Molecular Biological and Physiological Investigations of Heterotrophic Bacteria Associated with Marine Filamentous Cyanobacteria

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Table of contents

List of abbreviations

AAnP	aerobic anoxygenic phototrophic bacteria
AB	BBL™ Actinomyces Broth
AnAnP	anaerobic anoxygenic phototrophic bacteria
ASN_{III}	artificial seawater nutrient culture medium of half salinity
EPS	exopolysaccharides
FISH	fluorescence *in situ* hybridisation
MB	Difco™ Marine Broth culture medium
ME	meat extract
n.d.	not determined
PEP	phosphoenolpyruvate
PSU	practical salinity units
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
YE	yeast extract

Abstract

Cyanobacteria have long been known to live in coexistence with heterotrophic bacteria. However, to date little is known about the functionality of these associations.

Oscillatoria brevis strain Bo10 and *Nodularia harveyana* strain Bo53, two benthic filamentous cyanobacteria from the Baltic Sea, were chosen for investigation. First, the composition of the heterotrophic community within the cultures of both cyanobacteria was investigated. On this account, 51 heterotrophic strains were isolated and phylogenetically characterised, 30 from the *Nodularia* and 21 from the *Oscillatoria* culture. Both communities were dominated by *Alphaproteobacteria* (10 out of the 24 isolates tested from *Nodularia* and 8 out of 20 from *Oscillatoria*), followed by *Bacteroidetes* bacteria (7/24 and 7/20 respectively) and *Gammaproteobacteria* (3/24 and 3/20 respectively). Four heterotrophic strains were chosen for further investigations: a red (strain Bo53-33), pink (Bo10-20), and colourless one (Bo10-19), grouping with *Porphyrobacter*, *Roseobacter*, and *Rhodobacter* respectively (all *Alphaproteobacteria*), and a yellow-pigmented one (Bo10-09) that grouped with *Muricauda* (*Bacteroidetes*). The *Porphyrobacter* and *Roseobacter* isolates were shown to belong to the group of the so called "aerobic anoxygenic phototrophic bacteria" (AAnP). For further investigations specific fluorescence *in situ* hybridisation probes were designed for the genus *Muricauda* and for the family *Erythrobacteraceae*, comprising the genus *Porphyrobacter*.

The mutual influence of the cyanobacteria and the heterotrophs was investigated with all four heterotrophic bacterial strains. An improved method was developed to prepare axenic cultures of *Nodularia* Bo53 and *Oscillatoria* Bo10, which then were mixed with single pure cultures of the four different heterotrophs to examine the growth behaviour of both partners. A detrimental effect was determined only for *Oscillatoria* with increasing amounts of heterotrophs added, but not for *Nodularia*. The abundances of heterotrophs within the cyanobacterial cultures were found to be self-regulated. It could be shown that in all cases distinct new heterotrophic communities developed in the cyanobacterial cultures in the course of the experiment. The occurrence of *Porphyrobacter*- and *Roseobacter*-related bacteria as well as *Muricauda* was studies with ten further cyanobacterial cultures from the culture collection of the department "Marine Mikrobiolgie". A relation between heterotrophic occurrence and cyanobacterial origin, morphology, or diazotrophy could not be observed for

any of the groups tested, with the exception that *Muricauda* could not be found on unicellular cyanobacteria.

Additionally, the heterotrophs were characterised concerning their morphological, biochemical, and physiological properties with respect to their possible function for such connections. Three of the heterotrophs were shown to be able to live on cyanobacterial exopolysaccharides. This behaviour could not be demonstrated for *Rhodobacter*. Based on pigment analysis, 14 different carotenoids were determined in the *Porphyrobacter* isolate, five in the *Roseobacter* isolate, and one in the *Muricauda* isolate. *Porphyrobacter* and *Roseobacter* possessed bacteriochlorophyll *a* as well. The results obtained for *Rhodobacter* suggest that this strain might comprise a new species.

Zusammenfassung

Koexistenzen zwischen Cyanobakterien und heterotrophen Bakterien sind seit langem bekannt. Trotzdem weiß man bis heute relativ wenig über die Zusammenhänge innerhalb dieser Gemeinschaften.

Für die Untersuchungen in der vorliegende Arbeit wurden die zwei aus der Ostsee stammenden benthischen filamentösen Cyanobakterien *Oscillatoria brevis* Stamm Bo10 und *Nodularia harveyana* Stamm Bo53 ausgewählt. Zuerst wurde die Zusammensetzung der heterotrophen Gemeinschaft in beiden Kulturen untersucht. Dabei wurden 51 heterotrophe Stämme isoliert und phylogenetisch charakterisiert, 30 davon aus der *Nodularia*-Kultur und 21 von *Oscillatoria*. Beide Gemeinschaften wurden von Alphaproteobakterien dominiert (10 von den 24 untersuchten Isolaten von *Nodularia* und 8 von 20 im Fall von *Oscillatoria*), gefolgt von *Bacteroidetes*-Bakterien (jeweils 7/24 und 7/20) und Gammaproteobakterien (jeweils 3/24 und 3/20). Vier Isolate wurden für weitere Untersuchungen ausgewählt: jeweils ein rotes (Bo53-33), ein rosa-farbiges (Bo10-20) und ein farbloses (Bo10-19), welche in die Genera *Porphyrobacter*, *Roseobacter* und *Rhodobacter* (alle drei *Alphaproteobacteria*) eingeordnet wurden, sowie ein gelbes (Bo10-09), welches dem Genus *Muricauda* (*Bacteroidetes*) zugeordnet wurde. Für das *Porphyrobacter*- und *Roseobacter*-Isolat konnte jeweils die Zugehörigkeit zur Gruppe der "aerob anoxygenen phototrophen Bakterien" (AAnP) nachgewiesen werden. Für die weiteren Untersuchungen wurden spezifische Fluoreszenz-*in situ*-Hybridisierungs-Sonden für den Genus *Muricauda* und für die Familie *Erythrobacteraceae*, welche auch den Genus *Porphyrobacter* einschließt, entwickelt.

Mit allen vier heterotrophen Isolaten wurden die gegenseitigen Beeinflussungen von Cyanobakterien und Heterotrophen untersucht. Es wurde eine verbesserte Methode entwickelt, um axenische Cyanobakterienkulturen herzustellen. Diese wurden dann einzeln mit den Reinkulturen der Heterotrophen versetzt. In den Ansätzen wurde das Wachstumsverhalten beider Partner analysiert. Es konnte gezeigt werden, dass die Heterotrophen einen schädlichen Einfluss auf das Wachstum von *Oscillatoria* hatten, nicht aber auf *Nodularia*. Ferner wurde festgestellt, dass sich die Abundanzen der Heterotrophen in den Cyanobakterienkulturen selbst regulierten. Im Laufe des Experiments entwickelten sich neue heterotrophe Gemeinschaften in den cyanobakteriellen Kulturen. Zehn weitere Cyanobakterienkulturen aus der Stammsammlung der Abteilung Marine Mikrobiologie wurden auf das Vorkommen von *Porphyrobacter*, *Roseobacter* und *Muricauda* hin

untersucht. Allerdings konnten keine Zusammenhänge zwischen dem Vorkommen von Heterotrophen und der Herkunft der Cyanobakterien, sowie deren Morphologie oder ihrer Diazotrophie beobachtet werden. Eine Ausnahme bildete *Muricauda*. Dieser Organismus kam in keiner der untersuchten unizellulären Cyanobakterienkulturen vor.

Zusätzlich wurden die Heterotrophen auf verschiedene morphologische, biochemische und physiologische Eigenschaften hin untersucht, um weitere Rückschlüsse zu erhalten, die auf eine Interaktion zwischen Cyanobakterien und Heterotrophen hindeuten könnten. Für drei der Heterotrophen konnte gezeigt werden, dass sie in der Lage sind, von den cyanobakteriellen Exopolysacchariden zu leben. Lediglich für *Rhodobacter* konnte ein derartiges Verhalten nicht nachgewiesen werden. Mittels Pigmentanalyse wurden in *Porphyrobacter* Bo53-33 14 verschiedene Carotinoide gefunden, in dem *Roseobacter* Bo10-20 fünf verschiedene und eines in dem *Muricauda* Bo10-09. In den *Porphyrobacter*- und dem *Roseobacter*-Isolaten wurde zusätzlich noch Bacteriochlorophyll *a* nachgewiesen. Die Untersuchungsergebnisse von *Rhodobacter*, deuten darauf hin, dass es sich bei diesem Stamm um eine neue Spezies handelt.

1 Introduction

1.1 The Baltic Sea

The Baltic Sea, located in Northern Europe, is the world's largest brackish water environment. It developed as a huge fresh-water lake about 14,000 years ago when the glaciers of the last glacial period melted. During its history several salt and fresh water phases alternated, before it became brackish (Bursa 1968; Schiewer 2008). The water temperature increased from early to modern times as well (von Storch and Omstedt 2008).

Nowadays, the Baltic Sea is a shallow semi-enclosed intra-continental shelf area made up of a series of large basins comprising a "microtidal" system. The average daily tidal range is 15 cm, but seiches, caused by air pressure variations or influence of the wind, can lead to changes in sea level of up to 4 m (Schiewer 2008). The Baltic surface salinity ranges between 6 - 8 practical salinity units (PSU; corresponding to 6-8 ‰) in the central Baltic Sea Proper and 2 - 3 PSU in the northernmost parts (Larsson et al. 2001; Wasmund and Uhlig 2003). The deep-water (below 60 m) salinity is higher and can increase to 10 – 13 PSU in the Baltic Proper (Schiewer 2008; Stal et al. 2003). Due to this permanent isohaline, the lower-salt surface water and the saltier bottom water are constantly separated. This cuts the deeper basins off from the supply of atmospheric oxygen and can lead to anoxic regions below a depth of 130 m (Schiewer 2008). In summer, the surface layer heats up, leading to a thermally stratified water body (Stal et al. 2003). New fresh water coming from rivers and less salty lakes is then kept at the surface by the low salinity and the thermal stratification (Schiewer 2008). Due to its huge north-south extension of more than 1,200 km, the Baltic Sea exhibits a strong temperature gradient from the north to the south (Schiewer 2008). The average temperature in the upmost layer in summer typically ranges from 15 to 18 °C. At exceptionally calm and warm weather conditions, an additional thin warm surface layer with temperatures of up to 22 °C can develop (Stoń et al. 2002; Wasmund and Uhlig 2003). By the end of fall, the water temperature decreases to mean temperatures of 3 to 4 °C (Mašín et al. 2006) and during winter, the eastern and northern part is regularly covered with ice (Schiewer 2008). Near Warnemünde at the German Baltic coast, the mean annual temperature is approximately + 8.4 °C.

Due to the fact that all countries in the catchment area are developed industrial states and some of them also possess highly developed agricultural systems, the Baltic Sea is strongly influenced by anthropogenic pollution. In this context, it is mainly nitrogen and phosphorus

that is of interest, but also other industrially produced allochthonous substances occur (Schiewer 2008). Since the exchange of water masses with the North Sea is limited, most of the introduced substances remain trapped in the Baltic Sea and accumulate in the sediments, which has recently led to the endangerment of the coastal biotopes in particular (Schiewer 2008). The nitrogen and phosphorus eutrophication has also resulted in an increased occurrence of cyanobacteria (Kahru et al. 1994; Larsson et al. 1985).

Shallow shore areas are characterised by extensive mixing of the water-sediment interface by tides or wind stress. Resuspension of sediment particles into the water column is followed by periods of sedimentation. Sharp changes in salt content occur, caused by flooding incidents or through river water inputs. But also variations in light intensity or temperature (Schiewer 2008), nutrient as well as oxygen concentrations can emerge from the shallowness of the water at the shores. Microorganisms, found in these regions, are faced with these pronounced fluctuations and need to be able to adapt to them.

The Salzhaff, where the cyanobacteria investigated in this study derived from, is a relatively enclosed water body with an area of approximately 21 km² and a depth of 2.3 to 10 m. Its average temperature in summer is around 20 °C. The opening to the Baltic Sea is about 1.5 km wide and 4 m deep and accounts for an intensive exchange of water. The fresh water inflow and thereby the anthropogenic pollution is low in this area. It is typically well mixed and therefore well supplied with oxygen. Nevertheless, oxygen deficiency periods have been observed as well. Recently, massive local occurrences of cyanobacteria, such as *Spirulina* and *Oscillatoria*, caused by increasing eutrophication have been observed in this area (Schiewer 2008).

All these changing conditions are circumstances that the Baltic organisms had and still have to adapt to. Especially the prokaryotes are sufficiently adaptive organisms showing different strategies to cope with these relatively hostile conditions.

1.2 Bloom-forming and benthic Baltic Sea cyanobacteria

Blooms are defined to be mass occurrences of microalgae (Stal et al. 2003). Cyanobacterial blooms are aggregations of cyanobacteria that mainly occur in eutrophic lakes and seas. The cyanobacteria involved develop in large numbers to form loose, visible aggregates that may cover large areas. Some blooms release substances toxic to fish and other organisms. But even blooms of non-toxic cyanobacteria can cause fish kills by excluding light, necessary for

photosynthesis in the lower water layers and thereby preventing release of oxygen, or by depletion of the oxygen in the cause of their decay (Stal et al. 2003).

Cyanobacteria are ancient organisms (Xiong 2007) which might have occurred in the Baltic Sea from early times (Dippner and Vuorinen 2008) and which survived and adapted to the changing conditions that emerged in former as well as in modern times. But even though, they have occurred from primordial times on, there is evidence that, due to recently increased nutrient contamination and global warming, cyanobacterial blooms have augmented and gained in importance for the whole ecosystem (Dippner and Vuorinen 2008; Wasmund and Uhlig 2003).

Two blooms typically occur in the Baltic Sea annually. The spring bloom (March till May) is generally dominated by dinophytes and/or diatoms under participation of chlorophytes, cryptophytes, euglenophytes, and cyanobacteria. The autumn bloom (September till October), which is much more diverse, is dominated by cyanobacteria accompanied by dinophytes, cryptophytes, and chlorophytes as other main contributors. The cyanobacteria mainly found in these blooms are *Aphanizomenon*, *Merismopedia*, *Gomphosphaeria*, *Microcystis* (Stal et al. 2003; Stoń et al 2002), *Anabaena* (Halinen et al. 2008), and *Synechococcus* (Stal et al. 2003), as well as *Nodularia spumigena* (Kahru et al. 1994; Stal et al. 2003). However, the compositions are known to vary considerably from year to year (Dippner and Vuorinen 2008). The cyanobacterial blooms are not triggered primarily by a surplus of nitrogen, since most of the bloom-forming cyanobacteria are diazotrophs. They are generally limited by phosphorus and iron (Sivonen et al. 2007; Stal et al. 1999). The blooms are typically set off by the picoplanktonic cyanobacteria, which are followed by the filamentous ones (Schiewer 2008), and they generally end in the depletion of inorganic nutrients, especially nitrogen (Stoń et al. 2002; Wasmund and Uhlig 2003).

Since these bloom-forming planktonic cyanobacteria are mainly of public interest, the benthic mat-forming ones remained largely unexplored so far. These benthic mats are defined to be multilayer vertically stratified microbial communities, which are usually dominated by phototrophic bacteria. In most cases, cyanobacteria are the main mat-forming organisms (Stal et al. 1985). These mats represent complex ecosystems enclosing photoautotrophic, photoheterotrophic, chemoautotrophic, and heterotrophic microorganisms (algae and bacteria). However, in comparison to other ecosystems, within mats, the microbial and chemical zonations and thus also the nutrient cycles occur on much smaller scales (Canfield and Des Marais 1993). Halinen and co-workers (2008) and Sivonen and co-workers (2007) presumed the benthic cyanobacteria to be more diverse than planktonic ones, and even though

they do not produce microcystins or nodularins, they seem to contain other potentially harmful cytotoxins (Surakka et al. 2005). But apart from that, their ecological characteristics are not well-known yet.

Nodularia is a diazotrophic heterocystous cyanobacterium. During bloom events, *N. spumigena* forms aggregates as large as 10 cm in diameter (Stal et al. 2003). Due to the possession of gas vesicles and its formation of aggregates, *N. spumigena* together with *Aphanizomenon* floats to the surface during periods of calm weather and can form thick surface accumulations (Walsby et al. 1997), which then can be transported by wind over long distances and thus become widely distributed (Stal et al. 2003). But in the Baltic Sea, the two benthic species *N. sphaerocarpa* and *N. harveyana* occur as well (Lyra et al. 2005; Stal et al. 2003). However, *N. harveyana*, which was used in the present study, does not possess gas vesicles and is normally found in shallow coastal waters, where it forms the microbial mats and only occurs occasionally but in much lower numbers than *N. spumigena* (Stal et al. 2003).

Oscillatoria, the other genus used in the present study, can be found generally to a much lesser extent in the Baltic Sea. Major occurrences of *Oscillatoria* species seem to be more local events as described by Schiewer for the Salzhaff (2008). *Oscillatoria* is a mainly planktonic cyanobacterium that is known to fix nitrogen but does not form heterocysts (Carpenter and Price 1976).

Cyanobacterial growth in general is known to be influenced by nutrient availability, salinity, turbulence, and temperature (Dippner and Vuorinen 2008; Stal et al. 2003). But it is as well strongly influenced by the abundance of various heterotrophic bacteria (Paerl and Fulton 2006). On the other hand, all cyanobacteria produce and excrete also labile organic substances and thus represent nutrient-rich hotspots in the Baltic water which attract heterotrophic bacteria.

1.3 Cyanobacteria and heterotrophic bacteria

Heterotrophic bacteria can be found attached to cyanobacterial trichomes as well as imbedded in the mucopolysaccharide layer surrounding the trichomes (Nausch 1996; Paerl et al. 1989) or unicellular cyanobacteria (Brunberg 1999). It has been shown that the bacterioplankton during blooms is controlled primarily by the availability of labile dissolved organic carbon produced by the phytoplankton (Heinänen et al. 1995). But these partnerships seem to provide advantages for both partners: While the cyanobacteria supply the heterotrophs with organic

substances, the latter ones provide remineralised nutrients (Tuomainen et al. 2006). It is known that the associations can range from the general presence of heterotrophs in the surroundings and in the mucilaginous sheaths of the cyanobacteria to highly specific associations (Paerl and Gallucci 1985). But even though cyanobacterial blooms have intensively been studied within the last decades, the exact role of the cyanobacteria-associated heterotrophs has remained virtually unexplored so far (Tuomainen et al. 2006).

Most cyanobacteria are able to fix atmospheric nitrogen and provide oxygen and organic matter, which might support heterotrophic growth, especially in nutrient deficient environments (Hietanen et al. 2002). Marine waters are generally considered to be nitrogen limited (Capone 2000). Therefore, living on nitrogen-fixing organisms seems to be an advantage for non-nitrogen-fixing heterotrophic bacteria. Larsson and Hagström (1982) showed that in the Baltic Sea a substantial part of the energy necessary of heterotrophic growth derived from phytoplankton exudates, and they suggest the existence of very effective bacterial mechanisms for the use of exudates as substrates for growth. Even a bacterial chemotactic response to the concentration gradient surrounding phytoplankton has been proposed (Larsson and Hagström 1982; Paerl and Gallucci 1985). Various investigations indicate that bacteria living attached to particles show higher activity (measured by thymidine incorporation rates) and higher exoenzyme concentrations than free-living ones (Bidle and Fletcher 1995; Griffith et al. 1994). However, there are also studies contradicting these findings (Hietanen et al. 2002; Nausch 1996).

Bidle and Fletcher (1995) described significant differences between the composition of free-living and particle-associated bacterial communities. Variations among different particle-associated communities were low compared with those among the free-living ones. This indicates that attached associations may comprise very characteristic assemblages of microorganisms which are specifically adapted to growth on particles, for example by their substrate utilisation capabilities or by attachment characteristics (Bidle and Fletcher 1995; Cole 1982). Other studies however lead to the assumption that bacteria living attached to phytoplankton might not necessarily be functionally distinct to free-living ones (Worm et al. 2001).

