 | EF554364 | 94% | *Ulvibacter antarcticus* strain IMCC3101 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Ulvibacter* |
| KK_A03_T_200806 | 7 | 1280 | AY162109 | 94% | Bacteroidetes bacterium GMD16C10 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Ulvibacter* |
| TEM104_34 | 1 | 480 | DQ109924 | 84% | Uncultured Saprospiraceae bacterium clone E48E07cD | Bacteroidetes | Sphingobacteria | Sphingobacteriales | not defined | not defined |
| TEM219_34 | 1 | 579 | EU925904 | 92% | Uncultured bacterium clone 130H27 | Bacteroidetes | Sphingobacteria | Sphingobacteriales | Saprospiraceae | not defined |
| KK_D18_T_200706 | 3 | 1222 | EF515584 | 92% | Uncultured bacterium clone 30e06 | Bacteroidetes | Bacteroidia | Bacteroidales | Prevotellaceae | *Prevotella* |
| TEM224_11 | 1 | 588 | AB362273 | 100% | *Bacillus hwajinpoensis* | Firmicutes | Bacilli | Bacillales | Bacillaceae | *Bacillus* |
| KK_D09_T_200706 | 1 | 832 | FJ380956 | 99% | *Staphylococcus capitis* strain BQEP2-01d | Firmicutes | Bacilli | Bacillales | Staphylococcaceae | *Staphylococcus* |
| TEM104_41 | 1 | 343 | EU005290 | 94% | Uncultured alpha proteobacterium clone G1-47 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM104_43 | 1 | 385 | EU005290 | 87% | Uncultured alpha proteobacterium clone G1-47 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM104_58 | 1 | 516 | EU005290 | 99% | Uncultured alpha proteobacterium clone G1-47 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM104_39 | 1 | 462 | EU005290 | 99% | Uncultured alpha proteobacterium clone G1-47 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM104_44 | 1 | 458 | EU005290 | 97% | Uncultured alpha proteobacterium clone G1-47 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM104_56 | 3 | 434 - 513 | DQ839253 | 95 - 96% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM219_36 | 2 | 562 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM223_03 | 2 | 562 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM219_08 | 1 | 558 | DQ839253 | 97% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM223_24 | 1 | 561 | DQ839253 | 98% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM224_01 | 3 | 553 - 562 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM291_12 | 1 | 452 | DQ839253 | 96% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM291_24 | 2 | 504 - 506 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM228_04 | 1 | 531 | AF114485 | 97% | *Roseovarius crassostreae* isolate CV919-312 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM224_14 | 2 | 500 | DQ870539 | 95 - 99% | Uncultured Rhodobacteraceae bacterium isolate DGGE gel band B29 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM226_09 | 2 | 539 - 562 | DQ870539 | 94 - 95% | Uncultured Rhodobacteraceae bacterium isolate DGGE gel band B29 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM104_42 | 2 | 325 - 563 | EU573108 | 95 - 96% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM291_33 | 1 | 487 | EU573108 | 93% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_A02_T_200806 | 17 | 1228 | EU573108 | 99% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM224_18 | 3 | 502 - 562 | EU573108 | 95 - 97% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM194_01 | 2 | 517 | EU799843 | 98% | Uncultured bacterium clone 1C227505 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM104_59 | 1 | 510 | EU005307 | 99% | Uncultured alpha proteobacterium clone G3-45 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| TEM201_22 | 1 | 512 | AF359535 | 99% | Marine bacterium ATAM407_56 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Phaeobacter* |
| TEM201_23 | 1 | 517 | EU799890 | 96% | Uncultured bacterium clone 1C227558 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM228_33 | 1 | 562 | AJ505788 | 96% | Alpha proteobacterium GRP21 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM290_23 | 1 | 474 | AB302376 | 97% | Alpha proteobacterium C09 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM290_26 | 1 | 499 | AM945581 | 96 | Rhodobacteraceae bacterium MOLA 351 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM290_27 | 1 | 460 | FJ202569 | 96 | Uncultured bacterium clone SGUS469 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM291_01 | 1 | 470 | EF618676 | 97% | *Roseobacter* sp. DJHH4 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM223_05 | 1 | 509 | DQ486490 | 97% | Rhodobacteraceae bacterium DG1250 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM290_22 | 1 | 499 | EU052732 | 97% | *Sulfitobacter* sp. MH178 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM291_20 | 1 | 477 | EU365589 | 99% | *Sulfitobacter* sp. BSw20064 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM223_16 | 1 | 562 | DQ683726 | 100% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM223_17 | 1 | 561 | DQ683726 | 99% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| KK_D30_T_200706 | 7 | 583 | AJ532580 | 97% | *Sulfitobacter* sp. isolate BIO-7 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM225_27 | 2 | 562 | AF359531 | 100% | Marine bacterium ATAM173a_16 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |

**Tab. 3 (cont.)**

| DGGE band | Number of Sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| TEM225_30 | 1 | 560 | AF359531 | 99% | Marine bacterium ATAM173a_16 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM224_20 | 1 | 562 | AF359531 | 99% | Marine bacterium ATAM173a_16 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM228_27 | 1 | 419 | AJ534230 | 95% | *Sulfitobacter* sp. HEL-78 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM291_39 | 1 | 475 | AJ534230 | 99% | *Sulfitobacter* sp. HEL-78 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| TEM222_28 | 1 | 468 | AJ534230 | 94% | *Sulfitobacter* sp. HEL-78 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM223_23 | 1 | 562 | AF468356 | 96% | Arctic sea ice bacterium ARK10029 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM224_09 | 1 | 562 | AJ534225 | 96% | *Jannaschia helgolandensis* isolate HEL-43 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Jannaschia* |
| TEM222_07 | 1 | 562 | AJ534223 | 96% | *Methylarcula* sp. DT-12 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| TEM224_22 | 1 | 562 | AJ534223 | 99% | *Methylarcula* sp. DT-12 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| TEM228_07 | 4 | 510 - 561 | AJ534223 | 98 - 99% | *Methylarcula* sp. DT-12 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| KK_D16_T_200706 | 2 | 1276 | AF468373 | 96% | Arctic sea ice associated bacterium ARK10207 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| KK_D20_T_200706 | 1 | 981 | AF468375 | 98% | Arctic sea ice bacterium ARK10226 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| TEM290_24 | 1 | 494 | AY576770 | 96% | *Ruegeria* sp. 3X/A02/236 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| TEM220_06 | 1 | 562 | AF513928 | 97% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM220_05 | 2 | 510 - 562 | AF513928 | 97 - 98% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM224_21 | 1 | 562 | AF513928 | 96% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM226_07 | 1 | 561 | AF513928 | 96% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM226_08 | 1 | 562 | AF513928 | 97% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| TEM228_06 | 1 | 472 | AF513928 | 96% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_A11_T_200806 | 4 | 1215 | DQ372842 | 94% | Uncultured bacterium clone NH10_05 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Pelagicola* |
| KK_A13_T_200806 | 2 | 1223 | AY772092 | 97% | *Marinosulfonomonas methylotropha* | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_D04_T_200706 | 9 | 666 | CP000264 | 96% | *Jannaschia* sp. CCS1 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_D10_T_200706 | 5 | 943 | EU005760 | 95% | Uncultured marine bacterium clone KG_A3_120m56 1 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_D12_T_200706 | 5 | 893 | AM945591 | 98% | Rhodobacteraceae bacterium MOLA 361 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_D19_T_200706 | 4 | 987 | AF359530 | 98% | Marine bacterium ATAM407_62 1 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_D08_T_200706 | 3 | 890 | AM936911 | 98% | Uncultured *Caulobacter* sp. clone CM14H2 | Proteobacteria | Alphaproteobacteria | Caulobacterales | Caulobacteraceae | not defined |
| TEM228_30 | 1 | 518 | AB369041 | 99% | Uncultured bacterium clone PT-QTL-B41 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylobacteriaceae | *Methylobacterium* |
| TEM222_29 | 2 | 536 - 586 | EU794201 | 99% | Uncultured *Delftia* sp. clone EMP_K41 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| TEM222_33 | 1 | 525 | EU794201 | 96% | Uncultured *Delftia* sp. clone EMP_K41 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| KK_D03_T_200706 | 1 | 783 | AY294215 | 88% | *Bdellovibrio* sp. MPA 1 | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bdellovibrionaceae | *Bdellovibrio* |
| TEM194_24 | 2 | 504 - 520 | AY426613 | 97% | Uncultured *Thiothrix* sp. clone UP23b | Proteobacteria | Gammaproteobacteria | Thiotrichales | Thiotrichaceae | *Thiothrix* |
| KK_A15_T_200806 | 1 | 1256 | DQ383802 | 95% | Ewingella americana isolate PRC120 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | not defined |
| TEM291_11 | 1 | 475 | AY165598 | 95% | Uncultured Arctic sea ice bacterium clone ARKXVI/2-136 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| TEM291_16 | 2 | 504 | EU000245 | 99% | *Psychrobacter maritimus* strain KOPRI_22337 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_D38_T_200706 | 1 | 1050 | AF468396 | 98% | Arctic sea ice bacterium ARK10139 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A01_T_200806 | 46 | 1304 | DQ064620 | 98% | Arctic seawater bacterium Bsw20461 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A06_T_200806 | 3 | 1264 | AF468390 | 98% | Arctic sea ice bacterium ARK10052 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A08_T_200806 | 1 | 1191 | EF101547 | 95% | *Psychrobacter aquimaris* strain KOPRI24929 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A16_T_200806 | 1 | 1240 | AJ748270 | 98% | *Psychrobacter psychrophilus* isolate CMS 32 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A19_T_200806 | 2 | 968 | AJ313425 | 99% | *Psychrobacter nivimaris* type strain 88/2-7 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_A20_T_200806 | 1 | 1258 | AJ309940 | 99% | *Psychrobacter submarinus* strain KMM 225 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_D01_T_200706 | 26 | 861 | EF101544 | 98% | *Psychrobacter nivimaris* strain KOPRI24925 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_D02_T_200706 | 3 | 859 | AJ849371 | 99% | *Pseudoalteromonas* sp. isolate HAL34 | Proteobacteria | Gammaproteobacteria | Alteromonadales | Pseudoalteromonadaceae | *Pseudoalteromonas* |
| KK_D05_T_200706 | 2 | 587 | FJ015025 | 99% | Halomonadaceae bacterium M48-6.6 | Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae | *Cobetia* |
| KK_D33_T_200706 | 1 | 887 | FJ153024 | 94% | Uncultured bacterium clone TX4CB_155 | Proteobacteria | Gammaproteobacteria | Legionellales | Legionellaceae | *Legionella* |
| KK_D06_T_200706 | 1 | 1033 | EF667054 | 98% | *Vibrio fischeri* strain Ipeal.1.1 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Aliivibrio* |
| KK_D36_T_200706 | 1 | 727 | X56576 | 99% | *V. alginolyticus* | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| KK_D07_T_200706 | 34 | 1078 | AY292947 | 98% | | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_D17_T_200706 | 1 | 973 | AB239480 | 97% | *Photobacterium phosphoreum* strain LC1-238 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| KK_D23_T_200706 | 1 | 1253 | AY435156 | 99% | *Photobacterium phosphoreum* strain RHE-01 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| TEM220_08 | 1 | 565 | EF188432 | 99% | Uncultured eukaryote clone 246, chloroplast | Cyanobacteria | Cyanobacteria | n. a. | Chloroplast | *Streptophyta* |
| TEM291_37 | 1 | 479 | FJ002211 | 93% | *Thalassiosira gravida* isolate C140 | Cyanobacteria | Cyanobacteria | n. a. | Chloroplast | *Bacillariophyta* |

