Impact of environmental factors on bacterioplankton communities

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Gutachter

Prof. Dr. Klaus Jürgens

Leibniz-Institut für Ostseeforschung

Sektion Biologische Meereskunde

Prof. Dr. Jakob Pernthaler

Universität Zürich

Institut für Pflanzen- und Mikrobiologie

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Summary

Aquatic bacteria are main drivers of biogeochemical cycles and contribute predominantly to organic matter and nutrient recycling. As a high biodiversity is assumed to stabilize ecosystem functioning, it is necessary to understand the bacterial community dynamics and their structuring factors. It is known that different taxa are dominant across different habitats and seasons. This indicates an occurrence of species sorting by community structuring environmental factors. A first attempt for the understanding of bacterial distribution is to test for a correlation between microbial composition and measured environmental variables. In order to get further insights into the impact of environmental factors on bacterial communities, this thesis assessed the influence of major structuring drivers by using 16S rRNA gene amplicon sequencing, bacterial bulk parameters and interdisciplinary approaches in laboratory experiments and field studies.

In a field study in the Benguela upwelling system, the influence of different levels of primary production and the planktonic succession on bacterial community composition and its development was investigated. Community analysis revealed a clustering of different microbial assemblages along aging upwelled water. This zonation was mainly driven by phytoplankton composition and abundance and the spatial differences were comparable with a temporal succession that occurs during phytoplankton blooms in temperate coastal waters. A dominance of *Bacteroidetes* and *Gammaproteobacteria* was observed during algal blooming and high abundance of "*Pelagibacterales*" was found in regions with low algal abundance. Overall, this study highlightes the strong impact of quality and quantity of phytoplankton and nutrients on the bacterial communities.

A laboratory experiment with Baltic Sea water was performed to better understand the potential impact of rising temperature and CO_2 on planktonic bacteria. The development of the bacterial community composition was followed in bifactorial mesocosm experiments during a diatom bloom in autumn and a phytoplankton bloom in

summer. The results confirmed that phytoplankton succession and temperature were the major variables structuring the bacterial community. The impact of CO_2 on the broad community was weak but high-resolution community analyses revealed a strong effect on specific bacterial groups, which might play important roles in specific organic matter degradation processes.

The response of bacterial communities to a disturbance by a saline intrusion could be investigated during a major Baltic inflow event. Community structuring factors were dominated by mixing of the inflow water with the former bottom water. Although the inflow had a selecting effect on the bacterial community, some immigrated taxa showed increased potential activity and seem to profit from changing environmental conditions. These results suggest a potential impact of inflow events on bacterial functions and therefore on biogeochemical processes.

Altogether, the results confirm the strong structuring effects of environmental conditions on bacterial community composition. Furthermore, high-resolution sequencing enabled an identification of specific affected taxa, which in turn give first clues for the impact of the investigated factors on specific bacterial functions.

Zusammenfassung

Aquatisch Bakterien sind Hauptbestandteil von biogeochemischen Kreisläufen und spielen eine wichtige Rolle für das Recycling von organischer Substanz und Nährstoffen. Da angenommen wird, dass eine hohe Artenvielfalt die Funktion von Ökosystemen stabilisiert, ist es notwendig die Dynamik bakterieller Gemeinschaften und die strukturierenden Faktoren zu verstehen. Es ist bekannt, dass verschiedene Taxa in verschiedenen Lebensräume und Jahreszeiten dominieren. Dies deutet auf ein „species sorting“ durch die strukturierenden Umweltfaktoren hin. Ein erster Ansatz zum Verständnis der bakteriellen Verteilung ist die Untersuchung auf Korrelationen zwischen der Zusammensetzung mikrobieller Gemeinschaften und gemessenen Umgebungsvariablen. Um weitere Einblicke in die Auswirkungen von Umweltfaktoren auf die Bakteriengemeinschaften zu erhalten, wurde in dieser Arbeit der Einfluss der wichtigsten strukturierenden Faktoren unter Verwendung von 16S rRNA Gen Amplikon Sequenzierung, bakterieller Bulk-Parameter und interdisziplinärer Ansätze in Laborexperimenten und Feldstudien untersucht.

In einer Feldstudie im Benguela Auftriebssystem, wurde der Einfluss verschiedener Entwicklungsstufen der Primärproduzenten und der Plankton Sukzession auf Zusammensetzung und Entwicklung der Bakteriengemeinschaft untersucht. Gemeinschaftsanalysen ergaben Gruppierungen unterschiedlicher mikrobieller Gemeinschaften entlang alternden Auftriebswassers. Diese Zonierung wurde hauptsächlich von Phytoplankton Zusammensetzung und Abundanz angetrieben und die räumlichen Unterschiede waren vergleichbar mit einer zeitlichen Sukzession, die während Phytoplanktonblüten in gemäßigten Küstengewässern auftritt. Eine Dominanz von *Bacteroidetes* und *Gammaproteobacteria* wurde während der Algenblüte beobachtet und eine hohe Abundanz von "*Pelagibacterales*" wurde in Regionen mit geringer Algen

Abundanz gefunden. Insgesamt unterstreicht diese Studie die starke Wirkung von Qualität und Quantität von Phytoplankton und Nährstoffen auf die Bakteriengemeinschaften.

Um die möglichen Auswirkungen von Erwärmung und erhöhter CO_2 Konzentrationen zu verstehen, wurde ein Laborexperiment mit Ostseewasser durchgeführt. Die Entwicklung der Zusammensetzung der Bakteriengemeinschaft wurde in bifaktorialen Mesokosmosexperimenten während einer Kieselalgenblüte im Herbst und einer Phytoplanktonblüte im Sommer untersucht. Die Ergebnisse bestätigten, dass Phytoplanktonsukzession und Temperatur den größten Einfluss auf die Bakteriengemeinschaft haben. Die Auswirkungen von CO_2 auf der gesamte Gemeinschaft war schwach, aber hochauflösende Gemeinschafts-Analysen konnten eine starke Wirkung auf bestimmte Bakteriengruppen aufgedecken, die eine wichtige Rolle bei dem Abbau organischen Materials spielen könnten.

Die Reaktion der Bakteriengemeinschaften auf eine Störung durch Eindringen von Salzwasser konnte im Rahmen eines großen Einstromereignisses in der Ostsee untersucht werden. Gemeinschaftsstrukturierende Faktoren wurden von der starken Durchmischung des Einstromwassers mit dem ehemaligen Bodenwasser dominiert. Obwohl der Einstrom einen Selektionseffekt auf die Bakteriengemeinschaft hatte, zeigten einige eingewanderte Taxa eine erhöhte potentielle Aktivität und scheinen von sich ändernden Umweltbedingungen zu profitieren. Diese Ergebnisse deuten auf einen möglichen Einfluss von Einstromereignissen auf bakterielle Funktionen und damit auf biogeochemische Prozesse hin.

Insgesamt bestätigen die Ergebnisse die starken strukturierenden Effekte der Umweltbedingungen auf die bakterielle Gemeinschaftszusammensetzung. Darüber hinaus ermöglichte hochauflösende Sequenzierung eine Identifizierung spezifischer betroffener Taxa, die wiederum einen ersten Anhaltspunkt für die Auswirkungen der untersuchten Faktoren auf bestimmte Bakterienfunktionen liefern.

Introduction

The role of bacteria in aquatic systems

Aquatic ecosystems account for >70% of the Earth's surface and provide various services for human populations (Costanza *et al.*, 1997). Planktonic microorganisms dominate these ecosystems in terms of their abundance and biomass. A liter of sea water contains approximately 10^6 eukaryotic cells (Brown *et al.*, 2009), 10^8 prokaryotic cells (Whitman *et al.*, 1998) and 10^9–10^{11} virus-like particles (Wilhelm and Matteson, 2008). The large and diverse pool of aquatic bacteria within the global ocean is estimated to consist of > 2×10^6 species (Curtis *et al.*, 2002) that carry out an enormous variety of metabolic functions (DeLong, 2009; Gilbert and Dupont, 2011; Ferrera *et al.*, 2015). The major biogeochemical processes of the planet are sustained by planktonic microorganisms (e.g., Falkowski *et al.*, 2008). Phytoplankton perform the majority of primary production in the ocean and nearly half of the net primary production on Earth (Field, 1998). Heterotrophic prokaryotes and protists together form the "microbial loop" (for details see Figure 1), contributing predominantly to organic matter and nutrient recycling (Azam *et al.*, 1983; Pernthaler, 2005; Pomeroy *et al.*, 2007). Higher biodiversity is assumed to be a strong stabilizing force for aquatic ecosystems to resist and recover from perturbation both by maintaining ecosystem functioning despite species loss and by diversifying the responses to this perturbation (Loreau *et al.*, 2001; Carrara *et al.*, 2015). Studies of the biodiversity of aquatic environments are therefore necessary for understanding, monitoring, and anticipating these processes and their sustainability (Duffy and Stachowicz, 2006).

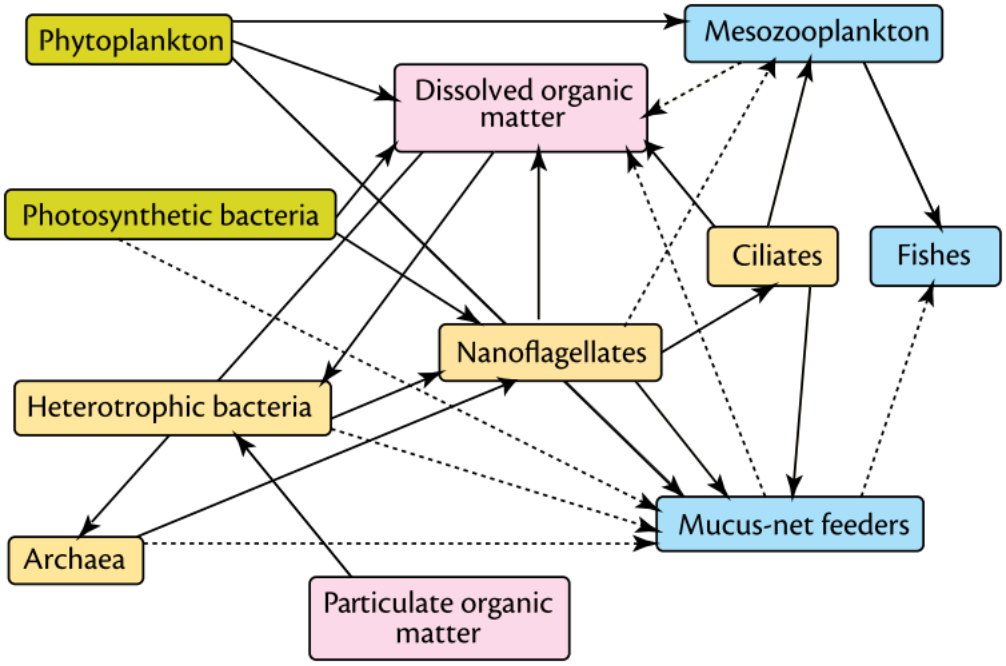

Figure 1: Simplified diagram of the ocean's food web, showing the dominant roles of the microbial loop. The major fluxes of carbon and energy are shown as continuous lines; fluxes usually of lesser magnitude are delineated by broken lines. Other than mesozooplankton and fish (all blue boxes), the boxes represent organisms that are a part of the microbial loop (green = photosynthetic and yellow = heterotrophic) (from Pomeroy *et al.*, 2007).

Phylogenetic diversity of aquatic bacteria

In contrast to the complexity of higher life forms, the morphology of bacteria is too simple to be used in their identification. The diversity of aquatic bacteria was previously elucidated by microscopy studies of isolates from agar plates, followed by physiological and biochemical tests. However, it is now appreciated that this isolation approach promotes the growth of specific bacteria and that these cultured species represent only a minor fraction of the existing diversity. Staley and Konopka (1985) called this phenomenon the ''great plate count anomaly,'' a problem that was subsequently addressed by the development of culture-independent techniques, which opened up new possibilities to assess the community structure of aquatic bacteria. These studies rely on the use of genetic markers, such as rRNA, to provide information about the phylogenetic relationships between species. The first attempts to characterize environmental samples by studying rRNA, based on its conserved function and universality among living species,

were made in the 1980s (e.g., Stahl et al., 1984, 1985). Initially, 5S rRNA molecules were used to analyze the bacterial community, but the information content in its ~120 nucleotides is relatively small. The average bacterial 16S rRNA has a length of 1,500 nucleotides, which provides sufficient information for reliable phylogenetic analyses (Pace *et al.*, 1986).

Analyses of the clone sequences of aquatic samples using a 16S rRNA-based approach confirmed the large number of uncultured species (Giovannoni, 1990). Following cloning studies revealed high quantities of the *Alphaproteobacteria* lineages SAR11, *Roseobacter* and SAR86 and the *Gammaproteobacteria* lineage SAR86 (Giovannoni and Rappé, 2000). However, traditional DNA-sequencing (Sanger *et al.*, 1977) can only sequence individual specimens and is thus both time-consuming and limited in its ability to identify low-abundance members of bacterial communities. With the advent of next-generation sequencing (NGS) platforms, it became possible to recover the high diversity in an environmental sample by the parallel sequencing of DNA from multiple templates. Using NGS it has become possible to characterize samples from coastal waters and from the surface waters of the open ocean (Rusch *et al.*, 2007; Teeling *et al.*, 2012).

Nonetheless, our accumulated knowledge of bacterial diversity has far outpaced our ability to assess the function of these organisms, given the slow advances in methods to culture representative marine bacteria. An alternative strategy to reveal the ecological functions of bacterial populations is to analyze their patterns of distribution in the ocean along spatial gradients.

Microbial biogeography

Microbial biogeographical studies explore the spatial and temporal distributions of microbes, their diversity and dispersal patterns, and their species interactions in different environments (Martiny *et al.*, 2006). Another application is Experimental biogeography, using mesocosm experiments to test Predictions from theories in biogeography.

Depending on the chosen approach, investigations of bacterial diversity will yield different types of information. For example, an analysis of species composition and relative abundance at a single site will indicate the extent of resource partitioning

between species; if these parameters are studied at multiple sites, then insights into the spatial and temporal organization of these resources will be obtained.

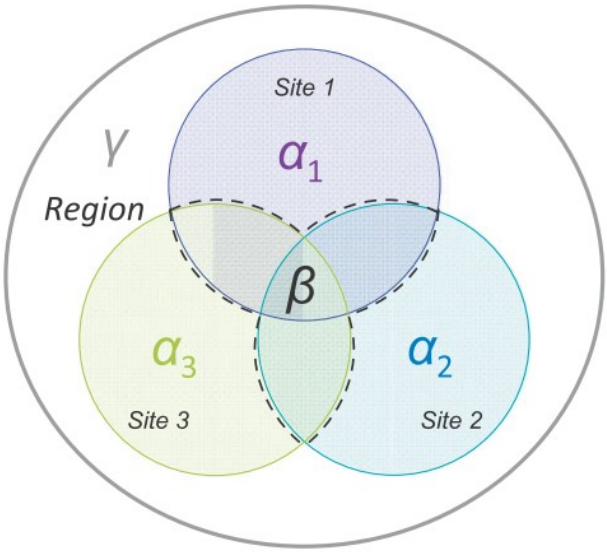

Figure 2: Scheme of alpha (colours), beta (black dotted line) and gamma (grey) diversity (from Zinger *et al.*, 2012).

The diversity within a single location or sample is referred to as alpha diversity and it is often measured as species richness (i.e., the number of species) rather than as species evenness (the extent of species dominance) (Figure 2). However, species richness is strongly sensitive to the sampling effort and requires standardized samples or the use of estimators, such as Chao1 or ACE, that correct undersampling biases. Evenness is less affected by undersampling biases and is usually assessed with Simpson's or Pielou's indices or rank abundance curves (review in Magurran, 2004)

Beta diversity is the difference in diversity or community composition between two or more locations or samples and it is determined by considering species composition alone. It makes use of incidence data and associated metrics, such as the Jaccard or Sorensen similarity indices; alternatively, it may be based on evaluations of the relative abundances of species using Bray–Curtis or Morisita–Horn dissimilarity measures (Ramette, 2007; Anderson *et al.*, 2011).

Gamma, or regional diversity is similar to alpha diversity but it is applied to a larger area, one that encompasses the many units under study.

What controls bacterial diversity in aquatic ecosystems?

The results of biogeographical studies have shown that there are differences in the dominant microbial taxa across habitats and over seasons (Nemergut *et al.*, 2011; Caporaso *et al.*, 2012). Baas-Becking (1934) postulated that, with respect to microorganisms, "everything is everywhere, but the environment selects." This was later formulated as "species sorting" in a metacommunity framework (Holyoak *et al.*, 2005) and it implies that all microbial groups and species are present ubiquitously, but their responses to external factors determine their abundance at a certain location. However, the "everything is everywhere" hypothesis has recently been challenged as molecular evidence has revealed a high degree of cryptic diversity, restricted dispersal, and biogeographic patterns in a variety of prokaryotes (Martiny *et al.*, 2006). A recent conceptual synthesis in community ecology (Vellend, 2010) grouped all processes affecting community assembly into four basic categories: (i) diversification, in which diversity changes by the generation of new genetic variation, (ii) dispersal, in which diversity is determined by the movements of organisms across space, (iii) selection, in which changes in community structure are caused by deterministic fitness differences between taxa, and (iv) drift, in which the relative abundances of the different taxa within a community stochastically change through time.

As the relative importance of these four categories cannot be easily determined, a first approach is to test for a correlation between microbial composition and measured environmental variables as evidence for contemporary selection. Several recent studies have shown that different habitat types (lakes, soil, seawater, living hosts, etc.) harbor different species of bacteria (Chaffron *et al.*, 2010; Tamames *et al.*, 2010; Nemergut *et al.*, 2011). Thus, bacteria are not randomly distributed over the globe; rather, species sorting seems to occur at the global scale, with community-structuring occurring in the form of factors well known as top-down effects (e.g. grazing, viral attack) (Jürgens and Matz, 2002; Pernthaler, 2005; Wilhelm and Matteson, 2008) and bottom-up effects (depth, salinity, temperature, pH, hydrological conditions, organic matter, phytoplankton interactions, and other biological and physical factors) (Field *et al.*, 1997; Crump *et al.*, 2003; Kent *et al.*, 2007; Fuhrman *et al.*, 2008; Herlemann *et al.*, 2011; Fortunato *et al.*, 2013) (Figure 3). On temporal scales, seasonal succession and the annual reassembly of communities have

been shown in both freshwaters and marine waters (Fuhrman *et al.*, 2006; Carlson *et al.*, 2009; Crump *et al.*, 2009; Andersson *et al.*, 2010). The following section describes the impact of the major structuring drivers on bacterial activity and community composition.

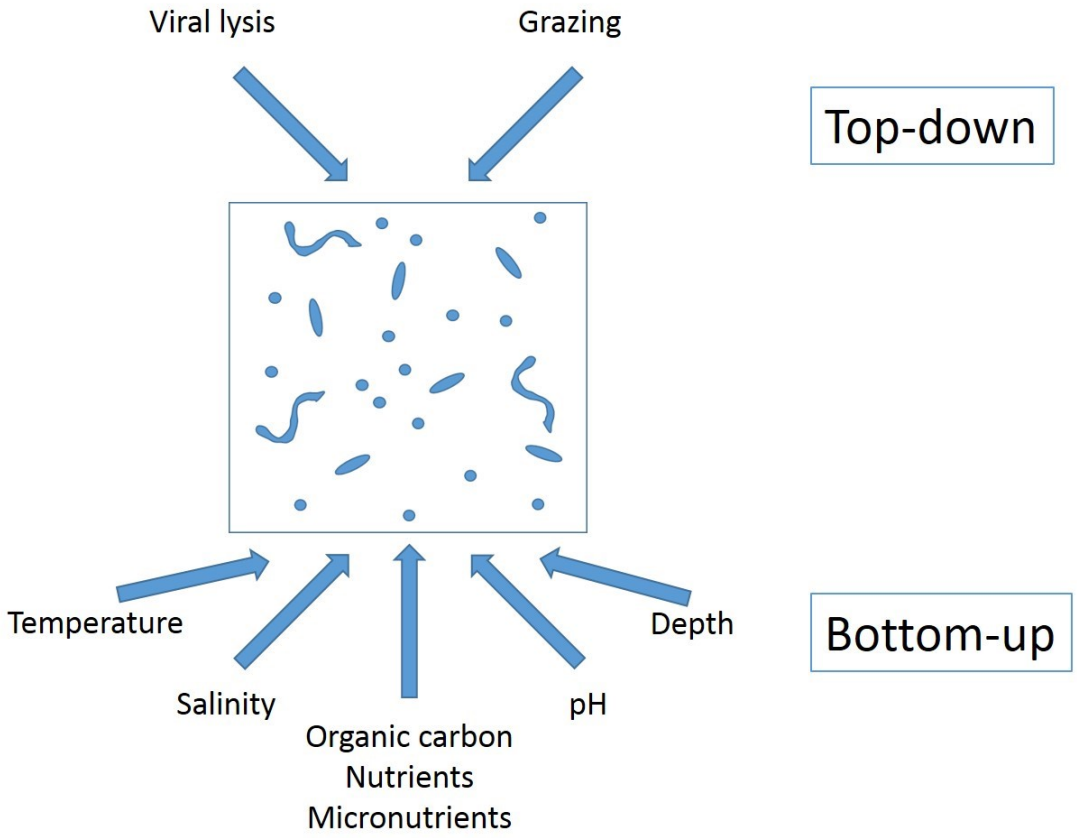

Figure 3: Simplified diagram of major bottom-up and top-down factors acting on bacterioplankton community composition and abundance.

i) Primary production

The proposed coupling between total bacteria and phytoplankton communities has frequently been examined in terms of the extracellular release of photosynthetically produced dissolved organic carbon (DOC) (Cole *et al.*, 1988; Mou *et al.*, 2008; Teira *et al.*, 2009). Studies have shown that 30–50% of the bacterial carbon demand under many oceanographic conditions can be met by the direct excretion of photosynthetically fixed carbon in situ (Baines and Pace, 1991). Thus, heterotrophic prokaryote abundance and production usually covary with phytoplankton biomass (measured as chlorophyll a) and primary production (Gasol and Duarte, 2000; Morán *et al.*, 2002; Lefort, 2012; Lekunberri

et al., 2012). Furthermore, Sarmento et al. (2012) demonstrated that DOC originating from different phytoplankton species differentially stimulates the major phylogenetic groups of heterotrophic prokaryotes.

Teeling et al. (2012) published the first high-resolution sequencing study of bacterial populations during and after a phytoplankton bloom along the island of Helgoland in spring 2009. Based on the high taxonomic resolution achieved in that study, the main findings were that pre-bloom bacteria were dominated by *Alphaproteobacteria*, mainly from the SAR11 and Roseobacter clades. During the early bloom phase, the abundances of *Bacteroidetes*, which consisted mostly of *Flavobacteria,* increased whereas *Gammaproteobacteria* was stimulated later on, during bloom decay.

In addition to coastal regions, upwelling regions are sources of mono-specific phytoplankton blooms in the ocean. In a bloom, a diverse community of phytoplankton shifts to a community dominated by only a few members (Painting, 1993a; Hansen *et al.*, 2014). It has therefore been proposed that if the two groups of organisms are specifically associated then bacterial diversity will likewise plummet during phytoplankton blooms, with a few members of the bacterial community becoming dominant. These specific bacterial groups should be readily detectable in bloom waters and otherwise absent or present only in reduced numbers comparable to those of the host phytoplankton (see Chapter 1 for details).

ii) pCO_2

The parameters that describe marine carbonate chemistry are pH, the partial pressure of carbon dioxide (pCO_2), dissolved inorganic carbon (DIC), and total alkalinity (TA) (Figure 4). An increase in the CO_2 concentration in the ocean leads to an increase in both pCO_2 and DIC and a drop in pH (Schulz *et al.*, 2009). Thus, assuming an ocean pH of 7.65, H_2CO_3 and CO_2 (aq) will increase by 300% and HCO_3^- by 9%, whereas CO_3^{2-} will decrease by 56% compared with present day concentrations (Hurd *et al.*, 2009; Schulz *et al.*, 2009). The reason for this imbalanced change in the components of DIC is the formation of more HCO_3 by the combination of released protons from dissolved H_2CO_3 with CO_3^{2-} (Schulz et al 2009). Therefore, ocean acidification will lead to a decrease in pH and CO_3^{2-} and an increase in pCO_2, CO_2 (aq), H_2CO_3, and HCO_3^-. However, these pH values

and carbonate system parameters are global; at smaller regional and temporal scales, the variability is much higher due to events such as phytoplankton blooms, stratification, and upwellings (Feely *et al.*, 2008; Hofmann *et al.*, 2011; Joint *et al.*, 2011).