The heterotrophic community on *Nodularia*, comprising mainly *Alpha-*, *Beta-*, and *Gammaproteobacteria*, as well as *Actinobacteria* (Salomon et al. 2003), has only roughly been investigated so far. Tuomainen and co-workers (2006) additionally found *Bacteroidetes* bacteria and Gram-positives. It has been demonstrated in particular for *N. harveyana* that this cyanobacterium does not produce the toxin nodularin. However, the production of compounds

with antimicrobial features has been detected (Lyra et al. 2005; Pushparaj et al. 1999). This also indicates a very specifically adapted community on this cyanobacterium, since the heterotrophs, particularly beneficial for the cyanobacterium, need to be resistant to these compounds. The heterotrophic community on *Oscillatoria* has not been investigated so far.

1.4 Aerobic anoxygenic phototrophic bacteria

1.4.1 Characterisation and physiology of aerobic anoxygenic phototrophic bacteria

One group of bacteria that is known to live often in association with cyanobacteria are the so called aerobic anoxygenic phototrophic bacteria (AAnP) (Jiao et al. 2007; Waidner and Kirchman 2007).

AAnP were discovered 1979 in the sediments of Tokyo Bay, Japan (Shiba et al. 1979). Even though the oxygenic photosynthesis of plants and cyanobacteria and the bacterial anaerobic anoxygenic photosynthesis had been known for a long time, this was the first time that the third pathway of photosynthesis had been described for AAnP (Karl 2002; Shiba et al. 1979). AAnP seem to be ubiquitous in the euphotic zone of the ocean (Kolber et al. 2000; Kolber et al. 2001) and seem to represent a large fraction of the prokaryotic community, irrespective of the trophic status of the water masses (Lami et al. 2007).

Members of these phototrophic bacteria are obligate aerobes with unusually high concentrations and a great variety of carotenoids, low cellular contents of bacteriochlorophyll *a*, and while containing photosynthetic reaction centres and light harvesting complex I, they often lack light harvesting complex II (Yurkov and Beatty 1998). Like anaerobic anoxygenic phototrophic bacteria (AnAnP), they do not use water as photosynthetic electron donor so that no oxygen is produced (Karl 2002). However, they are not able to use their bacteriochlorophyll for anaerobic photosynthetic growth (Nishimura et al. 1996). AAnP synthesise their bacteriochlorophyll *a* in the presence of oxygen and carry out photosynthesis under oxygenic conditions (Yurkov and Beatty 1998). Bacteriochlorophyll synthesis is inhibited by light (Beatty 2002; Nishimura et al. 1996; Yurkov and Gemerden 1993), but growth rates increase in the light due to its utilisation as additional energy source (Yurkov and Gemerden 1993), indicating that AAnP are well adapted to live in environments with alternating light/dark conditions. Kolber and co-workers (2001) found out that AAnP are able to control the expression of their photosynthetic apparatus. The authors described the AAnP

to be facultative phototrophs, switching to a mostly heterotrophic metabolism under organic-rich conditions, where photosynthesis presumably offers fewer advantages. However, Yurkov and Beatty (1998) characterised these organisms to mainly grow heterotrophically and to use light as an additional source of energy when organic carbon is scarce. In any case, the possibility to live phototrophically and heterotrophically (photoheterotrophy) allows AAnP to operate with significantly lower carbon requirements than obligate heterotrophs (Goericke 2002). When living heterotrophically, most AAnP exhibit chemoorganoheterotrophy (Yurkov and Beatty 1998) or mixotrophy (chemolithoheterotrophy) (Swingley et al. 2007).
AAnP do not possess a Calvin cycle and are unable to feed on inorganic carbon. Nevertheless, under deficiency conditions they are able to fix small amounts of atmospheric carbon in a light-depending manner (Kolber et al. 2001; Yurkov 2006), with the help of pyruvate-orthophosphate dikinase, phosphoenolpyruvate (PEP) carboxylase, or other functionally diverged carboxylases (Swingley et al. 2007; Yurkov and Beatty 1998). A light-stimulated reverse citric acid cycle may also account for some carbon fixation (Yurkov and Beatty 1998). Data from Kolber and coworkers (2001) indicate that AAnP contribute significantly to the global carbon cycle, but those from Goericke (2002) and Schwalbach and Fuhrman (2005) suggest only a minor role.

1.4.2 Evolution of AAnP

It has been proposed that all proteobacteria descended from a common purple photosynthetic bacterial ancestor (Xiong et al. 2000), and it is assumed that the first AAnP evolved from AnAnP after the accumulation of oxygen in the earth's atmosphere (Beatty 2002) or after the oceans became enriched of dissolved organic carbon (Jiao et al. 2007). Beatty (2002) and Woese (1987) suggested that phototrophy is ancestral in all *Proteobacteria* and that non-phototrophs arose from the loss of photosynthesis. The loss of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) genes was maybe caused by a combination of RuBisCO inhibition by oxygen and the decreasing need for carbon assimilation (Swingley et al. 2007). However, it is questionable whether it is possible to draw unambiguous conclusions from modern existing bacteria onto potential ancestors, since the possibility of lateral gene transfer needs to be taken into consideration as well (Nagashima et al. 1997; Raymond et al. 2002). This aspect is also supported by the fact that the *puf* genes, coding for the photosynthetic apparatus, were found to be located on plasmids (Pradella et al. 2004).

1.4.3 Taxonomy, phylogeny, and distribution of AAnP

After only few alphaproteobacterial AAnP had been discovered initially (Shiba et al. 1979), some years ago Béjà and co-workers (2002) described the group of AAnP to be much more diverse than expected. Nowadays, it is known that the group mainly consists of *Alphaproteobacteria* including the six marine genera *Erythrobacter*, *Roseobacter*, *Citromicrobium*, *Rubrimonas*, *Roseovarius*, *Roseivivax* and the six freshwater genera *Erythromicrobium*, *Roseococcus*, *Porphyrobacter*, *Acidiphilum*, *Erythromonas*, *Roseateles*, and *Sandaracinobacter*, as well as the two soil genera *Craurococcus* and *Paracraurococcus* (Yurkov 2006). Additionally, some *Gammaproteobacteria* (Csotonyi et al. 2008; Hu et al. 2006) and one betaproteobacterium (Mašín et al 2006; Suyama et al. 1999; Suyama et al. 2002) have been found as well. In most of these genera, the AAnP are intermixed with both phototrophic and non-phototrophic bacteria.

AAnP are presumed to constitute as much as 10% of all microbial cells in the oceans (Cottrell et al. 2006; Kolber et al. 2000; Kolber et al. 2001) and seem to be widely distributed (Lami et al. 2007). This wide distribution is probably due to the fact that phototrophy in AAnP is combined with a wide range of other metabolic capacities (Lami et al. 2007). However, in oligotrophic situations, their photosynthesis provides an advantage in competition with strictly heterotrophic organisms (Beatty 2002). Additionally, AAnP have been found often to live in association with phytoplankton. Schwalbach and Fuhrman (2005) as well as Sieracki and co-workers (2006) described a coupling between algal blooms and the numbers of AAnP occurring. Dinoflagellates, for example, represent an important ecological niche for AAnP. Since dinoflagellates are phototrophic organisms themselves and can swim actively, they probably provide suitable light conditions for the associated bacteria (Allgaier et al. 2003). AAnP can also use the DMSP produced by dinoflagellates as readily available carbon source (Yurkov and Csotonyi 2008). But AAnP also have been found often to live attached to particles (Waidner and Kirchman 2008), other phytoplankton, algae, and sea grasses (Shiba et al. 1979; Shiba et al. 1991). However, the exact function of these interactions has not been investigated so far.

1.5 Objective of the thesis

Associations between cyano- and heterotrophic bacteria have been known for a long time, but the interactions between both organisms have not been investigated in detail yet.
Therefore, the approaches of the present study were

i) to identify the heterotrophic bacteria living on two different filamentous cyanobacteria,
ii) to find out which influences single phylogenetically and physiologically different bacteria have upon the cyanobacterial strains examined and vice versa, and
iii) to analyse what factors exactly determine the mutual influences.

Therefore, i) heterotrophs from marine filamentous heterocystous and non-heterocystous cyanobacterial cultures were supposed to be isolated, phylogenetically characterised by means of various molecular biological methods, and a selection of distinct isolates was assumed to be sequenced. The sequence data was also to be used to design specific rRNA probes for the different heterotrophs for further investigations. It was planned ii) to develop a method to obtain axenic cyanobaceria. Cyanobacteria, treated with this method, were supposed to be used to check the influence that heterotrophic bacteria and cyanobacteria have upon each other. In this regard, also the occurrences and abundances of heterotrophs in these and as well in other cyanobacterial cultures of the department's culture collection were to be examined. The heterotrophs iii) were supposed to be tested regarding various biochemical and physiological properties that might have an influence upon their association with cyanobacteria.

2 Material and Methods

All cyanobacteria used in this study were taken from the culture collection of the "Marine Mikrobiologie" department at Bremen University. They had been isolated from shallow coastal water sediments of the Salzhaff near Boiensdorf, situated at the German Baltic Sea coast (see Figure 1) between 1992 and 1994 (Rethmeier 1995). Both cyanobacterial strains, the heterocystous *Nodularia harveyana* strain Bo53 and the non-heterocystous *Oscillatoria brevis* strain Bo10, are filamentous and were chosen to compare two organisms which derived from the same sampling site, but yet revealed a considerably different way of living.

Fig. 1. Map of the Baltic Sea (Mairs Geographischer Verlag 1994). The arrow indicates the sampling site of the cyanobacteria.

It was the aim of the study to isolate and analyse the heterotrophic community in two different filamentous cyanobacterial cultures and to elucidate their role inside the community.

To determine the heterotrophic community within the cyanobacterial cultures, heterotrophic cultures were obtained by streaking the supernatant of the cyanobacterial culture (before and after ultrasonication) onto solid media. This cultivation dependent approach was chosen to allow further investigations on the influence of the isolated heterotrophs upon their host cyanobacterial strains.

In the course of the identification of the associated bacteria living in culture with the cyanobacteria mentioned above, 51 different heterotrophic strains were obtained which were phylogenetically characterised by fluorescence *in situ* hybridisation (FISH), fingerprinting methods, and sequencing of the 16S rRNA genes. Four phylogenetically and physiologically different strains, one non-coloured (strain Bo10-19) and two differently coloured (strains Bo10-20 and Bo53-33) *Alphaproteobacteria* together with one coloured *Bacteroidetes* bacterium (strain Bo10-09), were chosen for further comparable investigations to study their possibly different influences on cyanobacterial growth behaviour. Afterwards, an improved method was developed to obtain axenic cyanobacterial cultures. The axenic cultures were mixed with single pure cultures of the heterotrophic strains mentioned above to study the influence of the heterotrophic bacteria upon the cyanobacterial growth behaviour and vice versa.

Pigment analyses, proofs for production of catalase and extracellular enzymes, and utilisation of carbon sources were accomplished as well to characterise the heterotrophic bacteria and to get information on their possible role in their coexistence with cyanobacteria. More detailed information on material and methods used can be found in the manuscripts (see chapter 3).

3 Manuscripts

Explanation to my own contribution to each manuscript.

Manuscript I

Hube AE, Heyduck-Söller B, Fischer U (2009) Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture. Syst Appl Microbiol 32:256-265

This manuscript describes and compares the composition of heterotrophic bacteria living attached to and in culture with the two different cyanobacterial strains examined.
I developed the experimental set-up for this investigation and carried out the practical work. Laboratory work was supported by the second author. The manuscript was written in discussion with both other authors.

Manuscript II

Hube AE, Fischer U (2009) Interactions between heterotrophic bacterial and cyanobacteria. Submitted to "Aquatic Microbial Ecology" in November 2009.

In the second manuscript results concerning the influence of four different heterotrophic bacteria upon cyanobacteria and vice versa are presented.
The idea for the experimental set-up was designed by me. I also conducted all the laboratory work. The data were analysed and the manuscript written in discussion together with the second author.

Manuscript III

Hube AE, Heyduck-Söller B, Fischer U (2009) Characterisation of phylogenetically different heterotrophic bacteria isolated from marine filamentous cyanobacteria. Ready to be submitted to “Aquatic Microbial Ecology”.

This manuscript describes a variety of biochemical and physiological properties of the heterotrophic bacteria in order to get information on the possible nature of influences, heterotrophic bacteria carry out upon cyanobacteria.
All experiments were developed and conducted by me. The pigment analysis was supported by the second author. The manuscript was prepared in discussion with both other authors.

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Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture ☆

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Abstract

Fifty-one heterotrophic bacterial strains were isolated from the marine cyanobacterial cultures of heterocystous *Nodularia harveyana* strain Bo53 and non-heterocystous *Oscillatoria brevis* strain Bo10. Fluorescence *in situ* hybridisation and fingerprinting methods were used for a preliminary taxonomical classification of 44 of the 51 isolates. The strains obtained from Bo53 were mostly *Alphaproteobacteria* (10/24), followed by *Bacteroidetes* (7/24), and *Gammaproteobacteria* (3/24). The affiliation of the isolates originating from Bo10 was dominated by *Alphaproteobacteria* (8/20) and *Bacteroidetes* (7/20), followed by *Gammaproteobacteria* (3/20). The 16S rRNA genes of four selected isolates were sequenced. A red-coloured bacterium from Bo53 grouped with the alphaproteobacterial genus *Porphyrobacter*, while the other three strains, obtained from Bo10, belonged to the alphaproteobacterial genera *Roseobacter* (pink) and *Rhodobacter* (colourless), and to the genus *Muricauda* (yellow) of *Bacteroidetes*. The findings indicated that the aerobic anoxygenic phototroph *Porphyrobacter* and its relatives only occurred in Bo10 culture, whereas members of the *Roseobacter* clade and the *Bacteroidetes* bacterium *Muricauda* sp. seemed to be more ubiquitous.

Keywords: *Oscillatoria brevis*; *Nodularia harveyana*; *Porphyrobacter*; *Roseobacter*; *Rhodobacter*; *Muricauda*

Introduction

It is well known that cyanobacteria often live in association with heterotrophic bacteria [6,7,25], but the composition of these heterotrophic communities has not been intensively studied yet. Cyanobacteria might provide a perfect habitat for heterotrophic bacteria within their polysaccharide- and peptide-containing envelopes. On the other hand, heterotrophs might be able to affect cyanobacteria either positively by providing beneficial metabolites or negatively by excreting algicidal substances [29].

Filamentous cyanobacteria can be divided into those which possess a heterocyst and those which do not. Heterocysts are differentiated cells which contain the oxygen sensitive N_2-fixing enzyme nitrogenase, lack the O_2-producing photosystem II, and, by means of their thick cell wall, lower oxygen diffusion into the cell to stabilise the N_2-fixing system. Non-heterocystous nitrogen fixing cyanobacteria overcome this inhibitory effect by a temporal separation of N_2-fixation and oxygenic

Abbreviations: AAnP, aerobic anoxygenic phototrophic bacteria; FISH, fluorescence *in situ* hybridisation; PAR, photosynthetically active radiation.

☆The 16S rDNA sequences of the heterotrophic bacteria were deposited in the GenBank® database under the following accession numbers: Strain Bo53-33: EU839360, strain Bo10-20: EU839359, strain Bo10-19: EU839358, strain Bo10-09: EU839357.

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photosynthesis together with other oxygen protection mechanisms [33].

It may be an additional advantage, especially for heterocystous cyanobacteria, that heterotrophic bacteria might lower the oxygen partial pressure so that the nitrogenase is better protected against the harmful effect of oxygen. It has been shown that some heterotrophs are specialised to live in contact with heterocysts, using cyanobacterial excretion products on the one hand and enhancing cyanobacterial nitrogenase activity by consuming oxygen on the other hand [24].

Salomon and co-workers [29] and Delucca and McCracken [7] could demonstrate that the associated heterotrophic community of the main bloom forming heterocystous *Nodularia spumigena* influenced the growth behaviour of the phototroph partner. They reported either stimulation or inhibition or no effect at all [7,29]. Concerning the heterotrophic community on non-heterocystous cyanobacteria, such as *Oscillatoria*, it could be demonstrated that the growth rates of the phototrophs were all positively affected by the heterotrophs [7,14].

To our knowledge, the community structure of heterotrophs and heterocystous or non-heterocystous cyanobacteria has never been compared before. Therefore, it was the aim of the present work to examine comparatively the heterotrophic communities of cultures of nitrogen-fixing (*Nodularia harveyana* strain Bo53) and non-nitrogen-fixing (*Oscillatoria brevis* strain Bo10) filamentous cyanobacteria by applying molecular biological methods for their taxonomical grouping.

Materials and methods

Cultivation of cyanobacteria

The filamentous cyanobacteria *Oscillatoria brevis* strain Bo10 and *Nodularia harveyana* strain Bo53 were taken from the culture collection of the ''Marine Mikrobiologie'' department at Bremen University. Both organisms originated from sediments of shallow coastal waters of the Baltic Sea (Boiensdorf, Germany), sampled between 1992 and 1994 [27]. Cyanobacteria containing sediment samples were streaked onto ASN_{III} (see below) containing agar plates [28]. Repeated transfers were performed until single filaments or colonies could be picked under a stereomicroscope with a sterile Pasteur pipette to pass unicyanobacterial isolates into liquid ASN_{III} medium (stock culture). Approximately 3 ml of these stock cultures were regularly transferred into 50 ml Erlenmeyer flasks containing 17 ml of medium every four weeks. The cyanobacteria were cultivated at 21 °C and a photon flow density of either 15 to 20 $\mu E\,m^{-2}\,s^{-1}$ photosynthetically active radiation (PAR) (*Oscillatoria*) or ~10 $\mu E\,m^{-2}\,s^{-1}$ PAR (*Nodularia*) in ASN_{III} medium [28] of half salinity containing 12.5 g/l NaCl, 1 g/l $MgCl_2 \times 6H_2O$, 0.25 g/l KCl, 1.75 g/l $MgSO_4 \times 7H_2O$, 0.25 g/l $CaCl_2 \times 2H_2O$, 0.75 g/l $NaNO_3$ (only for *O. brevis*), 0.12 g/l Na_2CO_3, 0.01 g/l $K_2HPO_4 \times H_2O$, 1.5 mg/l Fe-NH_4-citrate, 5 mg/l vitamin B_{12}, and 0.5 ml of a trace metal mix solution according to Rippka et al. [28].

Isolation of heterotrophic bacteria

To obtain free-living heterotrophs, 50 µl aliquots of either undiluted cyanobacterial culture or 1:10, 1:100, and 1:1000 dilutions were directly streaked onto solid media. The remaining culture was washed three times with medium (centrifugation: 5 min at 2,250 × g and 21 °C) and then subjected twice to ultrasonication at room temperature for 1 min (Elma® Transsonic Digital, highest intensity) to detach heterotrophic bacteria from aggregates. After this treatment, undiluted and diluted 50 µl supernatants (see above) were streaked onto agar plates. The following media were used: ASN_{III} enriched with either 1% or 0.1% (w/v) meat or yeast extract, Difco™ Marine Broth undiluted or 1:10 and 1:100 diluted, and BBL™ Actinomyces Broth. The agar plates were incubated at 21 °C for 4 to 10 days. Colonies which macroscopically appeared to be distinguishable from each other were used to obtain pure cultures by repeated plating on agar plates. Pure cultures were tested for their Gram staining behaviour and then frozen in stocks containing 17.4% glycerol.