TEM = field-caught *Temora longicornis*

# Tab. 4

| DGGE band | Number of sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| CEN222_21 | 1 | 516 | AF197049 | 99% | *Arthrobacter* sp. SMCC ZAT031 | Actinobacteria | Actinobacteria | Actinomycetales | Micrococcaceae | *Arthrobacter* |
| CEN228_29 | 1 | 572 | DQ521508 | 95% | Uncultured bacterium clone ANTLV2_C06 | Actinobacteria | Actinobacteria | Actinomycetales | Propionibacteriaceae | not defined |
| CEN194_14 | 4 | 533 - 578 | FJ196065 | 99 - 100% | *Polaribacter* sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| CEN291_07 | 1 | 487 | DQ870529 | 87% | Uncultured *Mesonia* sp. isolate DGGE gel band B21 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Mesonia* |
| CEN201_48 | 3 | 477 - 511 | DQ683726 | 96 - 97% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CEN222_24 | 1 | 562 | DQ683726 | 99% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CEN225_12 | 1 | 561 | DQ683726 | 99% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CEN226_11 | 1 | 561 | AJ534228 | 99% | *Sulfitobacter* sp. HEL-76 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CEN219_31 | 3 | 561 - 562 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CEN228_01 | 3 | 559 - 561 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CEN291_19 | 1 | 496 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CEN291_32 | 1 | 502 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CEN219_25 | 1 | 562 | DQ870539 | 97% | Uncultured Rhodobacteraceae bacterium isolate DGGE gel band B29 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CEN219_26 | 1 | 562 | AJ534223 | 99% | *Methylarcula* sp. DT-12 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Loktanella* |
| CEN219_27 | 1 | 564 | EU375058 | 96% | Uncultured *Rhodobacter* sp. clone Rc22 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| CEN228_02 | 1 | 560 | EU375058 | 98% | Uncultured *Rhodobacter* sp. clone Rc22 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Paracoccus* |
| KK_B05_C_2008060 | 5 | 1210 | EU573108 | 99% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_E01_C_2007060 | 29 | 1213 | EU573108 | 99% | Bacterium enrichment culture clone EB39.6 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| KK_B04_C_2008060 | 3 | 1273 | EU491168 | 99% | Uncultured bacterium clone P9X2b7A12 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| KK_B01_C_2008060 | 32 | 1261 | DQ064620 | 98% | Arctic seawater bacterium Bsw20461 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_B02_C_2008060 | 2 | 1241 | EF101547 | 98% | *Psychrobacter aquimaris* strain KOPRI24929 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_B03_C_2008060 | 1 | 1257 | AJ748270 | 98% | *Psychrobacter psychrophilus* isolate CMS 32 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_B07_C_2008060 | 1 | 1247 | EF101551 | 98% | *Psychrobacter pulmonis* strain KOPRI24933 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_E02_C_2007060 | 5 | 1287 | AF468396 | 99% | Arctic sea ice bacterium ARK10139 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_B10_C_2008060 | 4 | 1258 | EF101544 | 99% | *Psychrobacter nivimaris* strain KOPRI24925 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* |
| KK_B09_C_2008060 | 1 | 1277 | AJ874364 | 99% | *Vibrio splendidus* strain 01/246 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| KK_B13_C_2008060 | 1 | 1287 | AY292947 | 99% | *Photobacterium leiognathi* strain RM1 | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Photobacterium* |
| CEN201_11 | 1 | 516 | AM940305 | 99% | Uncultured bacterium | Cyanobacteria | Cyanobacteria | n.a. | Chloroplast | *Streptophyta* |

CEN = field-caught *Centropages* sp.

**Tab. 5**

| DGGE band | Number of Sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| CAL222_01 | 1 | 518 | EU834273 | 91% | *Microbacterium phyllosphaerae* strain DS56 | Actinobacteria | Actinobacteria | Actinomycetales | Propionibacteriaceae | *Tessaracoccus* |
| CAL223_13 | 1 | 571 | DQ521508 | 98% | Uncultured bacterium clone ANTLV2_C06 | Actinobacteria | Actinobacteria | Actinomycetales | | |
| CAL201_16 | 2 | 526 - 578 | FJ196065 | 99 - 100% | *Polaribacter* sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| CAL194_06 | 1 | 553 | FJ196065 | 96% | *Polaribacter* sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| CAL222_14 | 1 | 580 | FJ202110 | 97% | Uncultured bacterium clone SGUS1259 | Bacteroidetes | Flavobacteria | Flavobacteriales | Cryomorphaceae | *Brumimicrobium* |
| CAL222_13 | 1 | 581 | AM295256 | 98% | *Lewinella persicus* type strain ATCC 23167T | Bacteroidetes | Sphingobacteria | Sphingobacteriales | Saprospiraceae | *Lewinella* |
| CAL201_20 | 1 | 509 | EF418067 | 98% | Uncultured bacterium clone BHS-1656 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | not defined |
| CAL201_26 | 1 | 545 | EU419931 | 100% | *Exiguobacterium* sp. RW44 | Firmicutes | Bacilli | Bacillales | Bacillaceae | *Exiguobacterium* |
| CAL205_07 | 1 | 587 | AB362273 | 95% | *Bacillus hwajinpoensis* | Firmicutes | Bacilli | Bacillales | Bacillaceae | *Bacillus* |
| CAL219_19 | 3 | 561 - 562 | DQ839253 | 99 - 100% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CAL225_19 | 1 | 560 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CAL223_14 | 1 | 561 | DQ870539 | 98% | Uncultured Rhodobacteraceae bacterium isolate DGGE gel band B29 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CAL224_08 | 1 | 558 | AF513928 | 95% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| CAL222_09 | 1 | 537 | AF513928 | 93% | Uncultured Rhodobacter group bacterium clone LA1-B32N | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Oceanicola* |
| CAL201_17 | 1 | 515 | AF359534 | 96% | Marine bacterium SCRIPPS_732 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CAL219_20 | 1 | 560 | AF359531 | 100% | Marine bacterium ATAM173a_16 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CAL223_12 | 1 | 512 | DQ683726 | 99% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CAL201_18 | 1 | 514 | AJ534230 | 99% | *Sulfitobacter* sp. HEL-78 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| CAL194_07 | 1 | 530 | AJ505788 | 96% | Alpha proteobacterium GRP21 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| CAL223_15 | 1 | 564 | AJ505788 | 96% | Alpha proteobacterium GRP21 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| CAL225_29 | 2 | 510 - 562 | EU346575 | 96% | Marine sponge bacterium PLATEdelicii-(2)-29 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| CAL219_21 | 1 | 528 | AF359531 | 97% | Marine bacterium ATAM173a_16 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| CAL291_06 | 1 | 469 | AY580880 | 95% | Uncultured gamma proteobacterium clone PI_4q4f | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | *Vibrio* |
| CAL222_11 | 1 | 652 | L81939 | 99% | Calanus pacificus 18S ribosomal RNA gene | Copepod | n. a. | n. a. | n. a. | *n. a.* |
| CAL222_11 | 1 | 652 | DQ839259 | 99% | Uncultured Calanus sp. isolate DGGE band NS14 | Copepod | n. a. | n. a. | n. a. | *n. a.* |

CAL = field-caught *Calanus helgolandicus*

# TAB. 6

| Copepod genus | DGGE band | Number of Sequences | Sequence length [bp] | Nearest relative sequence [Acc. No.] | Similarity | Bacterial Identity | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudo-/Paracalanus* sp. | PSE222_25 | 1 | 584 | AY494657 | 98% | Uncultured *Turicella* sp. clone ACTINO9E | Actinobacteria | Actinobacteria | Actinomycetales | Corynebacteriaceae | *Turicella* |
| *Pseudo-/Paracalanus* sp. | PSE219_06 | 1 | 520 | DQ660959 | 99% | Uncultured bacterium isolate DGGE gel band 49 | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | not defined |
| *Pseudo-/Paracalanus* sp. | PSE219_09 | 2 | 529 - 577 | FJ196065 | 99% | *Polaribacter* sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| *Pseudo-/Paracalanus* sp. | PSE291_34 | 1 | 494 | EU799843 | 97% | Uncultured bacterium clone 1C227505 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | |
| *Pseudo-/Paracalanus* sp. | PSE201_27 | 1 | 510 | DQ683726 | 98% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| *Pseudo-/Paracalanus* sp. | PSE291_35 | 1 | 497 | EU641637 | 99% | Uncultured Comamonadaceae bacterium clone LW9m-3-69 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| *Pseudo-/Paracalanus* sp. | PSE201_07 | 1 | 538 | EU794201 | 99% | Uncultured *Delftia* sp. clone EMP_K41 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Delftia* |
| *Euterpina* sp. | EUT201_08 | 1 | 522 | AF197049 | 100% | Arthrobacter sp. 'SMCC ZAT031' | Actinobacteria | Actinobacteria | Actinomycetales | Micrococcaceae | *Arthrobacter* |
| *Euterpina* sp. | EUT194_19 | 3 | 528 - 531 | FJ196065 | 96 - 100% | Polaribacter sp. BSw20012b | Bacteroidetes | Flavobacteria | Flavobacteriales | Flavobacteriaceae | *Polaribacter* |
| *Euterpina* sp. | EUT194_20 | 1 | 514 | EU799843 | 97% | Uncultured bacterium clone 1C227505 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Thalassobacter* |
| *Euterpina* sp. | EUT219_07 | 1 | 540 | DQ234169 | 97% | Uncultured Rhodobacteraceae bacterium clone DS086 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| *Euterpina* sp. | EUT222_35 | 1 | 561 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| *Euterpina* sp. | EUT224_05 | 1 | 561 | DQ839253 | 99% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |
| *Euterpina* sp. | EUT224_06 | 3 | 514 - 562 | EU780363 | 98% | Uncultured alpha proteobacterium clone 4DP1-N24 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | not defined |
| *Euterpina* sp. | EUT225_03 | 1 | 499 | DQ683726 | 95% | *Sulfitobacter marinus* strain SW-265 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Sulfitobacter* |
| *Candacia* sp. | CAN291_04 | 1 | 384 | EU861202 | 93% | Uncultured alpha proteobacterium clone pl14F09 | Proteobacteria | Alphaproteobacteria | not defined | not defined | not defined |
| *Candacia* sp. | CAN291_14 | 1 | 469 | DQ839253 | 96% | Uncultured alpha proteobacterium isolate DGGE band NS8 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Roseovarius* |

# CHAPTER IV

# BACTERIAL TRANSFER THROUGH TROPHIC LEVELS?- DOES P-LIMITATION MAKE ANY DIFFERENCE?

# TRANSFER OF BACTERIA THROUGH TROPHIC LEVELS? DOES P-LIMITATION MAKE ANY DIFFERENCE?

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**Keywords:** bacteria; copepod; DGGE; *Rhodomonas salina*, *Acartia tonsa*; *Pleurobrachia pileus*; phosphorus limitation; trophic transfer;

## ABSTRACT

Different kinds of bacteria can be associated with zooplankton, such as copepods and jellyfish. To date little is known about the origin and character of these bacterial assemblages. It is assumed that they can derive either from food item uptake or from the surrounding water. However, it is not understood whether the bacteria can be transferred from one trophic level to the next and whether they can establish associated with a new host organism. Furthermore, it is not known whether the associated bacterial assemblages are different when the nutrient availability for the hosts is different.