Recent studies of the effects of ocean acidification have suggested that low pH and decreased carbonate availability will strongly impact calcifying microorganisms (Dupont *et al.*, 2010; Hendriks *et al.*, 2010) whereas the resulting increase in carbon availability will have a fertilizer effect on primary production (Riebesell *et al.*, 2007; Liu *et al.*, 2010; Joint *et al.*, 2011). The associated changes in phytoplankton dynamics and composition will affect phytoplankton-derived organic matter, by increasing transparent exopolymer particles and increasing CO_2 and temperature (e.g., Engel, 2002; Biermann et al., 2014; Endres et al., 2014; Engel et al., 2014). Concerning microbial processes, nitrogen fixation, cyanobacterial photosynthesis and elemental ratios were shown to be affected (Liu et al 2010). In contrast, less is known regarding heterotrophic bacteria.

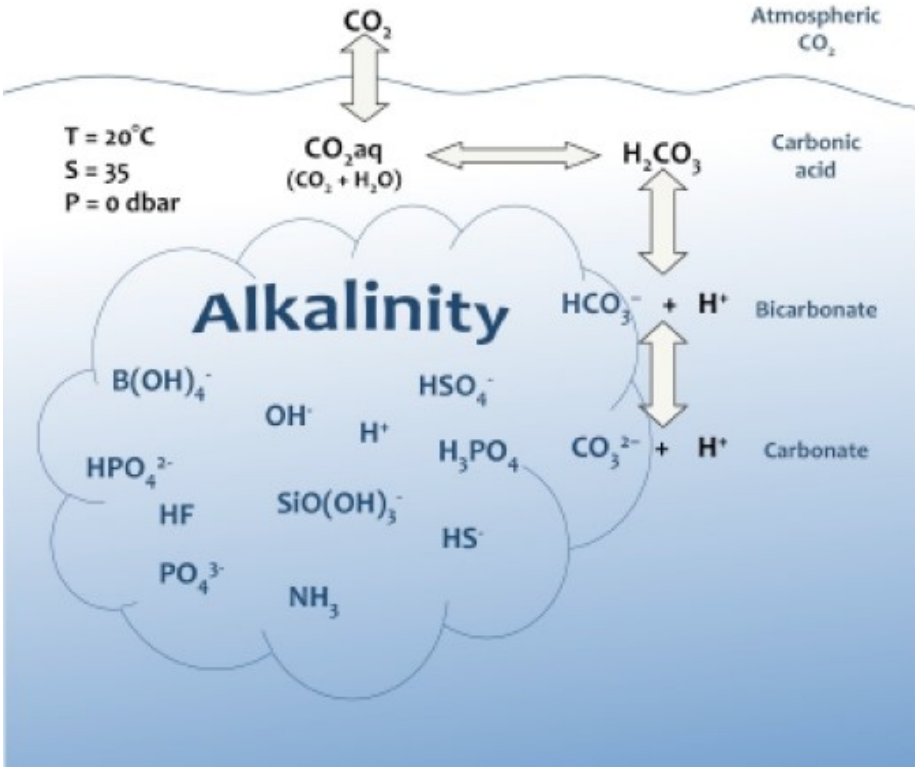

Figure 4: Schematic of carbonate system species in seawater and some of the equilibrium reactions that occur among them. Seawater total alkalinity is commonly defined as "the excess base" in seawater, or the sum of excess proton acceptors, and its component ions are illustrated in light blue (from Cooley *et al.*, 2012).

However, Joint et al. (2011) recently argued that microbe-dependent processes will not substantially change in a more acidic ocean, as marine microbes already experience large regional, temporal, and depth-dependent pH variability. In freshwater lakes, the pH ranges are even wider. This view is challenged by a meta-analysis of studies on the microbial responses to ocean acidification, which identified nitrogen fixation, cyanobacterial photosynthesis, and elemental ratios as affected by changes in seawater carbonate chemistry (Liu *et al.*, 2010).

The results concerning other microbial processes and especially those of heterotrophic bacteria have often been inconsistent. Some studies have reported no effect on bacterial activity or abundance (Allgaier *et al.*, 2008; Krause *et al.*, 2012; Newbold *et al.*, 2012; Teira *et al.*, 2012; Oliver *et al.*, 2014; Hartmann *et al.*, 2015) but others have presented evidence of increased abundance and activity, probably mediated by the stimulation of phytoplankton blooms (Grossart *et al.*, 2006; Endres *et al.*, 2014) or of decreased bacterial production (Motegi *et al.*, 2013). The effects of CO_2 on bacterial community composition were recently investigated in controlled mesocosms with natural bacterioplankton assemblages. Those studies showed either no (Newbold *et al.*, 2012; Oliver *et al.*, 2014) or only weak effects, because of shifts in phytoplankton dynamics (Allgaier *et al.*, 2008; Arnosti *et al.*, 2011; Roy *et al.*, 2013). In their study of freshwater lakes, Ren et al. (2015) found that in an increase in pH leads to a decrease in overall bacterial diversity, including shifts to taxa of higher adaptation potential. Interestingly, increased rates of hydrolytic extracellular enzymes under increased CO_2 have been described by several authors (Grossart *et al.*, 2006; Tanaka *et al.*, 2008; Piontek *et al.*, 2010, 2013; Maas *et al.*, 2013; Endres *et al.*, 2014) and a recent metatranscriptome study found that low pH induced the enhanced expression of genes encoding proton pumps (Bunse *et al.*, 2016). The impact of increased pCO_2 on bacterial processes is summarized in Table 1 (see Chapter 2 for details).

Table 1: Impact of increased pCO_2 on bacterial processes. (+) indicates an increase, (-) a decrease and (o) no change in the respective process. TEP: transparent exopolymer particles; BPP: bacterial protein production; BA: bacterial abundance; BCC: bacterial community composition

Process	Impact	References
TEP production	+	(Engel, 2002; Biermann *et al.*, 2014; Engel *et al.*, 2014)
BPP	o	(Allgaier *et al.*, 2008; Krause *et al.*, 2012; Newbold *et al.*, 2012; Teira *et al.*, 2012; Oliver *et al.*, 2014; Hartmann *et al.*, 2015)
	+	(Grossart *et al.*, 2006; Endres *et al.*, 2014)
	-	(Motegi *et al.*, 2013)
BA	o	(Allgaier *et al.*, 2008; Krause *et al.*, 2012; Newbold *et al.*, 2012; Teira *et al.*, 2012; Oliver *et al.*, 2014; Hartmann *et al.*, 2015)
	+	(Grossart *et al.*, 2006; Endres *et al.*, 2014)
BCC	Weak effect	(Allgaier *et al.*, 2008; Arnosti *et al.*, 2011; Roy *et al.*, 2013)
	No effect	(Newbold *et al.*, 2012; Oliver *et al.*, 2014)
Enzyme activity	+	(Grossart *et al.*, 2006; Tanaka *et al.*, 2008; Piontek *et al.*, 2010, 2013; Maas *et al.*, 2013; Endres *et al.*, 2014; Bunse *et al.*, 2016)

iii) Temperature

It is well established that temperature is a critical determinant of bacterial growth (Nedwell, 1999; Pomeroy and Wiebe, 2001; Apple *et al.*, 2006), and climate change studies have found that bacterial activity (Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009; von Scheibner *et al.*, 2014) and community composition (Hall *et al.*, 2009; Adams *et al.*, 2010; Dziallas and Grossart, 2011; von Scheibner *et al.*, 2014) are stimulated by an increase in temperature. The impact of increased temperature on bacterial processes is summarized in Table 2 (see Chapter 2 for details).

Likewise, temperature modulates the metabolic activity of aquatic bacteria in many systems. A metadata analysis in freshwater, coastal, and marine habitats found a strong relationship between the bacteria-specific growth rate and temperature (White *et al.*, 1991). In addition, temperature-related limitations to growth have been demonstrated in rivers (Freese *et al.*, 2006), coastal systems (Sherr *et al.*, 2001), and marine systems (Longnecker *et al.*, 2006). Temperature can also interact with carbon

availability and substrate affinity to control bacterial activity. At colder temperatures, bacteria begin to lose substrate affinity, possibly due to the stiffening of their lipid membranes, which leads to carbon limitation despite available sources in the environment (Nedwell, 1999).

Table 2: Impact of increased temperature on bacterial processes. (+) indicates an increase and (o) no change in the respective process. TEP: transparent exopolymer particles; BPP: bacterial protein production; BA: bacterial abundance; BCC: bacterial community composition

Process	Impact	Studies
TEP production	+	(Piontek *et al.*, 2009; Biermann *et al.*, 2014)
BPP	+	(Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009; von Scheibner *et al.*, 2014)
BA	o	(Lindh *et al.*, 2013)
	+	(Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009; von Scheibner *et al.*, 2014)
BCC	Strong effect	(Hall *et al.*, 2009; Adams *et al.*, 2010; Dziallas and Grossart, 2011; von Scheibner *et al.*, 2014)

At suboptimal temperatures bacteria may be present either at low densities or at higher densities but in relatively inactive states; as conditions become favorable, their dominance is likely to increase. In natural communities, the optimal temperature for a community can shift seasonally (Tison *et al.*, 1980), such that the relative abundances of bacterial populations with different optimal temperatures and substrate affinities will shift as well (Ogilvie *et al.*, 1997). In addition to preferred temperature ranges, bacterial populations vary in their enzymatic capabilities and preferred carbon substrates; thus, changes in community structure resulting from temperature shifts will also change the potential levels of activity.

iv) Salinity

Salinity is one of the most important factors structuring microbial communities and their distribution (Lozupone and Knight, 2007; Nemergut *et al.*, 2011). The considerable differences between marine and freshwater bacterial communities suggest

an evolutionarily-driven grouping into distinct marine and freshwater phylogenetic clusters (Logares *et al.*, 2009).

Shifts in bacterial community composition along a salinity gradient have been documented in various estuaries (e.g., Bouvier and Giorgio, 2002; Kirchman *et al.*, 2005; Fortunato and Crump, 2011). These studies documented drastic shifts, from the prevalence of *Betaproteobacteria* and *Actinobacteria* in freshwater regions to the dominance of *Alphaproteobacteria* in higher salinity regions. Differences in bacterial abundances have also been demonstrated along the salinity gradients of the Rhone estuary and the Columbia River (Crump *et al.*, 1999; Troussellier *et al.*, 2002). The sharp phylogenetic succession that occurs in freshwater to saltwater transition regions is accompanied by profound physiological changes at the bacterial community level (del Giorgio and Bouvier, 2002). However, the available data on bacterial diversity have led to inconsistent conclusions. In some studies, freshwater and marine bacterioplankton communities were shown to mix along estuarine gradients, suggesting that bacterioplankton in the estuarine zone comprises a mixture of the two communities (Troussellier *et al.*, 2002; Kirchman *et al.*, 2005; Zhang *et al.*, 2006). In a study of bacterial community composition along the Baltic Sea salinity gradient, Herlemann *et al.* (2011) observed significant differences between the composition of the bacterial community in brackish water and the compositions of its freshwater and marine counterparts. The authors concluded that the brackish waters of the Baltic Sea are occupied by a diverse combination of freshwater and marine clades specifically adapted to the conditions of their habitat.

Aims of the dissertation

To add to current knowledge on the impact of important environmental factors on bacterial communities, this thesis addresses three main questions:

i) How does bacterial community composition change along an upwelling-induced productivity gradient? Is bacterio-phytoplankton coupling the strongest structuring factor?

ii) How does bacterial community composition change with increased pCO_2 and temperature? How strong are phytoplankton-mediated indirect effects?

iii) How does bacterial community composition change with a saline intrusion which builds up a salinity gradient that ranges from marine to brackish conditions?

To answer question (i), we combined NGS of the 16S rRNA gene and a multivariate correlation approach to the communities present along the productivity gradient of the Northern Benguela upwelling system **(Chapter 1)**. The study was carried out as part of an interdisciplinary research project that examined succession processes during an upwelling event. Data on a broad range of bacterial parameters were combined with data obtained by other researchers from this project. We were thus able to describe for the first time the development of bacterial community composition along a productivity gradient in aging upwelled water, to estimate the degree of bacterio-phytoplankton coupling, and to relate it to other community-structuring factors.

To estimate the impact of increased pCO_2 and temperature on bacterial community composition, we conducted two bifactorial mesocosm experiments using natural Baltic Sea plankton communities **(Chapter 2)**. A great effort was made to describe bacterial diversity, the potentially affected key taxa, and their relation to phytoplankton and organic matter dynamics.

The influence of changing salinity on the structure of the bacterial assemblage was analyzed by taking advantage of a major inflow of highly saline water from the North Sea into the Central Baltic Sea **(Chapter 3)**. Again, NGS of 16S rRNA and of the 16S rRNA gene were used to follow the development of the bacterial community along the inflowing water body. An interdisciplinary approach was adopted to trace back the origins of the

respective water samples in order to estimate mixing effects and to compare the respective bacterial communities with the natural communities existing at the potential origin of the inflow samples.

Chapter 1

Zonation of bacterioplankton communities along aging upwelled water in the northern Benguela upwelling

Abstract

Upwelling areas are shaped by enhanced primary production in surface waters, accompanied by a well-investigated planktonic succession. Although bacteria play an important role in biogeochemical cycles of upwelling systems, little is known about bacterial community composition and its development during upwelling events. The aim of this study was to investigate the succession of bacterial assemblages in aging upwelled water of the Benguela upwelling from coastal to offshore sites. Water from the upper mixed layer at 12 stations was sampled along two transects from the origin of the upwelling to a distance of 220 km. 16S rRNA gene amplicon sequencing was then used in a bacterial diversity analysis and major bacterial taxa were quantified by catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH). Additionally, bacterial cell numbers and bacterial production were assessed. Community statistical analysis revealed a reproducible zonation along the two transects, with four clusters of significantly different microbial assemblages. Clustering was mainly driven by phytoplankton composition and abundance. Similar to the temporal succession that occurs during phytoplankton blooms in temperate coastal waters, operational taxonomic units (OTUs) affiliated with *Bacteroidetes* and *Gammaproteobacteria* were dominant during algal blooming whereas "*Pelagibacterales*" were highly abundant in regions with low algal abundance. The most dominant heterotrophic OTU (9% of all reads) was affiliated with "Pelagibacterales" and showed a strong negative correlation with phytoplankton. By contrast, the second most abundant heterotrophic OTU (6% of all reads) was affiliated with the phylum *Verrucomicrobia* and correlated positively with phytoplankton. Together with the close relation of bacterial production and phytoplankton abundance, our results showed that bacterial community dynamics is strongly driven by the development and composition of the phytoplankton community.

1.1. Introduction

Ocean margins and upwelling systems in particular are sites of enhanced primary production and organic matter export. The Benguela upwelling, one of the most intensive upwelling systems worldwide (Nelson and Hutchings, 1983), is characterized by constant upwelling of nutrient rich water from deeper layers with maximum upwelling between August

and October and a minimum between January and March (Shannon and Nelson, 1996). Upwelling is driven by southeast trade winds and the resulting Ekman offshore transport. Many studies on the Benguela upwelling have focused on the succession of phytoplankton and zooplankton. Their results have shown that the upwelling of nutrients leads to a characteristic phytoplankton succession in which the early bloom is dominated by diatoms or dinoflagellates (e.g., Barlow, 1982; Pitcher et al., 1998) that, after nutrient depletion, are replaced by other phytoplankton groups (Brown and Hutchings, 1987). As the bloom declines, there is an increase in nano- and mesozooplankton biomass and therefore in the grazing impact of flagellates and copepods (Painting, 1993a).

Investigations of the role of prokaryotes in upwelling systems have shown a significant correlation between bacterial and primary production (e.g., Painting, 1993b; Wiebinga et al., 1997; Cuevas et al., 2004) which indicates the importance of bacteria as decomposers of organic matter in these systems. Most studies have focused on prokaryotic bulk parameters such as cell abundance or production while the few that have examined upwelling-induced changes in prokaryotic community composition either lacked sufficient resolution (Kerkhof *et al.*, 1999; Suzuki *et al.*, 2001; Alonso-Gutiérrez *et al.*, 2009; Teira, Nieto-Cid, *et al.*, 2009), were limited to one sampling station, or focused on metabolic processes (Cury *et al.*, 2011; Zeigler Allen *et al.*, 2012). Nonetheless, their findings provided the first clues in upwelling systems of the frequent occurrence of *Bacteroidetes*, *Roseobacter*, and the gammaproteobacterial clade SAR86 as well as specific associations between bacteria and phytoplankton.

As a perennial upwelling system, the northern Benguela provides optimal conditions to investigate bacterial community development along aging upwelled water during the successive stages of a phytoplankton bloom. Hansen *et al.* (2014) analyzed samples taken in parallel to those collected for this study and found a shift in phytoplankton community composition, with dinoflagellates dominating coastal stations and diatoms dominating the phytoplankton community located approximately 50 km offshore.

The present study was part of an interdisciplinary research project examining successive processes in the coastal Benguela upwelling system during strong upwelling in late winter. Applying an Eulerian approach, we sampled a transect at a 45° angle to the upwelling current, which led to a projection of aging upwelled water along the transect (Mohrholz *et al.*, 2014). To determine whether, as expected, the bacterial community responds to upwelled water of different ages, we used next-generation sequencing of partial 16S rRNA genes to

describe the changes in community composition. The data were analyzed for indications of the major drivers of changes in the bacterial community. This study is the first to demonstrate the remarkable differences in the bacterial communities of the Benguela upwelling and that they are mainly triggered by the abundance and quality of phytoplankton. On a spatial scale, our findings also confirm the successional bacterial pattern that occur during phytoplankton blooms.

1.2. *Material and Methods*

Sampling

Samples were taken between 10 and 220 km off the coast of Namibia during a cruise of the R/V M.S. Merian in the northern Benguela upwelling region (Figure 5). The transect was sampled two times consecutively with a time interval of four days (transect 1: 27.08.2011–30.08.2011; transect 2: 30.08.2011–02.09.2011). Samples were taken at depths of 5 m and 20 m using a rosette water sampler comprising 24 10-L free-flow bottles. Profiles of temperature, salinity, oxygen, and chlorophyll fluorescence were measured using a CTD SBE911+ combined with the bottle sampler rosette. Water samples for DNA analysis were filtered onto 0.22-µm pore-size white polycarbonate filters. DNA was extracted according to Weinbauer *et al.* (2002). Chlorophyll *a* (Chl-a) concentrations were determined according to Hansen *et al.* (2014).

Prokaryotic cell number and activity measurements

Prokaryotic cells were counted using a flow cytometer (FacsCalibur, Becton Dickinson, Heidelberg, Germany) following the method of Gasol *et al.* (1999). Calculations were performed using the software program "Cell Quest Pro," plotting the emission fluorescence of SYBR Green I (488 nm) vs. the side scatter. Picocyanobacteria were similarly counted on the basis of their signature in a plot of orange (FL2) vs. red (FL3) fluorescence.

The incorporation of ^{3}H-leucine (140 Ci mmol^{-1}) was measured to estimate heterotrophic bacterial productivity in 10-mL water samples. Triplicate samples were incubated at a final concentration of 100 nM for at least 1 h at the *in situ* temperature in the dark. Incorporation was stopped by fixing the cells with formaldehyde (5% v/v). A fourth sample, serving as a blank, was fixed for at least 10 min prior to the addition of the

radioactively labeled substrate. The samples were filtered onto 0.22-µm polycarbonate filters (Millipore), which were then placed in 4 mL of scintillation cocktail. The incorporated substrate was counted in a scintillation counter (Packard). Bacterial carbon production was calculated from ^{3}H-leucine incorporation according to Simon *et al.* (1987), using a leucine mol% value of 7.3 and a carbon conversion factor of 0.86.

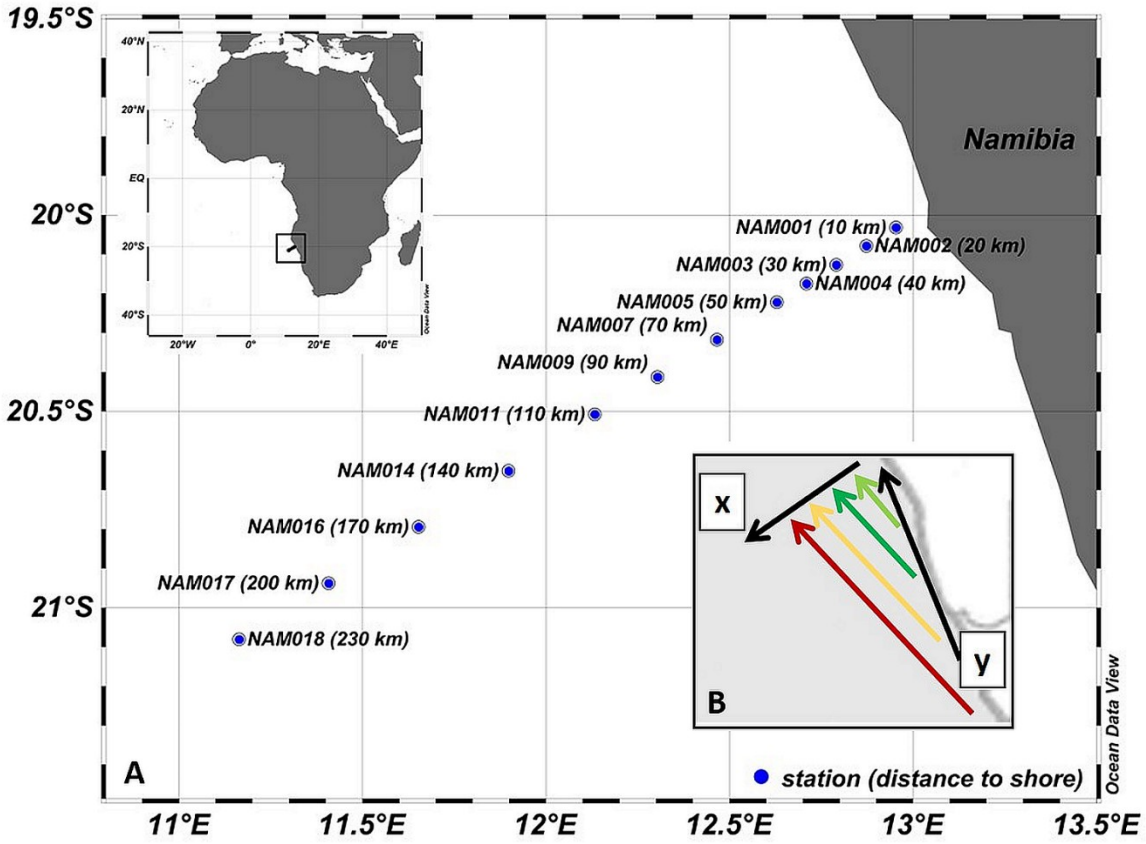

Figure 5: (A) Transect along the northern Benguela upwelling, showing the locations of the stations and their distance to the shore. **(B)** Scheme of the Eulerian approach used in this study, with the coastal parallel current vector y and the normal to the coast vector x that corresponds to the transect. The colored vectors indicate the projection of the upwelled water during different developmental stages on the transect.

Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) and cell counting

CARD-FISH was carried out using the protocol of Pernthaler *et al.* (2002), with modifications. Before digestion, the filters were incubated in 0.01 M HCl for 10 min to inactivate undesirable peptidases. Bacterial staining was carried out using the horseradish-peroxidase-labeled FISH probes EUBI-III (Daims *et al.*, 1999), VER47 (Buckley and Schmidt, 2001), and SAR11-486 (Fuchs *et al.*, 2005). For signal amplification, tyramide labeled with the

fluorescent dye carboxyfluoresceine was used. Total cell numbers were estimated by 4',6'-diamidino-2-phenylindole (DAPI)- staining of the probe-labeled samples.

DAPI and EUB I-III stained cells were counted using an automated system coordinated with the epifluorescence microscope AxioImager (Zeiss, Germany) and in combination with a Colibri LED unit and a charge-coupled device camera (AxioCam MRm, Zeiss, Germany). Images were acquired using a 100× Plan-Apochromat objective (Zeiss) and the Zeiss multi-band filter set 62HE. Automatic processing of the samples was achieved with the Visual Basic for Application module of AxioVision 4.6 (Zeiss, Germany) together with comprised automated sample recognition and localization, multichannel image acquisition, image processing, and cell counting routines (Zeder and Pernthaler, 2009). VER47- and SAR11-486 stained cells were counted manually at the same microscope using a 63× Plan-Apochromat objective and the same filter set. For each sample, at least 1000 DAPI-stained cells in at least ten independent microscopic fields were counted, excluding cells that exhibited autofluorescence (590 nm). Heterotrophic nanoflagellates (HNFs) were also counted manually using filter set 02 (Zeiss, Germany). A minimum of 100 cells per filter were counted at a magnification of 630×.

Bacterial community composition

For bacterial diversity analysis, hypervariable regions 3–5 (V3–V5) of the 16S rRNA gene were used to generate PCR amplicons, as described by Herlemann *et al.* (2011), but with a modification of 30 PCR cycles. Sequencing was performed by Eurofins MWG GmbH using 454 GS-FLX sequencer (Roche). The denoising tool Acacia (Bragg *et al.*, 2012) was used to correct amplicon pyrosequencing errors. Primer sequences were trimmed from the reads and the sequences were clipped 400 bp downstream of the primer. Reads shorter than 400 bp (excluding the primer) and/or containing Ns were excluded. Reads that were found only once in the sample set were removed from the analysis. Sequences were aligned and clustered at 97% identity into operational taxonomic units (OTUs), as described by Herlemann *et al.* (2011), using the pyrosequencing pipeline at RDP (Cole *et al.*, 2009). The online tool Decipher (Wright *et al.*, 2012) was used to identify and remove chimeric sequences in the remaining OTUs. The abundances of the resulting OTUs were normalized using the relative proportions of individual OTU reads from all sample reads. Sequences have been deposited in the European Nucleotide Archive (ENA), with the study accession: http://www.ebi.ac.uk/ena/data/view/PRJEB8816.

Statistics

A non-hierarchical clustering method, k-means clustering (MacQueen, 1967), was used to reveal similar spatial distribution patterns in the relative abundances of the 25 most frequent OTUs. According to the most pronounced distribution patterns, OTUs were clustered into five groups. Sequence results from the whole sequence data were analyzed using principal coordinate analysis (PCoA) with the Bray-Curtis index of dissimilarity based on the normalized abundance data. A cluster analysis was used to identify bacterial community clusters in the PCoA plot and a subsequent ANOSIM analysis was performed to confirm significant differences between the clusters. Spearman rank correlation analysis was used to compare PCoA coordinates with environmental parameters to determine their link to community separation. All statistical analysis were performed using the PAST software package version 2.17c (Hammer *et al.*, 2001).

1.3. *Results*

Hydrographic data showed that this study was performed during a period of constant upwelling and the offshore transport of nutrient-rich shelf water in front of Namibia (Mohrholz *et al.*, 2014; Nausch and Nausch, 2014). Since the mixed-layer depth was at least 20 m along the whole transect, for all analyses in this study the mean values from the 5-m and 20-m samples were used to describe bacterial community development in the upper mixed layer. Upwelling resulted in an increase in phytoplankton development, characterized by a high abundance of dinoflagellates, beginning 40 km offshore, at station NAM004, and peaking 70 km offshore, at station NAM007, where diatoms dominated (Hansen *et al.*, 2014). A cold-water filament moving offshore influenced the remote stations (NAM016 to NAM018,) causing minor differences between transects 1 and 2, namely, a more pronounced Chl-a peak at station NAM007 in transect 2 and a shift of the offshore Chl-a filament from station NAM017 in transect 1 to station NAM018 in transect 2. Therefore, the developmental stage of the upwelled water was classified to determine the validity of a description of successive processes along the transect or whether it would be affected by mesoscale dynamics such as filaments and eddies. The progressive aging of the water masses from station NAM001 to NAM014 was confirmed by Mohrholz et al. (Mohrholz *et al.*, 2014), who used salinity,

temperature and oxygen concentrations to calculate the relative ages (pseudoages) of the water masses.

Prokaryotic abundance and activity

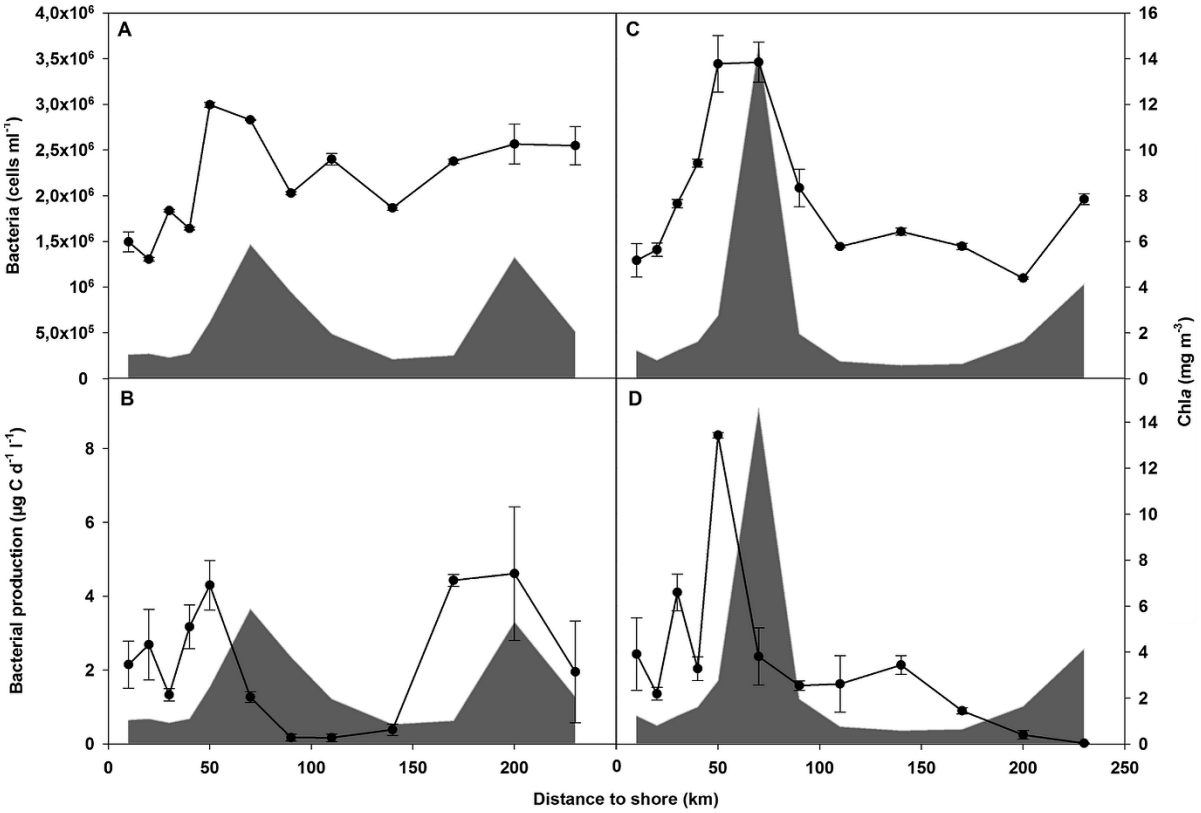

Figure 6: Prokaryotic abundance in transect 1 **(A)** and transect 2 **(C)** and bacterial protein production in transect 1 **(B)** and transect 2 **(D)** in the upper 20 m (lines). Chlorophyll-a concentration (grey area) in relation to the distance from the coast is also shown.

Prokaryotic abundance (PA) ranged from 1.3 × 10^6 cells mL^{-1} at the near-shore station (NAM001, transect 2) to a peak of 3.5 × 10^6 cells mL^{-1} at a distance of 70 km from the coast (NAM007, transect 2) (Figure 6A, C). The increase in PA over the first 70 km was followed by a decrease until 90 km, remaining relatively constant, at 1.5–2 × 10^6 cell mL^{-1}, thereafter. PA correlated significantly with Chl-a concentrations ($p < 0.05$). As most stations with high Chl-a concentrations were dominated by diatoms (Hansen *et al.*, 2014), there was a significant positive correlation between PA and diatom biomass ($p < 0.05$). Further analysis showed significant positive correlations between PA and NO_2 ($p < 0.05$) and a marginally significant correlation with the abundance of HNFs ($p < 0.1$) (Table 3).

Synechococcus cell numbers ranged from 6.5×10^6 cells L^{-1} at the near-shore stations of transect 1 to 5.8×10^7 cells L^{-1} in the region of low Chl-a concentration of transect 2 (NAM014). The *Synechococcus* distribution pattern was in good accordance with bacterial protein production (BPP) but the trend was not significant (data not shown). *Prochlorococcus* cells were not detected in either transect.

BPP increased in parallel with PA over the first 50 km, from 2.3 µg C d^{-1} L^{-1} ± 0.2 µg C d^{-1} L^{-1} at the most near-shore station (NAM001; means of transect 1 and 2) to 6.33 µg C d^{-1} L^{-1} ± 2.88 µg C d^{-1} L^{-1} at station NAM005 (Figure 6B, D). However, the peak in BPP occurred before the peak in Chl-a concentration in areas with high nutrient levels. At a distance of 70 km (NAM007), where PA was high and Chl-a concentrations reached a maximum, BPP strongly decreased to 1.82 µg C d^{-1} L^{-1} ± 0.78 µg C d^{-1} L^{-1}. A second but smaller increase in BPP, to 2.67 µg C d^{-1} L^{-1} ± 2.5 µg C d^{-1} L^{-1}, was measured at transect 1 at a distance of 170 km (NAM016). Bacterial production was positively correlated to nutrients (NO_3, PO_4, DIN) ($p < 0.05$) and was marginally significant correlated with the abundance of HNF ($p < 0.1$) (Table 3).

Table 3: Correlation (Spearman, r_s) of bacterial parameters and environmental variables from both transects. PA, prokaryotic abundance; BPP, bacterial protein production; Chl-a, chlorophyll-a; Dino, dinoflagellates; HNF, heterotrophic nanoflagellate cell number; ns, not significant.

Parameter	PA r-, p-value	BPP r-, p-value
NO_2	0.49, <0.05	ns
NO_3	ns	0.47, <0.05
PO_4	ns	0.52, <0.05
NH_4	ns	ns
Chl-a	0.71, <0.05	ns
Diatom[a]	0.57, <0.05	ns
Dino[a]	ns	ns
HNF	ns	ns

[a]Expressed as biomass.

Bacterial diversity

Pyrosequencing generated 150,006 raw sequence reads. After denoising and the removal of sequence reads present only once in all samples and of chimeric sequences, a total

of 125627 reads remained. These sequences were clustered into 1335 OTUs at an average sequence identity of 97% per OTU.

Table 4: Taxonomic affiliation of the 25 most abundant operational taxonomic units (OTUs) along transect 1 and transect 2. Abundance rank and averaged relative abundance of the respective OTUs are shown.

OTU no.	OTU	Transect 1 (rank)	Transect 1 (%)	Transect 2 (rank)	Transect 2 (%)
1	Cyanobacteria - Synechococcus	2	8.2	1	10.8
2	Alphaproteobacteria - Candidatus Pelagibacter	1	8.3	2	9.8
3	Verrucomicrobia - Persicirhabdus	3	5.3	3	5.5
4	Gammaproteobacteria - SAR86 clade	4	4.1	4	3.4
5	Gammaproteobacteria - Oceanospirillales	5	3.5	6	2.3
6	Actinobacteria - OCS155 marine group	7	2.5	5	2.6
7	Bacteroidetes - Flavobacteriaceae	6	2.6	8	2.2
8	Gammaproteobacteria - OM60(NOR5) clade	8	2.2	7	2.2
9	Alphaproteobacteria - Rhodobacteraceae	9	2.2	9	1.9
10	Bacteroidetes - Formosa	13	1.3	10	1.9
11	Alphaproteobacteria - Roseobacter clade DC5-80-3	10	1.7	13	1.4
12	Bacteroidetes - Flavobacteriaceae	12	1.5	11	1.4
13	Alphaproteobacteria - Rhodobacteraceae	11	1.7	20	1.0
14	Bacteroidetes - Formosa	14	1.3	12	1.4
15	Bacteroidetes - NS4 marine group	15	1.3	16	1.2
16	Gammaproteobacteria - E01-9C-26 marine group	18	1.1	17	1.2
17	Bacteroidetes - Gaetbulibacter	17	1.1	18	1.1
18	Gammaproteobacteria - SAR86 clade	16	1.2	21	1.0
19	Chloroplast	22	1.0	14	1.3
20	Bacteroidetes - NS2b marine group	19	1.1	15	1.2
21	Verrucomicrobia - Roseibacillus	23	0.9	19	1.1
22	Alphaproteobacteria - SAR11 clade	21	1.0	22	0.9
23	Bacteroidetes - VC2.1 Bac22	20	1.0	25	0.8
24	Bacteroidetes - Ulvibacter	24	0.9	23	0.8
25	Alphaproteobacteria - AEGEAN−169 marine group	25	0.8	24	0.8

Analysis of the normalized abundance data showed that the 25 most abundant OTUs contained more than 50% of all reads and were identical in both transects, although in a different rank order (Table 4). The most abundant OTUs in both transects were those of common marine bacterial groups, including two OTUs affiliated with "Pelagibacterales" (10% of the total read abundance), one OTU from *Cyanobacteria* family II (9.5%), which includes the genera *Prochlorococcus* and *Synechococcus*, one OTU identified as *Verrucomicrobia* genus *Persicirhabdus* (5.4%), and two OTUs from the *Gammaproteobacteria* SAR86 cluster (4.9%)

(Figures S1 and S2). The highest diversity occurred within the *Flavobacteriaceae*, represented by eight of the 25 most abundant OTUs.

Spatial patterns

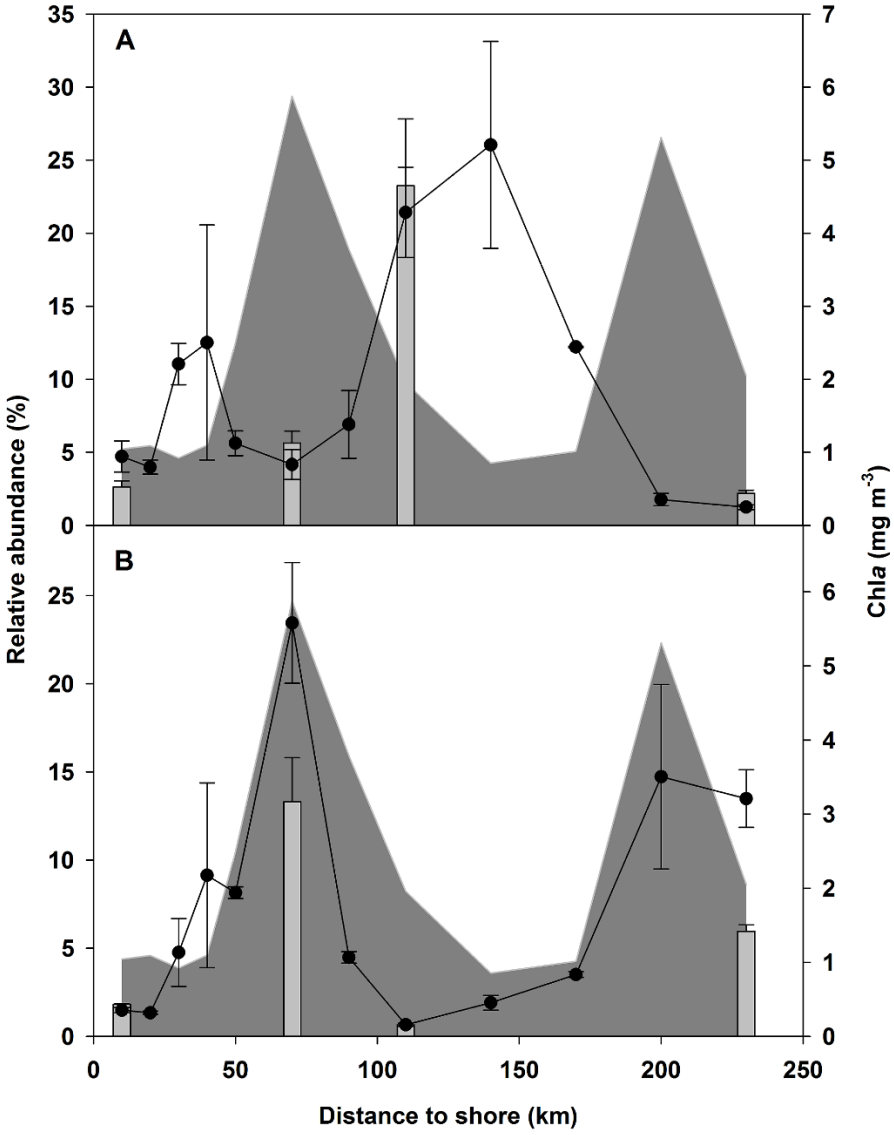

Figure 7: Abundance profiles of "Pelagibacterales" **(A)** and *Verrucomicrobia* **(B)** as the average of the upper 20 m of transect 1. Dots indicate the relative amounts of the respective reads from all reads obtained from pyrosequencing, bars indicate the relative amounts of respective cells from all bacterial cells detected by CARD-FISH. The chlorophyll-a concentration is indicated by the grey area.

Some of the most abundant OTUs showed contrary patterns with respect to their relative abundances along the transects. For example, the abundances of OTUs affiliated with "Pelagibacterales" correlated negatively with those of *Verrucomicrobia* ($p < 0.05$). "Pelagibacterales" were dominant at stations with low Chl-a concentrations, but at stations

with high Chl-a concentrations their relative abundances decreased whereas verrucomicrobial OTUs increased to as high as 23% of all bacterial reads (Figure 7), resulting in a significant positive correlation with Chl-a ($p < 0.05$). To validate the proportion of these abundant OTUs revealed by pyrosequencing, CARD-FISH was performed for a subset of eight samples from stations related to four clusters identified, as described below, by PCoA (5-m and 20-m samples from NAM001, NAM007, NAM011, and NAM018). The proportions of "Pelagibacterales" and *Verrucomicrobia* cells from all bacteria calculated from the direct cell count using CARD-FISH showed similar trends to sequence proportions derived from pyrosequencing (Figure 7). However, for *Verrucomicrobia* the CARD-FISH-derived abundance was lower than that determined by pyrosequencing (CARD-FISH n = 8, average 5% of EUB, pyrosequencing average 10% of the total bacterial community) whereas for "Pelagibacterales" the abundances determined by the two methods were the same (CARD-FISH n = 8, average 8% of EUB, pyrosequencing average 8% of the total bacterial community). Linear regression analysis of the pyrosequencing- and CARD-FISH-derived abundances, including all samples, showed that their relationship was significant ($n = 16$, $R^2 = 0.77$, $p < 0.01$).

K-means clustering was used to detect characteristic patterns of OTU abundance along the transects (Figure 8). The 25 most abundant OTUs could thus be assigned to five clusters with different patterns that were recognizable in both transects. The clustering approach mainly grouped OTUs according to their correlation with Chl-a. In both transects, cluster 1 and cluster 4 were mainly represented by OTUs that had a significant positive correlation with Chl-a ($p < 0.1$), including OTUs affiliated with *Synechococcus*, *Verrucomicrobia* genus *Persicirhabdus*, and several *Flavobacteriaceae*. Cluster 2 and cluster 3 comprised several OTUs that had a significant negative correlation with Chl-a, including "Pelagibacterales" and SAR86 clade OTUs. Cluster 5 contained OTUs that showed no correlation with Chl-a. Interestingly, cluster 4, which grouped OTUs that peaked in parallel during and after the bloom, was almost entirely represented in both transects by OTUs affiliated with *Flavobacteriaceae*.

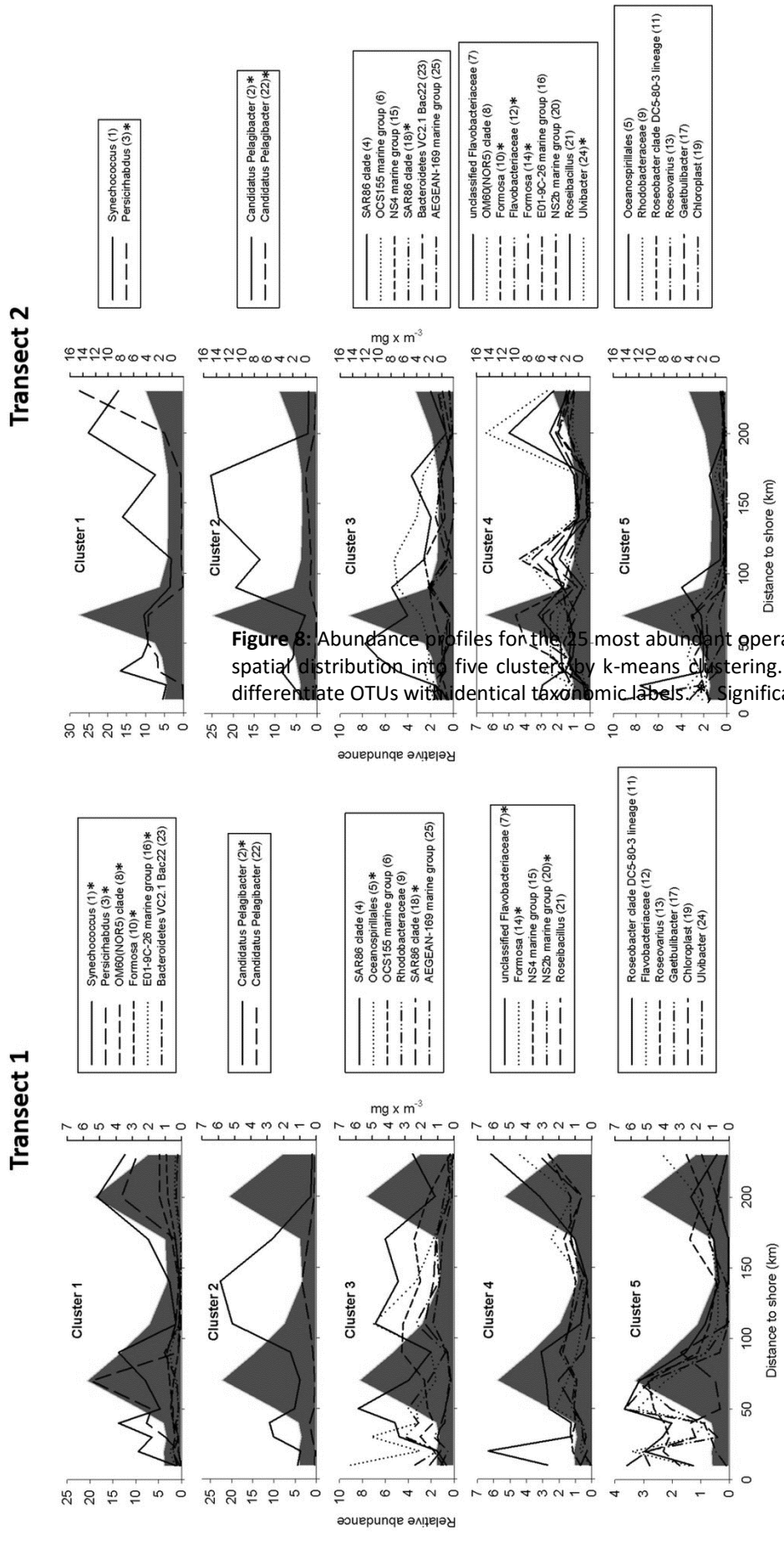

Figure 8: Abundance profiles for the 25 most abundant operational taxonomic units (OTUs) along the t
spatial distribution into five clusters by k-means clustering. The grey area indicates the chlorophyll-
differentiate OTUs with identical taxonomic labels. *) Significant correlation with the chlorophyll-a con

Table 5: Significant relationships between environmental parameters and principal coordinate analysis (PCoA) ordination of the normalized abundance of all OTUs for transect 1 and transect 2. The number of data (n), Spearman correlations (r_s), and significance values (p) are shown. Chl-a, chlorophyll a; DIN, dissolved inorganic nitrogen.