Fluorescence *in situ* hybridisation

The respective cultures were fixed in 4% formaldehyde for one hour on ice and filtrated on polycarbonate filters (GTBP black, 0.2 µm pore size, Millipore). Thereafter, the cells were washed with PBS buffer (22.8 g/l NaCl, 1.334 g/l $NaH_2PO_4 \times H_2O$, 3.8 g/l Na_2HPO_4). Fluorescence *in situ* hybridisation (FISH) was carried out as described by Glöckner and co-workers [10]. The probes and the formamide concentrations in the hybridisation buffer used are given in Table 1. Hybridised bacterial samples were analysed with a Zeiss Axiolab or a Zeiss Axioskop epifluorescence microscope.

DNA extraction and PCR amplification of 16S rRNA genes

DNA extraction was carried out either with the PrestoSpin D Bug kit (Molzym), according to the manufacturer's instructions, or by phenol chloroform extraction, modified after Mikolajczak and co-workers [20] and Neilan [23]. To remove potential RNAs, the

Table 1. Description of oligonucleotide probes.

Probe name	Probe sequence (5′-3′)	Specificity	Formamide concentration (%)	Reference
EUB338	(GCTGCCTCCCGTAGGAGT)	*Bacteria*	0	[2]
ARCH915	(GTGCTCCCCCGCCAATTCCT)	*Archaea*	0	[32]
ALF968	(GGTAAGGTTCTGCGCGTT)	*Alphaproteo bacteria*	20	[22]
GAM42a[a]	(GCCTTCCCACATCGTTT)	*Gammaproteo bacteria*	35	[18]
BET42a[b]	(GCCTTCCCACTTCGTTT)	*Betaproteo bacteria*	35	[19]
CF319a	(TGGTCCGTGTCTCAGTAC)	Bacteroidetes group	35	[19]
ROS537	(CAACGCTAACCCCCTCC)	Marine alpha cluster	35	[8]
ERY150	(CCGAAGACATTATCCGGT)	*Erythro bacteraceae*	20	This study
MUR88	(GTTCCATACGCGTTCCGC)	Muricauda sp.	70	This study

[a]Used together with competitor BET42a (GCCTTCCCACTTCGTTT).
[b]Used together with competitor GAM42a (GCCTTCCCACATCGTTT).

samples were afterwards incubated with 1 µl RNase A (30 mg/ml, Molzym) at 37 °C in a water bath for 1 h. RNase activity was inactivated at 65 °C for 10 min. The reaction mixture (50 µl total volume) for the PCR contained: 5 µl 10 × PCR buffer without $MgCl_2$ (Invitrogen), 4 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.4 mM of each primer, 0.001 mg/ml bovine serum albumin, 0.04 U/µl Ampli*Taq* Gold™ polymerase (Applied Biosystems), 10–20 ng DNA (except for strains Bo10-20 and Bo53-33, where the DNA was diluted to 0.1–0.2 ng, respectively, and 0.01–0.02 ng for strain Bo10-19), and it was filled with sterile distilled water. The following primer pairs were used: 8F (5′-AGAGTTTGATCMTGGC-3′) and 1507R (5′-TACCTTGTTACGACTT-3′) [21] for strain Bo10-09, R1n (5′-GCTCAGATTGAACGCTGGCG-3′) [34] and DG74 (5′-AGGAGGTGATCCAACCGCA-3′) [11] for strain Bo10-19, and 8F and 1542R (5′-AGAAAG-GAGGTGATCCARCC-3′) [15] for strains Bo10-20 and Bo53-33. The PCR reaction was started at 95 °C for 15 min to activate the polymerase, followed by 35 cycles for 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C. The reaction was completed by a 5 min extension at 72 °C. The PCR products were electrophoresed at 100 V on a 1% agarose gel prepared with 1 × TBE buffer [30] (pH 8.0), stained with ethidium bromide (0.5 µg/ml), and analysed with a Biometra Fluo_Link Transilluminator.

Fingerprinting methods

Amplified ribosomal DNA restriction analysis (ARDRA) was carried out by using a 25–50 ng 16S rRNA gene amplicon and 3 U of the restriction enzymes AluI, Hin6I or HpaII (all Fermentas) in a total reaction volume of 10 µl. The incubation was conducted for 3 h at 37 °C. Random amplified polymorphic DNA PCR (RAPD-PCR) was carried out with 10–20 ng of undiluted genomic DNA and the primers CRA22 (5′-CCGCAGCCAA-3′) and CRA23 (5′-GCGATCCC-CA-3′) [23] (the 50 µl reaction volume contained: 5 µl 10 × PCR buffer without $MgCl_2$ (Invitrogen), 3 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.4 µM of each primer, 1 mg/ml bovine serum albumine, 0.02 U/µl Platinum® *Taq* DNA polymerase (Invitrogen), and it was filled with sterile distilled water). PCR amplification involved a 2 min denaturation at 95 °C followed by 30 cycles at 94 °C for 20 s, 45 °C for 30 s, and 72 °C for 60 s. Thereafter, a 5 min extension at 72 °C was performed. The products of both fingerprinting methods were electrophoresed at 60–100 V on a 2% agarose gel prepared with 1 × TBE buffer [30] (pH 8.0). Staining and analysis were performed as described above. The gels were evaluated with the computer programme TotalLab TL120 (Nonlinear Dynamics Ltd.).

Purification and sequencing

Purification of 16S rRNA gene PCR products was carried out with the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's protocol. For sequencing, 30 µl PCR product aliquots with a concentration of at least 10 ng/µl were prepared. The DNA concentration of the PCR products was estimated in a 1% agarose gel using the MassRuler™ DNA Ladder Mix (Fermentas). Sequencing was conducted by GATC Biotec AG, Konstanz, Germany. The following primers were used: 517F (CCAGCAGCCGCGGTAATAC), 1099F (GCAACGAGCGCAACCC), 534R (ATTAC-CGCGGCTGCTGGC), and 803R (CTACAAGGG-TATCTAATCC) [39]. The four overlapping partial sequences obtained were assembled with the computer programme ChromasPro Version 1.34 (Technelysium Pty Ltd) and the consensus sequence was checked with the BLAST database (National Centre for Biotechnology Information, US National Library of Medicine), as well as with the database of the Ribosomal Database

Project II, Release 9.50 (Centre for Microbial Ecology, Michigan State University). Phylogenetic trees were constructed with the programme provided by the Ribosomal Database Project II and calculated with the neighbour-joining algorithm.

Probe design

Probes were designed by using the 16S sequence data of the heterotrophic strains and sequences from the Hugenholtz database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Primrose version 2.17 served as a computer programme. The specificity of the probes was reverified by comparison with the database of the National Centre for Biotechnology Information (NCBI), US National Library of Medicine, Bethesda, USA (http://www.ncbi.nlm.nih.gov), the Hugenholtz database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), the database of the Ribosomal Database Project II (http://rdp.cme.msu.edu), and the SILVA database (www.arb-silva.de). The accessibility of the newly designed probes was checked with data published by Behrens and co-workers [3] and Fuchs and co-workers [9]. Other properties of the probes were checked with the programme Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html).

The optimal formamide concentration for the hybridisation buffer was determined for both probes, as described by Hugenholtz and co-workers [16].

Results

A total of 30 heterotrophic bacterial strains were successfully isolated from the *Nodularia harveyana* Bo53 culture, of which two strains were red and six were light or dark yellow coloured. Red pigmented isolates grew in Difco™ Marine Broth from the undiluted and the 1:1000 diluted supernatant of the cyanobacterial culture. The yellow appearing ones were obtained from undiluted as well as from the 1:100 diluted supernatant in Difco™ Marine Broth and ASN_{III} with 0.1 and 1% meat extract. The colourless isolates grew from all dilutions performed in Difco™ Marine Broth, ASN_{III} with 1% meat extract and 0.1 or 1% yeast extract, and BBL™ Actinomyces Broth. Twenty-one heterotrophic strains were obtained from the *Oscillatoria brevis* Bo10 culture. Two of them were pink and grew only from the 1:100 dilution of the supernatant on ASN_{III} with 1% meat extract, while five yellow pigmented ones grew from all dilution rates on Difco™ Marine Broth. The remaining colourless isolates were obtained from all supernatant dilutions and could be cultivated on Difco™ Marine Broth, ASN_{III} with 1% meat or yeast extract, or BBL™ Actinomyces Broth.

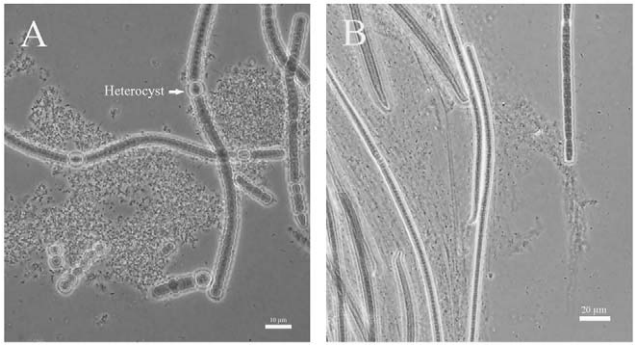

Fig. 1. Photomicrographs of (A) filamentous heterocystous *Nodularia harveyana* (strain Bo53) and (B) filamentous non-heterocystous *Oscillatoria brevis* (strain Bo10) with attached heterotrophic bacteria, respectively.

All red and pink isolates originated only from aggregates, while colourless and yellow colonies originated both from the supernatant and from the attached cells. The attachment of heterotrophic bacteria to the filamentous cyanobacteria *N. harveyana* strain Bo53 (A) and *O. brevis* strain Bo10 (B) is illustrated in Fig. 1. No Gram-positive bacteria were found from among the heterotrophic isolates.

FISH was applied first with 24 isolates from the Bo53 and 20 from the Bo10 culture by using the probes for Bacteria (EUB338) and Archaea (ARCH915). All strains hybridised well with the first mentioned probe, but not with the other one. Application of a set of group probes (see Table 1) indicated that most of the isolates belonged to *Alphaproteobacteria* (41.7% of isolates from Bo53 and 40% of the isolates from Bo10), the *Bacteroidetes* (29.2% or 35%, respectively), and *Gammaproteobacteria* (12.5% or 15%, respectively). *Betaproteobacteria* could not be found. As can be seen from Tables 2 and 3, the *Alphaproteobacteria* showed much more diversity in pigmentation than the *Bacteroidetes* group in which only yellow colonies occurred.

Nine yellow colonies were separated into two groups by ARDRA, whereas two did not group with any other colonies. Ten of the red, pink, and colourless isolates could be clustered into three groups. The results obtained with the three restriction enzymes applied were consistent (data not shown). Due to the fact that the primers used did not bind to the DNA of any red, pink or colourless isolate, RAPD-PCR was evaluated only for the yellow ones. The results were not in accordance with those of ARDRA. The fingerprints of the RAPD-PCR revealed that six yellow isolates still clustered into two groups, whereas the remaining isolates did not comprise any cluster (see Figs. S1–S3). From this analysis, it can be deduced that the following

Table 2. Summary of amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA PCR (RAPD-PCR), fluorescence *in situ* hybridisation (FISH), and 16S rRNA gene sequencing results of heterotrophic bacterial isolates obtained from *Oscillatoria brevis* cultures.

Occurrence and colour of heterotrophic isolates	Strain designation	Group arrangement based on ARDRA (I–V) and RAPD-PCR (i, ii)	Classification by	
			FISH[a]	16S rDNA sequencing[a]
Free-living, colourless	Bo10-04	III	AP	–
Free-living, colourless	Bo10-08	III	AP	–
Free-living, colourless	Bo10-12	III	AP	–
In aggregates, colourless	Bo10-18	III	AP	–
In aggregates, colourless	Bo10-19	III	AP	*Rhodobacter* sp.
In aggregates, pink	Bo10-20	V	AP	*Roseobacter* sp.
In aggregates, pink	Bo10-23	V	AP	–
Free-living, yellow	Bo10-09	I, i	BG	*Muricauda* sp.
In aggregates, yellow	Bo10-22	I, i	BG	–
Free-living, yellow	Bo10-07	II, ii	BG	–
Free-living, yellow	Bo10-10	II, ii	BG	–
Free-living, yellow	Bo10-13	II	BG	–

[a]AP = *Alphaproteobacteria*, BG = *Bacteroidetes* group, – = not determined.

Table 3. Summary of amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA PCR (RAPD-PCR), fluorescence *in situ* hybridisation (FISH), and 16S rRNA gene sequencing results of heterotrophic bacterial isolates obtained from *Nodularia harveyana* cultures.

Occurrence and colour of heterotrophic isolates	Strain designation	Group arrangement based on ARDRA (I–V) and RAPD-PCR (i, ii)	Classification by	
			FISH[a]	16S rDNA sequencing[a]
Free-living, colourless	Bo53-39	No group	AP	–
In aggregates, red	Bo53-33	IV	AP	*Porphyrobacter* sp.
In aggregates, red	Bo53-47	IV	AP	–
Free-living, yellow	Bo53-38	I, i	BG	–
In aggregates, yellow	Bo53-41	I, i	BG	–
Free-living, yellow	Bo53-45	I	BG	–
In aggregates, yellow	Bo53-34	II	BG	–
Free-living, yellow	Bo53-37	No group	BG	–
Free-living, yellow	Bo53-40	No group	AP	–

[a]AP = Alphaproteobacteria, BG = Bacteroidetes group, – = not determined.

summarized isolates might be one single species: (i) Bo53-38, Bo53-41, Bo10-09, and Bo10-22; (ii) Bo10-10 and Bo10-07; (iii) Bo10-04, Bo10-12, Bo10-18, Bo10-19, and Bo10-08; (iv) Bo53-33 and Bo53-47; and, (v) Bo10-20 and Bo10-23, although they showed diverse colony morphology after having been isolated as pure cultures.

Sequencing of 16S rRNA genes was performed with only one selected representative of each isolate colour (see Tables 2 and 3). In agreement with FISH, phylogenetic sequence analyses assigned the isolates Bo10-19, Bo53-33, and Bo10-20 into the *Rhodobacter, Porphyrobacter*, and *Roseobacter* group within the *Alphaproteobacteria*, respectively, while isolate Bo10-09 belonged to the *Muricauda* genus of the *Bacteroidetes* group. The closest validly described relatives of the examined strains were as follows: strain Bo10-19 and *Rhodobaca bogoriensis* strain LBB1 with 95% similarity, 94% similarity for strain Bo10-20 and *Sulfitobacter dubius* strain KMM 3554T, and 99% similarity for strain Bo53-33 and *Porphyrobacter dokdonensis* strain DSW-74, as well as strain Bo10-09 and *Muricauda aquimarina* strain SW-63.

The phylogenetic trees shown in Figs. 2 and 3 were calculated with the neighbour-joining algorithm based

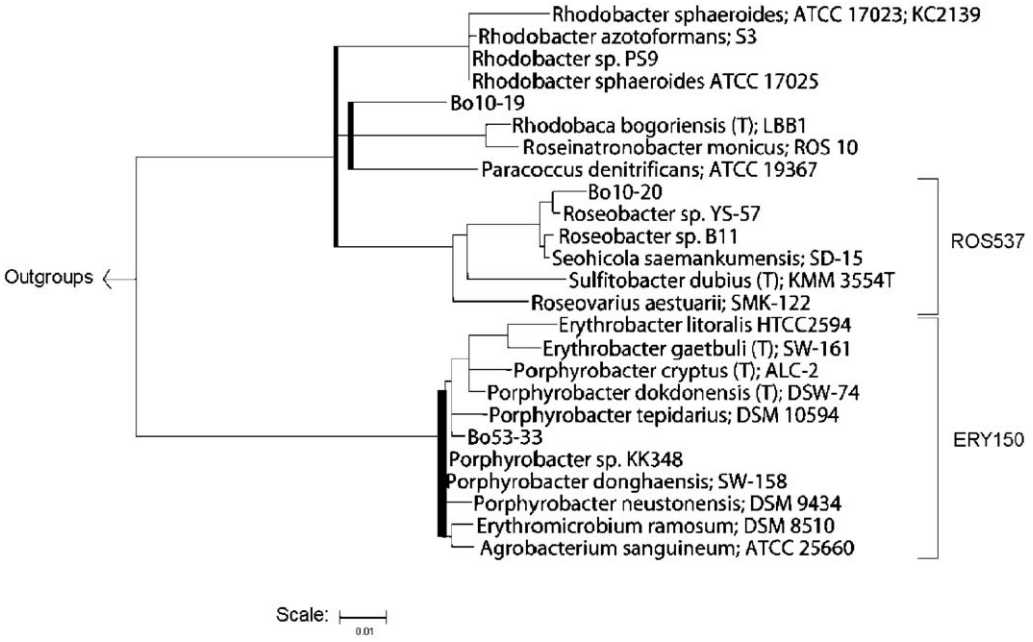

Fig. 2. Phylogenetic relationship between the 16S rRNA gene sequences of the isolates Bo10-19, Bo10-20, Bo53-33 and closely related *Alphaproteobacteria* (calculated with the neighbour-joining algorithm). Outgroups were a gammaproteobacterium, a *Bacteroidetes* bacterium, and a Gram-positive bacterium. The bar indicates 1% estimated sequence divergence.

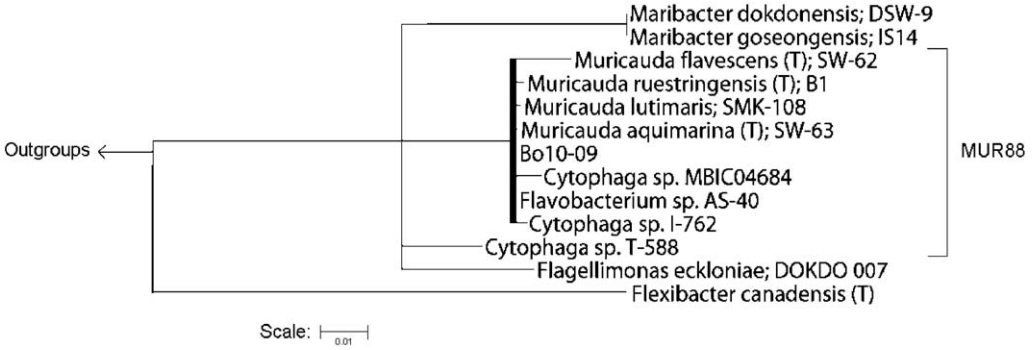

Fig. 3. Phylogenetic relationship between the 16S rRNA gene sequences of the isolate Bo10-09 and closely related bacteria of the *Bacteroidetes* group (calculated with the neighbour-joining algorithm). Outgroups were an alphaproteobacterium, a gammaproteobacterium, and a Gram-positive bacterium. The bar indicates 1% estimated sequence divergence.

on a nearly full-length sequence (~1500 bp). As can be seen, the isolates mentioned above fitted very well into the cluster of the appropriate group. Strains Bo10-19 and Bo10-20 had <97% 16S rRNA sequence similarity to their closest relatives. Therefore, these strains likely represent new species of the respective genera.