In this study we compared the bacterial assemblages of three different organisms by polymerase chain reaction-based denaturing gradient gel electrophoresis (DGGE) using a tri-trophic feeding experiment. The cryptophyte *Rhodomonas salina* served as the primary producer while the calanoid copepod *Acartia tonsa* and the ctenophore *Pleurobrachia pileus* were the primary and secondary consumers, respectively. To investigate whether phosphate limitation has any effect concerning the composition of the associated bacterial assemblages, we conducted this experiment both in nutrient-enriched and in phosphorus-depleted sea water. We discovered that the associated bacterial assemblages were distinct for the three different hosts. There were some bacterial phylotypes which were found associated with animals rather than with the algae or in the culture water. We detected no differences in the community composition between nutrient-enriched and phosphorus-depleted samples.

## INTRODUCTION

Marine bacteria can colonise the surfaces of algae (e.g. Grossart et al. 2005; Sapp et al. 2007a) and also surfaces and the intestine of zooplankton organisms such as copepods (e.g. Sochard et al. 1979; Hansen and Bech 1996; Møller et al. 2007; Brandt et al. 2010) and jellyfish (e.g. Schuett and Doepke 2009). For copepods it is known that their body appendages, the intersegmental regions and the regions around the mouth and anus are the parts which are generally colonised (Huq et al. 1983; Carman and Dobbs 1997) by different kinds of bacteria (e.g. Sochard et al. 1979; Hansen and Bech 1996; Møller et al. 2007). The gut lining can also provide a favourable surface for some bacteria (Carman and Dobbs 1997). The 'sloppy feeding' behaviour (Møller et al. 2003; Møller 2007) of copepods feeding on large food items and the nutrient-leakage from faecal pellets (Møller et al. 2003) offer nutrient-rich particles which can attract bacteria and would encourage them to stay in the vicinity of copepods or even to attach to them. Replenishment of bacteria on the zooplankton can arise from the water column as well as from the food. Whether the food-particle associated bacteria can survive the gut passage and establish on the new host remains unknown. Even when the surface bacteria were stripped off the food by the hosts feeding instruments and were conferred to the new host surface they have to handle the new conditions to establish but they could obviously be outcompeted by previous established bacterial community (e.g. Rao et al. 2006).

The transfer of nutrients such as carbon, nitrogen and phosphorus across trophic levels in food webs has been the subject to numerous studies over the last six decades (Persson et al. 2007), but to our knowledge only one study has investigated the transfer of bacteria across two phylogenetic levels (Tang et al. 2009) to date.

Furthermore, the development of associated bacterial assemblages under nutrient limitation for different hosts and the transfer of these bacteria through the trophic levels remains unknown. In the case of phosphorus limitation, the preferred environment of bacteria from a nutritional point of view would be in the vicinity of micro- or mesozooplankton.

Phosphorus limitation occurs in the late phase of a phytoplankton spring bloom in temperate waters (Schoo et al. 2010). Phytoplankton then becomes

progressively more phosphorus-limited and zooplankton which feeds on it has a surplus of carbon (high C:P ratio). The phytoplankton itself and the herbivorous zooplankton have to get rid of this surplus carbon. This requires needs energy. This expenditure both in terms of carbon excretion and energy could make the organisms more attractive for bacterial pathogens which could easier afflict them. Carbon excretion into the water may attract bacteria which then remain close by.

In this study we compared the bacterial assemblages of three different organisms by DGGE in a tri-trophic feeding arrangement. The cryptophyte *Rhodomonas salina* served as the primary producer while the calanoid copepod *Acartia tonsa* and the ctenophore *Pleurobrachia pileus* were the primary and secondary consumers, respectively. To investigate whether bacteria were transferred across the three trophic levels, the bacterial community fingerprints of the three organisms and additionally of the culture-water were compared. With subsequent sequencing of prominent bands the predominant bacterial phylotypes could be assigned to band classes. To investigate whether phosphate limitation affects the composition of the bacterial communities associated with the organisms we conducted this experiment both in nutrient-enriched and in phosphorus-depleted sea water in parallel.

## MATERIALS AND METHODS

A tri-trophic feeding experiment was designed with the cryptophyte *Rhodomonas salina* as primary producer, the calanoid copepod *Acartia tonsa* as primary consumer and the ctenophore *Pleurobrachia pileus* as secondary consumer.

### EXPERIMENTAL SET UP

The seawater used in the experiments was taken from the North Sea at Helgoland Roads (54°11.3’ N, 7°54.0’ E) in one event. It was filtered through a sterile 0.2 µm filter and stored dark and cold until use.

#### PRIMARY PRODUCER *RHODOMONAS SALINA*

Stock cultures of the cryptophyte *R. salina* were cultivated in f/2 medium (Guillard and Ryther 1962). For the experimental treatments *R. salina* was

cultured in f/2-enriched seawater and under phosphorus-limited conditions (without addition of phosphorus) (Guillard and Ryther 1962). The algae therefore had access only to the phosphorus present in the seawater at the time of filtration (1.4 µmol/l Schoo et al. 2010). The algae were cultured at 17 °C under a 16:8 light:dark cycle. To assure a constant food quality, every day new cultures of *R. salina* were inoculated for both treatments. For results on algal growth rates and determination of algal densities see Schoo et al. (2010).

#### PRIMARY CONSUMER *ACARTIA TONSA*

Copepod eggs were obtained from a long-term laboratory culture of the calanoid copepod *A. tonsa*. For the experimental treatments copepods were reared from egg to the fifth naupliar stage in four-litre plastic bags at a density of about 3000 individuals/l in nutrient-free artificial seawater adjusted to a salinity of 31 (salt: hw-marinemix professional, Wiegandt GmbH, Krefeld, Germany). After hatching the animals were kept at 18 °C under a 16:8 light:dark cycle. They were first fed with *R. salina* 24 hours after hatching. To avoid food quantity effects copepods were fed 10,000 cells of *R. salina* (in each treatment) per individual and day. To assure a constant food quality for the ctenophore *P. pileus* each day two new copepod cultures were started. To ensure that the copepods from different treatments were in the same developmental stage and had the same size the phosphorus-limited copepods were one day older than the f/2-copepods, because Schoo et al. (2010) and Malzahn et al. (2010) showed that the phosphorus-limited copepods displayed a delayed development which resulted in a time-lag of ~ 1 day compared to the copepods reared on f/2-algae.

#### SECONDARY PRODUCER *PLEUROBRACHIA PILEUS*

The ctenophore *P. pileus* was captured from the sea water at Helgoland Roads. The individuals were transferred to flow-through tanks and kept at ambient temperatures in filtered seawater. Prior to the experiment the individuals were starved for five days and only ctenophores of the same size range (10-15 mm) were used in the experiment. They were kept separately in one-litre glass bottles containing filtered seawater. During the experiment the water was changed daily to remove the uneaten copepods. *P. pileus* were fed 500

copepods per ctenophore daily for nine days. The copepods were mixed along a phosphorus-gradient, resulting in five treatments with differing P-content (F/2, ¾ F/2, ½ F/2, ¼ F/2, -P) and a treatment with ctenophores which starved during the experiment. These treatments were randomly assigned to the ctenophores (for more details see Schoo et al. 2010). From these six treatments three ctenophores of each treatment were investigated using DGGE.

## SAMPLING DURING THE EXPERIMENT

### WATER

For all three trophic levels the culture-water was investigated. 25 ml were prefiltered through 10 and 3 µm membrane filters in succession. To investigate the free-living bacteria fraction of the culture-water the flow-through was filtered through a 0.2 µm membrane filter.

#### *RHODOMONAS SALINA*

Because of the size of the algal cells (approx. 7 µm) the algae cultures were prefiltered with a 10 µm filter and sampled with a 3 µm filter to investigate the bacterial communities attached to the algal cells. After filtration of 25 ml the filters were washed twice with sterile seawater, dried and stored at – 20 °C until further analysis.

#### *ACARTIA TONSA*

Copepods were sieved through a 100 µm gauze sieve and 50 ml sterile seawater was added to all copepods. Copepods were counted (see Schoo et al. 2010) and a defined volume of 3 ml was filtered through a 10 µm membrane filter. The copepod filters were washed twice with sterile seawater and then stored at -20 °C until further analysis.

#### *PLEUROBRACHIA PILEUS*

The ctenophores were sampled using a 100 µm gauze sieve and washed twice with sterile seawater before being transferred into individual sterile tubes. The ctenophores were frozen at – 80 °C until further analysis.

## ANALYSIS

### DNA EXTRACTION

The filters were cut and put into sterile reaction tubes with addition of 700 µl STE buffer (6.7 % saccharose, 50 mM Tris, 1 mM EDTA, pH 8). Cell lysis was accomplished by incubating the samples with 18.4 µl lysozyme (10 mg / ml) (for all filter samples except copepod filters) for 30 min at 37 °C with gentle shaking at 400 rpm. Next, 73.4 µl EDTA-Tris (250 mM, 50 mM) and 44 µl SDS-Tris-EDTA (20 %, 50 mM, 20 mM) were added and samples were incubated for 60 min at 50 °C with gentle shaking. After centrifugation at 13,000 rpm for 10 min the supernatant was transferred into a new sterile reaction tube. The salt concentration was increased by adding a 1/10 vol. of NaCl (5 M) and proteins were removed using 1 vol. phenol-chloroform (1:1). DNA was then precipitated by overnight incubation with isopropanol at -20 °C. Precipitates were washed with 75 % ethanol and dried in a laminar flow cabinet for 15 min prior to resuspension with 30 µl sterile water. DNA extracts were stored at -20 °C until further analysis.

Copepod filters were cut and put into sterile reaction tubes. With addition of 350 µl STE buffer they were treated with pellet pestle using pellet pestle motor (Kontes, Vineland, NJ, USA) for 30 s. After addition of 350 µl STE buffer cell lysis was accomplished by incubating the samples with 100 µl lysozyme for 30 min at 37 °C with gentle shaking. Following steps were carried out as described for the filters above.

The ctenophores were freeze-dried and homogenised with a pellet pestle before adding 370 µl STE buffer and 100 µl lysozyme. After 30 min at 37 °C with gentle shaking 50 µl Tris-EDTA and 30 µl SDS-Tris-EDTA were added. The following steps of the analysis were the same as described above.

### PCR CONDITIONS

PCR-amplification of 16S rRNA gene fragments for DGGE was performed using the bacteria specific primers 341 with GC-clamp (P3) (Muyzer et al. 1993) with a 40-bp GC-rich sequence at the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3') (Muyzer et al. 1995).