		Transect 1			Transect 2		
Coordinate	**Variable**	**n**	**r_s**	**p**	**n**	**r_s**	**p**
1							
	Chl-*a*	12	-0.76	<0.01	12	0.6	0.03
	Diatom biomass	9	-0.66	0.05			
	PO_4				12	0.59	0.04
	Temperature				12	-0.63	0.03
	Salinity				12	-0.80	<0.01
	Pseudoage				12	-0.63	0.03
2							
	Salinity	12	-0.86	<0.01			
	Dinoflagellate biomass	9	-0.66	0.05			
	NO_3	12	0.94	<0.01	12	0.62	0.03
	PO_4	12	0.88	<0.01	12	0.63	0.03
	SiO_4	12	0.92	<0.01	12	0.67	0.02
	DIN	12	0.94	<0.01	12	0.63	0.03
	Temperature	12	-0.97	<0.01	12	-0.62	0.03
	O_2	12	-0.79	<0.01	12	-0.70	0.01
	Distance	12	-0.90	<0.01	12	-0.72	0.01
	Pseudoage	11	-0.95	<0.01	12	-0.59	0.04

To identify a general structure in the distribution of OTUs along the transects, the relative abundances of all OTUs were subjected to a PCoA (Figure 9). Correlation analysis with environmental variables indicated that in both transects the first axis distinguished samples with low vs. high Chl-a values. The first axis divided samples from transect 2 by temperature and salinity (Table 5). In the second axis, samples from both transects were divided based on inorganic nutrient levels, temperature, and distance to shore. The analysis showed a zonation along the transects and a cluster analysis distinguished four bacterial community clusters defined at the OTU level (Figure S3). An ANOSIM analysis confirmed the significant differences between these four clusters (transect 1: $R = 0.83$, $p < 0.01$; transect 2: $R = 0.68$, $p < 0.01$). The four clusters were numbered according to their distance to the shore, with increasing distance from cluster 1 to cluster 4. Their major features are presented in Table 6. Even on a phylum level, differences between the four clusters were obvious and they were apparent in both transects. Thus, all clusters comprised the same dominating phyla (*Alphaproteobacteria, Bacteroidetes, Gammaproteobacteria, Cyanobacteria* and *Verrucomicrobia*) but their relative

abundances differed greatly among clusters 1–4 (Figure 9). Whereas there was no clear trend in the proportion of OTUs affiliated with *Cyanobacteria*, the relative abundance of *Gammaproteobacteria* sequences was highest in the near-shore cluster 1 (23%) and lowest in the off-shore cluster 4 (14.5% ± 2.5%). *Alphaproteobacteria* reads were most abundant in cluster 3 (37% ± 1%) but very low in cluster 4 (13.5% ± 4%). By contrast, the proportion of sequences affiliated with *Bacteroidetes* was highest in cluster 4 (28% ± 2.5%) and lowest in cluster 3 (18.5% ± 2.5%). The strongest variations were observed in the relative abundances of *Verrucomicrobia* sequences, which were very low in clusters 1 and 3 (2% ± 0.8 %) but much higher in clusters 2 and 4 (12.25% ± 4.5%).

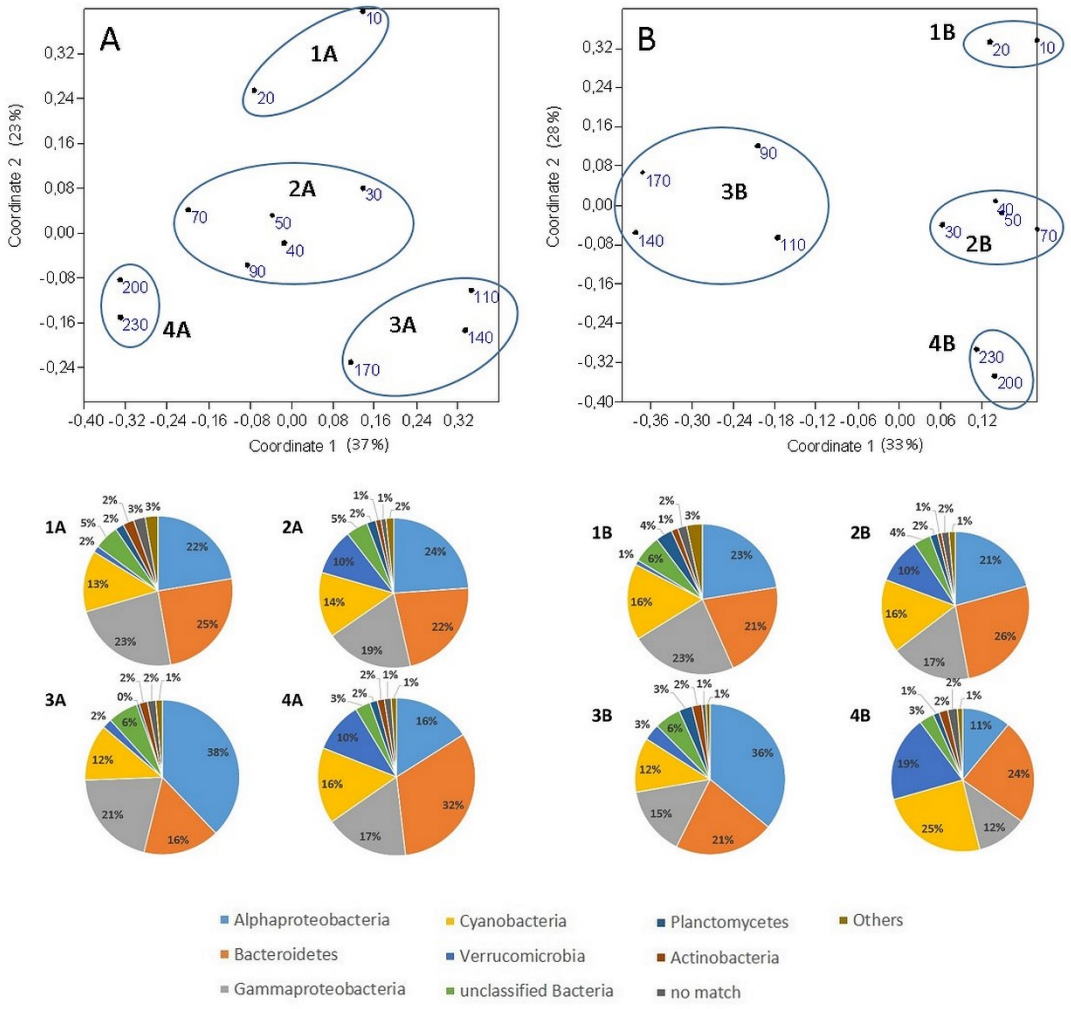

Figure 9: Principle coordinate analysis (PCoA) of the normalized abundance of all OTUs based on the Bray-Curtis dissimilarity. Each number corresponds to the distance to shore (in km) of the respective sample. The first three coordinates of the PCoA explained 71% of the variance (coordinate 1: 37%. 2: 23%. 3: 11%) in the community data of transect 1 **(A)** and 73% of the variance (coordinate 1: 33%. 2: 28%. 3: 12%) in the community data of transect 2 **(B)**. The pie charts show the relative abundances on the phylum level and refer to the respective clusters marked with blue ellipses in the PCoA plots. Stations were clustered according to the results of the Bray Curtis clustering analysis (see Figure S2).

Table 6: Major phytoplankton groups and OTUs (according to the values shown in Table 2) in the four zones determined by PCoA and the respective diversity measures.

Zone	**1**	**2**	**3**	**4**
Major phytoplankton	Dinoflagellates	Dinoflagellates/Diatoms	Dinoflagellates	Diatoms
Major OTUs	5, 1, 19, 2, 7	1, 2, 3, 4, 5	2, 1, 4, 6, 5	1, 3, 8, 7, 14
Shannon (H)	9.235	7.082	8.256	6.04
Richness (S)	862	888	868	514

1.4. Discussion

The northern Benguela is a highly productive, perennial upwelling system (Shannon *et al.*, 1986). The aim of this study, performed in late winter, when the upwelling is strongest, was to describe the development of the bacterial communities in aging upwelled water and to gain insight into the primary drivers of bacterial community composition. To follow successive developments we used an Eulerian approach, investigating water masses from different source areas and of different ages after the upwelling, rather than following the development of a single aging water mass (Langrangian approach). However, Mohrholz *et al.* (2014) found that most surface water along the transect stems from an upwelling band located along the coast and characterized by similar hydrographic conditions. Our data provide insights into the response of the bacterial community to different developmental stages after the upwelling and to an upwelling-induced phytoplankton bloom. During our study reproducible changes in PA, BPP, and BCC were determined in the two sampled transects. These changes were mainly caused by the abundance and quality of phytoplankton and to some extent resembled the bacterial succession that occurs in temperate water during phytoplankton blooms (e.g., Teeling et al., 2012).

Phyto-bacterioplankton coupling

The measured PA corresponded well to the Chl-a concentration along the transects, while BPP peaked closer to the shore, where nutrient concentrations were high, and nearly reached its minimum during the Chl-a peak . This resulted in a significant correlation between upwelled nutrients (NO_3, PO_4) and BPP such that bacterial growth at this location was not carbon-limited but was instead stimulated most likely by the upwelled inorganic nutrients. This conclusion is supported by an estimation of bacterial carbon requirements (data not

shown) based on BPP measurements and using a theoretical bacterial growth efficiency of 30%, as previously determined in other upwelling systems (Sorokin and Mikheev, 1979; Lønborg *et al.*, 2011). These calculations suggest that the estimated primary production (Fernández-Urruzola *et al.*, 2014) was sufficient to meet bacterial carbon demands along the entire length of each transect. It is also possible that BPP was stimulated by upwelled refractory dissolved organic carbon (DOC) from the deep-ocean which became more bioavailable due to photochemical transformations after reaching the surface (Benner and Biddanda, 1998). We therefore hypothesize that the initial upwelled bacterial community benefited from upwelled nutrients and photosynthetically derived organic carbon from the dinoflagellates that were dominant in this area (Hansen *et al.*, 2014).

The mean PA is in accordance with the bacterial numbers previously reported from the Benguela upwelling (Brown *et al.*, 1991) and from other upwelling regions (Wiebinga *et al.*, 1997; Barbosa *et al.*, 2001; Cuevas *et al.*, 2004). The low PA in the active upwelling is in accordance with the low cell numbers characteristic of deep water after the cells have been moved to the surface. A strong coupling between PA and the developing phytoplankton bloom after an upwelling has also been reported for the northwest Indian Ocean, the southern Benguela upwelling, and the Chilean upwelling (Painting, 1993a; Wiebinga *et al.*, 1997; Cuevas *et al.*, 2004).

The increase in BPP was comparable to that in previous reports from the northern and southern Benguela upwellings and the Chilean upwelling (McManus and Peterson, 1988; Painting *et al.*, 1989; Brown *et al.*, 1991). Several studies have confirmed the high level of bacterial activity in newly upwelled water (Sorokin and Mikheev, 1979; McManus and Peterson, 1988; Carvalho and Gonzalez Rodriguez, 2004). Their results are in accordance with the earlier observations of Vinogradov & Shushkina (1978) and Sorokin & Mikheev (1979), who suggested that upwelled water passes through heterotrophic and autotrophic phases as it moves offshore. McManus *et al.* (1988) hypothesized that bacteria in newly upwelled water are stimulated by the sinking substrates generated by herbivory in offshore waters and advected inshore during an upwelling.

The low level of BPP during the peak Chl-a concentration has several possible explanations. Hansen *et al.* (2014) observed, for the same transect, a significant change in phytoplankton community composition between the upwelling and the Chl-a maximum. Whereas coastal stations were dominated by dinoflagellates, approximately 50 km offshore

there was a drastic shift to a diatom-dominated community, which resulted in maximum Chl-a concentrations at a distance of 70 km. Our Eulerian approach prevented an assessment of the developmental stage of the diatom bloom during the Chl-a peak. However, the reduction in BPP might have been linked to the young age of the diatom bloom, when less dissolved organic carbon (DOC) is produced such that bacteria must attach to phytoplankton cells in order to hydrolyze the dissolved organic matter (Smith *et al.*, 1995). Furthermore, the high abundance of HNF (data not shown) and their marginally significant correlation with bacterial abundance would have led to a predominant reduction of active cells by grazing (Jürgens and Matz, 2002).

In contrast to our observations, Painting *et al.* (1993b) and McManus *et al.* (1988) reported an increase in bacterial production during and after the peak of primary production. This discrepancy might reflect the fact that we did not sample the diatom post-bloom and therefore missed a second peak in BPP. However, phytoplankton, bacteria, and the control of the latter by bacterivores are closely related, as shown in simulations of different biomass relationships in plankton communities after an upwelling (Moloney and Field, 1991). Sarmento *et al.* (2012) were therefore able to show that DOC originating from different phytoplankton species differentially stimulated heterotrophic prokaryotes. Furthermore, an increase in BPP during the diatom post-bloom might have been hindered by the dynamic conditions in this area, with potentially high HNF grazing and sinking of diatom cells. This conclusion is supported by the rapid decrease in PA 90 km offshore, probably due to predation by bacterivores, as demonstrated in a microcosm simulation of the upwelling in the southern Benguela (Painting *et al.*, 1989).

Zonation of bacterioplankton communities

The influence of phytoplankton development on PA and BPP was even more obvious when the shifts in BCC were considered. The dominance of the taxonomic groups *Alphaproteobacteria* clade SAR11, *Cyanobacteria* clade GpIIa, *Gammaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia* was in accordance with previous studies in the eastern Atlantic Ocean (Alonso-Sáez and Arístegui, 2007; Friedline *et al.*, 2012) and indicative of a large-scale stable community structure. However, the upwelling induced spatial variability in the structure of the bacterial assemblage, as suggested by other studies of upwelling systems

(Baltar *et al.*, 2007; Alonso-Gutiérrez *et al.*, 2009; Teira, Nieto-Cid, *et al.*, 2009; Zeigler Allen *et al.*, 2012).

Parallels in abundance patterns were identified using k-means clustering of the 25 most abundant OTUs, assuming that similar distributions along the transect indicated similar lifestyles. Five distinct clusters were present in both transects, consistent with the strong effects of the presence and absence of phytoplankton, possibly related to copiotrophic or oligotrophic lifestyles. Interestingly, most of the phyla were exclusively represented in either a positively or a negatively correlated cluster, which suggests that different taxonomic groups are influenced by different growth-controlling factors (Gasol *et al.*, 2008).

A PCoA of OTU abundance revealed four clusters of microbial assemblages along the transects. A correlation analysis showed that Chl-a concentrations had a significant impact on zonation as the driver separating the microbial assemblages. This finding was consistent with the observed spatial changes in BCC that are typical for the different temporal stages of phytoplankton bloom development along coastal waters (e.g., Gilbert et al., 2012; Teeling et al., 2012). The characteristics of the four zones were as follows:

Zone 1 comprised freshly upwelled water with low Chl-a concentrations, the dominance of dinoflagellates, high nutrient concentrations, and a low PA. Both the high Shannon index and the high richness indicated the high microbial diversity in this zone , which was probably caused by the mixing of upwelled bacteria from deeper waters with the coastal surface community. This conclusion is supported by the high relative abundance of an OTU with high similarity to sequences derived from oxygen minimum zones and affiliated with *Oceanospirillalespa*. These sequences were reported to be common in the mesopelagic zone and in the dark ocean (Swan *et al.*, 2011; Friedline *et al.*, 2012). The high abundance of other *Gammaproteobacteria* and *Bacteroidetes* in the upwelling zone was in agreement with the findings of Baltar *et al.* (2007) in their study of a coastal transition zone and indicated the stimulation of *Gammaproteobacteria* by upwelled nutrients (Eilers *et al.*, 2000; Alonso-Sáez and Arístegui, 2007).

Zone 2 was characterized by blooming phytoplankton; however, it could be divided into a section with high abundance of dinoflagellates and high prokaryotic abundance and production, all of which occurred at stations with lower Chl-a concentrations, and a second section characterized by the dominance of diatoms, a high PA, and low levels of BPP, occurring at stations with high Chl-a concentrations (Hansen *et al.*, 2014). We found both a high diversity

and a high relative abundance of OTUs affiliated with *Bacteroidetes* class *Flavobacteria*, the abundance of which is known to increase during phytoplankton blooms (Teeling *et al.*, 2012; Williams *et al.*, 2012), especially of diatoms (Pinhassi *et al.*, 2004; Grossart *et al.*, 2005). These bacteria have been identified as initial degraders of complex organic matter (Kirchman, 2002; Arnosti, 2011; Gómez-Pereira *et al.*, 2012). An abundant OTU affiliated with *Gammaproteobacteria* clade SAR86 was present in the first section of zone 2, consistent with earlier studies in which the SAR86 group was highly abundant at coastal stations of the Benguela upwelling system (Morris *et al.*, 2012). Suzuki *et al.* (2001) hypothesized that the SAR86 group is stimulated by macronutrients in upwelled water. This is supported by the potential for proteorhodopsin-based ATP generation (Dupont *et al.*, 2012). Zone 2 was also characterized by a dramatic increase in the relative abundance of *Verrucomicrobia*, mainly because of a single OTU affiliated with *Persicirhabdus,* verrucomicrobial subdivision 1, which became the dominant OTU as Chl-a concentrations reached a maximum. *Verrucomicrobia* subdivision 1 is frequently found in marine bacterial communities (Bano and Hollibaugh, 2002; Freitas *et al.*, 2012). Although the type species *Persicirhabdus sediminis* has been isolated (Yoon *et al.*, 2008), little is known about the function of these organisms. Recent studies provided evidence of the high polysaccharide activity of *Verrucomicrobia,* in which polymer degradation may be more efficient than that by members of *Bacteroidetes* (Martinez-Garcia *et al.*, 2012). Friedline *et al.* (2012) compared bacterial communities along the eastern Atlantic Ocean and found a similar strong shift to a high abundance of *Verrucomicrobia* during a diatom-dominated bloom. Similarly, in our study there was a significant positive correlation between *Persicirhabdus* and Chl-a concentrations.

Zone 3 was characterized by low Chl-a concentrations, a dominance of dinoflagellates, a low PA, low-level BPP, and a bacterial community shift on the phylum level to a dominance of *Alphaproteobacteria*, the largest proportion of which was made up by an OTU affiliated with “Pelagibacterales.” The ecological role of “Pelagibacterales” in the ocean and in the presence of phytoplankton blooms has been studied extensively (e.g., Morris et al., 2002, 2012; Fuchs et al., 2005; Teira et al., 2009a). “Pelagibacterales” dominate in oligotrophic conditions with low Chl-a concentrations and can be stimulated by phytoplankton-derived labile compounds made available by *Flavobacteria* (Williams *et al.*, 2012). This finding is in accordance with our observation of a high abundance of “Pelagibacterales” after the decay of the phytoplankton bloom. The co-occurrence of “Pelagibacterales” with the SAR86 group is

consistent with the report of Dupont *et al.* (2012), who suggested that "Pelagibacterales" and SAR86 are not metabolic generalists and can thus avoid competing for DOC by utilizing different compounds.

Zone 4 was characterized by higher Chl-a concentrations and a dominance of diatoms due to an invading water filament of younger upwelled water, which resulted in higher PA, increased BPP, and a bacterial community more closely related to the community in zone 2 than in zone 3. Consequently, the relative abundances of both *Verrucomicrobia* and *Flavobacteria* increased in zone 4.

Overall, the zonation of the microbial communities along the aging upwelled water in the Benguela system was stable, with the quality and quantity of phytoplankton and nutrients acting as the main drivers of the observed zonation. The spatial shifts in BCC observed in this study were comparable with the temporal succession stages of algal blooms in temperate seas. Thus, the perennial Benguela upwelling system provides ideal conditions for investigations of the mechanisms linking bacteria and phytoplankton in the ocean. For a more comprehensive understanding of the observed bacterial successions, future studies should analyze species-specific patterns in bacterial substrate utilization for an understanding of their functional role, as well as the influence of top-down effects on the bacterial community composition.

Chapter 2

Acidification and warming affect prominent bacteria in two seasonal phytoplankton bloom mesocosms

Abstract

In contrast to clear stimulatory effects of rising temperature, recent studies of the effects of CO_2 on planktonic bacteria have reported conflicting results. To better understand the potential impact of predicted climate scenarios on the development and performance of bacterial communities, we performed bifactorial mesocosm experiments (pCO_2 and temperature) with Baltic Sea water, during a diatom bloom in autumn and a phytoplankton bloom in summer. The development of bacterial community composition (BCC) followed well-known algal bloom dynamics. A principle coordinate analysis (PCoA) of bacterial OTUs (operational taxonomic units) revealed that phytoplankton succession and temperature were the major variables structuring the bacterial community whereas the impact of pCO_2 was weak. Prokaryotic abundance and carbon production, and organic matter concentration and composition were partly affected by temperature but not by increased pCO_2. However, pCO_2 did have significant and potentially direct effects on the relative abundance of several dominant OTUs; in some cases these effects were accompanied by an antagonistic role of temperature. Our results suggest the necessity of high-resolution BCC analyses and statistical analyses at the OTU level to detect the strong impact of CO_2 on specific bacterial groups, which in turn might also influence specific organic matter degradation processes.

2.1. Introduction

Climate change caused by an increase in anthropogenic carbon dioxide (CO_2) in the atmosphere is projected to increase seawater temperatures and acidification by a shift in carbonate chemistry. The atmospheric CO_2 concentration (pCO_2) will presumably increase from 390 ppm to 700 ppm by the end of the century (Intergovernmental Panel on Climate Change, 2013). In the past, the oceans absorbed up to one third of anthropogenic CO_2 (Sabine *et al.*, 2004), which reduced the surface ocean pH by 0.1 units (Caldeira and Wickett, 2003). According to predictions, a further pH decrease by 0.3–0.4 units can be expected (Feely *et al.*, 2009), accompanied by an increase in sea surface temperature by up to 6°C (Intergovernmental Panel on Climate Change, 2013). As stated by the Royal Society (Raven *et al.*, 2005), ocean acidification is likely leading to less robust marine ecosystems with higher vulnerability to other environmental changes.