Specific probes were designed for the genus *Muricauda* (MUR88) and for the family *Erythrobacteraceae* (ERY150), comprising the genus *Porphyrobacter* (see Table 1 and Figs. 2 and 3). The occurrence and location of these respective heterotrophic bacteria within both cyanobacterial cultures were verified with these new probes in the complete (Fig. 4A–D) as well as the fractionated (see the isolation of heterotrophs in "Materials and methods") culture media. Since it was not possible to design probes specific for only Bo10-20 or Bo10-19, the *Roseobacter* probe ROS537 was also applied [8] (see Table 1 and Figs. 2, 4E and F), and it was specific for Bo10-20 but only partly for Bo10-19 (one mismatch, approximately 10% of the cells showed a signal). Cells of the *Roseobacter* clade and *Muricauda* spp. were identified in both cyanobacterial cultures, whereas members of the *Erythrobacteraceae* were found only in the *Nodularia* culture. *Roseobacter* and members

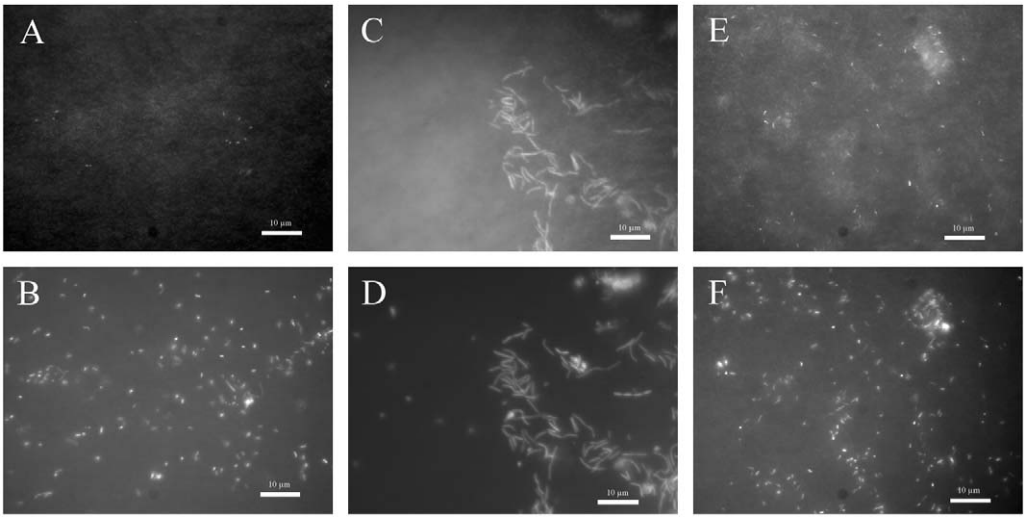

Fig. 4. Photomicrographs taken after fluorescence *in situ* hybridisation: Bacteria within *Nodularia* supernatant (A) stained with probe ERY150 and (B) with DAPI, an accumulation of *Muricauda* cells within *Oscillatoria* supernatant (C) stained with probe MUR88 and (D) with DAPI, and bacteria within *Nodularia* supernatant (E) stained with probe ROS537 and (F) with DAPI. Panels A and B, C and D, and E and F show identical microscopic fields, respectively.

of the *Erythrobacteraceae* occurred both as attached as well as free-living. This also applied to *Muricauda* in the *Oscillatoria* culture, but in the *Nodularia* culture it occurred only within the aggregates. Due to the fact that the heterotrophic bacteria occurred in huge aggregates attached to cyanobacterial filaments (Fig. 1), it was not possible to determine exactly the percentages of the different heterotrophic genera by counting FISH signals. Nevertheless, abundances of 1–10% were estimated for *Roseobacter*, *Erythrobacteraceae*, and *Muricauda*, respectively, in the *Nodularia* culture on the basis of FISH microphotographs (see Fig. 4). *Roseobacter* comprised approximately 10–20% and *Muricauda* less than 10% of all heterotrophs in the *Oscillatoria* culture.

Discussion

Probes

Probe ERY150 had 16.1% hits within the *Erythrobacteraceae* target group (2172 sequences in total) and 29 out group hits in SILVA [26] version 96. Out group hits were regarded as negligible, because most were bad sequences or *Planctomycetes*. Nevertheless, in the *Porphyrobacter* target group (61 sequences in total), which was most important for this study, it hit 78.7% of the sequences. Probe MUR88 hit 79.7% of the *Muricauda* target group (59 sequences in total). Probe ROS537 had 88% group hits (1080 sequences in total) and 31 out group hits in SILVA [26] version 93. All probes were regarded as specific.

Relationship between cyanobacteria and heterotrophic bacteria

Bacteria of the genera *Porphyrobacter* and *Roseobacter* belong to the so-called "aerobic anoxygenic phototrophic bacteria" (AAnP) group [36,41]. This group comprises bacteria that live mainly heterotrophically in an aerobic environment, but nevertheless are able to accomplish anoxygenic photosynthesis using the photosynthetic mechanism, which has only been known previously for purple sulphur bacteria. It has been shown that AAnP can be found attached to particles [37] or live in coexistence with algae [5,31] and cyanobacteria, for example, on dinoflagellates [1] or in cyanobacterial mats [36,42], much more often than expected. However, even though the existence of such associations has been known for quite a long time, they are not yet fully understood. For example, so far, it has not been proven whether AAnP express their pigments when living in association with phototrophs or whether they completely change to a heterotrophic way of living. For strains Bo10-20 and Bo53-33, the pigment composition was characterised but only after isolation, and bacteriochlorophyll *a* occurred in both isolates. As many as 14 different carotenoids were present in *Porphyrobacter* sp. Bo53-33, whereas in *Roseobacter* sp. Bo10-20 only one carotenoid was found (data not shown). From these findings, it was concluded that the AAnP growing in

association with cyanobacteria might indeed express their pigments. It is not possible to extract and subsequently analyse the AAnP's pigments from inside the cyanobacterial cultures because they will always be overlaid by the much more abundant cyanobacterial pigments.

For members of the *Roseobacter* lineage, it has long been assumed that they establish relationships with producers of organic carbon [1] or sulphur compounds (organic or inorganic) [5,36]. Another reason for such coexistences might be that the partners are phototrophs themselves and that AAnP are exposed to a suitable light intensity on their surface. However, this cannot explain why the *Roseobacter* isolates were found in both cyanobacterial cultures, while *Porphyrobacter* occurred only on the nitrogen-fixing *Nodularia*, even though both cyanobacterial strains were derived from the same shallow coastal water site in the Baltic Sea. The same behaviour was also observed with other *Nodularia* and *Oscillatoria* cultures from the department's culture collection (data not shown), which might imply a specific dependency. The detection of *Roseobacter* in the marine habitat contradicts the report of Hagström and co-workers [13] who did not find any *Roseobacter* or closely related isolates in the Baltic Sea, even though they also used a cultivation dependent [17] approach, as performed in the present study.

The genus *Muricauda* has been described only recently. Coexistence with filamentous cyanobacteria has not been mentioned yet, although it has been reported for some of their relatives [4]. To date, *Muricauda* has only been isolated from the German Wadden Sea [4] and from a salt lake near Hwajinpo Beach of the East Sea in Korea [40]. Bruns and co-workers [4] presume that the special appendages of the *Muricauda* cells might be used for attachment to a given substratum. Our results affirm this assumption, since *Muricauda* were often found within the aggregates. Other members of the *Bacteroidetes* group also live in association with cyanobacteria [7] or algae [12], and it is assumed that they are involved in particle degradation [8,38].

In contrast to the results of Salomon and co-workers [29] and Tuomainen and co-workers [35], we could not detect heterotrophs belonging to the *Betaproteobacteria* or to the phylum *Firmicutes* associated with nitrogen-fixing *Nodularia* species. On the other hand, although cyanobacteria-associated AAnP representatives were found in our study, the authors cited above did not find them [24,35]. These differences in the community structure might be due to different sampling sites. While our cyanobacterial strain originated from the benthos of coastal waters, the other strains derived from the open water column [29,35].

Future prospects

Cyanobacteria are not easily cultivable without accompanying heterotrophs. They often die when the abundance of heterotrophs decreases to a certain amount [25]. Since the present work provided heterotrophs from the phototrophic cyanobacteria community, it will now be possible to elucidate the potential influences of these bacteria on the cyanobacterial host.

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Appendix A. Supplementary materials

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.syapm.2009.03.001.

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Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture.

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Supplementary figures

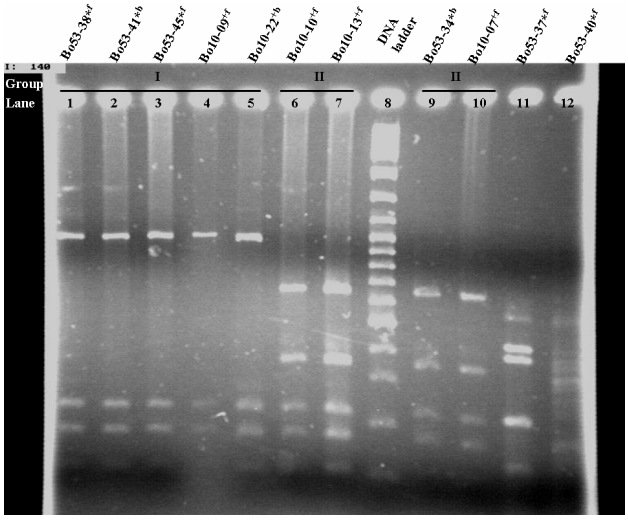

Fig. S1. ARDRA fingerprints of yellow heterotrophic isolates originating from *Oscillatoria brevis* or *Nodularia harveyana* cultures after digestion with the restriction endonuclease HIN6I and their arrangement into two different groups (I and II). * isolates obtained from *N. harveyana*, $^{+}$ isolates obtained from *O. brevis*, ^{f} free-living occurrence, ^{b} arranged in aggregates.

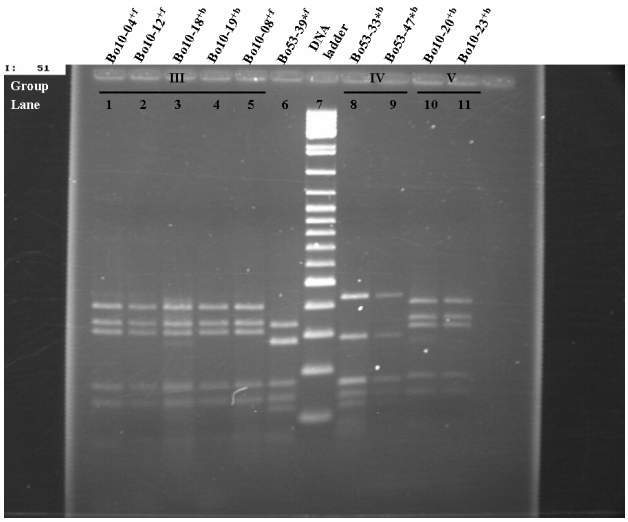

Fig. S2. ARDRA fingerprints of red, pink, and colourless heterotrophic isolates originating from *Oscillatoria brevis* or *Nodularia harveyana* cultures after digestion with the restriction endonuclease Hin6I and their arrangement into three different groups (III-V). The isolates in lanes 1- 6 are all colourless, those in lanes 8 and 9 are both red, and those in lanes 10 and 11 are both pink. * isolates obtained from *N. harveyana*, $^{+}$ isolates obtained from *O. brevis*, ^{f} free-living occurrence, ^{b} arranged in aggregates.

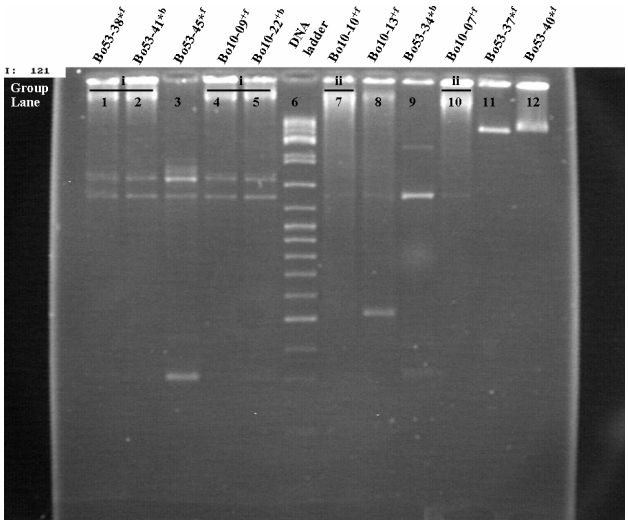

Fig. S3. RAPD-PCR fingerprints of yellow heterotrophic isolates originating from *Oscillatoria brevis* or *Nodularia harveyana* cultures after amplification using the primers CRA22 and CRA23 and their arrangement into two different groups (i, ii). * isolates obtained from *N. harveyana*, $^{+}$ isolates obtained from *O. brevis*, ^{f} free-living occurrence, ^{b} arranged in aggregates.

Interactions between heterotrophic bacteria and cyanobacteria

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ABSTRACT

Associations of cyanobacteria and heterotrophic bacteria have been observed for a long time, but such biocoenoses have not fully been understood so far. Here, we present some data, how heterotrophic bacteria and cyanobacteria influence each other's respective growth behaviour. Specific FISH probes were used to detect members of the family *Erythrobacteraceae* and the genera *Muricauda* and *Roseobacter* in various cyanobacterial cultures. For any of the heterotrophic groups, a dependence on cyanobacterial origin, morphology, or diazotrophy could be observed, except that *Muricauda* was not found together with unicellular cyanobacteria. Furthermore, an improved method to obtain virtually axenic cyanobacterial cultures was established and applied to cultures of the heterocystous *Nodularia harveyana* strain Bo53 and the non-heterocystous *Oscillatoria brevis* strain Bo10. Both of these axenic cyanobacterial species were mixed with single pure cultures of *Muricauda* sp., *Roseobacter* sp., and *Rhodobacter* sp., which had been obtained from the *Oscillatoria* culture, and *Porphyrobacter* sp., which had been obtained from the *Nodularia* one. All heterotrophic bacteria exhibited with increasing abundance only a negative effect on the growth of *Oscillatoria* but not on that of *Nodularia*.

KEY WORDS

Cyanobacteria-associated heterotrophs, aerobic anoxygenic phototrophic bacteria, *Muricauda*, *Roseobacter*, *Porphyrobacter*

INTRODUCTION

It has long been known that cyanobacteria often live in association with heterotrophic bacteria (Delucca & McCracken 1977, Cole 1982, Palinska et al. 1999). For some of the heterotrophs it has been shown that they are able to live without their phototrophic partner (Hube et al. 2009). However, there must be a reason for them to live in such associations. Palinska and co-workers (1999) demonstrated for cyanobacteria that it is not easily possible to keep axenic cultures alive. A lot has been speculated so far about such interferences (Paerl 1978, Larsson & Hagström 1982, Lignell 1990). Hietanen and co-workers (2002) suggested a mutualistic relationship with cyanobacteria providing substrates for the heterotrophs and the latter providing a source of remineralised nutrients for the phototrophs. Larsson and Hagström (1982) showed that in the Baltic Sea a substantial part of the energy, necessary for heterotrophic growth, derived from phytoplankton exudates and they assumed the existence of very effective bacterial mechanisms for the use of exudates as growth substrates. Sánchez and co-workers (2005) reported that heterotrophs living in the sheath of *Microcoleus chthonoplastes* are responsible for nitrogen fixation and hydrogen compound degradation, while *Microcoleus* provides a habitat and source of oxygen and organic matter. Up to now, the entire nature of these dependencies has not completely been understood. Recently, Hube and co-workers (2009) could demonstrate the occurrence of heterotrophic species of the genera *Muricauda*, *Porphyrobacter*, *Rhodobacter*, and *Roseobacter* living in association with the marine filamentous cyanobacteria *Nodularia harveyana* and *Oscillatoria brevis*. *Roseobacter* and relatives had been known for a long time to be aggressive colonisers, since they are able to form biofilms and even inhibit growth of other colonisers by producing antibacterial substances (Gram et al. 2002, Buchan et al. 2005, Bruhn et al. 2007). The ability to attach to phytoplankton had previously only been assumed for *Muricauda*, based solely on their morphology (Bruns et al. 2001). *Porphyrobacter* and *Roseobacter* belong to the aerobic anoxygenic phototrophic bacteria (AAnP). This group of bacteria has been known to comprise effective colonisers (Jiao et al. 2007, Waidner & Kirchman 2007), but to our knowledge, this never had been shown for *Porphyrobacter* itself. In either case, nothing is known so far about factors enabling heterotrophs to colonise cyanobacteria.

Therefore, the aim of this study was to investigate twelve cyanobacterial species, representing members of all five cyanobacterial subspecies as described by Castenholz (2001), concerning the occurrence of heterotrophic species and to find out what kind of influence the particular heterotrophs may have upon the cyanobacteria and vice versa.

MATERIALS AND METHODS

Isolation and cultivation of cyanobacteria and heterotrophs

All cyanobacterial strains (Table 2) were taken from the culture collection of the department "Marine Mikrobiologie" at Bremen University. With the exception of *Geitlerinema* sp. and *Fischerella ambigua*, all other strains originated from shallow coastal waters of the Baltic Sea and were sampled between 1992 and 1994 (Rethmeier 1995). Unicyanobacterial cultures were prepared as described by Hube and co-workers (Hube et al. 2009). *Geitlerinema* sp. derived from a North American Atlantic coast mangrove wood and *Fischerella ambigua* was provided by Prof. Gabriele M. König (University of Bonn) in 2000 and derived from a fresh water lake. The cyanobacteria were cultivated in ASN_{III} medium (Rippka et al. 1979) of half salinity at 21 °C and 1 µE m^{-2} s^{-1} photosynthetically active radiation (PAR) photon flow density for *Chroococcus*, *Synechocystis*, *Myxosarcina*, and *Geitlerinema*; 1 – 5 µE m^{-2} s^{-1} PAR for *Lyngbya*, *Anabaena*, *Oscillatoria laetevirens*, and *Nodularia* sp.; ~10 µE m^{-2} s^{-1} PAR for *Nodularia harveyana*; 10 – 15 µE m^{-2} s^{-1} PAR for *Fischerella ambigua*, and 15 - 20 µE m^{-2} s^{-1} PAR for *Oscillatoria brevis*. The medium for *Chroococcus*, *Synechocystis*, *Myxosarcina*, *Geitlerinema*, *Lyngbya*, and *Oscillatoria* contained 0.75 g/l $NaNO_3$, while the other strains were kept without an inorganically bound nitrogen source. *Fischerella ambigua* was cultivated in BG11 medium (Rippka et al. 1979) containing 1.5 g/l $NaNO_3$ and was continuously shaken at 70 rpm. Every four weeks, approximately 3 ml of the cyanobacterial cultures were transferred into 50 ml Erlenmeyer flasks containing 17 ml medium.

Heterotrophs used in this study and the source of their origins are documented in Table 1. Their isolation, identification, and cultivation have been described by Hube and co-workers (2009).

Fluorescence *in situ* hybridisation

Sample preparation and fluorescence *in situ* hybridisation (FISH) were carried out as described by Hube and co-workers (2009), using probes ERY150, MUR88 (Hube et al. 2009), and ROS537 (Eilers et al. 2001). Heterotrophic cell numbers in the FISH samples were determined by counting the signals in photomicrographs taken microscopically with a Zeiss Axioskop 2 epifluorescence microscope equipped with a Zeiss AxioCam MRm and the AxioVision 4.3 software.

Preparation of axenic cyanobacterial cultures

Biomasses of *Oscillatoria brevis* strain Bo10 and *Nodularia harveyana* strain Bo53 cultures of the stationary growth phase (approximately four weeks old) were divided into portions of between 20 and 230 mg wet weight and transferred into a test tube containing 2 ml of fresh medium each under sterile conditions. These samples were ultrasonicated at room temperature for 10 min with highest intensity (Elma® Transsonic Digital) and afterwards washed four times with culture medium by centrifugation (10 min at 9,000 *x g* and 20 °C). Only the *Nodularia* culture was ultrasonicated (see above) after each washing step. These steps were repeated until mainly single filaments could microscopically be observed. Then, the cyanobacteria were resuspended in 200 µl fresh culture medium and again ultrasonicated (see above) before they were centrifuged in a Percoll™ (GE Healthcare) solution, which was prepared as follows: 1 ml of 10 x concentrated culture medium was mixed with 9 ml of the Percoll™ solution. 1.8 ml of this mixture were pipetted into a 2 ml test tube, and the cyanobacterial sample (0.2 ml) was added on top. The samples prepared in this way were centrifuged for 3 h at 17,530 *x g* and 20 °C and with deceleration adjusted to the lowest intensity (Beckman GS-15R). After centrifugation, the Percoll™ solution on top was removed and the cyanobacteria band transferred into a new test tube, containing 5 ml of fresh culture medium. Subsequently, 0.61 µmol Rifampicin (stocksolution 10 mg/ml in distilled water) were added to the culture which was cultivated for 24 h under standard conditions (see above). Then, this culture was washed four times (see above) and the cell pellet suspended in 5 ml culture medium.