PCR mixtures with a volume of 50 µl contained 5 µl of 10 x Taq buffer (5 Prime, Hamburg, Germany), 8 µl of Master Enhancer (5 Prime) for initial PCRs and no enhancer for reamplification after DGGE, 200 µM dNTPs (Promega, Mannheim, Germany), 0.2 µM of each primer, 2U of Taq DNA polymerase (5 Prime) and either 2 µl of DNA both prior and after DGGE.

'Touchdown' PCR was performed as described by (2007b). PCRs were conducted in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) and PCR products were separated on 1.2% (w/v) agarose gels (50 min at 100 V in 0.5 x TBE).

Separated DNA was visualised by ethidium bromide (0.5 mg $l^{-1}$). Images were captured with a ChemiDoc XRS System (BioRad, München, Germany). The thickness and intensity of each band visualised were used to gauge the relative volume of the corresponding product used for DGGE (see below).

**DGGE**

All 16S rRNA gene amplicons were resolved on 6% (w/v) polyacrylamide gels in 0.5 x TAE buffer (20 mM TrisHCl, 10 mM acetic acid, 0.5 mM EDTA) with denaturing gradient of 15–55% urea/formamide (100% denaturant contains 7M urea and 40% formamide). Electrophoresis was performed at 60 °C and 150 V for 10 h (Sigler et al. 2004) using a DCode mutation detection system (BioRad). DGGE gels were stained with SYBRGold (Invitrogen, Karlsruhe, Germany). Imaging was performed with a ChemiDoc XRS System (BioRad). Prominent DGGE bands were excised, eluted in 50 µl PCR-water (Eppendorf) by gentle shaking at 37 °C for 60 min, reamplified and confirmed by an additional DGGE. As a marker for comparative analyses of all DGGE gels the combined PCR-amplicons (GC-341f / 907r) of four bacteria (*Polaribacter filamentus* DSM 13964, *Sulfitobacter mediterraneus* DSM 12244, *Arthrobacter agilis* DSM 20550, *Leifsonia aquatica* DSM 20146) were used.

**SEQUENCING AND PHYLOGENETIC ANALYSIS**

Predominant DGGE bands were excised, the DNA was eluted and reamplified using the primers 341f (without GC-clamp) (Muyzer et al. 1993) and 907r. PCR-products were checked on 1.2 % (w/v) agarose gels prior to sequencing. PCR products with the correct size (~566 bp) were excised from the agarose gels

and used for sequencing. DNA sequencing of PCR products was performed by Qiagen GmbH using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as used for reamplification.

Sequence data were checked for the presence of PCR amplified chimeric sequences by the CHECK_CHIMERA program (Cole et al. 2003). Nearest relatives for all sequences were searched using BLAST (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997). The ARB software package (http://www.arb-home.de, Ludwig et al. 2004) was used for phylogenetic analysis. After addition of sequences to the ARB 16S rDNA sequences database (release May 2005), alignment was carried out with the Fast Aligner integrated in the program and refined by comparison of closest relatives retrieved by BLAST. Sequences with more than 1300 nucleotides were used to calculate phylogenetic trees. The ARB 'parsimony interactive' tool was used to add partial sequences to respective trees. Phylogenetic relationships were deduced by the neighbour joining method including the correction algorithm of Felsenstein (1993).

#### STATISTICAL ANALYSIS

Analyses of DGGE fingerprints were carried out with Bionumerics 5 software package (Applied Maths NV, Sint-Martens-Latem, Belgium). Normalisation of DGGE gels was performed using a marker consisting of combined PCR-amplicons (GC-341f / 907r) of four bacteria (see above) with different GC-contents.

For sample comparison band matching analysis was carried out with Bionumerics 5 software (Applied Maths NV). Bands were assigned to classes of common bands within all profiles. Ordination techniques based on DGGE fingerprints were used to analyse the bacterial community at the phylotype level and the factors affecting specific bacterial phylotypes. Multivariate analysis of fingerprints was performed using the subroutines of non-metric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM) of the PRIMER 5 software suite (PRIMER-E Ltd., Plymouth, UK). ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA).

Analysis of similarity (ANOSIM; Primer; Jaccard distance measure, 999 permutations) was used to test differences in bacterial community composition of the free-living bacterial communities of the different culture water, and associated bacterial communities of the alga, copepod and ctenophore, as well as between the two investigated treatments.

### NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

Not yet available

## Results

### COMPARISON OF DGGE BANDING PATTERNS

We sampled algae and algal culture-water of eleven batches for each treatment (F/2 and –P) and copepods and copepod culture-water from four batches for each treatment (F/2 and –P). In the end three *P. pileus* individuals and their culture-water for each of the six treatments (F/2, ¾ F/2, ½ F/2, ¼ F/2, -P and starved) were sampled. Not from all sampled organisms and filters we could successfully yield DNA or PCR products. So in the comparison of DGGE banding patterns 68 samples were involved (for details see Tab. 1). For the different investigated organisms and culture-water samples the DGGE band numbers ranged between two and ten. 31 different band classes were assigned for statistical analysis.

### STATISTICAL ANALYSIS

Based on the comparison of DGGE banding patterns of the different samples or the two distinct treatments with phosphorus replete and limited samples, statistical analysis (ANOSIM) revealed that there were no differences between the treatments with complete nutrients and with phosphorus limitation, neither when algae-, copepod- or jellyfish-samples were tested separately nor when all samples were considered ($R = 0.014$; $P = 35.5\%$). The R value measures whether separation of community structure is found ($R = 1$), or whether no separation occurs ($R = 0$). R values > 0.75 are commonly interpreted as well separated, R values > 0.5 as separated, but overlapping, and $R < 0.25$ as barely separable (Clarke and Warwick 2001). In Figure 1 the nMDS plot illustrates that there was no separation between the two different treatments.

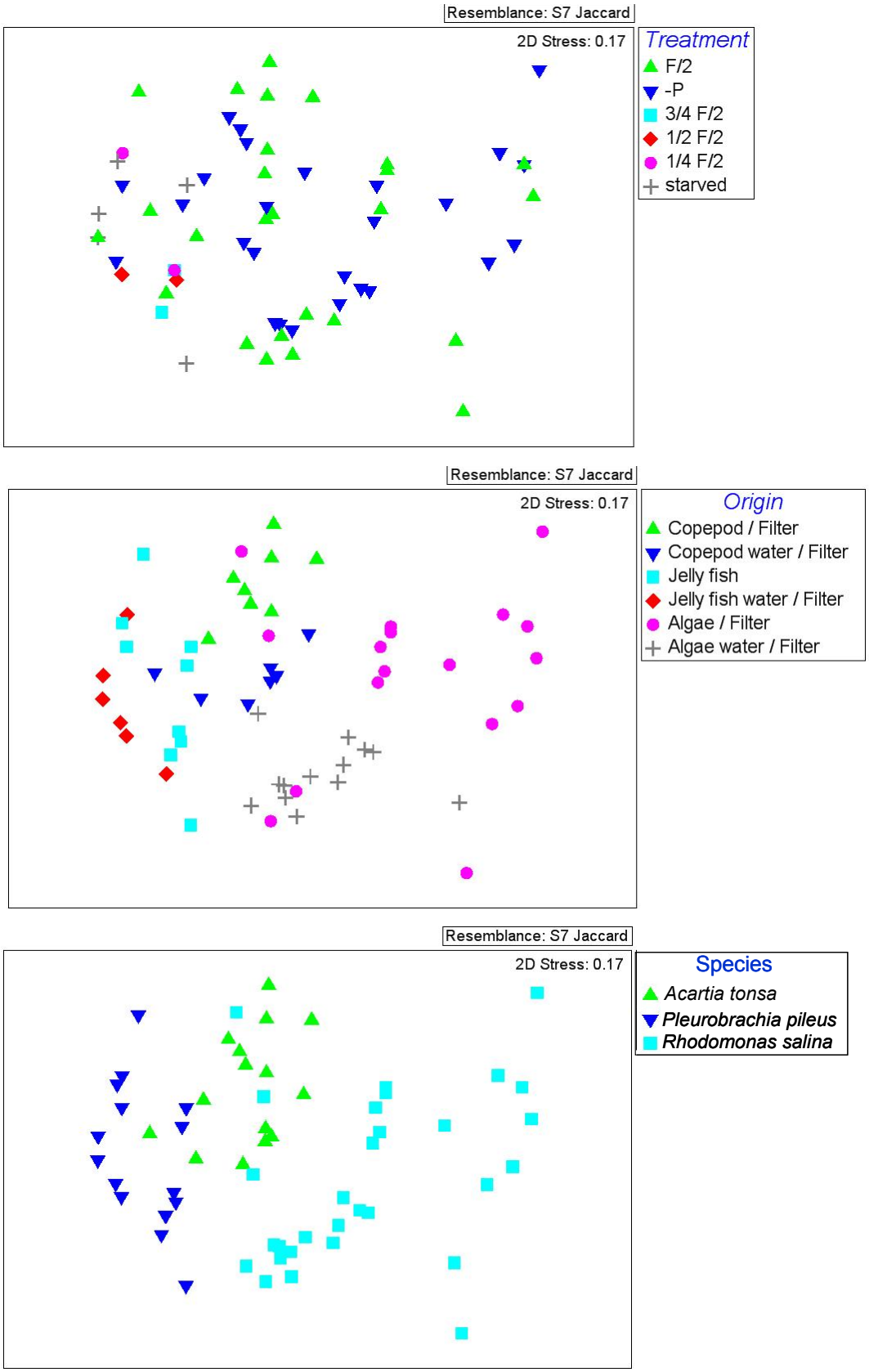


Fig. 1-3: nMDS plots based on Jaccard similarities of DGGE fingerprints of bacterial communities of all investigated samples (*R. salina, A. tonsa, P. pileus*, and the particular culture-water). Stress level in 3D = 0.12.

We found clear differences between free-living and associated bacterial communities. Algae-attached bacterial community is separated from those of the free-living fraction (Global R = 0.462; p = 0.1%), whereas the copepod-associated and culture-water bacterial communities were clearly separated (Global R = 0.7; p = 0.1%). Ctenophore and ctenophore culture-water bacterial communities are separated (Global R = 0.394; p = 0.3%). Figure 2 shows the nMDS plot based on these results. Figure 3 shows that the bacterial communities from the three different organisms (free-living in culture-water and associated with the organism) are separated (R = 0.386; p = 0.1%). In nMDS plots values of stress in the range 0.2-0.3 should be treated with a great deal of scepticism (Clarke and Warwick 2001).The stress level of the nMDS plots mentioned above (Fig. 1-3) showed a 2D stress level of 0.17 (3D of 0.12). This gives a potentially useful two-dimensional picture for interpretation (Clarke and Warwick 2001).

## PHYLOGENETIC ANALYSIS

Overall, we discovered 31 band classes. Ten band classes were exclusively detected for associated bacteria and five for free-living bacteria. An overview of the number of band classes with regard to associated or free-living bacteria is given in Figure 4. From that figure also emerges the number of band classes shared among the investigated organisms. Some band classes were exclusively detected for single organisms. Three band classes were detected exclusively for bacteria associated with *P. pileus* (1, 6 and 1 band(s)). There was no exclusive bacterial band class for *A. tonsa*, but two exclusively connected with bacteria associated with *R. salina* (8 and 2 bands) and one with free-living bacteria in the *R. salina* culture-water (13 bands).