Many recent studies have demonstrated the strong effects of warming and acidification on the dynamics of phytoplankton growth. Warming accelerates phytoplankton blooming, decreases total phytoplankton biomass, and triggers a shift towards pico- and nanophytoplankton (Keller et al., 1999; Sommer and Lengfellner, 2008; Lewandowska and Sommer, 2010; Sommer and Lewandowska; 2011 Brussard et al. 2013). This is assumed to have implications for the turnover of organic matter and biogeochemical cycling and might influence the ratio of production and respiration and the efficiency of the biological pump (Brussaard *et al.*, 2013). An increase in aquatic pCO_2 reduces the growth of calcifying taxa (Riebesell *et al.*, 2000; Kroeker *et al.*, 2013) but stimulates the growth of total phytoplankton biomass (Eggers *et al.*, 2014). The resulting differences in phytoplankton dynamics and composition affect phytoplankton-derived organic matter by increasing transparent exopolymer particles and increasing CO_2 and temperature (e.g., Engel, 2002; Biermann et al., 2014; Endres et al., 2014; Engel et al., 2014).

CO_2-induced changes in phytoplankton are expected to also impact bacterial activity and community composition, both of which play a major role in marine biogeochemical cycles and especially in the cycling of organic matter (e.g. Cole *et al.*, 1988; Azam, 1998). That temperature is a critical determinant of bacterial growth is well established (Nedwell, 1999; Pomeroy and Wiebe, 2001; Apple *et al.*, 2006). Climate change studies have found a stimulating impact of temperature on bacterial activity (Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009; von Scheibner *et al.*, 2014) and temperature-related shifts in community composition (Hall *et al.*, 2009; Adams *et al.*, 2010; Dziallas and Grossart, 2011; von Scheibner *et al.*, 2014). By contrast and as stated in a meta-analytical review (Liu *et al.*, 2010), the effect of increased pCO_2 on bacterioplankton is unclear. Some studies report no effect on bacterial activity or abundance (Allgaier *et al.*, 2008; Krause *et al.*, 2012; Newbold *et al.*, 2012; Teira *et al.*, 2012; Oliver *et al.*, 2014; Hartmann *et al.*, 2015) and others show increased abundance and activity, probably mediated by the stimulation of phytoplankton blooms (Grossart *et al.*, 2006; Endres *et al.*, 2014) or even decreased bacterial production after nutrient addition in a mesocosm experiment (Motegi *et al.*, 2013). Recent investigations into the effects of CO_2 on bacterial community composition in controlled mesocosms with natural bacterioplankton assemblages also found either no (Newbold *et al.*, 2012; Oliver *et al.*, 2014) or only weak effects because of shifts in phytoplankton dynamics (Allgaier *et al.*, 2008; Arnosti *et al.*, 2011; Roy *et al.*, 2013). A few studies assumed the pH sensitivity of specific bacterial

groups and proposed direct CO_2 effects on metabolic rates via alterations in the metabolic cycling of acids and bases (Coffin *et al.*, 2004; Witt *et al.*, 2011; Krause *et al.*, 2012). Ren et al. (2015) reported that in freshwater lakes an increase in pH leads to a decrease in overall bacterial diversity, including shifts to taxa of higher adaptation potential. That study as well as others on freshwater lake bacteria suggest that pH strongly influences bacterial community composition (Lindström *et al.*, 2005; Jezbera *et al.*, 2012), perhaps via the pH dependence of extracellular enzymatic reactions (Münster, 1991). In line with this are studies showing increased rates of hydrolytic extracellular enzymes under increased CO_2 (Grossart *et al.*, 2006; Tanaka *et al.*, 2008; Piontek *et al.*, 2010, 2013; Maas *et al.*, 2013; Endres *et al.*, 2014) and a recent metatranscriptome study in which low pH induced the energetically costly, enhanced expression of genes encoding proton pumps (Bunse *et al.*, 2016).

In contrast to studies of bacteria in freshwater systems, data on marine bacteria are scarce. In the Baltic Sea, only one previous study investigated the combined effect of warming and acidification on bacterioplankton (Lindh *et al.*, 2013). It showed the strong impact of warming on a Baltic Sea spring bloom bacterioplankton community and that acidification played only a minor additional effect. That study also assumed a masking of pH effects by temperature, but whether the observed combined responses were mediated by shifts in higher trophic levels or by direct effects on bacteria was unclear. Furthermore, in that study the bacterial community analysis was carried out using denaturing gradient gel electrophoresis (DGGE), which is limited in its sequencing depth and phylogenetic resolution.

In the present study, we re-examined the combined effects of warming and acidification on Baltic Sea bacterial assemblages during two seasonal phytoplankton blooms (spring and autumn) and used next-generation sequencing of partial 16S rRNA genes to describe changes in community composition. We also considered changes in bacterial bulk parameters and in bacterial substrate supply, in an attempt to distinguish between effects directly attributable to CO_2 and those mediated by changes in phytoplankton or organic matter dynamics.

2.2. *Material and Methods*

Experimental design

A detailed description of the experimental design can be found in Paul et al. (2015). In brief, the autumn and summer experiments consisted of 12 indoor mesocosms, each with a volume of 1400 L, that were set up in the temperature-controlled rooms of GEOMAR-Kiel. The mesocosms were filled with unfiltered water from the Kiel Bight in the Western Baltic Sea. The water contained the natural plankton community, however, mesozooplankton was added at a density of 20 individuals L^{-1}. The mesocosms were stirred by a propeller to avoid sedimentation and were illuminated by computer-controlled light units aligned to the seasonal light patterns. CO_2 was manipulated by a flow-through of air-CO_2 mixtures and by the addition of CO_2-saturated filtered mesocosm water.

Table 7: Target temperature and target pCO_2 values for the different treatments in the autumn and summer experiments.

	Autumn		**Summer**	
Treatment	**pCO_2 (ppm)**	**Temperature (°C)**	**pCO_2 (ppm)**	**Temperature (°C)**
Warm high	1400	15	2500/3000	22.5
Warm medium	-	-	1500/2000	22.5
Warm low	560	15	500/1000	22.5
Cold high	1400	9	2500/3000	16.5
Cold medium	-	-	1500/2000	16.5
Cold low	560	9	500/1000	16.5

Low CO_2 levels were chosen to represent the present-day annual minima of the Kiel Fjord. High CO_2 levels were set to 1400 ppm in the autumn experiment, the level predicted by an IPCC-scenario for the year 2100 (Intergovernmental Panel on Climate Change, 2013). In the summer experiment, the CO_2 levels were as high as 3000 ppm because the Kiel Fjord is an area with strong natural variations, where short-term upwelling can result in peak values of 4000 ppm in summer (Thomsen *et al.*, 2010). The temperature regimes were set to 3°C above and below the respective in situ temperature at the time of the experimental setup. As the

experiments were conducted in different seasons, they are referred to in the text as the autumn experiment (conducted 19.10.2012–12.11.2012) and the summer experiment (14.08.2013–13.09.2013). The temperature and pCO_2 conditions for the two experiments are summarized in Table 7. The treatment combinations from the autumn experiment were replicated three times; in the cold, low CO_2 treatment, the data from one mesocosm were excluded from further analysis because the light supply in that mesocosm failed for 72 h. For detailed descriptions of the CO_2- and temperature development during the autumn and summer experiments, see, respectively, Paul et al. (2015) and Paul et al. (2016).

Sampling

In both experiments, water samples for prokaryotic cell numbers, prokaryotic activity, and the dissolved organic carbon (DOC) concentration were taken every 2–3 days. Samples for bacterial diversity analyses were taken at two subsequent time points during the phytoplankton bloom peak [autumn experiment: day 11 (t_1) and day 14 (t_2), summer experiment: day 7 (t_1) and day 10 (t_2)] and at two subsequent time points during phytoplankton decay [autumn experiment: day 18 (t_3) and day 21 (t_4), summer experiment: day 21 (t_3) and day 24 (t_4)]. The time points were selected based on the respective chlorophyll-a concentrations [see Paul et al. (2015) for the autumn experiment and Paul et al. (2016) for the summer experiment]. Samples for amino acids analyses were taken between t_1 and t_4, and those for the molecular characterization of dissolved organic matter (DOM) on the day of filling, day 16, and day 21 (t_4), both during the autumn experiment.

Quantification of dissolved organic carbon

Samples for DOC analysis were filtered through combusted GF/F filters, collected in 20-mL combusted (8 h, 500°C) glass ampoules, and acidified with 80 µL of 85% phosphoric acid. They were stored at 2–4°C for up to 2 months until the analysis. DOC concentrations in the filtrate were determined by high-temperature catalytic oxidation using a Shimadzu TOC-VCSH analyzer equipped with a Shimadzu TNM-1 module. The DOC concentrations are the average values of quadruplicate measurements.

Molecular characterization of DOM

A 2-L sample from each mesocosm was filtered through combusted 0.7-µm GF/F filters (4 h, 400°C, Whatman), acidified to pH 2 with HCl (25%, analysis grade, Carl Roth), and extracted via solid-phase extraction (SPE) using a commercially available PPL resin (1 g, Agilent) following the guidelines published by Dittmar et al. (2008). The extract-containing cartridges were then rinsed with acidified ultrapure water (pH 2, HCl 25%, analysis grade, Carl Roth), dried with argon gas, and eluted with 500 mL of methanol (HPLC-grade, Sigma-Aldrich). The average extraction efficiency was 50.0±2.0%, determined on a carbon basis. Mass spectrometric analysis of the SPE extracts was done via Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) using a 15-Tesla Solarix system (Bruker Daltonics, USA) equipped with an electrospray ionization source (ESI, Bruker Apollo II) applied in negative ionization mode. All molecules were detected as singly charged ions. MatLab routines developed in the Dittmar laboratory in Oldenburg (Germany) were used to assign the molecular formulae and in further data processing. Peaks with intensities below the detection limit of the method (Riedel and Dittmar, 2014) were not considered for analysis. Molecular formulae were assigned to the masses based on the criteria of Koch et al. (2007) and Rossel et al. (2013), considering the elements C, H, O, N, S, and P. To test the reproducibility and stability of the FT-ICR-MS analysis, SPE-DOM from North Equatorial Pacific Intermediate Water (NEqPIW) was analyzed with the same settings twice a day, which allowed determination of the analytical variability (Green *et al.*, 2014).

Dissolved free and combined amino acids

To measure the concentrations and compositions of the dissolved free and hydrolyzable amino acids (DFAA and DHAA), 6-mL seawater samples were filtered through 0.45-µm filters (GHP membrane, Acrodisk, Pall Corporation) in combusted (8 h, 500°C) glass vials and stored frozen at −20°C. Amino acids were analyzed based on the methods of Dittmar et al. (2009) and Escoubeyrou and Tremblay (2014), with modifications. Samples for DFAA analysis were supplemented with borate buffer to achieve a pH of 8.5. To analyze the combined dissolved amino acids, 1 mL of sample and 1 mL of 30% hydrochloric acid (Merck, suprapure) were hydrolyzed in sealed ampoules at 100°C for 20 h. The hydrolysate was dried in a microwave under a pure nitrogen atmosphere and then washed twice with 0.5 mL of

ultrapure water to remove the HCl. Finally, the samples were re-dissolved in 1 mL of ultrapure water.

Amino acids were separated by HPLC (1260, Agilent) using a C18 column (Phenomenex Kinetex, 2.6 µm, 150 x 4.6mm) after in-line derivatization (2 min) of the samples with o-phtaldialdehyde and mercaptoethanol. Solvent A was 0.1 M sodium dihydrogen phosphate adjusted to pH 7 with sodium hydroxide and premixed with acetonitrile (19:1 v/v). Solvent B was acetonitrile. A linear gradient was run starting from 6% solvent B to 27% solvent B in 40 min at a flow rate of 0.8 mL/min. Standards were used for asparagine + aspartic acid (AsX), glutamine + glutamic acid (GlX), serine (Ser), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), isoleucine (Ileu), phenylalanine (Phe), and leucine (Leu). Standards were run at the beginning and after each fifth sample.

Prokaryotic cell number and prokaryotic carbon production

For bacterial cell counts, 4.5-mL samples were preserved with 200 µL of glutaraldehyde (1% v/v final concentration) and stored at −20°C for up to 6 months until measurement. A stock solution of SybrGreen I (Invitrogen) was prepared by mixing 5 µL of the dye with 245 µL of dimethyl sulfoxide (DMSO, Sigma Aldrich); 10 µL of the dye stock solution and 10 µL of Fluoresbrite microspheres (diameter 0.94 µm, Polysciences) were added to 400 µL of the thawed sample and incubated for 30 min in the dark. Samples with high counts were diluted 1:4 or 1:8 with ultrapure water. The samples were then analyzed at a low flow rate using a flow cytometer (FACS Calibur, Becton Dickinson) [45]. TruCount beads (Becton Dickinson) were used for the calibration and in combination with Fluoresbrite YG microsphere beads (1.00 µm, Polysciences) for absolute volume calculation. Calculations were done using the software program “Cell Quest Pro”.

The incorporation of ^{3}H-leucine (specific activity 140 and 100 Ci mmol^{-1} in the autumn and summer experiment, respectively) was determined to estimate prokaryotic carbon production. Three replicate samples were amended with the radiotracer at a saturating final concentration of 20 nmol L^{-1} and incubated for 1–2 h in the dark at the treatment temperatures. The incubations were stopped by the addition of trichloroacetic acid (TCA) at a final concentration of 5%. The samples were then processed using the centrifugation method of Smith and Azam (1992). Briefly, the samples were centrifuged at 14,000×g and the remaining cell pellet was washed twice with 5% TCA. After resuspension of the cell pellet in

scintillation cocktail (Ultima Gold AB, Perkin Elmer), leucine incorporation was measured by liquid scintillation counting. Leucine incorporation was converted into bacterial carbon production according to the method of Simon and Azam (1987), using a leucine mol% value of 7.3 and a carbon conversion factor of 0.86.

Bacterial community composition

Water samples for DNA analysis were filtered onto 0.22-µm pore-size white polycarbonate filters. DNA was extracted according to Weinbauer et al. (2002). For bacterial diversity analysis, hypervariable regions 3–5 (V3–V5) of the 16S rRNA gene were used to generate PCR amplicons for 454 pyrosequencing, as described by Herlemann et al. (2011), but with a modification of 30 PCR cycles. Sequencing was performed by Eurofins MWG GmbH using 454 GS-FLX sequencer (Roche). The standard operating procedure for mothur described in Schloss et al. (2011) was used for further sequence processing. In brief, the raw reads were denoised, trimmed, quality-filtered, and aligned with the Silva database (Quast *et al.*, 2013). Sequences were grouped in operational taxonomic units (OTUs) on a 97% level. The number of sequences in each sample was normalized by randomly selecting the number of sequences present in the smallest sample. A heat map of relative OTU abundances on the phylum level was created using heatmap.2 in the gplots package in the R statistical language (R Development Core Team, 2012). The sequences were deposited in the European Nucleotide Archive (ENA) and can be accessed at: http://www.ebi.ac.uk/ena/data/view/PRJEB12113.

Statistics

We decided to transform The CO_2 treatment gradient ranging from 500 to 3000 ppm in the summer experiment to three CO_2 levels (low, medium, high) in order to obtain results that were comparable with those of the autumn experiment by applying the same multivariate tests.

Prokaryotic abundance and prokaryotic carbon production were analyzed for significant temperature and pCO_2 effects using an analysis of variance with repeated measures (rmANOVA) with SPSS statistics (IBM). A p value < 0.05 according to Greenhouse-Geisser was considered to indicate statistical significance.

OTU abundance data were square-root transformed and beta-diversity was calculated with the Bray-Curtis coefficient. From these data, a principal coordinate analysis (PCoA) was

performed and a Spearman correlation analysis of the PCoA coordinates with the experimental parameters was used to detect drivers for the separation of samples in the PCoA. A repeated measures permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2006) was applied to the OTUs based on the Bray-Curtis dissimilarity matrix, to test the individual CO_2 treatments and the temperature treatments. These analyses were performed using the software PRIMER6 with the PERMANOVA+ addon (PRIMER-E).

The make.lefse command in mothur was used to create a linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.*, 2011) input file from the mothur shared file. This was followed by a LEfSe (http://huttenhower.sph.harvard.edu/lefse/) to test for discriminatory taxa between all treatments. With a normalized relative abundance matrix, LEfSe uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa in the different treatments and performs a LDA to estimate the effect size of each feature. A significance alpha of 0.05 and an effect size threshold of 2 were used for all biomarkers discussed in this study.

For statistical analysis, the DOM data were normalized to the sum of the peak intensities. Based on the normalized peak intensities of the 6152 most intense peaks with assigned molecular formulae for each sample, a Bray-Curtis dissimilarity matrix was calculated using the software package R (Version 3.0.2, package "vegan" (Oksanen *et al.*, 2013)). The amino acid composition data from both the CO_2 treatments and the temperature treatments were further tested using a PERMANOVA. The average distance to each group centroid was calculated based on a matrix of Euclidean distances. These analyses were done using the software PRIMER6 with the PERMANOVA+ addon (PRIMER-E).

2.3. Results

Prokaryotic abundance and activity

Phytoplankton development differed in the two experiments. While a complete bloom succession occurred in the autumn experiment (Figure 10A), the phytoplankton biomass in the summer experiment was already high on day 1 and decreased thereafter (Figure 10E). Prokaryotic abundance (PA) ranged from $2.0 \pm 0.3 \times 10^5$ cells mL^{-1} to $1.8 \pm 0.5 \times 10^6$ cells mL^{-1} in the autumn experiment (Figure 10B) and from $2.0 \pm 0.4 \times 10^6$ cells mL^{-1} to $4.9 \pm 0.1 \times 10^6$ cells mL^{-1} in the summer experiment (Figure 10F). PA developed in parallel, with only minor

variations in all treatments. An increase in temperature resulted in significantly lower PAs in both experiments (Table 8), with the lowest abundances measured at the last three sampling dates (t-tests, $p < 0.05$). Especially in the autumn experiment, an earlier and lower PA peak was detected in the warm vs. the cold treatments. While CO_2 did not significantly impact PA, the correlation between PA and phytoplankton carbon in the autumn experiment was significant (rmANOVA, $p < 0.05$).

Table 8: Differences in the development of bacterial abundance and activity between treatments in the autumn and summer experiments (**r**epeated measures ANOVA).

	Autumn		**Summer**	
Factor	**Abundance**	**Activity**	**Abundance**	**Activity**
Time	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time × Temperature	**< 0.01**	**< 0.01**	**< 0.01**	**0.01**
Time × pCO_2	0.66	0.38	0.25	0.48
Time × Temperature × pCO_2	0.92	0.65	0.23	0.41

Significant results ($p < 0.05$) are shown in bold.

Prokaryotic carbon production (PCP) ranged from 2.0 ± 0.6 µg C d^{-1} L^{-1} to 26.4 ± 12.9 µg C d^{-1} L^{-1} in the autumn experiment (Figure 10C) and from 3.6 ± 0.1 µg C d^{-1} L^{-1} to 33.1 ± 4.0 µg C d^{-1} L^{-1} in the summer experiment (Figure 10G). Although PCP increased over time during the autumn experiment, it was highest at the beginning of the summer experiment and then progressively decreased, in parallel with phytoplankton biomass. Corresponding to the PA, there were only minor variations in the PCP in all treatments but temperature had a significant impact (Table 8). The differences were more pronounced in the autumn experiment, in which PCP was significantly higher in the cold treatments at the last three sampling dates. There was no significant impact of CO_2 on PCP whereas the correlation between PCP and phytoplankton carbon was significant in both experiments ($p < 0.05$).

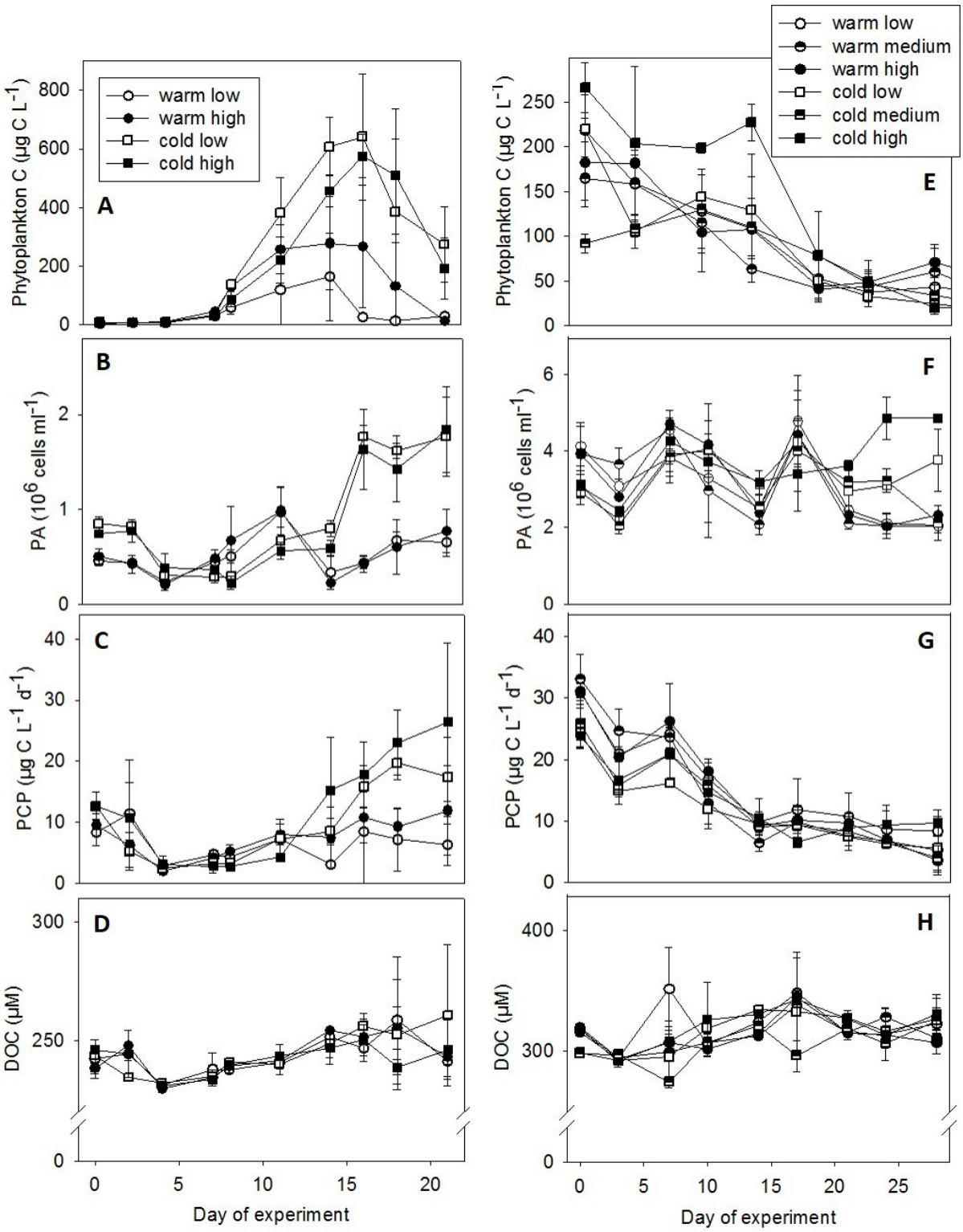

Figure 10: Phytoplankton biomass development [from Paul et al. (2015) and Paul et al. (2016) for the autumn and summer experiments, respectively], prokaryotic abundance (PA), prokaryotic carbon production (PCP), and the dissolved organic carbon (DOC) concentration in the autumn (A, B, C, D) and summer (E, F, G, H) experiments.

Dissolved organic matter (DOM) concentration and composition

There was no clear trend in dissolved organic carbon (DOC) concentrations and no difference between treatments in both experiments (rmANOVA, $p > 0.05$). DOC concentrations ranged from 229 ± 1.4 µmol/L to 260-± 29.7 µmol/L during the autumn experiment (Figure 10D) and from 274 ± 0.3 µmol/L to 351-± 34.5 µmol/L during the summer experiment (Figure 10H). During the former, concentrations of dissolved free amino acids (DFAA) ranged from 7.5 ± 5.0 nmol/L to 44 nmol/L and those of dissolved combined amino acids (DCAA) from 674 ± 67.6 nmol/L to 1049 ± 212.6 nmol/L (see Figure S5 in the Supplementary Material). There was no significant difference in the DFAA or DCAA concentrations in response to temperature or CO_2, or their interaction term (rmANOVA, $p > 0.05$). However, regarding the amino acid composition, a repeated measures PERMANOVA identified temperature, but not CO_2, as a significant driver of changes in the composition of DFAA ($df = 1$, pseudo-$F = 4.0$, $p < 0.05$) and DCAA ($df = 1$, pseudo-$F = 7.7$, $p < 0.01$).