Preparation of cyanobacteria-heterotroph mixtures

This mixture was prepared directly after the above mentioned cyanobacterial cultures *Oscillatoria brevis* strain Bo10 and *Nodularia harveyana* strain Bo53 had been made axenic. The cell numbers of the heterotrophs were determined with a Neubauer improved haemocytometer. Each axenic cyanobacterial culture was divided into two nearly equal parts. These parts were mixed either with 1,000 or 10,000 heterotrophic cells per µg cyanobacterial wet weight of single two days old heterotrophic cultures (Table 3). Cyanobacterial cultures supplemented with mean volumes of heterotrophic culture medium served as control. Studies performed in this way were evaluated after two weeks of incubation under standard conditions (see above) by macroscopic comparison and fluorescence *in situ* hybridisation analysis, including determination of heterotrophic cell numbers. Each experiment was conducted three times (later referred to as three parallel assays).

RESULTS

Twelve different cyanobacterial cultures were investigated concerning the occurrence of *Muricauda* sp., *Roseobacter* sp., and members of the *Erythrobacteraceae*. As can be seen in Table 2, *Muricauda* and the *Erythrobacteraceae* could each be detected in 50% of the cultures tested (in 3 out of the 8 none-nitrogen-fixing cyanobacterial cultures and in 3 out of the 4 nitrogen-fixing ones in both cases), but *Roseobacter* only in one third (3 out of the 8 none-nitrogen-fixing cultures and 1 out of the 4 nitrogen-fixing ones). *Muricauda* was not found in any of the three unicellular cyanobacterial cultures tested, whereas *Erythrobacteraceae* bacteria could be found in one and *Roseobacter* in two of them. Of the filamentous cyanobacterial cultures, *Muricauda* sp. was the dominant heterotroph (66.7%), followed by the *Erythrobacteraceae* bacteria (55.6%), and *Roseobacter* sp. (22.2%).

The macroscopic evaluation of the growth behaviour of axenic cyanobacteria mixed with single heterotrophic cultures showed almost no influence of the heterotrophic bacteria upon *Nodularia*. Even within the control assay (no addition of heterotrophs), good cyanobacterial growth was observed. For the *Oscillatoria* culture, a toxic effect of the heterotrophs was detectable, especially in the approaches with 10,000 heterotrophic cells per µg cyanobacteria. Within the control assays, a cyanobacterial decay could be observed as well. None of the heterotrophs revealed an outstanding influence within the cyanobacterial cultures. They seemed to influence the cyanobacteria to similar degrees (Table 3).

Occurrence respectively reappearance of each heterotroph within the cyanobacterial cultures was checked after 14 days of incubation within one of the three parallel assays. The presence of *Porphyrobacter*, *Muricauda*, and *Roseobacter* was proved with the probes mentioned in the legend to Table 4. The numbers of the respective heterotrophs were counted within the cyanobacterial cultures they had been added to at the beginning of the experiment. It could be shown that in all cases a mixed heterotrophic community developed in the cyanobacterial cultures in the course of the experiment. In the *Oscillatoria* and *Nodularia* cultures total numbers of between 8.2×10^5 and 6.65×10^6 respectively 7.6×10^5 to 5.59×10^6 cells were found (obtained by counting the DAPI stained cells, data not shown). The cell numbers of the heterotrophic bacteria which had been added at the beginning of the experiment decreased. As can be seen in Table 4, the amount of *Porphyrobacter* and *Roseobacter* cells showed widely diverse values ($1.22 \times 10^4 – 3.24 \times 10^5$ in the case of *Porphyrobacter* and 1.86×10^4 - 1.08×10^6 that of *Roseobacter*) at the end of the experiment, independent of the amount of bacteria added and the respective cyanobacterial culture, while *Muricauda* reached cell numbers

between 4.01 x 10^4 and 1.26 x 10^6 in these cultures. Even though these total cell numbers did not show a significant difference between the diverse heterotrophs, a calculation on percental base shows that *Muricauda* cells stabilised to a level of 1.58 – 1.88% of the heterotrophic community in the *Nodularia* culture and of 18.9 - 23.4% in the *Oscillatoria* culture. In the case of *Porphyrobacter* and *Roseobacter*, no stable percentages could be observed.

DISCUSSION

Preparation of axenic cyanobacterial cultures

Although known to be difficult, it has been tried several times to detach filamentous cyanobacteria from their contaminants (Vaara et al. 1979, Kim et al. 1996, Vázquez-Martínez et al. 2004, Choi et al. 2008). Additional challenges occur for some of the filamentous ones which form tight aggregates together with their accompanying heterotrophs and which are difficult to access with common methods. Even though approaches of getting axenic cyanobacteria succeeded (Vaara et al. 1979, Shirai et al. 1989, Bolch & Blackburn 1996, Kim et al. 1999, Vázquez-Marínez et al. 2004, Choi et al. 2008), none of the authors reported to be able to keep the axenic cultures over a longer period alive.

By combining parts of the methods already described, we developed special adjusted methods suitable for our strains (see MATERIALS AND METHODS). Since in case of the *Oscillatoria* cultures the heterotrophs and cyanobacterial filaments seem to be comparatively loosely connected our developed procedure does not need to be repeated very often with this culture. However, most heterotrophs live in large tightly organised aggregates surrounding the cyanobacterial filaments in *Nodularia* cultures. Therefore, the procedure has to be repeated several times with this culture to disperse the aggregates to make the heterotrophs accessible for antibiotics. To circumvent the problem of keeping cultures axenic and alive, it is necessary to prepare the axenic cultures directly before running the experiment.

Influence of heterotrophic bacteria upon cyanobacteria

It has been reported before that some heterotrophic bacteria inhibit cyanobacterial growth by competition for limiting nutrients, lysis of cyanobacterial cells, and modification of the environment (Rhee 1972, Cole 1982, Drakare 2002). On the other hand, some heterotrophic bacteria can also stimulate cyanobacterial growth by nutrient regeneration, vitamin production, and maintenance of low oxygen concentrations in the vicinity of the heterocyst

(Paerl 1978, Cole 1982, Paerl et al. 1989). Delucca and McCracken (1977) demonstrated a stimulating effect of various heterotrophs upon *Oscillatoria*. These authors were also able to show a positive impact, as well as no reaction at all after addition of various heterotrophs to *Anabaena flos-aquae*. Paerl (1978) also described a bacteria-promoted enhancement of heterocystous *Anabaena* growth rates in most of his studies.

In the present study, we were able to show clear differences between the growth behaviour of *Nodularia* and *Oscillatoria* after the addition of heterotrophs. The fact that the absence or addition of heterotrophs only seem to have an effect on *Oscillatoria* but not on *Nodularia* might be due to the latter one's diazotrophy. While for *Nodularia* unlimited atmospheric nitrogen is present, *Oscillatoria* needs to compete with the heterotrophs for available bound nitrogen sources.

Moreover, we were able to show for *Oscillatoria* that the addition of higher amounts of heterotrophs caused a deterioration of its growth which did not happen when lower amounts were added. However, none of the heterotrophs seems to have a conspicuously stronger influence on the cyanobacteria than the others. We therefore assume for *Oscillatoria* that single heterotrophic colonisers are rather detrimental. When *Oscillatoria* cultures grow with their natural composition of heterotrophic colonisers, the latter might prevent each other from gaining an exceedingly strong influence.

Influence of cyanobacteria on heterotrophic bacteria

For some cyanobacteria it has been shown that they are able to inhibit bacterial growth, for instance by alteration of the macroenvironment or production of antibiotics (Østensvik et al. 1998). The latter has been reported for *Nodularia harveyana* which exhibited antibacterial activity upon Gram-positive bacteria (Pushparaj et al. 1999). But cyanobacteria can also stimulate bacterial growth by supplying nutrients (Paerl 1978) in the form of exudates (Larsson & Hagström 1982, Lignell 1990), by the disposal of soluble compounds during growth (Cole 1982, Cole et al. 1982), or by the decomposition of cyanobacterial cells. In oligotrophic environments, heterotrophs even depend on phytoplankton-produced organic carbon to a certain extent (Drakare 2002).

Another advantage of living attached to cyanobacteria has been reported especially for the AAnP. Cyanobacteria are phototrophs, so they might be able to transport the AAnP towards zones with appropriate light conditions by means of buoyancy regulations (Walsby et al. 1995). Kolber and co-workers (2001) additionally supposed a nutrient cycle for AAnP, tightly linked to that of oxygenic phototrophs. We detected *Erythrobacteraceae* in various

cyanobacterial cultures, independently of the cyanobacterial origin, their morphology, or utilisation of atmospheric or bound nitrogen sources. The factors regulating such colonisation still remain unknown. *Porphyrobacter* originally did not occur in our *Oscillatoria* cultures. But when we added our heterotrophic strain Bo53-33 to *Oscillatoria* strain Bo10, *Porphyrobacter* survived well, even though the cell numbers varied considerably after two weeks (see Table 4). *Porphyrobacter* may originally have occurred in *Oscillatoria*-associated communities but was eliminated by other heterotrophs in the cultures for unknown reasons.

By means of their cell morphology it has been assumed for *Muricauda* sp. that they might colonise particles or phytoplankton (Bruns et al. 2001), but to our knowledge this has never been investigated. In the present study, *Muricauda* was detected only on some of the filamentous cyanobacteria investigated but not on the unicellular ones. Furthermore, no relation to cyanobacterial diazotrophy or to the origin could be observed. The fact that *Muricauda* was the only strain which seemed to have become a stable part of respective communities in the cyanobacterial cultures after two weeks of incubation, might indicate that they are fast colonisers.

Roseobacter is known to be an effective and aggressive coloniser (Bruhn et al. 2007, Buchan et al. 2005, Gram et al. 2002). However, our investigations revealed that it does not occur in all cyanobacterial cultures examined. But a relation to cyanobacterial diazotrophy, origin, or morphology could not be demonstrated either. Since *Roseobacter's* cell number had not settled to a stable level after the incubation either, this may indicate that it emerges as a stable part of the community rather slowly.

These findings indicate that *Erythrobacteraceae*, *Muricauda* as well as *Roseobacter* aim at certain unknown factors when colonising cyanobacteria which still needs to be elucidated.

Neither the cyanobacterial morphology nor the use of atmospheric or bound nitrogen sources seems to play a role in the distribution of the heterotrophic bacteria. Since none of the heterotrophic bacteria tested could be found to be ubiquitously present in all cyanobacterial cultures tested, other dependencies seem to exist than the ones mentioned above.

In the culture of *Geitlerinema* sp., the only cyanobacterial strain from the Atlantic Ocean, *Erythrobacteriaceae* as well as *Muricauda* occurred (Table 2). This might indicate an ubiquity of these two groups in marine cyanobacteria-associated communities. In the *Fischerella ambigua* culture, the only fresh water cyanobacterial strain, none of the heterotrophs tested could be found. Therefore, they might not occur in fresh water cyanobacteria-associated communities.

We could demonstrate that heterotrophs reappeared in high amounts in the cyanobacterial cultures they were not added to within two weeks after our axenic cyanobacterial cultures had been mixed with single heterotrophic cultures. This finding indicates that i) it was not possible to remove all heterotrophs from these filamentous cyanobacteria, even by using an adjusted combination of different purification methods and ii) it is not possible to keep filamentous cyanobacterial cultures axenic without further treatment. A distinct, even though not yet fully developed, new heterotrophic community is capable to form itself within two weeks.

In conclusion, the relationship between *Nodularia* and its heterotrophic partners might be of commensal behaviour, being of benefit for the heterotrophs, while that between *Oscillatoria* and heterotrophs seems to be more complex, having a kind of symbiotic character but only within certain limits.

Future prospects

It has been shown before that in some cases cyanobacteria respond differently to single- than to multiple-bacterial cultures which they were exposed to (Delucca & McCracken 1977, Cole 1982). On this account, results gained with the addition of only one single bacterial strain need to be handled with caution. Continuative experiments should therefore include testings concerning the effect of combinations of heterotrophic bacteria on cyanobacteria. Furthermore, the effect of single bacterial components or dead bacteria on cyanobacteria needs to be studied, to find out more about the nature of the mutual influence.

Additionally, the abundances of different heterotrophic bacteria living in association with cyanobacteria still remain to be analysed in more detail. These investigations might give hint towards the importance of the heterotroph for the cyanobacterium as well as towards the relationship of the different heterotrophic partners among themselves.

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TABLES

Table 1. Heterotrophic strains used in the mixed culture experiment and source of isolation

Heterotrophic strain	Source
Muricauda strain Bo10-09	*Oscillatoria brevis* strain Bo10
Rhodobacter strain Bo10-19	*Oscillatoria brevis* strain Bo10
Roseobacter strain Bo10-20	*Oscillatoria brevis* strain Bo10
Porphyrobacter strain Bo53-33	*Nodularia harveyana* strain Bo53

Table 2. Occurrence of members of the family *Erythrobacteraceae* and the genera *Muricauda* and *Roseobacter* in various cyanobacterial cultures of the culture collection of the Department of "Marine Mikrobiologie" at Bremen University (Germany) by using the probes ERY150, MUR88, and ROS537. + = present, - = not present.

Cyanobacterial strain and classification subsections* (given in parentheses)	*Erythro-bacteraceae*	*Muricauda* sp.	*Roseo-bacter* clade
Unicellular			
Chroococcus turgidus Bo1 (I)	-	-	+
Synechocystis sp. Bo79 (I)	+	-	-
Myxosarcina chroococcoides AH149 (II)	-	-	+
Filamentous without heterocysts			
Geitlerinema sp. Flo1 (III)	+	+	-
Lyngbya semiplenta Fä8 (III)	+	-	-
Oscillatoria brevis Bo10 (III)	-	+	+
Oscillatoria laetevirens Bo17 (III)	-	+	-
Filamentous with heterocysts			
Anabaena flos aquae Hi35 (IV)	+	-	+
Anabaena variabilis Hi4 (IV)	-	+	-
Nodularia harveyana Bo53 (IV)	+	+	-
Nodularia sp. Fä19 (IV)	+	+	-
Fischerella ambigua (V)	-	-	-

* Subsections I-V according to Castenholz (2001)

Table 3. Macroscopic evaluation on the growth behaviour of axenic cyanobacteria mixed with different amounts of single heterotrophic cultures after two weeks of incubation.
+ + + cyanobacteria alive; + + cyanobacteria alive, but less green; + cyanobacteria mostly alive; + - cyanobacteria half-dead; - cyanobacteria almost dead. The evaluation was conducted following the method of Berg and co-workers (2009).

Added heterotrophs	*Oscillatoria brevis* Bo10	*Nodularia harveyana* Bo53
10,000 heterotrophic cells/µg cyanobacteria		
Muricauda Bo10-09	+ -	+ + +
Rhodobacter Bo10-19	-	+ + +
Roseobacter Bo10-20	-	+ + +
Porphyrobacter Bo53-33	+ -	+ +
Control	-	+ + +
1,000 heterotrophic cells/µg cyanobacteria		
Muricauda Bo10-09	+	+ + +
Rhodobacter Bo10-19	+	+ + +
Roseobacter Bo10-20	+ +	+ + +
Porphyrobacter Bo53-33	+	+ + +
Control	+ +	+ + +

Table 4. Occurrences of heterotrophs within cultures of *Oscillatoria brevis* strain Bo10 and *Nodularia harveyana* strain Bo53 two weeks after their addition to virtually axenic cyanobacterial cultures. Occurrences of *Porphyrobacter*, *Muricauda*, and *Roseobacter* were proved by using the probes ERY150, MUR88, and ROS537, respectively. In all cyanobacterial cultures, the occurrence of each heterotroph was checked, in order to find out any reappearance. The numbers of the respective heterotrophs were only counted within the cyanobacterial cultures they had been added to at the beginning of the experiment. + detected; - not detected. The numbers given represent the total of the respective cells counted, while the percentage quotation represents the share of the respective cells in all DAPI-stained cells counted.

Amount of the respective heterotrophic cells added to each cyanobacterial culture	Cyanobacterial cultures and applied probes					
	Oscillatoria			*Nodularia*		
	ERY150	ROS537	MUR88	ERY150	ROS537	MUR88
10,000 heterotrophic cells/µg cyanobacteria[†]						
Muricauda Bo10-09	+	+	4.60×10^5 (23.4%)	+	+	4.58×10^4 (1.88%)
Rhodobacter Bo10-19	+	+	+	+	+	+
Roseobacter Bo10-20	+	4.18×10^5 (9.5%)	-	+	1.08×10^6 (19.3%)	+
Porphyrobacter Bo53-33	3.03×10^5 (5.6%)	+	-	3.24×10^5 (11.7%)	+	+
Control	+	-	-	+	+	+
1,000 heterotrophic cells/µg cyanobacteria[‡]						
Muricauda Bo10-09	+	-	1.26×10^6 (18.9%)	+	+	4.01×10^4 (1.58%)
Rhodobacter Bo10-19	+	+	+	+	+	+
Roseobacter Bo10-20	+	1.86×10^4 (1.2%)	+	+	1.65×10^5 (18.5%)	+
Porphyrobacter Bo53-33	1.57×10^5 (19.2%)	-	-	1.22×10^4 (1.59%)	-	+
Control	+	-	+	+	+	+

Total heterotrophic cells added to each cyanobacterial approach at the beginning:
[†] $7 \times 10^8 – 4 \times 10^9$ and $2 - 3 \times 10^8$ cells to cultures of *Oscillatoria* and *Nodularia* respectively.
[‡] $1.1 – 1.3 \times 10^8$ and $2 - 3 \times 10^7$ cells to cultures of *Oscillatoria* and *Nodularia* respectively.

Characterisation of phylogenetically different heterotrophic bacteria isolated from marine filamentous cyanobacteria

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ABSTRACT

Little is known about the connection of bacteria accompanying filamentous cyanobacteria and their phototrophic partners. Morphological, biochemical, and physiological properties of four bacteria (*Porphyrobacter* Bo53-33, *Roseobacter* Bo10-20, *Muricauda* Bo10-09, and strain Bo10-19, closely related to *Rhodobacter*) deriving from two distinct cyanobacterial cultures were investigated to highlight different aspects that might contribute to such a relationship. Fourteen different carotenoids were detected in *Porphyrobacter*, five in *Roseobacter*, and only one in *Muricauda*. *Porphyrobacter* and *Roseobacter* contained bacteriochlorophyll *a* as well. Flexirubin could not be detected. The physiological investigations showed that *Porphyrobacter* had only a very restricted potential with respect to its utilisation of carbon substrates. On the other hand, *Muricauda* degraded all kinds of sugars, but none of the alcohols offered. Extracellular amylases were detected in *Porphyrobacter*, *Roseobacter*, and *Muricauda*. The latter one was also shown to excrete DNase. None of the isolates tested produced peptidases or lipases. All four strains were shown to possess a catalase. Many of the properties shown are beneficial when living in association with phytoplankton. Therefore, the compiled data set indicates a complex and multifarious adaptation to this way of living.

KEY WORDS

Porphyrobacter, *Roseobacter*, *Muricauda*, cyanobacteria-associated heterotrophs, aerobic anoxygenic phototrophic bacteria

INTRODUCTION

Cyanobacteria have long been known to live in associations with heterotrophic bacteria (Delucca & McCracken 1977, Cole 1982). However, the dynamics of these co-existences have not intensively been studied yet. To at least partially fill this knowledge gap, we characterised four different heterotrophic strains, isolated from associations with two unlike cyanobacterial isolates from the Baltic Sea. Of these heterotrophic strains, the phylogenetical identities and influence on the cyanobacterial growth behaviour have been investigated before (Hube et al. 2009, Hube & Fischer 2009).