Some of these band classes could be assigned to bacterial phylotypes. We successfully sequenced 16 prominent bands from different samples and we detected nine different bacterial phylotypes and one sequence of a *Rhodomonas* chloroplast (Tab. 2). We identified six different Alpha- and one Gammaproteobacteria, as well as two Bacteroidetes (Tab. 2).

Band class 20 (*P. pileus* associated bacteria) could be assigned to an uncultured Bacteriodetes bacterium (Tab. 2) formerly found associated with polar sea ice (Brown and Bowman 2001). Band classes 6 and 7 could be

dedicated to a chloroplast sequence of *R. salina* (Tab. 2) and shows up for *R. salina*- and *R. salina* culture-water samples as well as for copepod culture water-samples.

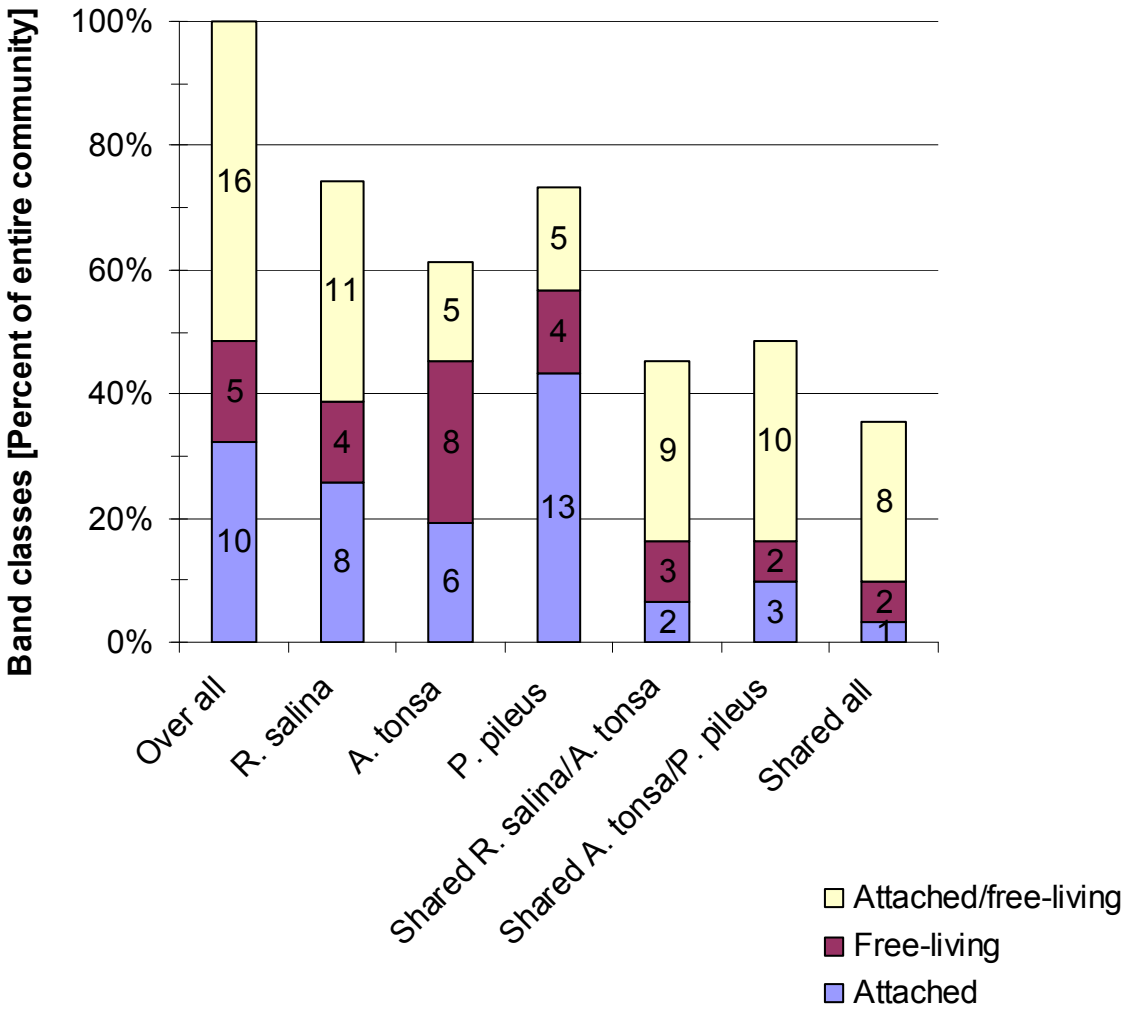


Fig. 4: Overview of band classes. Number of band classes and their appearance connected with the three investigated organisms *R. salina*, *A. tonsa* and *P. pileus* as well as the number of band classes which were shared by *R. salina* and *A. tonsa*, or A. *tonsa* and *P. pileus*, or shared by all three of them. Numbers of band classes which are affiliated with organism associated and free-living band classes are shown in the columns whereas the percentage of the whole community is given on the y-axis.

Band class 23 was assigned to a Gammaproteobacteria sequence, *Psychrobacter* sp. QJJN20, from China Sea surface water (EU438921) (Tab. 2). This band class occurred for all investigated organisms, both, associated as well as in the culture-water.

Tab. 1: Sampled and analysed samples from the different origins

| | F/2 | | 3/4 F/2 | | 1/2 F/2 | | 1/4 F/2 | | -P | | starved | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | sampled | in DGGE analysis | sampled | in DGGE analysis | sampled | in DGGE analysis | sampled | in DGGE analysis | sampled | in DGGE analysis | sampled | in DGGE analysis |
| *R. salina* | 11 | 10 | no | no | no | no | no | no | 11 | 10 | no | no |
| *R. salina* culture water | 11 | 7 | no | no | no | no | no | no | 11 | 9 | no | no |
| *A. tonsa* | 4 | 4 | no | no | no | no | no | no | 4 | 4 | no | no |
| *A. tonsa* culture water | 4 | 4 | no | no | no | no | no | no | 4 | 3 | no | no |
| *P. pileus* | 3 | 2 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 2 | 3 | 3 |
| *P. pileus* culture water | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 2 |

Tab. 2: Bacterial phylotypes associated with the investigated organisms R. salina, A. tonsa and P. pileus

| Band | Origin | Sequence length | Similarity [%] | Acession Number of nearest Genbank relative sequence | Describtion of nearest relative sequence | Affiliation |
|---|---|---|---|---|---|---|
| PB290_08 | *P. pileus* | 468 | 99 | AM905331 | *Ruegeria scottomollicae* strain LMG 24368 | Alphaproteobacteria |
| PB290_09 | *P. pileus* culture water | 472 | 99 | | | |
| PB290_10 | *R. salina culture water* | 476 | 97 | FJ202501 | Uncultured bacterium clone SGUS1277 | Alphaproteobacteria |
| PB290_11 | *R. salina culture water* | 476 | 95 | EF657811 | *Thalassobius* sp. 7PSW-6 | Alphaproteobacteria |
| PB290_14 | *A. tonsa* | 490 | 98 | EU542125 | Uncultured bacterium clone SAV01H01 | Alphaproteobacteria |
| PB290_16 | | 476 | 99 | | | |
| PB290_21 | *R. salina culture water* | 500 | 98 | AF367403 | Uncultured Rhodobacter group bacterium clone D129 | Alphaproteobacteria |
| PB290_18 | *R. salina* | 470 | 98 | AB307984 | Uncultured bacterium DGGE band KH03-34B | Alphaproteobacteria |
| PB290_04 | *A. tonsa* culture water | 332 | 99 | | | |
| PB290_06 | *P. pileus* | 336 | 96 | EU438921 | *Psychrobacter* sp. QJJN20 | Gammaproteobacteria |
| PB290_13 | *A. tonsa* | 476 | 98 | FJ196065 | *Polaribacter* sp. BSw20012b | Bacteroidetes |
| PB290_15 | | 487 | 99 | | | |
| PB290_05 | *P. pileus* | 293 | 97 | AF277556 | Uncultured bacterium SIC.B8012 | Bacteroidetes |
| PB290_12 | *R. salina culture water* | 496 | 100 | EF508371 | *Rhodomonas salina* strain CCMP1319 chloroplast | Chloroplast |
| PB290_17 | | 504 | 99 | | | |
| PB290_20 | *R. salina* | 501 | 99 | | | |

Band class 10 (*A. tonsa* associated and *R. salina* associated, as well as free-living in the *R. salina* culture-water) could be dedicated to a sequence of *Polaribacter* sp. (Bacteroidetes; Tab. 2). This sequence has already been found in Arctic sea water (FJ196065) and associated with adult North Sea copepods (Brandt et al. in prep.).

Band class 29 (*R. sativa*, *A. tonsa*, *P. pileus* and *P. pileus* culture-water) was assigned to an uncultured Alphaproteobacterium formerly found attached to aquatic angiosperms (Crump and Koch 2008).

Band class 21 was the only in common for all three investigated organisms simultaneously and band class 11 for the culture-water samples of the three organisms. Without distinguishing free-living and organism-associated bacterial community, the communities shared nine band classes (band classes 8, 14, 19, 22, 23, 24, 25, 26, 29) (Fig. 4).

## DISCUSSION

In this study we investigated the different bacterial community fingerprints of three marine organisms, *R. salina*, *A. tonsa* and *P. pileus* during a tri-trophic feeding arrangement with both nutrient replete and depleted batches in regard of the transfer of bacteria across trophic levels. For investigation whether nutrient limitation has any effect of the composition of the bacterial communities we did this with both P-limited and replete batches due to the fact that P-limitation in marine waters is a common feature (Schoo et al. 2010). We found no differences between the nutrient-replete or phosphorus-depleted treatments with our experimental settings. Although it could be supposed because the seasonal availability of nutrients such as phosphorus has strong direct effects on phytoplankton and zooplankton growth as well as on composition (Pinhassi et al. 2006; Schoo et al. 2010). Pinhassi et al. (2004) suggested that the qualitative and quantitative differences in phytoplankton community composition were important for structuring the composition of the bacterial assemblages, possibly due to differences in the stoichiometry of organic matter produced by different algae (Pinhassi et al. 2006). Many studies have shown that bacteria are superior competitors for phosphorus than algae (Danger et al. 2007a). Moreover, the more the algae are stressed by a lack of nutrients the more they release carbon rich compounds, such as DOC in order to attempt to re-balance

their carbon-nutrient composition (Danger et al. 2007a; Danger et al. 2007b) in order to attempt to re-balance their carbon to nutrient composition. Nutrient deficiencies place severe limits on vital metabolic activities directly linked to organismal fitness (Frost et al. 2008). The immune system of the organism can also thus be weakened. It could be conceivable that in P-depleted batches of all three organisms more bacterial phylotypes are associated with the 'hosts'. Our results repudiate this.

We found that *R. salina*, *A. tonsa* and *P. pileus* each had their distinct associated bacterial communities which were also different from those of the surrounding culture-water, although some bacterial phylotypes were shared. Furthermore, the free-living bacterial community of each culture-water sample was different.