Using ultrahigh-resolution mass spectrometry (15 Tesla Fourier-transform ion cyclotron resonance mass spectrometry, FT-ICR-MS), we analyzed the composition of the DOM in the autumn experiment. Over 6,000 molecular formulae corresponding to DOM compounds in the experiments were thus identified and the relative abundances of those compounds were followed over the course of the experiments. A statistical Bray-Curtis dissimilarity analysis of the molecular formulae and their respective signal intensities was performed to detect changes in the molecular composition of DOM (Figure 11). Until day 16, the molecular differences between the experiments were within analytical variability; only on day 21 (t_4) did the molecular DOM composition begin to diverge between experiments, but this divergence was independent of both temperature and pCO_2.

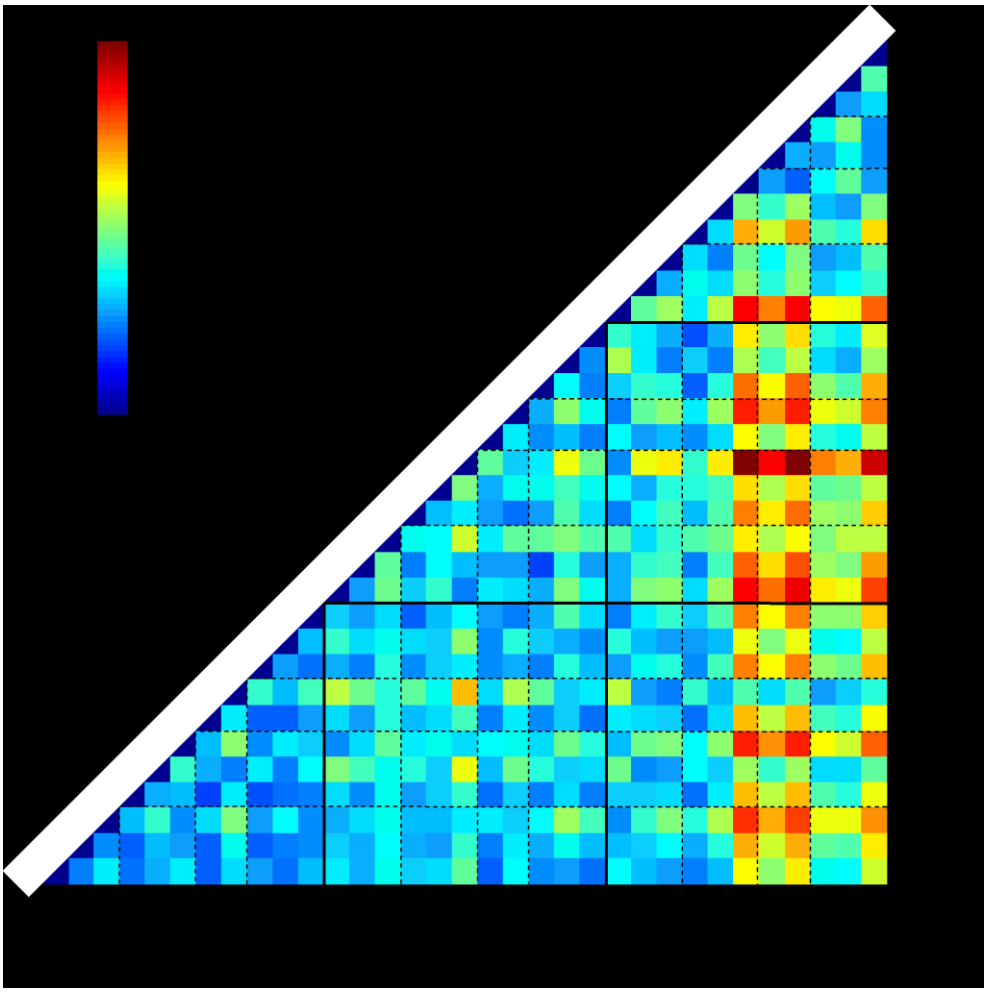

Figure 11: Bray-Curtis dissimilarity matrix based on the relative signal intensities of 6813 detected molecular formulae in the full range FT-ICR mass spectra from the autumn experiment. The color scale displays the dissimilarity between two samples. The samples were ordered first by the respective time point of the experiment and second by the temperature and pCO2 treatment. The analytical variability was determined by repeated measurements of a reference sample from the deep sea. Phytoplankton bloom and end are days 16 and 21, respectively.

Bacterial community response

Pyrosequencing generated 194,435 raw sequence reads from the autumn experiment and 141,514 from the summer experiment. Using the standard operating procedure for mothur, 163,591 quality sequences were obtained from the autumn experiment and 117,518 from the summer experiment. Sub-sampling to obtain the fewest sequences per sample (autumn experiment: 1,511 sequences, summer experiment: 1,395 sequences) resulted in 1,512 operational taxonomic units (OTUs), with 97% similarity across the entire sample set, in the autumn experiment and 2,752 OTUs in the summer experiment. The predominant phyla across all samples were *Bacteroidetes* (53.3% of all sequences), and *Alphaproteobacteria*

(27%) in the autumn experiment and *Bacteroidetes* (40.8%), *Alphaproteobacteria* (17.8%), *Cyanobacteria* (13.4%), and *Gammaproteobacteria* (10.8%) in the summer experiment. In both experiments, there was a shift from a high abundance of *Alpha*- and *Gamma*proteobacteria and *Bacteroidetes* during the bloom phase (t_1 and t_2) to a dominance of *Bacteroidetes* during bloom decay (t_3 and t_4) (Figure 12A, B).

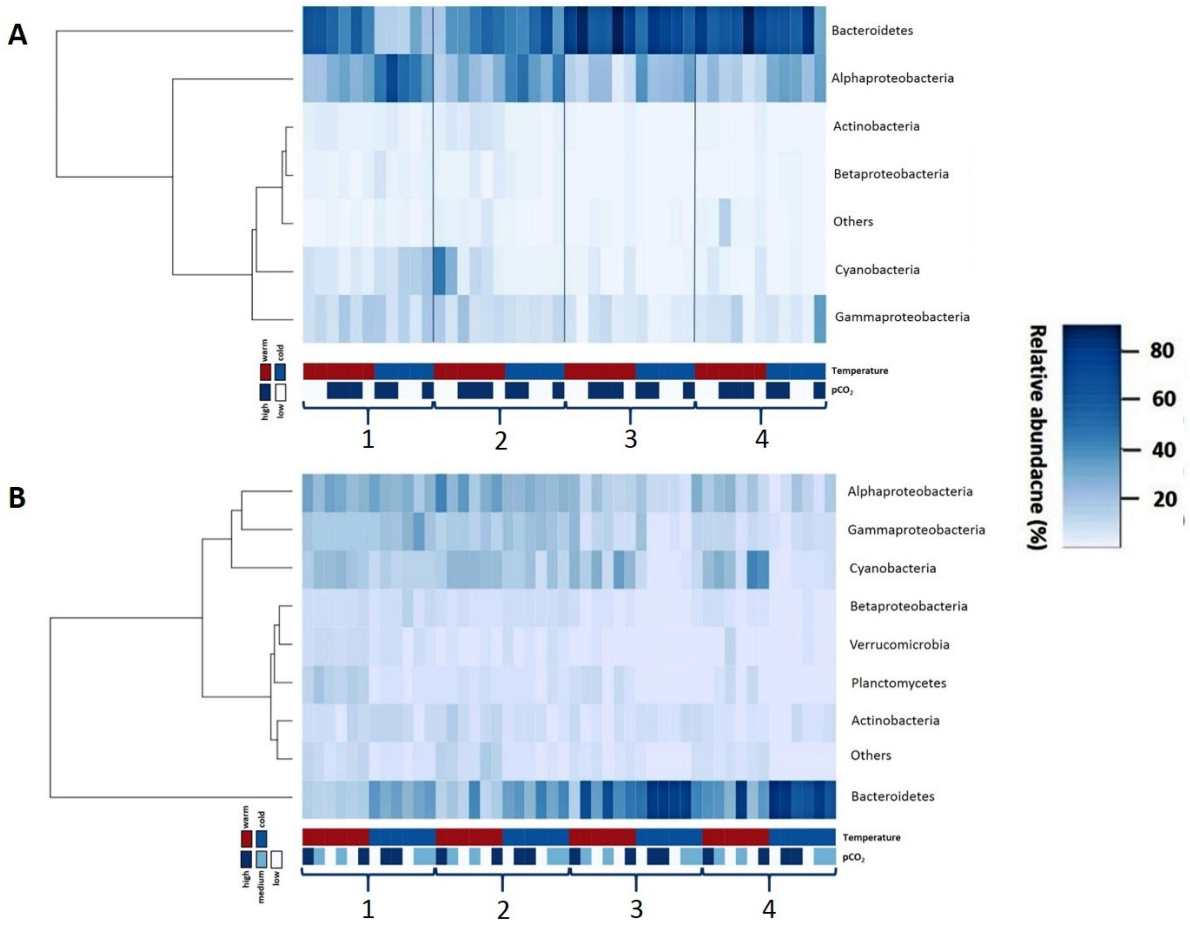

Figure 12: Heat map of the relative OTU abundance at the phylum level at the four sampled time points in the autumn (A) and summer (B) experiments. Vertical blue lines separate the sampling time points t_1–t_4. The dendrograms show the distances according to the relative abundance of the respective phylum. The color code below the heat map indicates the temperature and pCO_2 conditions in the respective sample.

Bacterioplankton community composition (BCC) at the OTU level was also strongly influenced by the temporal plankton succession and by temperature. A Spearman correlation analysis of the coordinates obtained in the principal coordinate analysis (PCoA) and the experimental parameters showed that temperature- and time (Day)-related differences in BCC aligned along coordinate 2 in both experiments, explaining 15.7% and 14.6% of the total variation, respectively (Figure 13A, B). The impact of CO_2 on BCC was weak. Correlation

analysis showed that coordinate 3 separated samples from different CO_2 conditions in the autumn experiment and explained 8.9% of the total variation. In the summer experiment, coordinate 6 correlated with CO_2, explaining 3.7% of the total variation.

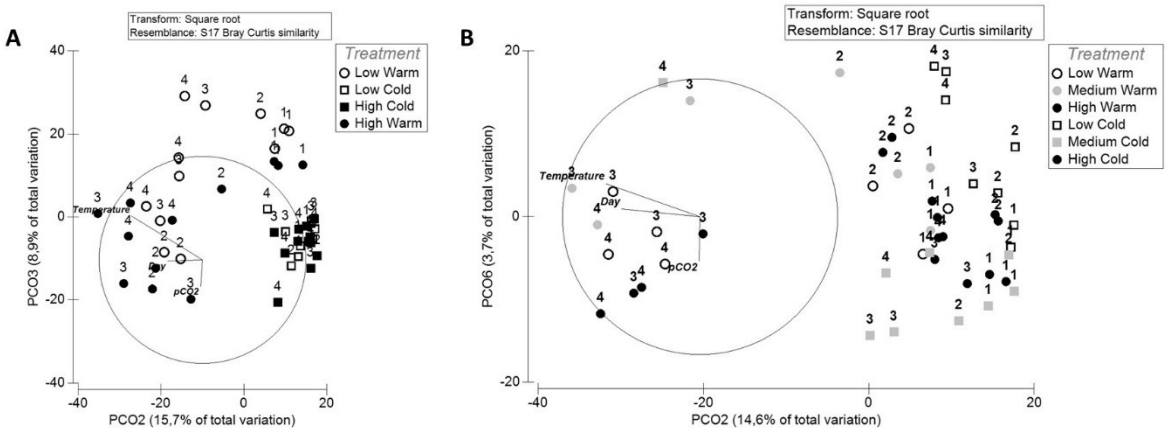

Figure 13: Principle coordinate analysis (PCoA) of OTU abundance based on the Bray-Curtis dissimilarity at the four sampled time points in all treatments during the autumn (A) and summer (B) experiments. Each PCoA shows the two axes with the highest correlation with the pCO_2 and temperature values. The numbers refer to the sampling time points t_1–t_4 and the lines indicate the correlations of the PCoA axes with the main experimental parameters in the correlation circle.

These relationships were confirmed by a PERMANOVA analysis, which showed that mainly temperature but also, to some extent, CO_2 significantly affected BCC (square root, Table 9). As the interaction effect of temperature and CO_2 was marginally significant in autumn and significant in summer (Table 9), a second PERMANOVA analysis was carried out to reveal the impact of CO_2 on BCC within the separate temperature treatments. A weak but significant effect of CO_2 on BCC also within these treatments was determined, except for the cold treatments in the autumn experiment (Table 9).

Table 9: Main tests for temperature and pCO_2 effects on bacterial community composition based on Bray-Curtis dissimilarities in the relative OTU abundance (repeated measures PERMANOVA). The displayed are tests for the factors 'pCO_2', 'temperature', 'day' and their interactions and the partitioning of multivariate variation. p-values were obtained using type III sums of squares and 999 permutations under a reduced model. d.f.: degrees of freedom, SS: sums of squares, Sq. root: square root of the component of variation.

Season	Temperature	Sources of variation	d.f.	SS	Pseudo F	p (perm)[1]	Sq. root
Autumn	**All**	**pCO_2**	1	2087	1.68	**0.0127**	6.29
		Temperature	1	8959	7.21	**0.0001**	19.02
		Day	3	13714	3.68	**0.0001**	17.66
		pCO_2 × Temperature	1	1680	1.35	0.0919	6.41
		pCO_2 × Day	3	3159	0.84	0.8588	-5.94
		Temperature × Day	3	7972	2.14	**0.0001**	16.29
		pCO_2 × Temperature × Day	3	2395	0.64	0.9976	-12.88
		Residuals	28	34761			35.23
		Total	43	75880			
	Warm	**pCO_2**	1	2655	1.90	**0.0123**	10.24
		Day	3	12657	3.02	**0.0001**	21.68
		pCO_2 × Day	3	3180	0.76	0.9434	-10.62
		Residuals	16	22372			37.39
		Total	23	40864			
	Cold	**pCO_2**	1	1266	1.23	0.2302	4.94
		Day	3	9392	3.03	**0.0002**	20.91
		pCO_2 × Day	3	2455	0.79	0.8927	-9.44
		Residuals	12	12390			32.13
		Total	19	25859			
Summer	**All**	**pCO_2**	2	4970	1.98	**0.0003**	8.70
		Temperature	1	13223	10.65	**0.0001**	22.34
		Day	3	19796	5.32	**0.0001**	21.13
		pCO_2 × Temperature	2	4548	1.83	**0.0003**	11.36
		pCO_2 × Day	6	7304	0.98	0.5632	-2.45
		Temperature × Day	3	9075	2.44	**0.0002**	17.24
		pCO_2 × Temperature × Day	6	6891	0.93	0.7566	-6.82
		Residuals	24	29795			35.24
		Total	47	95541			
	Warm	**pCO_2**	2	4375	1.82	**0.008**	11.09
		Day	3	13827	3.83	**0.0002**	23.82
		pCO_2 × Day	6	6486	0.90	0.8139	-7.84
		Residuals	12	14449			34.70
		Total	23	39137			
	Cold	**pCO_2**	2	5080	1.99	**0.0036**	12.56
		Day	3	15044	3.92	**0.0001**	24.95
		pCO_2 × Day	6	7709	1.00	0.4774	1.74
		Residuals	12	15347			35.76
		Total	23	43181			

[1] Significant results [p (perm) < 0.05] are shown in bold.

A linear discriminant analysis (LDA) effect size (LEfSe) was then applied to identify the discriminant taxa in the temperature and the CO_2 treatments. We focused on detecting distinct taxa for CO_2 levels within the warm treatments, since the PERMANOVA revealed a higher impact of CO_2 on BCC at warmer temperatures. Using the default logarithmic (LDA) value of 2, 15 OTUs were distinct to one temperature treatment in the autumn experiment and 40 OTUs in the summer experiment (see Table S1, S2 in the Supplementary Material). For

clarity, Figure 14A, B shows only the taxa (from phylum to OTU level) with LDA values > 4. In autumn, mainly *Bacteroidetes* were enriched in the warm treatments, and *Alpha*- and *Gammaproteobacteria* in the cold treatments. In summer, representatives of *Cyanobacteria* and *Bacteroidetes* were enriched in the warm treatments, and OTUs from *Alphaproteobacteria* and *Bacteroidetes* in the cold treatments. Highly abundant OTUs were detected among the enriched taxa, namely, *Polaribacter* in the warm treatments *and Loktanella* in the cold treatments in autumn and *Synechococcus* in the warm treatments and *Bacteroidetes* "NS3a marine group" in the cold treatments in summer (Figure 14).

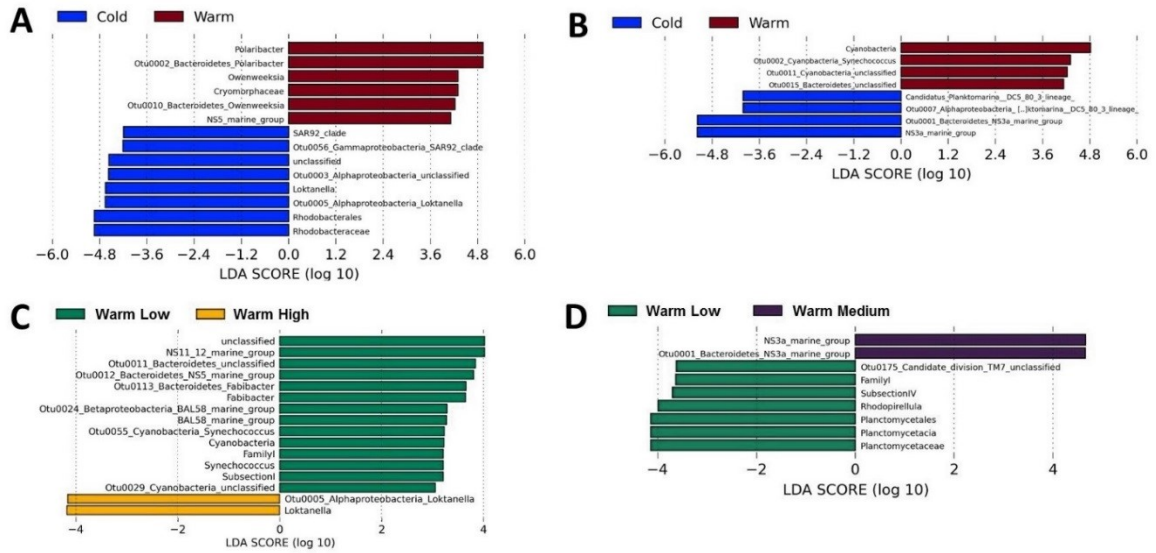

Figure 14: LEfSe results for the temperature treatments with LDA values > 4 for the autumn (A) and summer (B) experiments and for the CO2 treatments within the warm treatments with LDA values > 3.6 for the autumn (C) and summer (D) experiments.

Considerably fewer OTUs were enriched in one of the CO_2 treatments: seven OTUs were distinct to one of the warm CO_2 treatments in the autumn experiment and nine OTUs to one of the warm CO_2 treatments in the summer experiment, whereas only two OTUs were enriched in the cold CO_2 treatments in autumn and ten low-abundance OTUs were detected in summer. Figure 14C, D shows the taxa (from phylum to OTU level) with LDA values > 3.6. The bacterial taxa enriched by a high CO_2 level in the autumn experiment belonged to *Alphaproteobacteria* and those by a low CO_2 level to *Bacteroidetes*, *Betaproteobacteria*, and *Cyanobacteria.* In summer, OTUs belonging to *Planctomycetes*, *Cyanobacteria*, and *Candidate*

division TM7 were enriched in the low CO_2 treatments and *Bacteroidetes* in the medium CO_2 treatment.

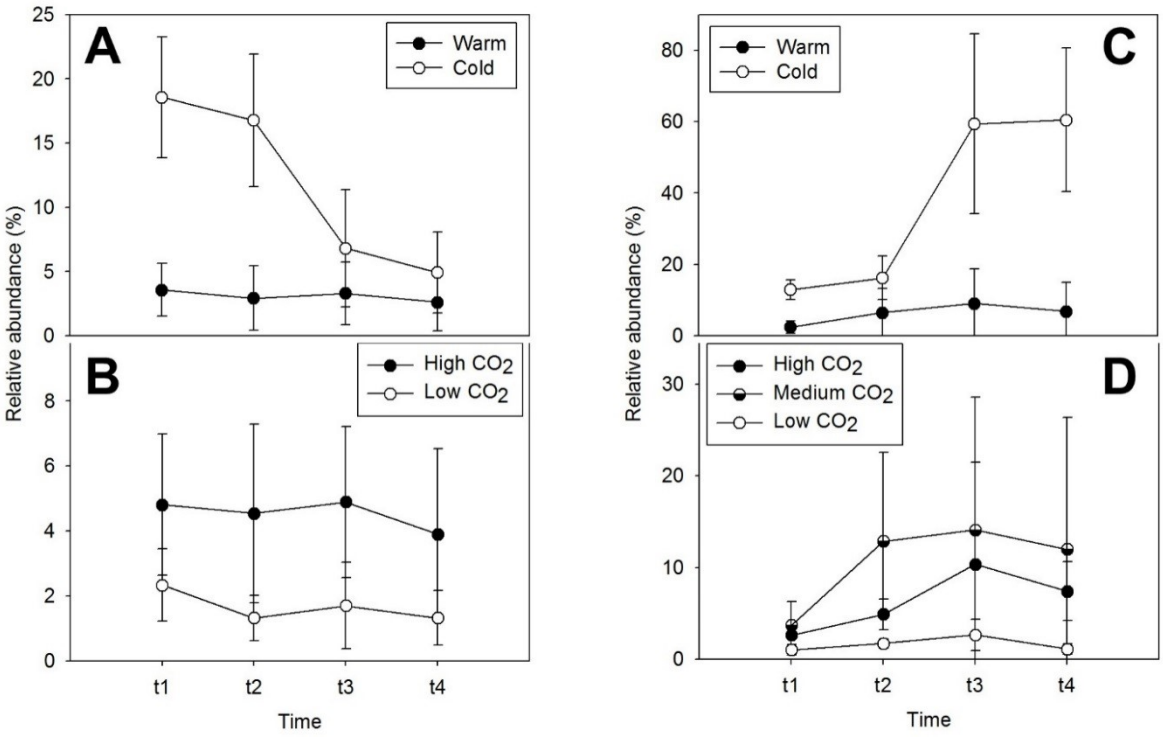

Figure 15: Relative abundance of OTU 0005 reads affiliated with *Alphaproteobacteria Loktanella* from the autumn experiment at the four sampled time points in the temperature (A) and warm CO_2 (B) treatments, and the relative abundance of OTU 0001 reads affiliated with *Bacteroidetes "*NS3a marine group" from the summer experiment at the four sampled time points in the temperature (C) and warm CO_2 (D) treatments.

Interestingly, particularly abundant OTUs were identified among the taxa enriched under warm, high/medium CO_2 conditions. In addition, in each experiment there was one taxon that was previously found to be enriched in the cold treatments, namely, *Loktanella* in the autumn experiment and *Bacteroidetes* "NS3a marine group" in the summer experiment. A comparison of the development in relative abundance showed the significantly higher abundances of both OTUs in the cold treatments and under the higher CO_2 conditions of the warm treatments (Figure 15). By employing a categorical design rather than a linear model for the summer experimental data, we were also able to confirm the significance of the enrichment of *Bacteroidetes* "NS3a marine group" under high CO_2 conditions, using a linear regression analysis (see Figure S4 in the Supplementary Material). In contrast to the abundant

OTUs in the warm CO_2 treatments, all enriched OTUs in the cold CO_2 treatments were of low abundance, with a maximum mean abundance of 1.1% for all OTUs.