One important group known to be living attached to cyanobacteria are the aerobic anoxygenic phototrophic bacteria (AAnP) (Yurkov & Beatty 1998, Wagner-Döbler & Biebl 2006, Hube et al. 2009). AAnP are a unique group of microorganisms in the marine environment whose ecological role is presently poorly understood. Little is known about their physiology and metabolisms and their role in energy flow and carbon cycling in the ecosystem. AAnP are known to live aerobically. Nevertheless, they are able to perform anoxygenic photosynthesis to complement heterotrophic metabolism (Yurkov 2006). In most of them, phototrophy is combined with a wide range of other metabolic capabilities (Yurkov 2006, Lami et al. 2007). Even though the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) has never been detected in any of them, some AAnP are able to fix small amounts of CO_2 via other pathways (Kolber et al. 2001, Swingley et al. 2007). Aerobic anoxygenic photosynthesis might constitute an advantage for the organism in carbon-limited environments (Buchan et al. 2005). Due to the presence of photopigments, less organic carbon sources are required for catabolic processes, hence more is available for growth (Yurkov & van Gemerden 1993). Since AAnP are known to be widely distributed (Kolber et al. 2000, Schwalbach & Fuhrman 2005) and possess a unique function (Yurkov & Beatty 1998), a better understanding of these microorganisms may change our current perspective on the energy and oxygen balance in the ocean. *Roseobacter* in particular was described by Swingley and co-workers (2007) to be a generalist, and the authors suggest a comparatively complex contribution to the global carbon cycle. In addition, the character of the connection between the AAnP and the cyanobacteria, which they are often found in association with, has also not been fully understood yet.

Muricauda sp., another heterotroph isolated from cyanobacterial cultures (Hube et al. 2009), which belongs to the *Bacteroidetes* group, is not capable of performing photosynthesis. For many of its relatives it is known that they are able to degrade natural polymers such as cell

wall components and exudates from algae (Johansen et al. 1999, Bruns et al. 2001). Nevertheless, concerning *Muricauda's* relation to its cyanobacterial partners, nothing is known so far.

Rhodobacter strain Bo10-19, to be characterised in the present study is an alphaproteobacterial isolate (Hube et al. 2009). Despite the close relationship to phototrophic purple bacteria and AAnP, strain Bo10-19 lives heterotrophically and does not possess considerable amounts of pigments. However, it seems to be to some extent specialised in living in association with cyanobacteria as well (Hube et al. 2009, Hube & Fischer 2009).

Based on these facts about the physiologically differing strains mentioned, we decided to investigate some of the strains' morphological, biochemical, and physiological properties to be able to specify their relationship with cyanobacteria.

MATERIALS AND METHODS

Isolation and cultivation

Heterotrophic bacteria were isolated from the cultures of *Oscillatoria brevis* strain Bo10 (*Muricauda* sp. strain Bo10-09, *Rhodobacter* sp. strain Bo10-19, and *Roseobacter* sp. strain Bo10-20) and *Nodularia harveyana* strain Bo53 (*Porphyrobacter* sp. strain Bo53-33) in 2005. Their isolation and identification, as well as their cultivation has been described by Hube and co-workers (2009).

Proof of slime capsules

Appearance of bacterial capsules was proved by i) mixing a bacterial culture scraped from an agar plate with either one drop of royal ink or a 10% nigrosin solution on a slide and ii) staining with crystal violet. For this procedure, bacterial cells were mixed with one drop of a skimmed milk powder suspension [100 g/l], dried, and thermal fixed on a slide, covered with one drop of 0.1% crystal violet and rinsed with a 20% copper sulphate solution. Then, the sample was air dried, embedded with one drop of immersion oil and afterwards covered with a cover slip. All preparations were evaluated by bright field microscopy (Zeiss Axiolab). Slime capsules appeared as clear zones around the organism and the dark blue background.

Pigment analysis

Cultures of 100 ml medium were grown at 21 °C under continuous light at a photon flow density of 10 – 15 µE m^{-2} s^{-1} photosynthetically active radiation (PAR) and shaken at 70 rpm for two days, centrifuged (6,500 x *g* at 10 °C for 15 min) and washed once in ASN_{III} culture medium (Rippka et al. 1979, Hube et al. 2009) without addition of meat extract and $NaNO_3$. Finally, cells were centrifuged at 8,500 x *g* and 10 °C for 15 min. The pellets were resuspended in 8 ml methanol and incubated at 4 °C in the dark for approximately 2.5 h. Afterwards, the samples were centrifuged again (17,530 x *g* at 4 °C for 5 min) and the supernatants stored in the dark on ice until further use. Pigments were separated by thin layer chromatography at room temperature for 1 h in the dark using Silica gel plates (pre-coated TLC plates SIL G-25 UV_{254}, Macherey-Nagel) and a mixture of acetone and n-hexane (40/60 v/v) as running solvent. The separated pigments were scraped off the plates, solved in ice cold methanol, and centrifuged (3,000 x *g* at 4 °C for 5 min). The supernatants were stored in the dark on ice until further use. The absorption spectra of the extracts were recorded with a Beckman DU®640 spectrophotometer from 200 – 900 nm.

In vivo spectra (200 – 900 nm) were obtained by resuspending the pellet in a 50% sucrose solution from 1 ml of a 2 days old centrifuged culture (15,000 x *g* at room temperature for 30 sec).

Identification of pigments was accomplished by Reversed Phase HPLC as described by Heuchert (2004). Pigments of each organism were extracted by adding 10 ml of an acetone methanol mixture (7/2 v/v) to the centrifuged cell pellets (8.500 x *g* at 10 °C for 15 min) obtained from 225 ml of four days old cultures. Immediately after the addition of the extraction solvent, cells were disrupted by ultrasonication (Elma® Transsonic Digital, highest intensity at 4 °C for 3 min). The samples were incubated and the pigments separated as described above for the thin layer chromatography, before they were applied to the HPLC treatment. After scraping them off the TLC plate, the pigments were resuspended in ice cold acetone methanol mixture (see above).

Presence of flexirubin was tested by applying one drop of 10% KOH solution onto agar plates overgrown with the used isolate (modified from Reichenbach et al. 1980). The colour of bacteria containing the pigment was supposed to change to red.

Anaerobic and autotrophic growth

Anaerobic and autotrophic growth behaviour of the heterotrophs was tested in six different approaches (i – vi) containing 10 ml of ASN_{III} culture medium modified as described by Hube

and co-workers (2009) for two weeks either in the dark (i and ii) or (iii-vi) under continuous light conditions (~ 1 µE m^{-2} s^{-1} PAR). The approaches were treated and supplemented as follows:

i) with 1% meat extract and degassed for 10 min with nitrogen

ii) with 1% meat extract, aerobically

iii) with 1% meat extract, degassed for 10 min with nitrogen

iv) with 1% meat extract, aerobically

v) with nitrate (0.75 g/l), aerated for 10 min with CO_2

vi) with nitrate (0.75 g/l), aerobically

Utilisation of carbon sources

Utilisation of various carbon sources was tested with api® 50 CH kits (Biomérieux). Bacterial cultures (20 ml) were grown in ASN_{III} culture medium (Rippka et al. 1979, Hube et al. 2009) enriched with 1% meat extract at 30 °C for two days. The bacterial cells were washed with a 0.9% NaCl solution and resuspended in a medium without additional carbon sources for the api® test kits. The medium used contained 13.19 g/l NaCl, 2.84 g/l $MgCl_2$ x 6 H_2O, 3.4 g/l $MgSO_4$ x 7 H_2O, 0.095 g/l $NaHCO_3$, 0.74 g/l $CaCl_2$ x $2H_2O$, 0.36 KCl, 0.5 g/l KH_2PO_4, 0.5 g/l NH_4Cl, 0.05 mg/l vitamin B_{12}, 0.05 mg/l riboflavin, 0.05 mg/l thiamine, 4.2 mg/l $FeSO_4$, 10.4 mg/l Na_2-EDTA, 0.06 mg/l H_3BO_3, 0.2 mg/l $MnCl_2$ x 4 H_2O, 0.38 mg/l $CoCl_2$ x 6 H_2O, 0.048 mg/l $NiCl_2$ x 6 H_2O, 0.02 mg/l $CuCl_2$ x 2 H_2O, 0.288 mg/l $ZnSO_4$ x 7 H_2O, 0.072 mg/l Na_2MoO_4 x 2 H_2O, 0.2 mg/l NaOH, 0.018 mg/l Na_2SeO_3 x 5 H_2O, 0.018 mg/l Na_2WO_4 x 2 H_2O, 0.414 mg/l NaH_2PO_4, 1.246 mg/l Na_2HPO_4, 0.04 mg/l 4-aminobenzoic acid, 0.02 mg/l D-(+)-biotin, 0.1 mg/l nicotinic acid, 0.05 mg/l Ca-D-(+)-pantothenate, 0.15 mg/l pyridoxine hydrochloride, 0.04 mg/l folic acid, and 0.02 mg/l lipoic acid. The resuspended bacteria were transferred into the microtubes of the api kits according to the manufacturer's instructions and incubated at 30 °C for seven respectively fourteen days. Acidification of the medium was evaluated after one, three, seven, and fourteen days of incubation.

Utilisation of fructose, galactose, and sucrose (0.5 mM final concentration) was additionally proved in 50 ml Erlenmeyer flasks, containing 20 ml of the medium described above or in a modified ASN_{III} culture medium as described by Hube and co-workers (2009). The ASN_{III} medium was prepared with 0.75 g/l Na_2NO_3 but without the addition of Na_2CO_3 and ferric ammonium citrate. Additionally, 0.18 g/l phenol red and varying carbon sources (see above) were added to both media and the pH was adjusted with 1N HCl or 1N NaOH to 8.

Catalase and extracellular enzyme activities

The presence of catalase was proved with the Bactident® Katalase Kit (Merck) according to the manufacturer's instructions.

Extracellular enzyme activities were detected by plating assays. ASN_{III} culture medium (Rippka et al. 1979, Hube et al. 2009) with 15 g/l agar was used for all tests. Amylase was tested as described by Smibert and Krieg (1981) with 2 g/l starch and 10 g/l peptone supplemented to the agar medium. Plates were incubated at 21 °C for 2 - 9 days. Then, plates were covered with Lugol's iodine (I_2:KI = 1:2) for 2 min, after the bacteria had been removed from the plates. In a positive test, colourless zones were visible after the iodine solution had been decanted. DNase activity was determined according to the method described by Schreier (1969) by adding 100 mg/l toluidine, 10 g/l meat extract, and 0.5 g/l DNA to the medium. Plates were incubated at 21 °C for up to 3 weeks. In a positive test, colonies were surrounded by a pink zone. Peptidase activity was proved as described by DeShazer and co-workers (1999) by adding 10 g/l yeast extract and 20 g/l skimmed milk powder to the agar medium. Evaluation was carried out after the plates had been incubated for up to 3 weeks at 21 °C. In a positive test, colonies were surrounded by a clear zone. Lipase activity was determined on agar medium plates supplemented with 10 g/l meat extract, 25 g/l olive oil, and 0.01 g/l rhodamine B according to the method of Kouker and Jaeger (1987). Plates were incubated for up to 4 weeks at 21 °C. Lipase-positive strains were identified by means of their fluorescence under UV irradiation (360 nm).

RESULTS

Morphological and biochemical characterisation

The AAnP strains Bo53-33 and Bo10-20 were both rod-shaped with 1.5 – 3.0 µm in length and ~ 0.5 µm in width. Both strains formed low-convex, circular, smooth colonies on agar plates. Colonies of Bo53-33 were brownish orange, whereas those of Bo10-20 were pink coloured. Bo10-19 had short rod shaped cells with 1.3 – 1.7 µm length and 0.4 – 0.5 µm width. Strain Bo10-19 cells formed thick milky colonies with a flattened, even rim on agar. Bo10-09 cells were rod shaped, 0.4 – 0.5 µm wide and 1.5 - 10.0 µm long. Single cells were able to form long chains and globular structures (Fig. 1). Its colonies were low-convex, circular, smooth, and yellow coloured. Capsules were observed for all four strains tested. All were motile and able to grow at 21 °C and at 30 °C.

Absorption spectra of methanol- or acetone/methanol extracts of strains Bo53-33, Bo10-20, and Bo10-09 revealed that they contain as pigments mainly the carotenoids zeaxanthin (or β,β-carotene) or spheroidenone besides a variety of other ones. The correct classification of the others was not possible with respect to available data from literature. Based on their retention times and absorption maxima, 14 respectively 5 pigments could be detected in the *Porphyrobacter* strain Bo53-33 and the *Roseobacter* strain Bo10-20 (Table 1, Fig. 2A). In addition to the carotenoids, bacteriochlorophyll *a* was found in these two strains as well. The *Muricauda* strain Bo10-09 (Fig. 2B) seems to contain only a yellow carotenoid (one maximum at 465 nm), which could not be identified more precisely by comparison with literature data. Flexirubin was not found in strain Bo10-09, the only organism tested for. The pigment composition of Bo10-19 was not examined because of its pale colour.

Physiological characterisation

As can be seen from Table 2, the *Muricauda* strain Bo10-09 can use many of the offered carbon sources for growth while the ability to do so is limited to only a few ones by the *Porphyrobacter* strain Bo53-33. Strain Bo10-09 has a preference for all kinds of sugars but not for alcoholic carbon sources. Strain Bo53-33 could use only a few of the carbon sources offered but seems to have a preference for disaccharides. When strain Bo53-33 was grown in liquid media in Erlenmeyer flasks, the organism could grow with fructose, galactose, and sucrose (data not shown) which was not the case in the api test kit. This test kit was not applied with strains Bo10-19 and Bo10-20, since preliminary tests had shown that the pH in their culture media did not change either.

Although strain Bo10-19 has a close relationship to purple non-sulphur bacteria, our strain could not grow anaerobically and autotrophically in the media applied (see Materials and Methods), growth of this is dependent on oxygen and organic carbon sources. Concerning enzymatic equipment, all four strains possessed catalase activity, whereas peptidases and lipases did not occur in any of the strains tested. Amylase was only absent in strain Bo10-19, while DNase was found only in Bo10-09.

DISCUSSION

All four strains investigated in this study have been isolated and first described to live on cyanobacteria by Hube and co-workers (2009). They had been characterised phylogenetically by means of various molecular methods. The strains Bo53-33 and Bo10-20 had been shown to belong to the AAnP affiliated alphaproteobacterial genera *Porphyrobacter* and *Roseobacter*. Strain Bo10-20 belongs to the *Bacteroidetes* genus *Muricauda*, and for Bo10-19 its close relationship to the alphaproteobacterial purple non-sulphur genus *Rhodobacter* had been shown.

The morphological observations of our AAnP strains largely correspond to former descriptions (Lafay et al. 1995, Hiraishi et al. 2002, Rainey et al. 2003, Yoon et al. 2004, Buchan et al. 2005, Yoon et al. 2006). These properties also apply to our *Muricauda* strain (Bruns et al. 2001, Yoon et al. 2008), including its ability to form chains and globular structures (Bruns et al. 2001, Müller et al. 2001). In contrast, the morphological characterisation of our strain Bo10-19 varied considerably compared to that of its next relatives *Rhodobaca* (Milford et al. 2000) and *Rodobacter* (Hiraishi & Ueda 1994). The cells of our isolate were noticeably larger and the colonies were lacking the typical yellow or brownish colour.

The main function of carotenoids in all photosynthetic organisms is to provide photooxidative protection. However, they also have a light-harvesting function (Britton 1995a, Armstrong 1997). Carotenoids harvest light of wavelengths between 460 and 550 nm, which can penetrate to about 50 m depth in eutrophic coastal waters, but up to 200 m depth in clear oligotrophic oceanic waters (Kolber et al. 2001, Ackleson 2003).

Although carotenoids are abundant in AAnP, they only play a minor role in harvesting light energy in these organisms (Yurkov & Beatty 1998, Kolber et al. 2001).

A variety of carotenoids has been described in *Porphyrobacter* strains (Hanada et al. 1997, Hiraishi et al. 2002, Rainey et al. 2003, Yoon et al. 2006, Rathgeber et al. 2007), but only few of them have been identified so far. Hanada and co-workers (1997) described the occurrence of carotenoid sulphates, derivates of hydroxyl-monoketo-ß-carotene, hydroxyl-ß-carotene as well as nostoxanthin and bacteriorubixanthinal for *Porphyrobacter tepidarius*. The latter one was also found in closely related *Erythrobacter* isolates, along with erythroxanthin sulphate, zeaxanthin, and ß,ß-carotene (Koblížek et al. 2003). Comparison with carotenoid absorption maxima from literature (Britton 1995b) showed that at least zeaxanthin and ß,ß-carotene are

likely to be present in the light yellow or orange TLC bands obtained from our strains as well. The occurrence of spheroidenone as major carotenoid has already been described for *Roseobacter* (Shiba 1991). This might as well be in accordance with our results, because one pigment (no. 19 in Table 1) shows a peak pattern similar to that of spheroidenone, compared with data from Britton (1995b). Additionally, Bruhn and co-workers (2005, 2007) as well as Gram and co-workers (2002) detected a brownish pigment in various *Roseobacter* strains, assuming that it has antibacterial potential and is produced when living in biofilms on marine snow and phytoplankton cells. This pigment was detected in the supernatant by measuring the absorbance at 398 nm. However, this pigment could not be detected in significant amounts in our *Roseobacter* strain. This might be due to inappropriate culture conditions or that our strain might not be able to produce this substance.

To our knowledge, the carotenoids in *Muricauda* have never been investigated before, but we were able to show that our strain Bo10-09 possesses at least one yellow carotenoid, even though we could not identify it precisely.

Although we were not able to qualitatively determine all pigments in our isolates, we could correctly determine the number of different carotenoids in our strains belonging to AAnP and *Muricauda* for the first time.

Nishimura and co-workers (1996) as well as Yurkov and Beatty (1998) described that the expression of photosynthetic pigments in AAnP can vary, due to the regulation by environmental parameters. The *puf* genes, encoding for bacteriochlorophyll *a*, even have been found to be coded on plasmids (Pradella et al. 2004). Additionally, Allgaier and co-workers (2003) reported the occurrence of *pufM* genes (encoding for the reaction centre) only in *Roseobacter* isolates deriving from dinoflagellate cultures and not in those deriving from water samples or biofilms. We were able to show the expression of bacteriochlorophyll *a* for our AAnP isolates when they were grown as pure cultures. Nevertheless, it was not possible to investigate the pigment expression of these strains when living in association with cyanobacteria. Since cyanobacteria are phototrophs themselves and are able to move to areas of suitable light condition, it is likely that they show photosynthetic activity when living associated. However, bright light restrains the production of bacteriochlorophyll in AAnP (Beatty 2002), and the availability of large amounts of carbon sources excreted by cyanobacteria might as well cause the AAnP to stop their photosynthetic activity. If this also applied to our isolates, we at least could proof that they are able to change back to a partly phototrophic way of living when separated from the cyanobacteria.

The enzyme catalase prevents cells from damage inflicted by harmful oxygen species (Halliwell & Gutteridge 1999). The ability to form this protective enzyme, which was found in all our heterotrophs tested, can be a special adaptation of bacteria living in oxygen rich environments, as in associations with phototrophs. Therefore, the heterotrophs might as well support cyanobacterial antioxidative defence (Hünken et al. 2008) and therefore be useful for the cyanobacteria.