Only one bacterial phylotype was associated with all three investigated organisms, while one free-living phylotype was common to the culture-water samples of the different organisms. However, the algae, the copepod and the jellyfish had nine different bacterial phylotypes in common, which occurred either associated to or free-living in the culture-water of the organisms. Thus, regarding the question whether some bacteria were transferred across the food chain these phylotypes are pivotal. Phylotypes 8, 14, 19, 22, 23, 24, 25, 26 and 29 are associated with the organisms and free-living. It seems that these bacteria are purely opportunistic. Phylotype 11 (which occurred in all culture-water samples) could represent a bacterium which is coincidently transferred with the algae via the copepods to the jellyfish cultures, whereas phylotype 21 seems to be either primarily associated with all of the three organisms or is transferred via feeding activity.

It is also possible that some bacteria are only transferred across one trophic level. So some bacteria may be only transferred from the cryptophyte to the copepod nauplius, or from the nauplius to the ctenophore. There are some indications that this may be occurring: Phylotype 29 was found attached to *R. salina*, occurred associated with *A. tonsa* and was discovered in both the associated and free-living parts of the *P. pileus* culture. Phylotype 17 was exclusively found associated with the two animals, whereas phylotype 26 was associated with the animals and occurred attached and free-living in the *R. salina* cultures.

Due to the fact that we did not discriminate between internal and external bacteria (for *A. tonsa* and *P. Pileus* analyses were carried out on tissue homogenate of whole animals) we cannot make a statement about whether the bacteria detected were ingested by or only attached to the animals. Both can, however, be connected with feeding activity.

Recently Tang et al. (2009) found with a two-trophic feeding experiment based on different algae-species and the adult copepod *Acartia tonsa*, using DGGE, that bacteria were transferred from algae to the copepod via feeding. They used axenic alga first and found no transfer; when using xenic algae, however, which were already populated by a natural bacterial community, there was a transfer of the bacteria from the algae to the copepod. We used the laboratory-reared alga *R. salina* which is a long-term 'labrat'. Therefore, the bacterial community attached to these cells is definitely not the naturally occurring one. There was enough time for selection processes. Additionally, the copepods were from a culture which had been reared in the laboratory for years. In the long time of laboratory rearing only bacteria which are really well adapted to the particular host and had outcompeted the rest would be likely to remain attached. So it seems likely that most of these Methuselah-bacteria did not have the ability to establish on a new host anymore.

DOM production by sloppy feeding of copepods depends on the relative size of the prey (Møller 2007). When copepods feed on very large prey items they seem to produce large amounts of DOM (Hasegawa et al. 2001; Møller 2007), while no DOM is produced when the prey is small (Møller and Nielsen 2001; Møller 2007). Møller (2007) stated that when fed with the relatively small *R. salina Acartia* sp. and *Temora* sp. produced no DOM by sloppy feeding but only by defecation, thus it is probably that not much DOM is produced during the experiment and so only bacteria which were capable of using chitin could be associated with the copepods, although the copepod nauplii were much smaller than adult copepods. It is not likely that long-term laboratory-reared *R. salina* has attached bacteria that have this ability.

Jellyfish may also stimulate bacterial growth directly through release of nutrients and DOM (Schneider 1989). Conversely, Titelmann et al. (2006) stated that it could be that bacteria were inhibited by jellyfish. Not much is known of the natural occurring bacterial assemblages of the ctenophore *P. pileus*. Thus we

can not state whether the detected bacteria belonged to the natural bacterial community of this ctenophore or whether they were transferred via feeding.
After sequencing of some prominent DGGE bands we found Alpha- and Gammaproteobacteria as well as Bacteroidetes. Tang et al. (2009) also found these three bacterial classes in their feeding experiment associated with different algae and *A. tonsa*. Many primary surface colonisers belong to the class of Alphaproteobacteria such as the family of Rhodobacterales (Jones et al. 2007; Dang et al. 2008; Porsby et al. 2008), which have already been found associated with different algae and copepods (Wagner-Döbler and Biebl 2006; Møller et al. 2007; Tang et al. 2009; Brandt et al. in prep.). Additionally, Gammaproteobacteria and Bacteriodetes are frequently found attached to marine organism and are thought to have chtitin-digesting properties, among others (e.g. Kaneko and Colwell 1975; Huq et al. 1983; Tamplin et al. 1990; DeLong et al. 1993; Cottrell and Kirchman 2000; Riemann et al. 2000; Heidelberg et al. 2002; Riemann et al. 2006).
For this experiment we used copepod nauplii. Copepods shed their carapace after each life stage, so they presumably lose their bacterial epibionts after each life stage and bacteria have to resettle them anew. Copepods also need to empty their gut when they moult because water enters the gut in order to expand the new exoskeleton (Clarke 1973 cited by Makino and Ban 2003), and possibly even shed also their gut membrane with all interior bacteria in that process. Hence, shortly after moulting very few bacteria will be found associated with a copepod. This explains why we had only a very small bacterial community associated with the nauplii. For adult copepods it could have been more likely to find bacteria which travel across the trophic levels.

## CONCLUSION

With regard to the bacterial communities associated with the primary producer *R. salina*, the first and secondary consumers *A. tonsa* and *P. pileus* we can conclude that in our tri-trophic feeding experiment all three organisms had distinct associated bacterial communities. Additionally, the particular free-living bacterial communities of the culture-water were different from each other and distinct from the organism-attached bacterial communities. Some bacterial phylotypes (band classes) were exclusively associated with one of the

investigated organisms and some with two or even three. We can only assume that these bacterial phylotypes were transferred across trophic levels due to our chosen settings with long-term laboratory reared *R. salina* and *A. tonsa*. Nevertheless, this feeding experiment over three trophic levels gives some indications that some bacteria can travel across the trophic levels by feeding activities.

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# CHAPTER V

# GENERAL DISCUSSION

# GENERAL DISCUSSION

This thesis aimed to provide detailed insights in consortia of copepods and associated bacteria. The most important findings from this thesis can be summarised as follows:

**Chapter II**: Comparison of different DNA-extraction techniques
An optimised method for investigation of the bacterial community of marine copepods on the basis of PCR and further analysis e.g. with DGGE was developed and applied to laboratory-reared as well as field-grown copepods. The bacterial communities of laboratory reared and free-living copepods were different. Bacteria seemed to be associated with copepods externally as well as internally.

**Chapter III**: The microbiome of North Sea copepods
Different copepod genera did not harbour different bacterial communities. A copepod-specific bacterial community was not identified but there seemed to exist some copepod-specific phylotypes. No seasonal changes of the bacterial community were detected. The copepod-associated and free-living bacterial community of the water column, however, differ from each other. Mainly four bacterial phyla were found associated with the field-grown copepods, Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. On genus level *Roseovarius* sp., *Sulfitobacter* sp., *Psychrobacter* sp. and *Photobacterium* sp. were the most abundant bacterial genera.

**Chapter IV**: Transfer of bacteria through trophic levels?
The bacterial communities of the long-term laboratory-reared cryptophyte *Rhodomonas salina* and the calanoid copepod nauplii (stage V) of laboratory-reared *Acartia tonsa* as well as the field-grown ctenophore *Pleurobrachia pileus* are clearly different. There is an indication that some bacterial phylotypes travel across trophic levels.

On the following pages these findings will be discussed in a broader context.

# METHODOLOGICAL APPROACHES

## MOLECULAR TECHNIQUES

Bacteria located on the copepod exterior, gut, faecal pellets and carcasses have been investigated with different methods in a number of previous studies (Harding 1973; Sochard et al. 1979; Nagasawa et al. 1985; Nagasawa and Nemoto 1988; Nagasawa 1992; Carman 1994; Delille and Razouls 1994; Kirchner 1995; Hansen and Bech 1996; Carman and Dobbs 1997; Tang 2005; Tang et al. 2006a; Tang et al. 2006b; Møller et al. 2007; Tang et al. 2009b) with culture-dependent and -independent techniques such as electron microscopy or molecular approaches.

Studies using culture-independent techniques like those by Tang and co-workers and Møller and co-workers (Tang et al. 2006a; Tang et al. 2006b; Møller et al. 2007) showed that there was no generally applicable DNA-extraction technique for copepod-bacteria consortia. Furthermore, Møller et al. (2007) reported that they had difficulties in extracting bacterial DNA from these consortia.

Hence, it was necessary to develop a suitable DNA-extraction technique to investigate the bacterial community of marine copepods (**Chapter II**). As already stated by Roose-Amsaleg and co-workers (Roose-Amsaleg et al. 2001), DNA extraction methods must be adapted for each individual sample type or each tissue. The best suited method determined was a modified phenol-chloroform DNA extraction with grinding the copepods as basis for further analysis of the whole bacterial community (internally and externally associated with copepods) by PCR, community fingerprinting and subsequent sequencing.

Community fingerprinting techniques such as randomly amplified polymorphic DNA (RAPD), single-stranded-conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), ribosomal intergenic spacer analysis (RISA), denaturing high performance liquid chromatography (DHPLC) as well as denaturing gel gradient electrophoresis (DGGE) and other molecular methods such as fluorescence *in situ* hybridisation (FISH) and cloning can provide useful insights into complex bacterial communities.

To provide comprehensive analysis in this thesis DGGE and cloning were combined to investigate the microbiome of North Sea copepods (**Chapter III**).

DGGE fingerprinting has been proven to be a powerful tool in a large number of studies on the structure and changes of a bacterial community regarding temporal, spatial or other parameters. This technique is convenient for the simultaneous analysis of numerous samples. It is well suited for the monitoring of whole communities, focusing on phylotypes for which the occurrence and/or the relative frequency is affected by environmental changes (Fromin et al. 2002). Additionally, DGGE offers the chance of subsequent sequencing of bands to identify the bacterial phylotypes. Nevertheless, due to methodological limitations only a relatively small part of the 16S rRNA gene can be analysed by DGGE and used as phylogenetic discriminator. It has already been shown that rDNA fragments of closely related bacteria are not fully resolved. Moreover, non-related sequences can co-migrate, which occurs often during analysis of a very complex bacterial community (Fromin et al. 2002 and references therein). Hence, it is likely that several 'species' are combined in one OTU (Ferrari and Hollibaugh 1999).

Muyzer et al. (1993) and Murray et al. (1996) reported that DGGE is sensitive enough to detect bacteria which make up only 1–2% of bacterial populations in the mixed assemblage of selected bacterial strains. Thus, the DGGE method tends to bias towards the more dominant groups within a community (v. Wintzingerode et al. 1997; Casamayor et al. 2000). Moreover, the detection limit of the DGGE method is not only affected by the relative abundance of a population but also by ribosomal RNA (rrn) operon copy numbers (Cottrell and Kirchman 2000a; Kan et al. 2006).

With DGGE only a few Gammaproteobacteria were found associated with the investigated marine copepods (**Chapter III**). This was also observed by Alónso-Sáez and co-workers (2007) who reported that Gammaproteobacteria were not detected in DGGE analysis. Castle and Kirchman (2004) carried out a comparative study between DGGE and FISH, and also showed that DGGE failed to detect the most abundant phylogenetic group detected by FISH in some samples. Besides primer specificity and other PCR biases (reviewed by v. Wintzingerode et al. 1997), these authors argued that high richness within groups could lead to an underestimation as compared with FISH, because different sequences would appear as different faint bands, which could be difficult to excise from the gels for sequencing. This could also be the fact with

our samples since not all emerged bands could be successfully cut, reamplified and sequenced. Nonetheless, substantial information about the species composition can be obtained from complex microbial communities by DGGE analysis (Muyzer and Smalla 1998).