2.4. Discussion

The impact of ocean acidification on bacterial communities remains controversial, as recent studies have reported conflicting results. The differences in their conclusions probably derive from the different sampling locations, seasons, sampling depth, and level of statistical analysis. The present study used high-resolution amplicon sequencing and a sensitive statistical analysis of the changes in bacterial community structure to examine the effects of ocean acidification on bacterial assemblages from a highly dynamic system in the Baltic Sea, one characterized by strong natural CO_2 variations (Thomsen *et al.*, 2010), during two different seasons. Furthermore, this study combines the effects of ocean acidification and warming in a factorial design. This design has been used only once before, in a bacterial community analysis by Lindh et al. (2013). However, because their study was based on DGGE, the results lack the high resolution of the analysis presented herein. Together with the sensitive statistical analyses, our high-resolution approach confirmed the overall strong impact of warming and, for the first time, allowed the detection of a significant CO_2 response by abundant taxa.

Influence of acidification and warming on bacterial abundance and activity

During the autumn experiment, bacterial abundance and activity followed well-known phytoplankton-induced dynamics. Cell number and activity development were coupled with phytoplankton growth, with an earlier peak in the warm than in the cold treatments, in accordance with the earlier and weaker phytoplankton biomass peak in the former (Paul et al. 2015). The different dynamics during the summer experiment reflected the different phytoplankton bloom dynamics. The blooming phase of the phytoplankton was defined as the period from day 0 to day 12 of its development and the post-bloom as the period from day 14 until the end of the summer experiment (Paul et al. 2016). Bacterial-phytoplankton coupling was only observed with respect to bacterial activity. In our study, despite the significant effect of temperature on bacterial abundance and activity in the summer experiment, it was not as clear as in the autumn experiment. The activity pattern in summer contrasted with that in autumn, perhaps because of the differences in bloom dynamics.

Although warming generally accelerates the occurrence of the phytoplankton bloom peak (Sommer and Lengfellner, 2008; Sommer and Lewandowska, 2011), it also results often in a decrease in total phytoplankton biomass (Sommer and Lengfellner, 2008; Lewandowska and Sommer, 2010) which is further amplified by more intensive grazing by copepods (Keller *et al.*, 1999; Lewandowska and Sommer, 2010). Previous experimental studies showed accelerated bacterial growth and activity and increased phyto-bacterioplankton coupling under warming (Wohlers *et al.*, 2009). Scheibner et al. (2014) also reported higher bacterial abundance and activity in spring bloom mesocosms at increased temperatures and a decoupling of phytoplankton and bacteria in cold treatments. While this pattern was confirmed in other mesocosm studies (Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009), according to Lindh et al. (2013) warming had no effect on bacterial abundance in a spring bloom mesocosm. By contrast, we found consistent bacteria-phytoplankton coupling in response to both temperature treatments whether in autumn or in summer, and a correlation between bacterial growth and phytoplankton development independent of temperature. The different results might be due to the different levels of grazing pressure or the different seasonal temperature conditions. In an early spring bloom, bacterial activity is low, such that the delay between phytoplankton blooming and the stimulation of bacterial activity might be too long to allow a strong coupling. By contrast, the bacterial communities in summer and autumn are already active and can immediately react to the changes in substrate supply afforded by the phytoplankton bloom.

The absence of an effect of acidification on bacterial abundance and activity is consistent with the observations of previous studies (Allgaier *et al.*, 2008; Krause *et al.*, 2012; Newbold *et al.*, 2012; Oliver *et al.*, 2014). While increases in abundance and activity in response to high CO_2 levels have been reported (Grossart *et al.*, 2006; Endres *et al.*, 2014), they were most likely related to indirect bacteria-phytoplankton coupling effects, as primary producers were stimulated by the higher CO_2 concentrations.

Influence of acidification and warming on bacterial community composition

The temporal bacterial succession followed the dynamics characteristic of phytoplankton blooms (Teeling *et al.*, 2012; von Scheibner *et al.*, 2014), with *Alpha*- and *Gammaproteobacteria* dominating during the bloom and *Bacteroidetes* during its breakdown. On a broad phylogenetic level, this trend was consistent in all treatments, with minor

variations. At an OTU identity level of 97%, temperature was the main community-structuring factor whereas CO_2 had a weak but significant influence. While temperature changes are known to induce shifts in bacterioplankton assemblages (Hall *et al.*, 2009; Adams *et al.*, 2010; Dziallas and Grossart, 2011; von Scheibner *et al.*, 2014), the results of studies on the influence of CO_2 are not consistent. Thus, weak changes (Allgaier *et al.*, 2008; Roy *et al.*, 2013), changes because of temporal shifts in the phytoplankton community composition (Arnosti *et al.*, 2011), and changes attributed to the progressive dominance of better-adapted bacteria (Ren *et al.*, 2015) have been described. Furthermore, there are also studies reporting no effect of increased CO_2 (Newbold *et al.*, 2012; Oliver *et al.*, 2014), effects on particle-associated bacteria only (Sperling *et al.*, 2013), and altered responses already at moderate changes in pH (Krause *et al.*, 2012).

Using the sensitive statistical test LEfSe at a high phylogenetic level we were able to detect several rare but also several abundant OTUs that were stimulated mainly by warming but in some cases also by an increase in CO_2 levels. Temperature-related abundance shifts can be explained by the stimulated growth of specific taxa and by the temperature dependence of phytoplankton bloom dynamics. However, some OTUs, such as one related to *Polaribacter*, was highly abundant at almost all time points in the warm treatments but nearly absent at all time points in the cold treatments. This finding was highly unexpected because *Polaribacter* is a psychrophilic genus (Gosink *et al.*, 1998) and warming is not known to enhance its growth. Another study found a strong increase in *Polaribacter*-related OTUs that was caused by nutrient enrichment (Baltar *et al.*, 2015), but in our study this possibility could be excluded because nutrient depletion developed in parallel in all treatments (Paul *et al.*, 2015). One potential reason for the high abundance of *Polaribacter*, besides warming, relates to the higher grazing pressure on phytoplankton in the warm treatments (Paul *et al.*, 2015), such that *Polaribacter,* which preferably utilizes labile organic carbon from the decaying phytoplankton bloom (Teeling *et al.*, 2012), would have been provided with an ample amount of labile organic carbon from the breakdown of phytoplankton.

In contrast to the numerous reports describing the effects of warming on bacterial taxa, few studies have examined the effects of CO_2. In their study of Arctic sediment communities, Tait et al. (2013) found an increase in *Halobacteria* OTUs with increasing CO_2. The only previous study to examine both warming and increased CO_2 levels reported the proliferation of specific rare phylotypes (Lindh *et al.*, 2013). In our autumn experiment, we

found a *Loktanella*-related OTU that was stimulated by higher CO_2 levels. *Loktanella* belongs to the *Rhodobacteraceae*, within the *Alphaproteobacteria* (Van Trappen *et al.*, 2004), and is member of the *Roseobacter* lineage. Interestingly, Meron et al. (2011) also found a shift to *Rhodobacteraceae* under high CO_2 in a coral-associated microbial community, whereas both Krause et al. (2012) and Witt et al. (2011) reported decreases in *Rhodobacteraceae* abundances with decreasing pH. The contradictory results might be due to the high diversity of the *Rhodobacteraceae*, whose members include representatives of the ubiquitous *Roseobacter* clade as well as species found in various habitats and occupying several ecological niches (see reviews by Buchan et al., 2005; Brinkhoff et al., 2008). Recent studies have highlighted the relevance of *Loktanella*-related strains in the re-mineralization of algal biomass (Hahnke *et al.*, 2013, 2014). This activity implies that a CO_2-mediated shift in *Loktanella* abundance would also impact organic matter cycling during the phytoplankton bloom.

In the summer experiment, the most abundant OTU was affiliated with the *Bacteroidetes* NS3a marine group and was enriched under medium to high CO_2 conditions. The NS3a marine group belongs to the *Flavobacteriaceae* family, which is abundant during phytoplankton blooms (Teeling *et al.*, 2012; Williams *et al.*, 2012) and plays a major role in the degradation of complex organic matter (Kirchman, 2002; Arnosti, 2011; Gómez-Pereira *et al.*, 2012). Thus, an enrichment in the *Bacteroidetes* NS3a marine group might also impact organic matter degradation processes, as the enhanced domination of one OTU implies a reduction of the overall diversity of the microbial community. Interestingly, none of the abundant OTUs were enriched under low CO_2 conditions. These results are in accordance with a recent study of Sperling et al. (2013), who showed a decrease in bacterial diversity at low pH. This result lends support to the hypothesis proposed by Ren et al. (2015), that low pH decreases both the richness and the evenness of bacterioplankton OTUs by the enrichment of better-adapted taxa (environmental filtering). Furthermore, a CO_2 effect on abundant OTUs occurred only in the warm treatments, in which, in addition, the effect on BCC was more pronounced. This indicates a coupling between warming and acidification. On the other hand, both of the abundant OTUs stimulated at higher CO_2 (*Loktanella* and NS3a marine group) were enriched in the cold treatments. Thus, warming and acidification can exert also opposing effects on abundant OTUs.

If exposed for longer durations to changing environmental conditions, physiological and genetic adaptations of microorganisms have to be expected. Genetic adaptation in response to continued acidification has been shown, for example for marine phytoplankton taxa(i. e., Lohbeck et al., 2012). However, the durations of our experiments were too short for conclusions about adaptation of the responding bacteria. To our knowledge there are so far no studies reporting adjustment to acidification by genetic changes of heterotrophic bacteria. There is evidence for morphological acclimation to the pH regulation by a Vibrio strain (Labare *et al.*, 2010) and the first metatranscriptome study on ocean acidification effects showed some biochemical acclimation on community level (Bunse *et al.*, 2016) but most studies have been conducted over too short periods (days to weeks) to give evidence for adaptation. In order to examine adaptive evolution as well as the underlying physiological reactions in more detail, bacterial taxa responding to acidification need to be isolated and cultured.

Direct vs. indirect effects

While the indirect effects of warming through shifts in phytoplankton bloom dynamics and composition are obvious, distinguishing between direct and indirect CO_2 effects is more difficult. Most earlier studies related CO_2 effects on bacteria to temporal and compositional changes in phytoplankton communities (e.g., Grossart et al., 2006; Arnosti et al., 2011; Piontek et al., 2013; Endres et al., 2014). However, in their autumn experiment Paul et al. (2015) found no effects of CO_2 on either blooming time or phytoplankton biomass whereas according to Sommer et al. (2015) changes in the composition of rare phytoplankton species were weak. In both studies, the only indications for CO_2 effects on phytoplankton were an interaction effect of CO_2 and temperature on phytoplankton biovolume and an increase in cell size with CO_2 enrichment (Sommer *et al.*, 2015) during the autumn experiment as well as an interaction effect of temperature and CO_2 on phytoplankton biomass because of reduced grazing in the summer experiment (Paul et al., 2016).

Changes in organic matter composition affect bacterial community composition, and organic matter originating from different phytoplankton species differentially stimulates heterotrophic prokaryotes (Pinhassi *et al.*, 2004; e.g. Sarmento and Gasol, 2012). Therefore, changes in the quantity and quality of DOM produced by the phytoplankton community could have altered bacterial community composition, thus representing an additional indirect effect of CO_2. However, we found no evidence of a relation between CO_2 concentrations and organic

matter composition and dynamics (concentration and composition of DFAA, DCAA, and low molecular weight DOM). This is supported by a recent study, which showed no effect of acidification on the composition of chromophoric DOM during a bloom of the coccolithophorid alga *Emiliania huxleyi* (Rochelle-Newall *et al.*, 2004). Similarly, a previous study in which the duration of the experiments was four times longer than those conducted in this work, found no effect of acidification on the composition of low molecular weight (<150 Da) DOM during phytoplankton blooms in a Swedish Fjord (Zark *et al.*, 2015). Although the entire bacterial substrate pool could not be covered by the analytics used in our study, those results together with our own suggest direct effects of acidification on the bacterial community, including the stimulation of several bacterial taxa at higher CO_2 levels. Potential direct effects can primarily be connected to the general dependence of enzymatic reactions, especially those of extracellular enzymes, on pH (Arrhenius, 1889; Münster, 1991). The impact of increased CO_2 on the reaction rates of hydrolytic extracellular enzymes is well established (Grossart *et al.*, 2006; Tanaka *et al.*, 2008; Piontek *et al.*, 2010, 2013; Endres *et al.*, 2014) and supports a direct impact of changing CO_2 conditions on bacterial communities. A recent metatranscriptome-based study also found evidence of a direct CO_2 effect, in the form of energetically costly pH homeostasis mechanisms with the ability to influence both the growth efficiency and the biogeochemical cycling of bacteria (Bunse *et al.*, 2016). These changes might remain undetectable in analyses based on bulk activities or cell abundance but become visible by resolving community shifts at the level of taxa that adapt to or benefit from the effects of high CO_2 concentrations. It is difficult to make clear distinctions between direct and indirect effects in such complex mesocosm experiments and therefore the respective results have to be considered only as indications for these effects. Ways to proof direct effects might be to isolate responding OTUs for acidification experiments on their pure cultures or to use single-cell activity techniques (e.g. Microautoradiography and Fluorescence in situ Hybridization (MAR-FISH) to follow responding OTUs with specific probes.

Concluding remarks

This study demonstrates that climate change can be expected to affect microbial abundance, activity, and composition. The strong impact of warming on bacterial communities is mediated mainly by phytoplankton and leads to shifts in the dominant bacterial groups. Our results also confirmed the generally low impact of acidification on

bacterial abundance, activity, and general community composition. However, we also identified potentially important organic-matter-degrading taxa that are sensitive to changes in pCO_2. The latter finding demonstrates the importance of high-resolution community analyses in environmental impact studies. Despite the apparent relevance of the affected taxa based on their high abundances and their roles in degrading the phytoplankton bloom, whether functional changes occur at the single-cell and/or community level remains to be elucidated. Future studies will need to focus on the impact of climate change on microbial community functioning, to determine whether functional redundancy can compensate for shifts in important taxa or whether these shifts result in the disruption of organic matter cycling.

Chapter 3

Response of Baltic Sea bacterial communities to a major saline inflow event

Abstract

Major Baltic Inflow (MBI) events carry highly saline water from the North Sea to the central Baltic Sea and affect the environmental conditions in the deep layer. To better understand the influence of MBIs on the Baltic Sea bacterial communities, we analyzed the community composition in the inflow water body and the uplifted former bottom water in the Bornholm and the Arkona basin and compared it with respective communities from non-inflow situations. 16S rRNA:rDNA ratios were analyzed for changes in potential activity of the whole community and individual taxa. The inflow water bacterial community had a high similarity with regular communities from the Bornholm and Arkona basin and with communities from the Belt Sea and the Western Baltic. Together with an increased diversity, this indicates a strong mixing of the inflow water. Community changes on phylum level were weak but significant differences were found on OTU level. These were accompanied by high variations in 16S rRNA:rDNA ratios, with increased potential activity of several potentially immigrated taxa. Our results suggest that some taxa immigrate with the inflow water and sustain a high level of potential activity. Together with indications for enhanced nitrification by immigrated nitrite oxidizers, this suggests a potential impact of MBIs on bacteria-mediated ecosystem services in the Baltic Sea.

3.1. Introduction

The Baltic Sea is a landlocked sea and the water exchange only takes place through the North Sea via the straits of the Kattegat and Skagerrak. The morphologic narrowing between the North Sea and Baltic Sea, as well as a pronounced basin and sill structure is restricting the water exchange. As a consequence of low seawater inflow and a strong freshwater input from rivers, rain and melting water, the Baltic Sea has an elongated, stable salinity gradient, extending from high salinities in the Skagerrak (salinity 25) to low salinity conditions in the Gulf of Bothnia (salinity < 5) (Reissmann *et al.*, 2009). Another result is a permanent halocline in the central Baltic Sea, which prevents vertical mixing and leads to permanent stratification. Consequently, the environmental conditions in the deeper layers of the Baltic Sea are mainly shaped by the lateral input of highly saline waters from the North Sea.

Major Baltic Inflow (MBI) events, forced by special atmospheric conditions, occur occasionally in the autumn and winter season and carry enough saline water to reach the central basins of the Baltic Sea (Matthäus and Franck, 1992). The winter inflow water salinity is most commonly between 17.5 and 18 and temperature ranges from 2 to 8°C, which is colder than the Baltic bottom water (Matthäus and Franck, 1992). It is also rich in oxygen, which is important for the ventilation of the Baltic Sea deep basins (Omstedt *et al.*, 2004).

In the last years, several studies have addressed the bacterial community compositions along the Baltic Sea salinity gradient (Riemann *et al.*, 2008; Herlemann *et al.*, 2011, 2014; Bergen *et al.*, 2014), as salinity is one of the most important factors in structuring microbial communities (Lozupone and Knight, 2007; Nemergut *et al.*, 2011). It is generally known that the dominant bacterial groups shift, from the prevalence of *Betaproteobacteria* and *Actinobacteria* in freshwater regions to dominance of *Alphaproteobacteria* in the higher salinity regions (e.g., Bouvier and Giorgio, 2002; Kirchman *et al.*, 2005; Fortunato and Crump, 2011). This trend was also confirmed in the Baltic Sea (Herlemann *et al.*, 2011). Furthermore, the authors reported the presence of an adapted bacterial community in the brackish waters of the Baltic Sea, which is significantly different from its freshwater and marine counterparts. However, so far there are no studies that investigated the influence of MBIs on the development of Baltic Sea bacterial communities.

In the present study, we describe the bacterial community composition of the inflow water body and compare it with the former bottom water community and with communities from non-inflow situations in 2008 and 2009. We used next-generation sequencing of partial 16S rRNA and 16S rRNA genes (16S rDNA) to describe differences in the community composition and to make assumptions about potentially active taxa. We also considered the amount of mixing in an attempt to better estimate the effects of changing environmental conditions on the bacterial community.

3.2. Material and Methods

Field sampling

Samples were taken in the Arkona basin, the Bornholm basin and the Gotland basin of the Baltic Sea (Fig. 16) during a cruise of the RV *Elisabeth Mann Borgese* in February 2015. Profiles of temperature, salinity, oxygen, and chlorophyll fluorescence were measured using

a CTD SBE911+ combined with the bottle sampler rosette. Sampling depths were chosen at each station in two depths according to the physical water mass characteristics. At each station one sample was taken from the inflow water body (temperature < 8°C, oxygen concentrations > 5ml L^{-1}) and one from the uplifted bottom water body (temperature > 8°C, oxygen concentrations > 5 ml L^{-1}). For details about the water mass characteristics and propagation see Mohrholz *et al.* (2015), Naumann *et al.* (submitted). Samples were taken using a rosette water sampler comprising 13 10-L free-flow bottles. Water samples for DNA analysis were filtered onto 0.22-µm pore-size white polycarbonate filters. DNA and RNA were extracted according to Weinbauer *et al.* (2002). Concentrations of inorganic nutrients were analysed as described by Grasshoff et al. (1983).

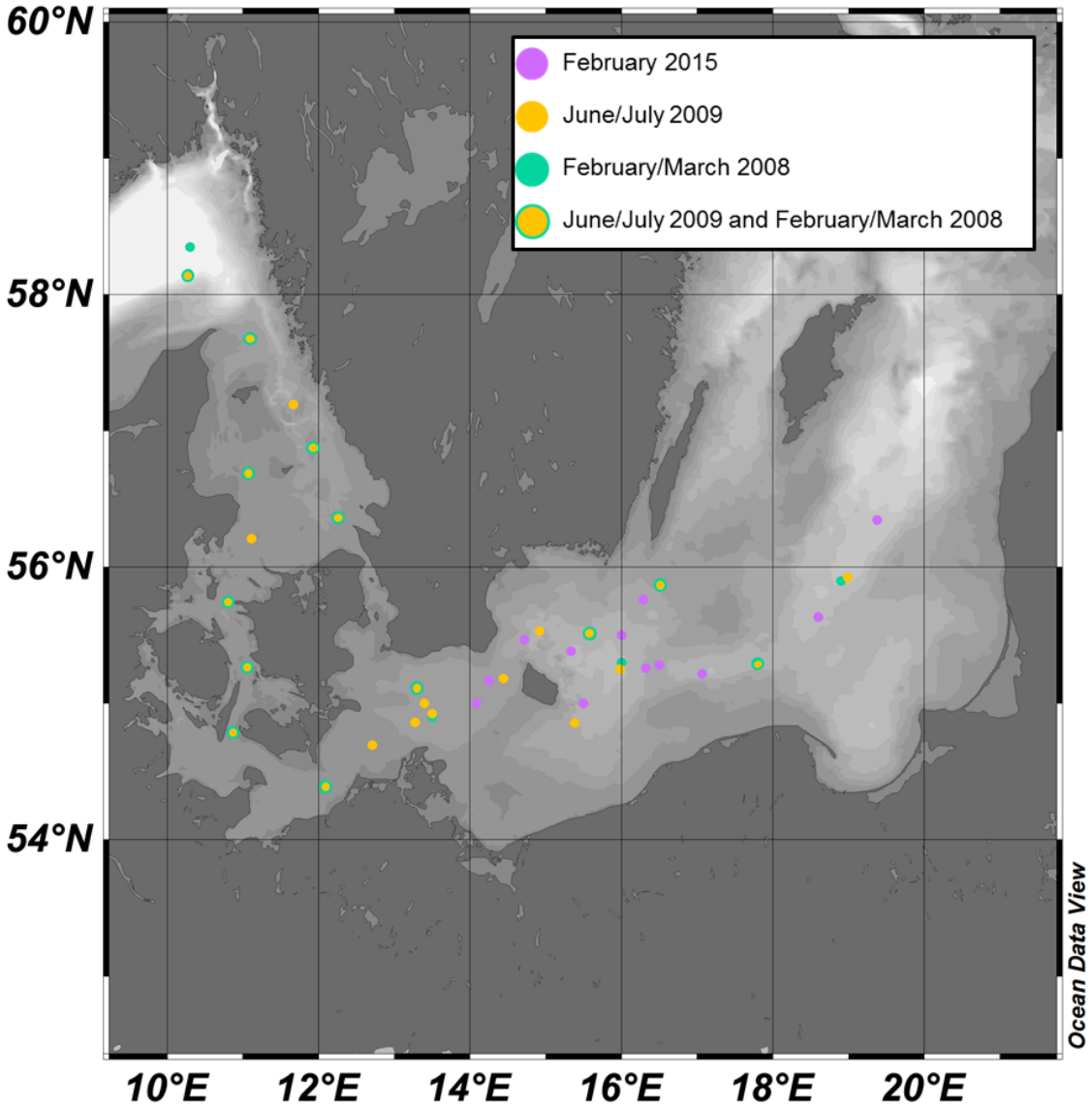

Figure 16: Map of the Baltic Sea with the respective sampling sites. Colours indicate the sampling time.

Reference samples for a non-inflow situation were taken accordingly on a cruise of the RV *Maria S Merian* in June/July (summer) 2009 and on a cruise of the RV *Alkor* in February/March (winter) 2008 along a transect from the Skagerrak to the Gotland basin (Fig. 1). DNA was extracted as described above. For a detailed description see Herlemann et al (2011, 2016).

Amplicon sequencing and sequence processing

For cDNA synthesis, DNA was removed from the RNA extracts by DNase I (Ambion/Applied Biosystems, Huntington, UK) digestion, following the manufacturer's protocol. RNA samples were then transcribed into cDNA by an RT-step using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) with the primer 1492r. DNA extracts and cDNA for bacterial diversity analysis were sent to LGC Genomics GmbH (Berlin, Germany) for further processing. The company used the primers Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACT ACHVGGGTATCTAATCC) (Herlemann *et al.*, 2011) for PCR amplification and sequences were generated on the Illumina MiSeq V3 platform in a 2x300 bp paired-end run.