Heterotrophic bacteria have long been known to live on carbon compounds released by primary producers (Azam et al. 1983). Cellular uptake rates of sugars (glucose) and amino acids (glutamate) of attached bacteria have been found to sometimes be higher than those of surrounding free-living bacteria (Kirchman & Mitchell 1982). Moreover, bacteria living on particles show higher activities and higher exoenzyme concentrations (Karner & Herndl 1992, Smith et al. 1992, Bidle & Fletcher 1995). In the present study, we tried to more specifically determine the carbon utilisation of the cyanobacteria associated heterotrophs and to draw conclusions concerning the correlation with excreted products of cyanobacteria.
The api® 50 CH test kit based on the fermentation or aerobic oxidation of the available carbon sources, resulting in the production of acids. Since a slight positive reaction (change of colour) was observed in the control assay without any carbon source after fourteen days, we decided to evaluate the test already after seven days of incubation. Additional studies with Bo53-33 as liquid culture in Erlenmeyer flasks have shown that in this case the pH in the culture medium does not necessarily change when the strain metabolises carbohydrates such as fructose, galactose, or sucrose. This might be an indication for the strains capacity to regulate the pH in its medium. Nevertheless, our *Porphyrobacter* strain Bo53-33 was shown to metabolise only some hexoses, disaccharides, and trisaccharides. Growth on pentoses, polysaccharides, amino acids, or alcohols could not be observed. These findings largely correspond to other descriptions of *Porphyrobacter* isolates (Fuerst et al. 1993, Hanada et al. 1997, Hiraishi et al. 2002, Rainey et al. 2003, Yoon et al. 2004, Yoon et al. 2006, Rathgeber et al. 2007). The *Muricauda* strain Bo10-09 was shown to be able to degrade almost all sugars, but none of the alcohols offered. These findings are partly in accordance with corresponding properties of other *Muricauda* isolates (Bruns et al. 2001, Yoon et al. 2005, Yoon et al. 2008). *Muricauda* generally seems to be able to live on di- and trisaccharides but not on alcohols. Our isolate was also able to degrade almost all monosaccharides it was tested for, whereas Bruns and co-workers (2001) and Yoon and co-workers (2005 and 2008) observed that their isolates only used a few of them.

Cyanobacteria are known to excrete organic compounds, such as amino acids, peptides, alkaloids, carbohydrates, and lipopolysaccharides (Paerl & Gallucci 1985). It is known that attached bacteria can degrade these organic compounds released by their hosts (Cole 1982, Cole et al. 1982, Kolber et al 2001). Sutherland and Tait (1992) described the occurrence of a xyloglucan, consisting of glucose and xylose (ratio 8:1), and another more complex polysaccharide containing uronic acid, glucose, xylose, and ribose (ratio 10:6:1:1) in *Anabaena flos-aquae*'s exopolysaccharides (EPS) and a complex proteoglycan containing uronic acids, glucose, xylose, mannose, galactose, and fucose as major components in *Nostoc calcicola*'s EPS. If these substances occur in the EPS of our *Oscillatoria* and *Nodularia* strains as well, *Muricauda* in the cyanobacterial cultures presumably lives on these carbon moieties, while *Porphyrobacter* seems able to utilise only the glucose of the EPS and maybe depends on other carbon sources as well. The findings that strains Bo10-09, Bo53-33, and Bo10-20 produce extracellular amylases corroborate this assumption. Only Bo10-19 seems to live on distinctly different carbon sources. Since neither peptidases nor lipases were found in any of our isolates, these heterotrophs do not seem to live on amino acids or lipids excreted by cyanobacteria.

All findings of the present study might shed some light on possible interactions between cyanobacteria and their attached heterotrophic bacteria. Even though Worm and co-workers (2001) stated that bacteria living attached to phytoplankton may not necessarily be functionally distinct to free-living ones, our results indicate that most of the properties tested might at least be of advantage when living in association with cyanobacteria. Additionally, our results indicate that the interaction between heterotrophs and their phytoplanktonic partners appears to be based not only on a single factor, but seems to be much more complex. Therefore, adjacent to the pigment analysis, investigations on the *pufM* genes and their expression need to be done to detect photosynthetic activity. Thus, it might as well be possible to draw more precise conclusions regarding the photosynthetic activity of the AAnP within the cyanobacterial cultures. It also would be useful to analyse the EPS of the *Nodularia* and *Oscillatoria* strains to get precise information of their composition and ratios of carbohydrates to each other.

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FIGURES

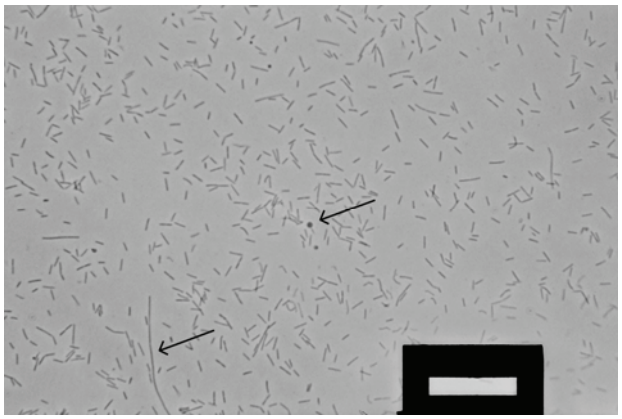

Fig. 1. Photomicrograph of *Muricauda* strain Bo10-09. Window inside the mask corresponds to 20 µm. The arrows indicate one globular structure and a chain formation of *Muricauda* cells.

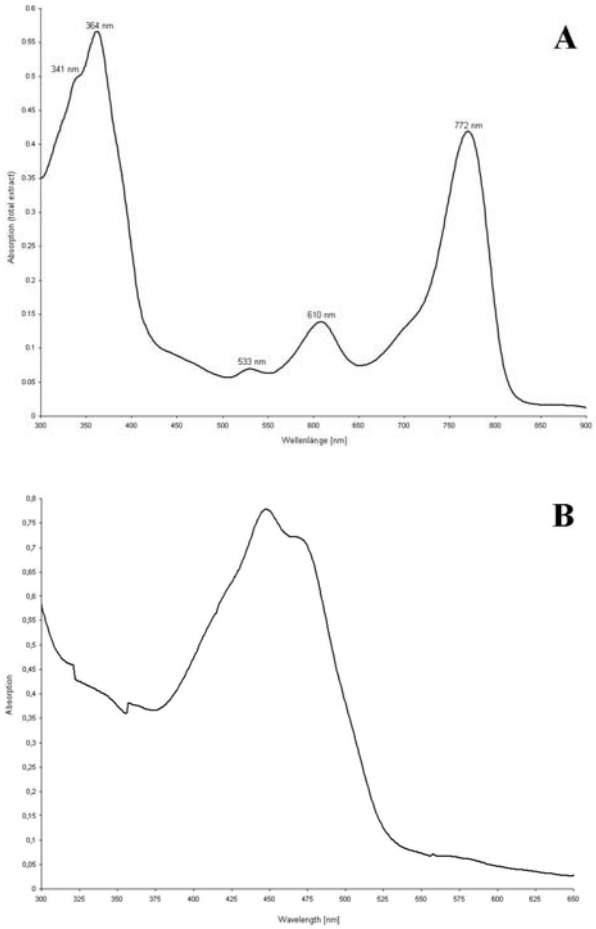

Fig. 2. Absorption spectra of methanol-extracts from *Roseobacter* strain Bo10-20 (A) and *Muricauda* strain Bo10-09 (B).

TABLES

Table 1. Properties of isolated carotenoids of *Porphyrobacter* strain Bo53-33 and *Roseobacter* strain Bo10-20, separated by TLC and detected by HPLC. Maxima and retention times were obtained by a diode array detector (DAD).

No.	Colour of the TLC band	Absorption maxima [nm] (main peaks are underlined)	Retention times recorded [min]	Potential identifications
Porphyrobacter strain Bo53-33				
1	Orange	381 414 419 424 458	1.05	n.i.
2	Light grey	400 409	1.58	n.i.
3	Light yellow	367 388 405 465	1.45	n.i.
4	Light yellow	429 458 479 487	5.44	zeaxanthin
5	Yellow	414 434 446 458 472	6.63	n.i.
6	Yellow	424 452 458 487	7.07	n.i.
7	Yellow	424 440 472 487 512	5.40	n.i.
8	Yellow	419 440 452 472	8.25	n.i.
9	Orange	381 396 405 434	1.27	n.i.
10	Orange	381 396 409 429 465	1.83	n.i.
11	Orange	429 446 452 465	2.34	zeaxanthin or ß,ß-carotene
12	Orange	434 446 465 487 503	4.06	zeaxanthin or ß,ß-carotene
13	Orange	446 472 503 512	6.66	n.i.
14	Pink	487 512 521 542	6.08	n.i.
Roseobacter strain Bo10-20				
15	Pink	429 446 465 495	6.23	n.i.
16	Pink	465 495 542 603	7.64	n.i.
17	Pink	495	8.25	n.i.
18	Pink	446 465 503 521	8.60	n.i.
19	Pink	487	13.43	spheroidenone

n.i. = no identification possible

Table 2. Utilisation of carbon sources by *Porphyrobacter* strain Bo53-33 and *Muricauda* strain Bo10-09 in the api® 50 CH test. Evaluation was carried out 14 (for Bo53-33) or 7 (for Bo10-09) days after inoculation. + acidification of the culture medium, - no acidification

Carbon source / strain	Bo53-33	Bo10-09
Control	-	-
Glycerol	-	-
Erythritol	-	-
D-Arabinose	-	-
L-Arabinose	-	+
D-Ribose	-	+
D-Xylose	-	+
L-Xylose	-	+
D-Adonitol	-	-
Methyl-ßD-Xylopyranoside	-	+
D-Galactose	-	+
D-Glucose	+	+
D-Fructose	-	+
D-Mannose	-	+
L-Sorbose	-	+
L-Rhamnose	+	+
Dulcitol	-	-
Inositol	-	-
D-Mannitol	-	-
D-Sorbitol	-	-
Mehtyl-αD-mannopyranoside	-	+
Mehtyl-αD-glucopyranoside	+	+
N-Acetylglucosamine	-	+
Amygdalin	-	+
Arbutin	-	+
Esculin / ferric citrate	-	-
Salicin	-	+
D-Cellobiose	-	+
D-Maltose	-	+
D-Lactose (bovine origin)	-	+

D-Melibiose	+	-
D-Sucrose	-	+
D-Trehalose	+	+
Inulin	-	+
D-Melezitose	-	+
D-Raffinose	+	+
Amidon (starch)	-	+
Glycogen	-	-
Xylitol	-	-
Gentiobiose	-	+
D-Turanose	+	+
D-Lyxose	-	+
D-Tagatose	-	+
D-Fucose	-	+
L-Fucose	-	-
D-Arabitol	-	-
L-Arabitol	-	-
Potassium gluconate	-	-
Potassium 2-ketogluconate	+	-
Potassium 5-ketogluconate	-	-

4 General Discussion

4.1 Characterisation of heterotrophic bacteria with respect to their association with cyanobacteria

To be able to compare various interactions that can occur between heterotrophs and cyanobacteria, four heterotrophic strains were chosen after their isolation to be investigated in more detail. Two of these strains, *Porphyrobacter* strain Bo53-33 and *Roseobacter* strain Bo10-20, were differing AAnP strains. Strain Bo10-19 was a colourless strain closely related to AAnP, while the coloured bacterium *Muricauda* strain Bo10-09 did not seem to be an AAnP.

4.1.1 Characterisation of *Porphyrobacter*

The genus *Porphyrobacter* was designated in 1993 by Fuerst and co-workers. It includes bacteriochlorophyll containing aerobes and branches with *Erythrobacter longus* within the *Erythrobacteraceae* (Lee et al. 2005) in the *Alphaproteobacteria*. All *Porphyrobacter* species described so far belong to the AAnP (Fuerst et al. 1993; Hanada et al. 1997; Hiraishi et al. 2002; Rainey et al. 2003; Rathgeber et al. 2007; Yoon et al. 2004; Yoon et al. 2006). But even though it has long been known that AAnP often live in association with phytoplankton, to my knowledge this has never been described before for *Porphyrobacter*. So far, only Navarro and co-workers (2008) detected a bacterial community containing *Porphyrobacter* and the unicellular cyanobacterium *Synechococcus* in the ephemeral hypereutrophic Mojave Dessert Playa Lake.

Porphyrobacter strain Bo53-33, described in the present study, derived from the cyanobacterial *Nodularia harveyana* strain Bo53 culture and showed highest 16S gene sequence similarity to *Porphyrobacter dokdonensis* (99% similarity to strain DSW-74^T (Yoon et al. 2006), see manuscript I). Yoon and co-workers (2006) isolated their *Porphyrobacter* strain from the Sea of Japan and did not observe any kind of association with phytoplankton. However, for the strain used in the present study, characteristics were found that are at least advantageous for this way of living. The extracellular amylase production and glucose utilisation (see manuscript III) for example enable the organism to metabolise amylose and hence live on readily available cyanobacterial exopolysaccharides (EPS). The high amount of carotenoids detected in strain Bo53-33 is advantageous when living in environments with an

increased requirement for a protection against photooxidation (Armstrong 1997; Kolber et al. 2001; Yurkov and Beatty 1998), as it is usual for phototrophic organisms. However, the cyanobacterial hosts, which generally move phototactically to light conditions that are approximately 1 – 10% of the daylight (Stal et al. 1985), might provide a certain protection as well.

The most significant properties were found when investigating the occurrence of *Porphyrobacter* and closely related species in various cyanobacterial cultures (see manuscript I and II). These organisms were only found in some cultures. In both *Nodularia* cultures tested they were detected, but in none of the *Oscillatoria* ones, even though these derived from the same sampling site as our *Nodularia harveyana* strain. But 14 days after the cultures of *Nodularia harveyana* strain Bo53 and *Oscillatoria brevis* strain Bo10 were made axenic, *Porphyrobacter* and relatives reoccurred in the Bo53 culture, but as well in the Bo10 culture cells were found sporadically (see manuscript II). This indicates that *Porphyrobacter* is able to live on both cyanobacteria's excretory products and is able to develop fast. However, in the naturally occurring community on *Oscillatoria* competitors might prevent the spread of *Porphyrobacter*. It can be assumed that the competitors grow more slowly than *Porphyrobacter* does and, therefore, would constrain the growth of the latter one in the formally axenic cultures within a certain time as well.

The fact that *Porphyrobacter* was also found in the culture of *Geitlerinema* strain Flo1, deriving from a mangrove area in Florida south of Miami, might be an evidence for an ubiquitous distribution and is in accordance with reports that *Porphyrobacter* isolates were obtained from various worldwide places, such as a brackish hot spring in Japan (Hanada et al. 1997), a hot spring in Portugal (Rainey et al. 2003), and the sea of Japan (Yoon et al. 2004; Yoon et al. 2006). *Porphyrobacter* could not be found in the culture of *Fischerella ambigua* from the department's culture collection, the only fresh water cyanobacterial culture tested. However, this does not necessarily mean that this heterotroph will not occur in any fresh water communities, since Fuerst and co-workers (1993) and Rathgeber and co-workers (2007) isolated their *Porphyrobacter* strains from freshwater.

In summary, there is evidence that strain Bo53-33 occurs in association with cyanobacteria and possesses some capabilities useful for a beneficial coexistence in such a community, but does not depend on such way of living. *Porphyrobacter* seems to be a fast growing, ubiquitously distributed organism, and does not seem to be specialised to a certain living condition. However, *Porphyrobacter* appears to be sensitive to interfere with other bacteria.

4.1.2 Characterisation of *Roseobacter*

The genus *Roseobacter* was established by Shiba in 1991 and belongs to the α-3 subclass of the *Proteobacteria*. This genus is physiologically heterogeneous and it is known to comprise some of the most abundant members of the AAnP, such as the first described species *Roseobacter litoralis* and *Roseobacter denitrificans* (Allgaier et al. 2003; Brinkhoff et al. 2008; Buchan et al. 2005; Oz et al. 2005). However, nowadays it is known that not all *Roseobacter* species are able to produce bacteriochlorophyll (Lafay et al. 1995). It is as well known that members of the *Roseobacter* clade dominate among marine phytoplankton-associated bacteria (Buchan et al. 2005). They are described to be effective colonisers that are able to form biofilms and even inhibit other bacteria by producing antibacterial substances (Bruhn et al. 2007; Gram et al. 2002).

Roseobacter strain Bo10-20, isolated during the present study (see manuscript I), derived from the culture of *Oscillatoria brevis* strain Bo10 and showed high 16S gene sequence similarities with some non-characterised *Roseobacter* clone sequences. However, the most closely related validly described species to strain Bo10-20 was *Sulfitobacter dubius* strain KMM 3554T (Ivanova et al. 2004) (94% similarity).

As previously described for *Porphyrobacter* (see chapter 4.1.1), the physiological and biochemical investigations of *Roseobacter* strain Bo10-20 also revealed characteristics that are useful when living in association with cyanobacteria, such as the possession of several carotenoids and the ability to produce extracellular amylases (see above). Nevertheless, it could be demonstrated that the occurrence of *Roseobacter* in the cyanobacterial cultures (see manuscript I and II) showed some noticeable differences between this heterotroph and *Porphyrobacter*.

Even though the *Roseobacter* clade has been described to be ubiquitously distributed in marine environments (Buchan et al. 2005; Wagner-Döbler and Biebl 2006), in the present study it was not possible to detect *Roseobacter* species in each of the cyanobacterial cultures tested. Among the ten marine Baltic cyanobacterial strains sampled, *Roseobacter* spp. could neither be detected in cultures of *Synechocystis*, *Lyngbya semiplenta*, *Oscillatoria laetevirens*, *Anabaena variabilis*, nor in any *Nodularia* culture. (But it must be mentioned that after the separation of attached and free-living heterotrophs from the *Nodularia harveyana* Bo53 culture, traces of *Roseobacter* cells were found in the supernatants (see manuscript I).) In the culture of *Geitlerinema* strain Flo1, *Roseobacter* was not found either. Additionally, in experiments with axenic cultures, this AAnP did not reappear in all of the *Oscillatoria brevis* cultures after 14 days of incubation. One explanation for the absence in some of the

cyanobacterial cultures might be a lack of certain essential nutrients. However, more likely is the explanation that certain competitors prevent the growth of *Roseobacter* as described for *Porphyrobacter* before (see above). The observation that *Roseobacter* appeared in almost all formally axenic *Nodularia harveyana* cultures, even though originally only scarcely found with this culture, is also in accordance with this assumption that its growth is often inhibited in natural heterotrophic communities, and as well with the observations for *Porphyrobacter* (see chapter 4.1.1).

Nevertheless, the observation that *Roseobacter* was not detected in the culture of the freshwater cyanobacterium *Fischerella ambigua* is in accordance with other reports for freshwater environments (Buchan et al. 2005).

In summary, it can be said that, even though *Roseobacter* strain Bo10-20 exhibited some benefiting features, it was not possible to maintain the behaviour of *Roseobacter* to be an aggressive, ubiquitously occurring coloniser. It became apparent that this organism reacts discriminative to various cyanobacterial cultures or the respective heterotrophic colonisers and in several cases its growth was inhibited.

4.1.3 Characterisation of strain Bo10-19

Strain Bo10-19 was a colourless alphaproteobacterium clustering with the *Rhodobacter* clade with highest 16S gene sequence similarity of only 95% to *Rhodobaca bogoriensis* (see manuscript I). It also derived from a culture of *Oscillaturia brevis* strain Bo10.

Rhodobaca bogoriensis is known to be a yellow to pink coloured purple non-sulphur bacterium, possessing bacteriochlorophyll *a* and carotenoids (Milford et al. 2000; Takaichi et al. 2001). To my knowledge, it has never been described living in coexistence with phytoplankton. However, Navarro and co-workers (2008) were at least able to detect *Rhodobaca* and *Rhodobacter* bacteria in samples from Mojave Desert Playa Lake (California) along with the unicellular cyanobacterium *Synechococcus*.

Strain Bo10-19 did not bear a strong resemblance with the photoheterotrophic *Rhodobaca bogoriensis* as described by Milford and co-workers (2000) and its relatives (Hiraishi and Ueda 1994). In contrast to *Rhodobaca bogoriensis*, strain Bo10-19 was colourless and grew exclusively chemoorganotrophically under aerobic conditions. Light did not seem to influence its growth behaviour (see manuscript III).