The method of cloning has its weaknesses, too. In this study, Bacteroidetes, an important group of the coastal marine bacterial community (Eilers et al. 2001), were found by DGGE-band-sequencing as well as in the clone libraries (**Chapter III**). Cottrell and Kirchman (2000a) stated that Alphaproteobacteria are generally overestimated and Bacteroidetes are underestimated in clone libraries. Hence, care must be taken when deducing the structure of a bacterial community from the use of just one analysis method. Thus, in this study, the combination of the two molecular techniques provided further insights into the bacterial assemblages of the investigated marine copepod genera, that could not have been gained from the use of one method alone. Some bacterial families and genera were only found by DGGE and some only by cloning. More Alphaproteobacteria were found by DGGE and only a few Gammaproteobacteria, whereas the cloning approach led to the assumption that Gammaproteobacteria were the predominant bacterial class. However, currently it cannot be judged whether DGGE or clone libraries represent the real *in situ* community composition.

## DIFFERENT NICHES

The phrase “everything is everywhere, but, the environment selects” (Baas Becking 1934) could mean in our context that, in dependence of further factors governing a specific marine environment (such as temperature, salinity), marine copepods offer a niche which can be realised by different functionally redundant bacteria.

Copepods themselves are functionally separated into different niches, since the body surface or the intestine represent different environments for different bacterial lifestyles (oxix/anoxic). Ideally, all these presumably different niches of a copepod should be investigated separately. For large polar copepods, such as *Neocalanus cristatus*, this is applicable (Dagg 1993) because of the big size of approximately 7 mm (Tsuda et al. 2001). North Sea copepods, however, are generally far smaller. Trials to dissect the gut without contaminating the content,

using *Acartia* sp. as most abundant genus in the North Sea, failed in this study, although other investigators such as Sochard et al. (1979) reported that they were successful in their study. Most other studies (see Tang et al. 2009a) relinquished dissecting the gut and investigated the whole copepod.

In this study a different way was attempted to distinguish between the exterior and interior of marine copepods (**Chapter II**). Copepod bacterial communities were investigated with and without grinding the copepod. This simple technical approach resulted in two different community fingerprints. Some bands only appeared in the DGGE banding pattern of ground copepods and therefore might represent intestinal bacteria.

This finding supports the results of Sochard and co-workers as well as Nagasawa and Nemoto (Sochard et al. 1979; Nagasawa and Nemoto 1988) who stated that bacteria are associated with the gut of copepods. In contrast, other authors like Gowing and Wishner (1986) did not find any bacteria associated with the copepods' gut.

Due to the fact that in the study of **Chapter II** copepods with empty guts were investigated, the outcome that there are additional bacteria when using ground copepods suggests that these bacteria could be enterics and not only transients. Enteric means in this context that these bacteria were attached to the gut lining whereas transient bacteria were ingested with food and excreted with faecal pellets.

## ASSOCIATED BACTERIA

### SPECIFICITY

In **Chapter III** different genera of highly abundant (*Acartia sp., Temora longicornis, Centropages sp*.) and rather rare North Sea copepods (*Calanus helgolandicus, Pseudo-/Paracalanus sp., Euterpina sp., Candacia sp*.) were investigated regarding their bacterial community structure using DGGE and cloning.

According to the DGGE banding patterns, it was obvious that no differences in the bacterial community of distinct copepods could be observed. This finding was surprising because the different copepod genera have different feeding behaviours and can also feed on different size classes of phyto- and zooplankton not only because of their differences in size. Most investigated

genera were herbivorous to omnivorous (*Acartia* sp., *Temora longicornis, Centropages* sp., *Pseudo-/Paracalanus* sp.), whereas *Euterpina* sp. represented a detrivorous to omnivorous genus (Greve et al. 2004). Moreover, the origin of the different copepod genera is different. *Candacia* sp., which was also investigated when this genus occurred at Helgoland Roads, is caught only sporadically in the Southern North Sea (Krause et al. 1995). *Candacia* sp. enters the North Sea mainly with the Atlantic inflow between the Shetlands and the Norwegian trench (Krause et al. 1995).

Due to vast seasonal differences in several abiotic and biotic factors (such as water temperature, nutrients, phytoplankton) at Helgoland Roads a clear seasonality in the composition of the bacterioplankton has previously been reported (Eilers et al. 2001; Gerdts et al. 2004). Sapp et al. (2007b) also showed that the bacterial community of the phycosphere displays seasonality at Helgoland Roads. Surprisingly, these results were not reflected in the bacterial community of the investigated copepods.

Crump et al. (2003) demonstrated that shifts in bacterioplankton were related to seasonal cycles in the source and lability of DOM. Similarly, succession in marine bacterioplankton assemblages occurred in response to seasonal shifts in water column stability and water temperature, suggesting that bacterioplankton community composition demonstrates an annual pattern of variability (Murray et al. 1998; Gerdts et al. 2004). Other studies have documented relationships between bacterioplankton community composition and seasonal dynamics of other members of the aquatic food web (Höfle et al. 1999; Fandino et al. 2001; Hahn and Höfle 2001; Arrieta and Herndl 2002). During phytoplankton blooms, large changes in numbers and phylogenetic shifts of the bacterial assemblage have been observed (Fandino et al. 2001; Yager et al. 2001; Sapp et al. 2007a). The main determinant of bacterial composition was the change in DOM supply mediated by different algal populations and the different temperature optima of bacterial populations (Mary et al. 2006).

However, for the bacterial community of marine copepods no significant dependency was observed on either biotic (different algae species) or abiotic (water temperature, salinity, silicate, phosphate, nitrogen, ammonium) factors, whereas bacteria in the pelagic show seasonality which is apparently connected

to the nutrient availability and temperature. A copepod, however, seems to serve as a 'nutrient station' throughout the year.

Heidelberg et al. (2002) detected seasonality in the abundance of bacteria associated with zooplankton in Chesapeake Bay. A larger proportion was found associated with zooplankton during the cooler months of the year. In the study of **Chapter III** a slight trend in the abundance of band classes could be detected, with increasing band class numbers from March to May and decreasing until September, resulting in fewer band classes in the winter months than in the others. This contradicts the findings in the Chesapeake Bay.

## THE MICROBIOME

In the study of **Chapter III** four bacterial phyla associated with the investigated copepods were detected; Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (in order of abundance). The most abundant bacterial classes (results of DGGE band sequencing and cloning pooled) were the Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Flavobacteria. The most abundant orders and families were Rhodobacterales, Pseudomonadales, Vibrionales and Rhodobacteraceae, Moraxellaceae, Vibrionaceae, respectively. *Roseovarius* spp., *Sulfitobacter* spp., *Psychrobacter* spp. and *Photobacterium* spp. were the most abundant bacterial genera, while *Vibrio* spp., *Loktanella* spp., *Polaribacter* spp., *Pseudoalteromonas* spp., *Delftia* spp., *Ulvibacter* spp. and *Sphingopyxis* spp. were also abundant (for details and less abundant bacterial classes, orders, families and genera see **Chapter III**).

Bacteria associated with marine copepods have been investigated in a number of previous studies at different sites, time points, with a vast variety of methods and different emphases such as the human health aspect (like the copepod-*Vibrio* association f.i. Huq et al. 1983; Huq et al. 1984; Dumontet et al. 1996). A multitude of studies only reported on associated bacteria but did not define them: Nagasawa (1988) *Cyclops abyssorum tatricus*; Nagasawa and Nemoto (1988) *Eucalanus bungii;* Nagasawa (1987) *Acartia* spp.; Nagasawa et al. (1987) *Acartia* spp., *Calanus cristatus*; Harding (1973) *Calanus finmarchicus*; Tang (2005) *Acartia tonsa*; Carman and Dobbs (1997) *Labidocera sp. Coullana sp. Pseudostenhelia wellsi*; Carman (1990) *Robertsonia* sp., *Zausodes arenicolus, Zausodes arenicolu*.

Other studies defined bacteria associated with copepods, such as Sochard et al. (1979). They found *Vibrio* spp., *Pseudomonas* spp. and *Cytophaga/ Flavobacteria* spp. (Bacteroidetes) associated with the surface and gut of different copepod species (*Acartia. tonsa, Pontellopsis regalis, Pleuromamma sp., Labidocera aestiva, Centropages furcatus*) from the Gulf of Mexico using culture techniques. Hansen and Bech (1996) found *Vibrio* spp., *Alteromonas* spp., *Photobacterium* spp, *Aeromonas* spp. and *Corynebacterium* spp. associated with the gut of *Acartia tonsa* and the same bacteria except for *Corynebacterium* spp. attached to the surface. Møller et al. (2007) found exclusively Rhobacteraceae associated with *Calanus helgolandicus* using DGGE. Recently, Tang et al. (2009a) found *Sulfitobacter* spp., *Roseobacter* spp., *Pseudoalteromonas* spp., *Vibrio* spp., *Yersinia* spp. and *Flavobacterium* spp. associated with *Acartia tonsa* using the same technique.

These four studies encompassing a period of 30 years showed only a small subset of the bacteria which were associated with copepods. Except for the Gammaproteobacteria *Yersinia* spp. all other bacterial genera were also found in the study of **Chapter III**, in part belonging to the abundant genera such as *Roseobacter* spp., *Sulfitobacter* spp., *Vibrio* spp., *Photobacterium* spp. and *Pseudoalteromonas* spp.

We did not find any *Vibrio cholerae* associated with the investigated copepods although a tight coupling between these bacteria and copepods was found for example in Chesapeake Bay and Bangladesh (Colwell et al. 1981; Huq et al. 1983; Huq and Colwell 1996; Colwell 2000; Colwell 2005; Huq et al. 2005). Although there have been cholera outbreaks in the Mediterranean Montanari et al (1999) did not find *Vibrio cholerae* associated with Mediterranean copepods by direct fluorescent-monoclonal antibody (DFA) staining. Whereas Gugliandolo et al. (2008) recovered *Vibrio cholerae* by PCR as adhering to copepods in August and October in the Straits of Messina (Italy).

However, we found some other human and aquatic animal pathogens in association with the investigated copepods (**Chapter III**) such as *Vibrio splendidus, Vibrio alginolyticus*. *Vibrio alginolyticus* cause soft tissue infections and is listed as human pathogen, whereas *Vibrio splendidus* is known to be a fish pathogen (Farmer III and Hickman-Brenner 2006). *Aliivibrio* spp. were found in the marine environment, often associated with animals; some species

are mutualistic symbionts or pathogens of marine animals (Urbanczyk et al. 2007). Some of these species have been associated with mortality in a wide range of marine animals, such as molluscs, fish, shrimps, and octopus (Beaz-Hidalgo et al. 2009).

All the bacteria found associated with adult copepods (**Chapter III**) can possess capabilities which are very specific but also manifold, due to the fact that the living copepod deliver different niches on the surface and the gut.

Inside the gut some bacteria might aid food digestion or deliver nutrients that the copepod cannot liberate, other might deduct nutrients from the food or only use resources that the copepod does not need. The gut bacteria could be transients or permanents, either ingested with the food and egested with faecal pellets or attached to the gut lining.