Neither extracellular amylases, nor DNases, peptidases, or lipases were found to be produced by strain Bo10-19 by testing with standardised plating methods (see manuscript III). This might indicate that within the cyanobacterial cultures this strain lives on very specific substances produced by the cyanobacteria. However, it should be noted that it was possible to cultivate strain Bo10-19 and related ones in pure culture on undefined media (see manuscript I).

Even though it was not possible to design a probe specific for strain Bo10-19, other experiments revealed that it is unlikely that Bo10-19-like bacteria occurred in *Nodularia harveyana* Bo53 culture as well. In the course of the strain's isolation from the cyanobacteria, several isolates with similar morphological properties were found in the *Oscillatoria brevis* Bo10 culture, being closely related to strain Bo10-19 when compared by fingerprinting methods. However, from the *Nodularia harveyana* Bo53 culture no similarly looking isolates were obtained (see manuscript I).

In conclusion, it can be said that strain Bo10-19 seems to be a heterotroph which is very specifically adapted to some particular living conditions such as association with *Oscillatoria brevis*. Since the 16S gene sequence similarity between *Rhodobaca bogoriensis* and strain Bo10-19 is only 95% and they do not share many other features either, strain Bo10-19 might comprise a new species. However, to pinpoint this assumption some more physiological investigations need to be performed.

4.1.4 Characterisation of *Muricauda*

The genus *Muricauda* was described in 2001 by Bruns and co-workers and belongs to the *Bacteroidetes* group. The genus comprises four species that are known to be marine, yellow pigmented, and strictly aerobic or facultatively anaerobic bacteria. Strains described so far are cosmpolitans and occurred e.g. in the German Wadden Sea (Bruns et al. 2001), the Sea of Japan (Yoon et al. 2005), the Yellow Sea, Korea (Yoon et al. 2008), and the Mediterranean Sea (Agogué et al. 2005). Even though it is known for some of their relatives (Delucca and McCracken 1977; Grossart et al. 2005), this way of living has never been shown for any of the *Muricauda* strains described. Nevertheless, Bruns and co-workers (2001) first observed appendages which they assumed to be meant for colonisation of given substrates.

Muricauda strain Bo10-09 was isolated from a culture of *Oscillatoria brevis* strain Bo10 and showed highest 16S gene sequence similarity with *Muricauda aquimarina* (Yoon et al. 2005)

(99%, see manuscript I). The biochemical and physiological investigations (see manuscript III) revealed that Bo10-09 contains one yellow carotenoid. Furthermore, this strain was able to metabolise almost all sugars offered, which seems to be advantageous when living on cyanobacterial EPS, but none of the alcohols tested. Extracellular enzymes detected were amylases and DNases. Especially the production of amylases is beneficial for the utilisation of cyanobacterial excretory products. Additionally, structures were observed by light microscopical observation studies that might be similar to those vesicle-like structures at the end of the appendages of *Muricauda ruestringensis* as described by Bruns and co-workers (2001) and Müller and co-workers (2001) (see manuscript III). If, as suggested by Bruns and co-workers (2001), the structures are used to attach to surfaces, they might represent a special equipment of *Muricauda* strain Bo10-09 to live on cyanobacteria. The results of our occurrence and growth experiments (see manuscript I and II) confirm these indications.

Muricauda was not found in any of the unicellular Baltic cyanobacterial cultures of the department's culture collection tested, but in most of the filamentous ones (both *Oscillatoria* strains Bo10 and Bo17 as well as both *Nodularia* strains Bo53 and Fä19). This might indicate special adaptations to the filamentous cyanobacteria, but needs further investigations. It is not surprising that *Muricauda* was found in the Atlantic *Geitlerinema* strain Flo1, but not in the freshwater strain *Fischerella ambigua*, since this genus is known to be ubiquitously distributed in marine environments but does not occur in freshwaters. Among the 14 days old formally axenic cyanobacterial cultures, *Muricauda* had reappeared in all of the *Nodularia harveyana* Bo53 cultures but only in six of the ten *Oscillatoria brevis* Bo10 cultures (see manuscript II). In all cultures to which *Muricauda* cells were added, they had reached stable abundances of 1 - 2% (*Nodularia harveyana* strain Bo53 cultures) and ~ 20 % (*Oscillatoria brevis* strain Bo10 cultures) after 14 days of cultivation, unlike *Porphyrobacter* and *Roseobacter* had in the same experiment. This might indicate that *Muricauda* is a more effective coloniser than the others are.

Rashidan and Bird (2001) suggested for some cyanobacteria-associated *Cytophaga* bacteria, which are closely related to *Muricauda* strain Bo10-09, to have a rather detrimental effect on the cyanobacteria. However, this could not be confirmed for *Muricauda* strain Bo10-09 which did not have noticeable effects different from those of the other heterotrophs tested (see manuscript II).

To sum up, the results obtained with *Muricauda* indicate that this organism is a fast and effective coloniser that is widely distributed among filamentous marine cyanobacteria-associated communities, but seems only in the case of the resettlement on *Oscillatoria brevis*

Bo10 cultures to be inhibited by some faster competitors. Compared with the three other heterotrophic strain investigated in this study (see chapter 4.1.1, 4.1.2, and 4.1.3), strain Bo10-09 seemed to be the one that is best adapted for living in association with cyanobacteria.

4.2 Characterisation of cyanobacteria with respect to their association with heterotrophs

Both cyanobacterial strains used for comparison (*Nodularia harveyana* strain Bo53 and *Oscillatoria brevis* strain Bo10) derived from the same sampling site at the German Baltic Sea coast and were sampled at the same time (Rethmeier 1995). *Nodularia harveyana* as well as *Oscillatoria* in general have been known before to be mat-forming cyanobacteria (de los Ríos et al. 2004; Stal et al. 1985; Stal et al. 2003). These cyanobacteria are known to produce large amounts of EPS which are involved in the formation of a matrix to embed and protect the mat organisms. Additionally, the cyanobacterial filaments form networks to stabilise the mats (de los Ríos et al. 2004; Paerl et al. 2000). These structures are known to contain the biocomplexity to allow complete nutrient cycles (Canfield and Des Marais 1993; Paerl et al. 2000). The budgets for the main nutrient cycles have already been identified. The main part of the carbon present within the mats derives from primary carbon fixation of oxygenic and anoxygenic phototrophs and minor parts of secondary carbon fixation of non-photosynthetic autotrophic and heterotrophic bacteria. The inorganic carbon is known to originate from aerobic and anaerobic respiration and heterotrophy within the mat as well as from the overlaying water layers. Oxygen is also known to be mainly produced and consumed within the mats, and this procedure is assumed for other nutrient elements as well (Canfield and Des Marais 1993). This means that within such consortia diverse metabolic processes occur that could inhibit each other, for example by the appearance of detrimental by-products (Paerl and Pinckney 1996). On this account, the temporal and spatial arrangements of the activities within the mats are crucial for its functionality.

In both cyanobacterial cultures examined in this study, the heterotrophs investigated occurred as free-living ones in the culture medium as well as associated with the cyanobacterial trichomes in form of large aggregates. By applying the new developed method to get axenic

cultures it became apparent that in the *Nodularia* culture the aggregates of heterotrophs seemed to be much more tightly attached to the filaments than in the *Oscillatoria* culture.

It has been known before that cyanobacteria die when the amount of heterotrophic associated bacteria decreases to a certain amount (Palinska et al. 1999). This was also observed for *Nodularia harveyana* strain Bo53 and *Oscillatoria brevis* strain Bo10 during the procedure to obtain axenic cyanobacterial cultures. Nevertheless, it could be shown that at least for *Oscillatoria brevis* strain Bo10 the addition of single heterotrophic pure cultures was also detrimental (see manuscript II), which might indicate that it is important for *Oscillatoria brevis* that none of the heterotrophs gets an exceedingly strong influence on this bacterial community.

The approximate composition of heterotrophs within the two cyanobacterial cultures was alike (see manuscript I). In both cultures, *Alphaproteobacteria* dominated the community of heterotrophs, followed by *Bacteroidetes* bacteria and *Gammaproteobacteria*. *Betaproteobacteria*, *Firmicutes*, or *Archaea* were found in neither of the cyanobacterial cultures, even though the occurrence of *Betaproteobacteria* and *Firmicutes* associated with *Nodularia* has previously been described (Salomon et al. 2003; Tuomainen et al. 2006). Detailed investigations with more specific oligonucleotide probes revealed that *Roseobacter*, *Erythrobacteraceae*, and *Muricauda* cells occurred to similar amounts of less than 10% within the *Nodularia* culture. A higher percentage of *Roseobacter* cells (10 – 20 %) existed within the *Oscillatoria* culture, whereas *Erythrobacteraceae* could not be found. Having made the two cyanobacterial cultures axenic, the reoccurrence of the heterotrophs within the cultures was checked after 14 days. In the *Nodularia* culture, all heterotrophs determined and investigated during the present study had reappeared, even though they did not exhibit the same percentages as found before. This is in contrast to the *Oscillatoria* culture, in which the heterotrophic composition had changed completely. *Erythrobacteraceae* appeared in all set ups, whereas *Roseobacter* and *Muricauda* could only be found in some of them. Since also the occurrences of the heterotrophs within other studied cyanobacterial cultures of the department's culture collection partially differed considerably to those in *Nodularia harveyana* strain Bo53 and *Oscillatoria brevis* strain Bo10, it became obvious that the heterotrophic composition does not necessarily depend on obvious features of the cyanobacterial cultures, such as origin of the strains, availability of nitrogen in the culture medium, or formation of heterocysts. It is much more likely that the heterotrophs depend either on very specific cyanobacterial excretory products or on their heterotrophic companions.

In summary, it seems that *Nodularia* has a much more stable heterotrophic community which is also much more difficult to detach in comparison to that of *Oscillatoria*, though *Oscillatoria* is much more sensitive to changes within the heterotrophic composition. *Oscillatoria* is known to be one of the cyanobacteria starting mat-formation (Stal et al. 1985). It is likely that consortia within developing young mats are not as stable as they are in older ones and that they undergo a succession. As a consequence, it makes sense that *Oscillatoria* does not seem to be specified to a certain heterotrophic company. However, it is astonishing that *Oscillatoria* reacts more intensely to changes than the heterocystous *Nodularia*. The latter is much more dependent on a stable community in order to be capable of protecting its heterocysts from evolving oxygen. This assumption is in coincidences with the results gained in the present study.

4.3 Conclusions

For cyanobacteria it had been proven before that they depend on at least some of their associated heterotrophic partners (Palinska et al. 1999). In the present study, it was shown that unique heterotrophic strains can as well have a detrimental effect on the cyanobacteria. In nature, this seems to be prevented by a self-regulation that the heterotrophic community exercises in the surroundings of *Nodularia harveyana* and *Oscillatoria brevis*. It has been shown for the heterotrophs investigated in the present study that they were shown to have some advantages when living in association with cyanobacteria, even though all of them belonged to genera known to occur free-living as well. Thus, it cannot be excluded that there are also heterotrophs that are dependent on living in those communities. In general, it can be said that the relations within these associations seem to be very complex and thus cannot be elucidated without performing a multitude of adjacent experiments. Additionally, it was demonstrated that differing cyanobacteria as well as heterotrophs showed various adaptations within the aggregates, and that it is not possible to predict the mutual effects only from the knowledge of common properties, neither for the cyanobacteria nor for the heterotrophs.

Up to now, our knowledge about the phytoplanktonic functions in the Baltic Sea had mainly been based on investigations of the conspicuous blooming events. Nevertheless, nowadays, in times of increasing anthropogenic inshore pollution, the mat-forming benthic cyanobacteria gain more and more influence (Schiewer 2008). To be able to estimate the effects that these

cyanobacteria might have for the whole ecosystem, it is important to have a close look on these organisms and their ecological characteristics. The data set compiled in the present study might at least provide first impressions of the complex inter-specific connections and functions within these habitats and thus will give a basis for further investigations.

4.4 Future prospects

The present study gave important information about the relationship between cyanobacteria and their heterotrophic associates. Nevertheless, there are still several aspects that remain ambiguous. Some adjacent experiments should be conducted which might be helpful to further illuminate the mutual connections.

The choice of a culture dependent set up for the identification of the heterotrophic bacteria might cause the non-discover of certain bacterial groups. To obtain a more intense view upon the compositions of the heterotrophic communities, it will be useful to additionally construct a clone library.

Being able to judge the influence of heterotrophs upon cyanobacteria, it is necessary to take into consideration that in nature the heterotrophic species composition within cyanobacterial aggregations undergoes seasonal changes (Mašín et al. 2006) and shifts during progression of a bloom as well (Rashidan and Bird 2001), whereas in lab cultures presumably a stationary community had developed. To get an impression about when certain defined heterotrophs appear, disappear, or remain constant in the community, it is necessary to take natural samples at different seasons for comparative investigations. These data would also give additional information about the interaction of the heterotrophs within the communities.

For a more significant survey about the abundances of heterotrophs in association with different cyanobacteria, it is indispensable to check many more deposited cultures for the occurrence of heterotrophs.

Another interesting approach to be conducted in the future would be to characterise the EPS of the *Nodularia harveyana* strain Bo53 and *Oscillatoria brevis* strain Bo10 and to compare the range of detected substances with respect to their utilisation by heterotrophic bacteria. These data might indicate a more definite connection between the cyanobacteria and their heterotrophic partners. Other physiological experiments, such as the examination of the influence of light intensity or oxygen availability on the heterotrophs' growth behaviour, may give valuable information on positive advantages for the heterotrophs when living in association with phytoplankton.

For a better understanding concerning the influence of heterotrophs upon cyanobacteria, it might be interesting to investigate the growth behaviour of the cyanobacteria after having been made axenic and when mixed with combinations of living heterotrophic bacteria, dead bacteria, or single bacterial components.

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6 Annex

Table 1. List of all heterotrophic strains obtained from the culture of *Oscillatoria brevis* strain Bo10. ASN_{III} = artificial seawater nutrient culture medium of half salinity (see manuscript I), ME = meat extract, YE = yeast extract, MB = Difco™ Marine Broth culture medium, AB = BBL™ Actinomyces Broth culture medium, n.d. = not determined

Isolate	Colony morphology	Cell morphology	Culture medium grown in
Bo10-01	Thick, milky, pale beige	Short rods	ASN_{III} + 1% ME
Bo10-03	Flat, milky, white	Rods of medium length	MB
Bo10-04	Thick, white	Short rods	ASN_{III} + 1% ME
Bo10-06	Thick, milky, pale beige	Rods of medium length	MB
Bo10-07	Lucent, yellow	Rods of medium length	MB, ASN_{III} + 1% ME
Bo10-08	White	Rods of medium or long length	AB
Bo10-09	Yellow orange	Long rods, formation of chains	MB, ASN_{III} + 1% ME
Bo10-10	Pale yellow	Rods of medium length	MB
Bo10-11	Thick, milky, pale beige	Rods of medium or long length	ASN_{III} + 1% ME
Bo10-12	Thick, beige or salmon	Short rods	ASN_{III} + 1% YE
Bo10-13	Yellow, slimy	Thin rods of medium length, formation of chains	MB, ASN_{III} + 1% ME
Bo10-14	Small, thin, milky, white, uneven edge	n.d.	ASN_{III} + 1% ME, ASN_{III} + 1% YE
Bo10-15	Thick, beige	Rods of medium length	MB, ASN_{III} + 1% ME
Bo10-16	Beige, greenish	Thick, short rods	MB

Bo10-17	Beige	Rods of medium length	BM
Bo10-18	Beige	Short rods	MB, ASN_{III} + 1% YE
Bo10-19	Thick, milky, pale beige, flattened even rim	Short rods	ASN_{III} + 1% ME
Bo10-20	Pink	Short rods	ASN_{III} + 1% ME
Bo10-21	Small, thin, milky, white, uneven rim	Rods of medium or long length	MB
Bo10-22	Yellow orange	Long rods, formation of chains	MB, ASN_{III} + 1% ME
Bo10-23	Pink	Rods of medium length	ASN_{III} + 1% ME

Table 2. List of all heterotrophic strains obtained from the culture of *Nodularia harveyana* strain Bo53. ASN_{III} = artificial seawater nutrient culture medium with halved salinity (see manuscript I), ME = meat extract, YE = yeast extract, MB = Difco™ Marine Broth culture medium, AB = BBL™ Actinomyces Broth culture medium

Isolate	Colony morphology	Cell morphology	Culture medium grown in
Bo53-01	Milky, white	Rods of medium length	MB
Bo53-03	Thick, white, lucent	Rods of medium length	MB, ASN_{III} + 1% YE
Bo53-05	Beige, uneven edge	Rods of medium length	ASN_{III} + 1% YE
Bo53-10	Thin, beige, even, uneven edge	Rods of medium length	ASN_{III} + 1% YE, ASN_{III} + 1% ME
Bo53-13	Thin, beige, even, uneven edge	Rods of medium length	ASN_{III} + 1% YE
Bo53-15	Very thin, clear	Rods of medium or short length	ASN_{III} + 1% YE, ASN_{III} + 1% ME
Bo53-16	Thin, beige, uneven centre	Long rods	ASN_{III} + 1% ME
Bo53-17	Thick, beige, lucent	Short rods	ASN_{III} + 1% ME

Bo53-19	Very thin, clear	Short or medium length rods	ASN_{III} + 1% YE, ASN_{III} + 1% ME
Bo53-20	Thick, pale beige	Long, thin rods	ASN_{III} + 1% ME
Bo53-21	Thin, beige, even	Short rods	ASN_{III} + 1% ME
Bo53-23	Thin, beige, even	Short rods	ASN_{III} + 1% ME
Bo53-31	Thick, salmon coloured centre, white edge	Rods	ASN_{III} + 1% YE
Bo53-32	Beige salmon coloured, uneven	Long rods	ASN_{III} + 1% YE
Bo53-33	Rubiginous	Short rods	MB, ASN_{III} + 1% YE
Bo53-34	Greenish yellow	Rods of medium length	MB, ASN_{III} + 1% YE
Bo53-36	Thick, beige	Short rods	ASN_{III} + 0.1% YE, ASN_{III} + 1% YE
Bo53-37	Dark yellow	Rods of short or medium length	ASN_{III} + 1% ME
Bo53-38	Orange, uneven edge	Very long thin rods, formation of chains	MB, ASN_{III} + 1% YE
Bo53-39	Milky, white	Rods of medium length	AB
Bo53-40	Orange	Short rods	ASN_{III} + 0.1% ME, ASN_{III} + 1% ME
Bo53-40-1	Rubiginous	Rods of short or medium length	ASN_{III} + 0.1% ME, ASN_{III} + 1% ME
Bo53-40-2	Beige	Very short rods	ASN_{III} + 0.1% ME, ASN_{III} + 1% ME
Bo53-40-3	Beige	Thick, rods of short or medium length	ASN_{III} + 0.1% ME, ASN_{III} + 1% ME
Bo53-41	Orange	Rods of medium length, formation of chains	MB, ASN_{III} + 1% YE
Bo53-44	Beige	Rods of medium length	ASN_{III} + 1% YE

Bo53-45	Yellow orange, lucent	Long thin rods, formation of chains	MB, ASN_{III} + 1% ME
Bo53-46	Beige	Long rods	ASN_{III} + 0.1% YE, ASN_{III} + 1% YE
Bo53-47	Rubiginous	Rods of medium or short length	MB, ASN_{III} + 1% ME
Bo53-49	White	Thick short rods	ASN_{III} + 1% YE

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Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel „Molecular Biological and Physiological Investigations of Heterotrophic Bacteria Associated with Marine Filamentous Cyanobacteria“

1) ohne unerlaubte Hilfe angefertigt habe
2) keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
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