Being associated with a copepod surface offers several potential benefits to the bacteria involved: They can use the DOM or DMSP from sloppy feeding or defecation (Møller and Nielsen 2001; Tang et al. 2001; Møller et al. 2003; Møller 2007) of the copepods (maybe preferably attached near the mouth, body appendages and near the anus) or the methane which can be produced by methanogenic bacteria inside the gut (Oremland 1979; de Angelis and Lee 1994) (presumably preferably attached to gut, near the anus) or they can digest the chitinous carapace of the copepod itself (Nagasawa 1987).

The bacteria which were found in the study of **Chapter III** can in part be allocated to these niches. Typical chitin digesting bacteria were identified among the Gammaproteobacteria and Bacteriodetes. Bacteroidetes, such as Flavobacteriaceae, are known to be chemoorganotrophic particle colonisers and particularly proficient in degrading various polymers such as cellulose, chitin and pectin, but also high molecular DOM (DeLong et al. 1993; Cottrell and Kirchman 2000b; Riemann et al. 2000; Kirchman 2002; Riemann et al. 2006). Additionally, some Gammaproteobacteria and Alphaproteobacteria, such as Vibrionaceae, Pseudoalteromonadaceae and some Sphingomonadaceae, are known to express chitinases for usage of chitin as carbon source (Nagasawa 1987; Bassler et al. 1991; Carman and Dobbs 1997; Zhu et al. 2007).

Methylotrophic Rhizobiales, Methylobacteriaceae, were also found in that study (**Chapter III**). Members of the *Roseobacter*-lineage play an important role in the

global carbon and sulphur cycles, amongst others, and are able to produce dimethylsulfide (DMS) (Wagner-Döbler and Biebl 2006).

Besides being abundant, ubiquitous, and frequently surface associated (Wagner-Döbler and Biebl 2006), the Roseobacter clade bacteria are physiologically active as well. They are the fastest utilisers of nutrient enrichments in coastal environments (Alonso-Sáez and Gasol 2007; Dang et al. 2008).

The Rhodobacterales group bacteria have been found to be rapid surface colonisers (Dang and Lovell 2000; Jones et al. 2007; Dang et al. 2008). The genus *Roseobacter* especially, which is closely related to the genus *Sulfitobacter*, is known for rapidly colonising surfaces (Berkenheger and Fischer 2004). Members of the Gammaproteobacteria also seem to be involved in the very early stages of colonisation of surfaces (Berkenheger and Fischer 2004).

Adult copepods do not moult again (Carman and Dobbs 1997). Until adulthood, however, copepods shed their carapace after each naupliar and copepodite stage. In this phase of life the bacterial community is disturbed by each shedding of the carapace and has to re-colonise the nauplius or copepodite and last the young recently moulted adult copepod. These different life stages could be the reason why so many primary surface colonisers were present associated with copepods when their terminal moult was endured.

## RECRUITMENT

At first sight bacterial communities associated with marine living surfaces seem broadly similar at phylum/class level to those found in the surrounding water, but they often appear quite different at genus or species level (Longford et al. 2007). A number of earlier studies on bacterioplankton and particle-associated bacterial communities such as DeLong et al. (1993) found that free-living and attached bacteria showed no identical rDNA types.

Eilers and co-workers (2001) identified Alpha- (*Roseobacter spp.*) and Gammproteobacteria (*Pseudoalteromonas spp., Alteromonas spp., Colwellia spp., Photobacterium spp.*), and members of the CF cluster (*Cytophaga spp., Polaribacter spp., Flavobacterium spp.*) in the water column of the North Sea near Helgoland. This seems to be compatible with the results in **Chapter III**. On a higher taxonomic level, however, differences become apparent. The

phylogenetic trees (**Chapter III**) illustrate that most of the copepod associated phylotypes did not cluster with the phylotypes of free-living bacteria but with particle/surface attached, or biofim bacteria. Interestingly, no exclusive cluster of copepod-associated bacteria was detected, although clusters emerged which included sequences from this study and those from Møller et al. (2007).

The observation that the copepods' body and the surrounding water share bacterial classes, but in different proportions, suggests an active exchange of bacteria. The different environments, however, tend to select for different bacterial groups (Sochard et al. 1979; Delille and Razouls 1994).

The copepod food could also be a source of bacteria. In the natural environment, food particles are quickly colonised by bacteria (see also Simon et al. 2002). As a consequence, copepod feeding would lead to an uptake of bacteria (Tang et al. 2009a). Whether these bacteria are digested or establish intestinal populations is still an open question since the gut transit time of copepods can vary between 166 and 24 min (Tirelli and Mayzaud 2005).

Conceptually, some researchers distinguish between transient and resident gut bacteria (Harris 1993). The former are regarded as the ones that were ingested but either digested or released through defecation; the latter are considered as the ones that permanently resident inside the gut (Tang et al. 2009a).

Concerning the bacterial community of copepod faecal pellets, the question remains as to whether the community reflects that of the copepod intestine (Gowing and Silver 1983) or indicates colonising by pelagic populations (Jacobsen and Azam 1984). There are several reasons why Lawrence et al. (1993) think that the bacteria they found in the faecal pellets were not enterics: Earlier studies did not find an enteric flora in *Calanus pacificus* (Honjo and Roman 1978; Gowing and Silver 1983; Jacobsen and Azam 1984) and Nott et al. (1985) suggested that *Calanus helgolandicus* did not harbour an enteric flora since the epithelium of the gut wall contributes the peritrophic membrane to the faecal pellet and since no bacteria were found in the empty guts of non-feeding copepods. That is why Lawrence et al (1993) stated that the bacteria egested into the faecal pellets were derived from the food.

In **Chapter IV** the transfer of bacteria across three trophic levels, from *Rhodomonas salina* via *Acartia tonsa* (naupliar stage V) to *Pleurobrachia pleus* was investigated in a tri-trophic feeding experiment using DGGE. It was obvious

that all three investigated organisms were colonised by different bacterial communities and only two bacterial phylotypes (band classes) were found associated with all three organisms and two and three were shared by *Rhodomonas salina* and *Acartia tonsa* and *Acartia tonsa* and *Pleurobrachia pileus*, respectively. These bacteria could have been transferred across the trophic levels, but this is not the only option. They could also already have been associated with these organisms initially.

Tang et al. (2009a) investigated the transfer of bacteria from different algal species to the adult copepod *Acartia tonsa* and found that *Pseudoalteromonas spp.*, *Sulfitobacter* spp. and *Roseobacter spp.* were absent in the initial samples, but were frequently present after the addition of xenic algae. The authors stated that these bacterial genera were delivered via food intake and were able to quickly establish a prominent presence associated with the copepod.

Tang et al. (2009a) stated that the composition of the bacterial community of *Acartia tonsa* depends on several factors: i) life history of the copepods; ii) the source of bacteria, and iii) the food that the copepods consumed. They hypothesised that the same copepod species would establish specific bacterial communities depending on the environmental conditions and food they were previously exposed to. Conversely, copepod species of different feeding habits would acquire different bacterial communities via food intake, even if they share the same environment (Tang et al. 2009a). Spatial and temporal variations in the food environment would therefore mediate changes in the copepod-bacteria interactions (Tang et al. 2009a). However, this possibility can be discounted using the results of the two years survey, due to the different food which was available during the different seasons, because of the seasonal succession pattern of different microalgae, but the bacterial community of the copepods did not reflect these seasonal changes (**Chapter III**).

Conversely, Tang et al. (2009a) stated that copepod species of different feeding habits would acquire different bacterial communities via food intake, even if they share the same environment. This can be refuted based on our results from different copepod genera with distinct feeding behaviour which we investigated in the study of **Chapter III**.

## CONCLUSION

Considering all results of the investigation of North Sea copepods it can be concluded that marine copepods are associated with bacteria of four different phyla: Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. *Roseovarius* spp., *Sulfitobacter* spp., *Psychrobacter* spp. and *Photobacterium* spp. were the most abundant bacterial genera, while *Vibrio* spp., *Loktanella* spp., *Polaribacter* spp., *Pseudoalteromonas* spp., *Delftia* spp., *Ulvibacter* spp. and *Sphingopyxis* spp. were also abundant. Some human and aquatic animal pathogens were found but no *Vibrio cholerae*. Besides some of the genera which have already been found associated with different copepods in other studies, this study provides a very much deeper insight into the bacterial community of marine copepods. This is the most detailed study so far, using different molecular techniques and different copepod genera in parallel over a period of two years. Nonetheless, no copepod- or even copepod genera-specific bacterial communities could be recognised but there seem to exist copepod-typical bacterial phylotypes. There seem to be no differences among the bacterial communities of different copepod genera. No seasonality of the bacterial assemblages associated with North Sea copepods could be detected. The bacterial community is neither affected by the investigated abiotic nor biotic environmental factors. The bacterial community of the copepods can most presumably be seen as a functional guild realising the chitin-rich 'copepod niche'.

The main resource for the copepod colonising bacteria seems to be the water column although on higher phylogentic levels the communities are different. The most important determinants of bacterial colonisation are presumably the release of nutrients via sloppy feeding, defecation and the chitinous surface itself. A copepod seems to serve as a 'nutrient hot spot' in the pelagic throughout the year.

## OUTLOOK

Copepods play a central role in the marine food web. They contribute to the microbial loop and serve as important food source for organisms of higher trophic levels. After the studies of this thesis there is an overview which bacteria can be associated with marine copepods in the German Bight, North Sea, but it

is only an assumption what their capabilities are and why they are associated with copepods. It is necessary to understand whether and if, what bacteria are important for the survival of the copepods. Are there necessary symbionts which are really needed? Do copepods need the bacteria for digesting the food? Or is the bacterial community a burden for the copepod? Are there bacteria which can cause copepod death so that there is a sharp decline of copepod abundance and organisms of higher trophic levels have to starve? Now we know that there are some aquatic animal pathogens associated with marine copepods. Can these bacteria be transferred to higher trophic levels by feeding on the copepods? The study of chapter IV and that by Tang and co-workers (2009b) give some hints in that this transfer is possible.

As a consequence, in future studies the genetic reservoir of these copepod colonising populations should be examined in detail by transcriptome or proteome analysis. The only focus on identity – concerning 16S units – does not provide sufficient autecological information and is therefore highly speculative.

It would be also helpful to locate the bacteria on different parts of the copepod. Therefore FISH could be used. The procedure to use whole copepods for FISH (which I already tried out during my PhD time) needs a lot of improvement. One option is to look only at parts of the copepod like only one antenna or a swimming leg.

A comparison of the copepod attached bacteria from different sample sites (tropical regions, polar regions, wadden sea) in parallel could be conducted to get to know more about spatial factors.

The question whether there exist an internal bacterial flora inside the copepods' gut could be approached using bigger polar copepods where it is easier to dissect the gut.

An important approach would be to investigate whether there exist differences of the bacterial community during the copepods' life cycle at every life stage directly after and before the moult occurs.

In context to the bacterial community also the other epi- and endobionts living associated with marine copepods, like algae and other protozoa should be investigated.

Altogether, although copepods and their associated bacteria and other epibionts have been investigated for decades until now, the interactions of these organisms are not understood to date.

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# DECLARATION

I herewith declare that this thesis is my own work and effort and has been written independently. Where other sources of information have been used, these have been cited and are shown in the references. Furthermore, I declare that this work has not been submitted to any University for the conferral of a Degree.

Petra Brandt

Helgoland, October 2010