Pairs of forward and reverse primer-trimmed sequences were combined using BBMerge 34.48 and processed with Mothur 1.35.1. In brief, the raw reads were quality-filtered, aligned to the 16S Mothur-Silva SEED r119 reference alignment (Quast *et al.*, 2013) and preclustered. Chimeras were eliminated with the uchime algorithm and sequences were taxonomically classified against the Silva reference classification. Sequences were grouped in operational taxonomic units (OTUs) on a 97% level and a BIOM format OTU table was created. We used QIIME 1.9.0 (Caporaso *et al.*, 2010) to filter out OTUs with one read only and to rarefy the data for the minimum sequence counts per sample.

DNA from the reference samples from 2008 and 2009 was amplified with the same primers Bakt_341F and Bakt_805R as described by Herlemann et al. (Herlemann *et al.*, 2011). Sequencing of the 2008 samples was performed by LGC Genomics GmbH and sequencing of the 2009 samples was performed by Eurofins MWG GmbH using 454 GS-FLX sequencer (Roche), respectively. For a detailed description of amplification and 454 sequencing see Herlemann et al. (2011, 2016).

For a comparison of the reference sequences with the inflow sequences, all sequences were processed together using the Silva NGS pipeline with standard settings (Quast *et al.*, 2013) The resulting OTU table was rarefied to the minimum sequence counts per sample and

converted into a BIOM format OTU table for further processing in QIIME 1.9.0 as described above.

Statistics

As the Gotland basin samples derived from anoxic conditions, which harbor distinct bacterial communities, we separated them from the rest of the samples and performed all statistical analyses separately. For all statistical tests, samples were grouped according to their origin. We used the QIIME script core_diversity_analyses.py to analyse alpha diversity and beta diversity between the different water masses and between the inflow samples and the reference samples. Additionally, we used a Bray-Curtis dissimilarity matrix based on square root transformed OTU abundance data to perform a principal coordinate analysis (PCoA) and to test for community similarity between inflow water and uplifted bottom water (ANOSIM test), both using the software PRIMER-E 6. We performed linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.*, 2011) (http://huttenhower.sph.harvard.edu/lefse/) to test for discriminatory taxa between water masses, between the inflow samples and the reference samples and between DNA and cDNA abundance. With a normalized relative abundance table, LEfSe uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa in the different treatments and performs LDA to estimate the effect size of each feature. A significance alpha of 0.05 and an effect size threshold of 3 were used for all biomarkers discussed in this study.

Physical numerical model

We used the General Estuarine Transport Model (GETM) in a multi nested downscaling framework, reaching from the North Atlantic (8 km resolution) to the Western Baltic Sea (600 m resolution, which is baroclinic eddy resolving). The numerical model provided hourly mean values of velocity, salinity and temperature at every grid point and reproduced this event in detail (Mohrholz et al. 2015, Gräwe et al. 2015). GETM has been succesfully used to simulate earlier inflow events and a statistical analysis of inflows in the western Baltic Sea (Burchard *et al.*, 2005; Gräwe *et al.*, 2013). For the application in this study the same model setup is used described in Gräwe (2015) to do a backtracking of the inflow water masses by using an age tracer. This tracer is starting at the sampling stations in the Bornholm gat (TF0145) and Slupsk channel (TFO222) to analyse the origin of this inflow water masses in more detail.

3.3. *Results*

Characterization of water masses by back trajectory

The origin of the sampled inflow water, estimated by numerical modelling, is shown by probability maps in Figure 17. Both sampling stations, where the back trajectory is done by age tracers, show equal results in the depicted time slices of the main inflow period (13th-25th December 2014) and pre-inflow stage of mid-October. The lower maps show the position of the sampled water in October indicating an area spanning from the Skagerak to the Danish Belt Sea, which is a common result for such events.

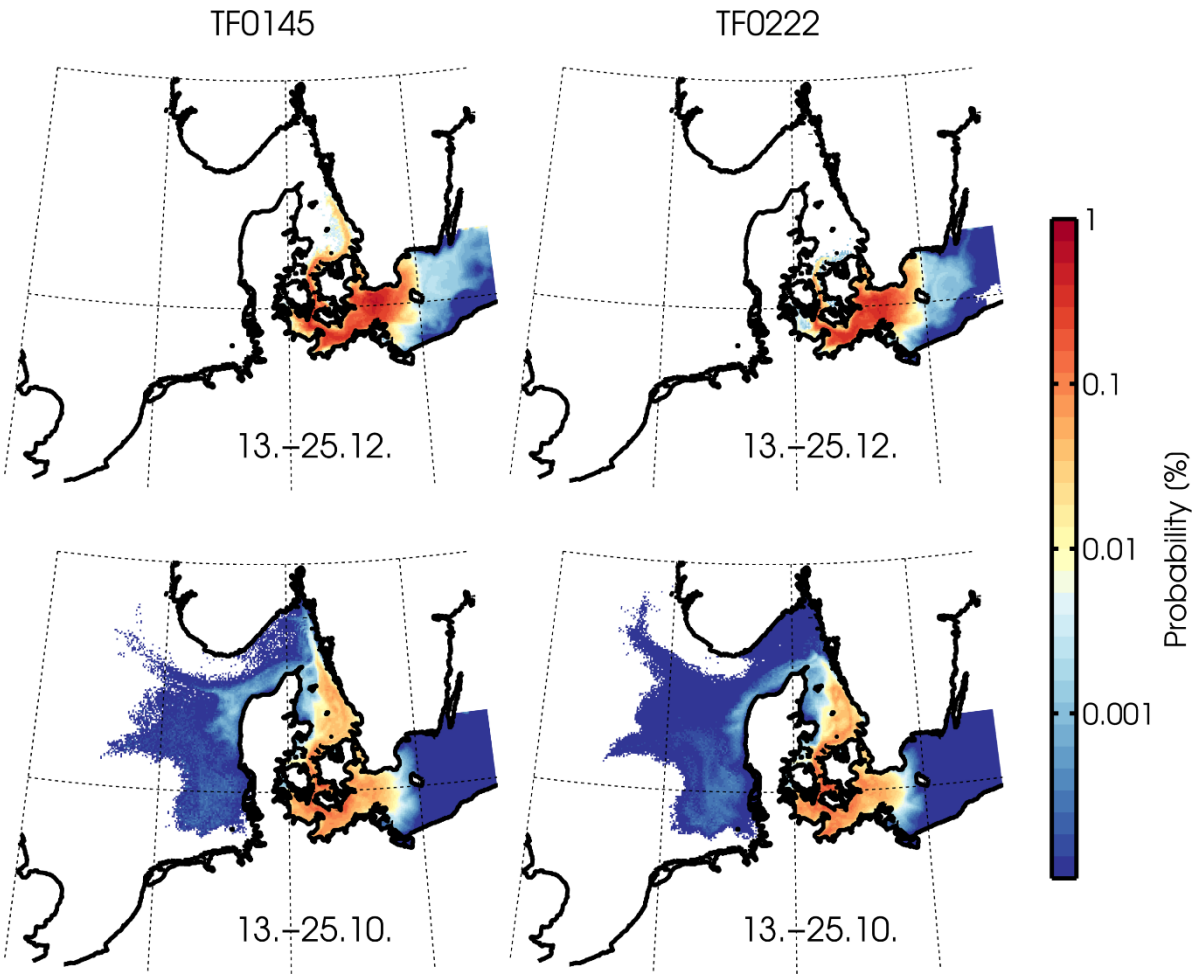

Figure 17: The origin of the sampled inflow water, estimated for one sampling stations in the Bornholm gat (TFO145) and one in the Slupsk channel (TFO222) for two different time points by numerical modelling.

Comparison of inorganic nitrogen concentrations from the inflow water bodies and from reference water

In a comparison of concentrations of nitrate, nitrite and ammonia in the Arkona basin and the Bornholm basin in samples from this study and reference samples from winter 2009, we found that the inflow water contained significantly lower concentrations of ammonia in both basins, lower concentrations of nitrite in the Arkona basin and higher concentrations of nitrate in both basins (Figure 18).

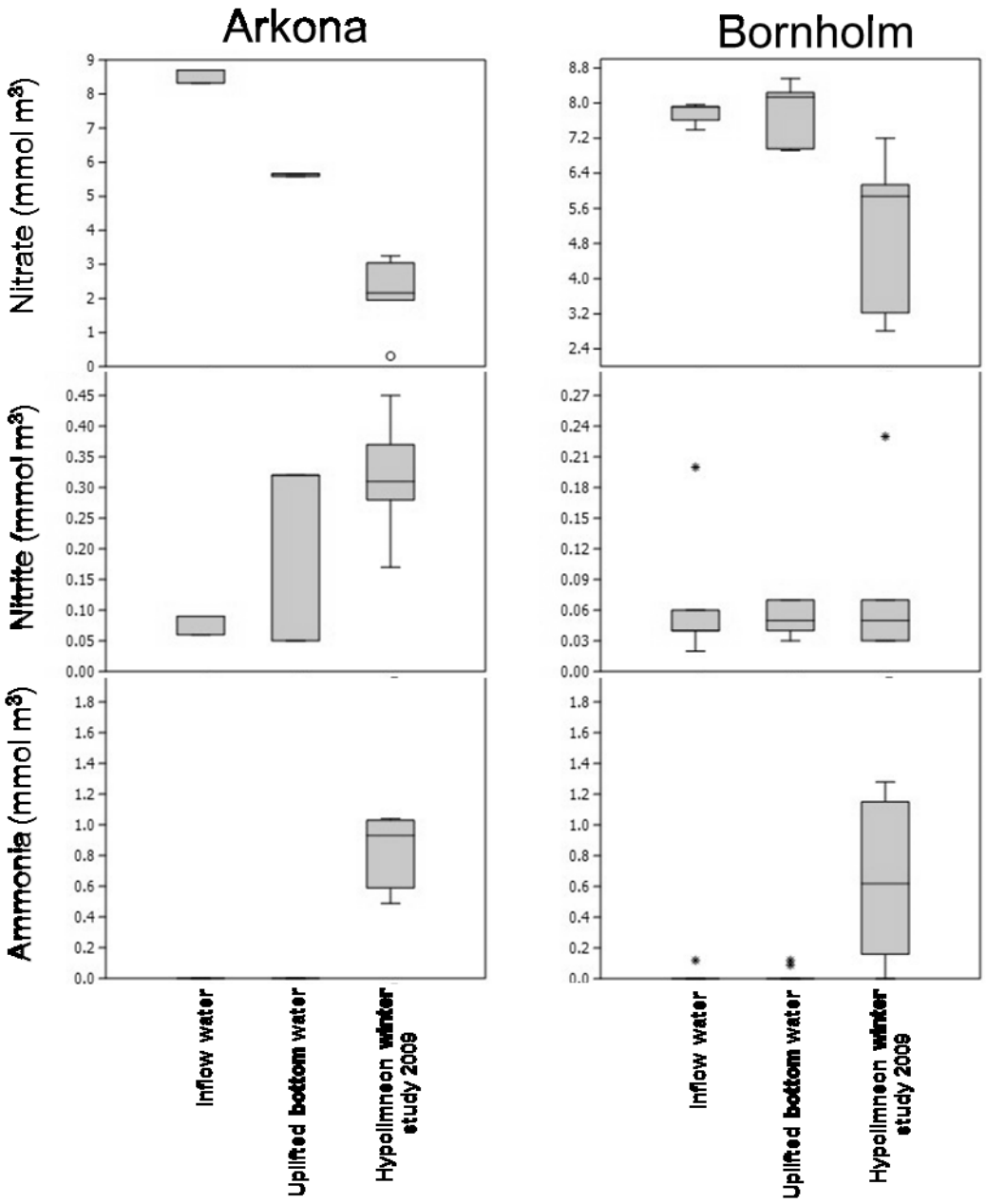

Figure 18: Concentrations of ammonia, nitrite and nitrate in the inflow water, the uplifted bottom water and hypolimneon samples from a non-inflow situation in 2009.

Taxonomic composition of microbial communities in the inflow water bodies

Derived from the 16S rDNA sequences, the Arkona basin and Bornholm basin bacterial communities were dominated by *Proteobacteria*, mainly *Alpha*- and *Gammaproteobacteria* (see Figure S6 in the Supplementary Material). Interestingly, the Bornholm basin community comprised a significant higher proportion of *Chloroflexi*.

We observed only minor differences between the inflow water communities and the uplifted bottom water communities on phylum level (see Figure S6 in the Supplementary Material). Most obvious changes were a higher proportion of *Proteobacteria* and a lower proportion of *Bacteroidetes* in the inflow water community of the Arkona basin. However, ANOSIM revealed significant differences between the inflow water communities and the uplifted bottom water communities on OTU level (R = 0.721). We identified 15 OTUs from various phyla with (LDA) effect size > 3 enriched in the inflow water (Figure 19).

The bacterial community from the Gotland basin anoxic bottom layer was dominated by *Epsilonproteobacteria* and few *Gammaproteobacteria* (see Figure S7 in the Supplementary Material). A significantly different community composition was observed in the deepest sample in the wester Gotland basin, with a lower proportion of *Epsilonproteobacteria* and high proportions of *Alphaproteobacteria*, *Chloroflexi* SAR202 clade and diverse other taxa.

Table 10: Relative phylum / class abundance from DNA reads and cDNA reads, respectively, and their cDNA:DNA ratio.

#OTU ID	rRNA	rDNA	cDNA/DNA
Bacteria;Acidobacteria	1.78280121	0.94519526	1.8861724
Bacteria;Actinobacteria	3.01017634	6.33032087	0.47551718
Bacteria;Bacteroidetes	3.7578653	10.2872896	0.36529207
Bacteria;Chloroflexi	11.1842616	9.63228588	1.16112227
Bacteria;Cyanobacteria	2.38095238	0.76382555	3.11714156
Bacteria;Firmicutes	1.90320827	0.09327585	20.4040843
Bacteria;Gemmatimonadetes	1.10891012	0.54514551	2.03415437
Bacteria;Lentisphaerae	2.64118698	1.51832352	1.73954163
Bacteria;Planctomycetes	5.53484036	12.8948678	0.42922816
Bacteria;Proteobacteria;Alphaproteobacteria	25.3748155	21.6171959	1.17382549
Bacteria;Proteobacteria;Betaproteobacteria	1.48566768	1.8997181	0.78204639
Bacteria;Proteobacteria;Deltaproteobacteria	10.5472695	3.82223696	2.7594494
Bacteria;Proteobacteria;Epsilonproteobacteria	9.08490639	8.83840478	1.02788983
Bacteria;Proteobacteria;Gammaproteobacteria	12.4524198	12.5621839	0.99126234
Bacteria;Proteobacteria;SPOTSOCT00m83	1.07006914	0.24977199	4.28418385
Bacteria;Verrucomicrobia	1.48566768	3.75072548	0.39610142

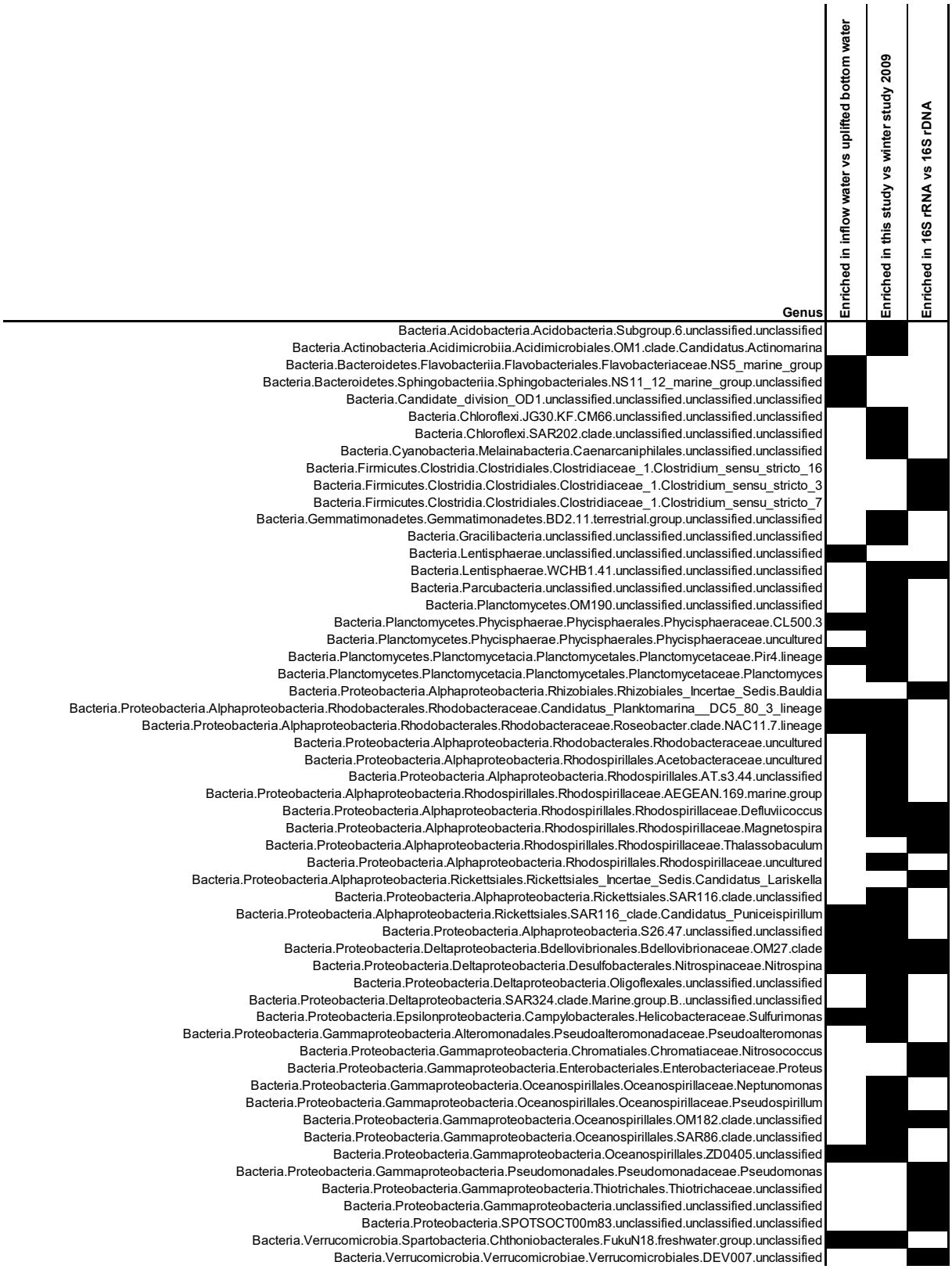

Figure 19: OTUs enriched in inflow water vs. uplifted bottom water, in this study vs a winter study from 2009 and in 16S rRNA vs 16S rDNA on genus level.

16S rRNA:rDNA ratios of bacteria in the inflow water bodies

The average 16S rRNA:rDNA ratio in the inflow water community was 1.4 and we found strong variations between the different phyla/classes. Highest ratios were found for *Firmicutes*, *Proteobacteria* class *SPOTSOCT00m83*, *Cyanobacteria* and *Deltaproteobacteria* and lowest for *Bacteroidetes, Verrucomicrobia* and *Planctomycetes* (Table 10). Interestingly, we found a significant positive linear correlation between the individual OTU 16S rRNA:rDNA ratios and salinity (r^2 = 0.48; p = 0.01) (see Figure S8 in the Supplementary Material). We identified 19 OTUs from various phyla with a significant higher amount of 16S rRNA sequences than 16S rDNA sequences and a (LDA) effect size > 3 (Figure 19). Two of these OTUs, related with *Deltaproteobacteria*, were also significantly higher abundant in the inflow water than in the uplifted bottom water.

Comparison of microbial community structures from the inflow water bodies and from reference water

A principle coordinate analysis (PCoA) analysis on OTU level of all oxic samples from this study and reference samples from 2008 and 2009 revealed a distinct community composition in the samples from this study (Figure 20). In addition, alpha diversity was significantly higher in the Arkona basin and Bornholm Basin samples from this study compared to all groups of reference samples (Figure 21). The significant difference between the inflow water community and the uplifted bottom water community, which was found by ANOSIM, is also detectable in the PCoA plot by a weak but clear separation of these samples from each other. Samples from the Arkona basin and the Bornholm basin from this study had a high similarity with reference samples from the Arkona basin and the Bornholm basin, respectively, and interestingly, all samples from this study also had high similarity with reference samples from the Western Baltic Sea and the Belt Sea (see Figure S9 in the Supplementary Material).

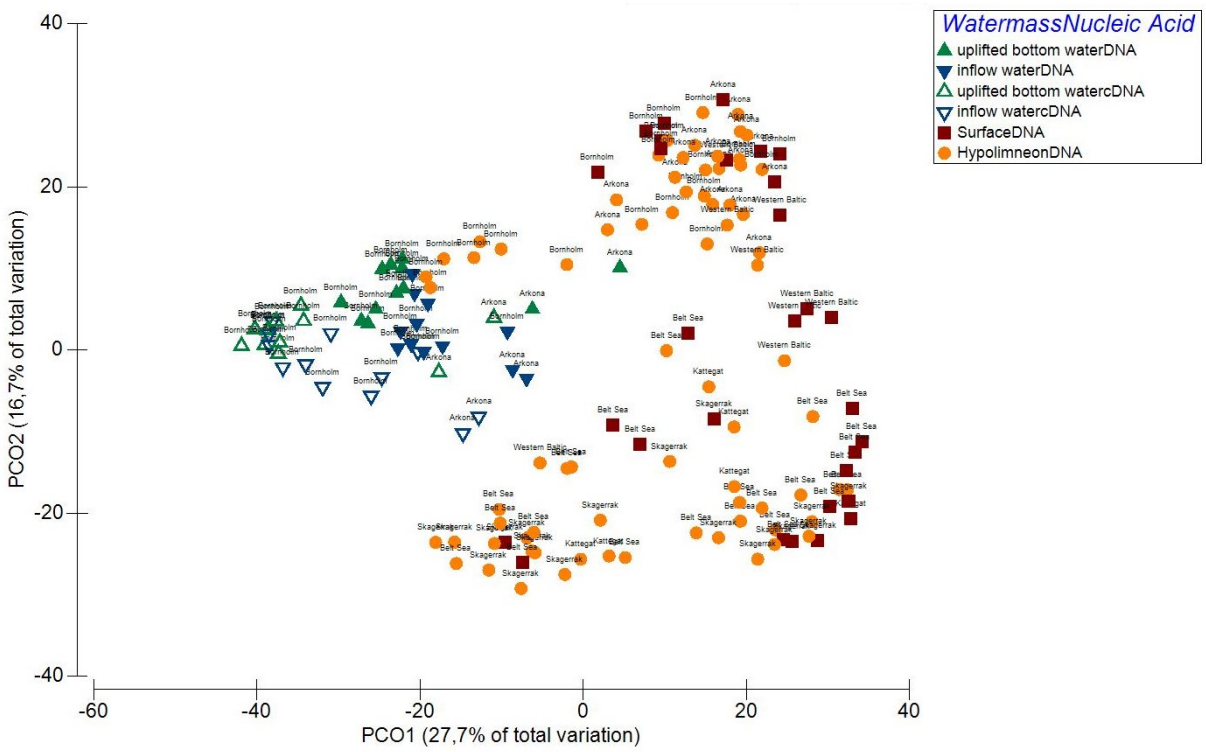

Figure 20: Principle coordinate analysis (PCoA) of OTU abundance based on the Bray-Curtis dissimilarity of DNA and cDNA samples in the inflow water, the uplifted bottom water and surface and hypolimneon samples from non-inflow situations in 2008 and 2009. The labels indicate the sample origin.

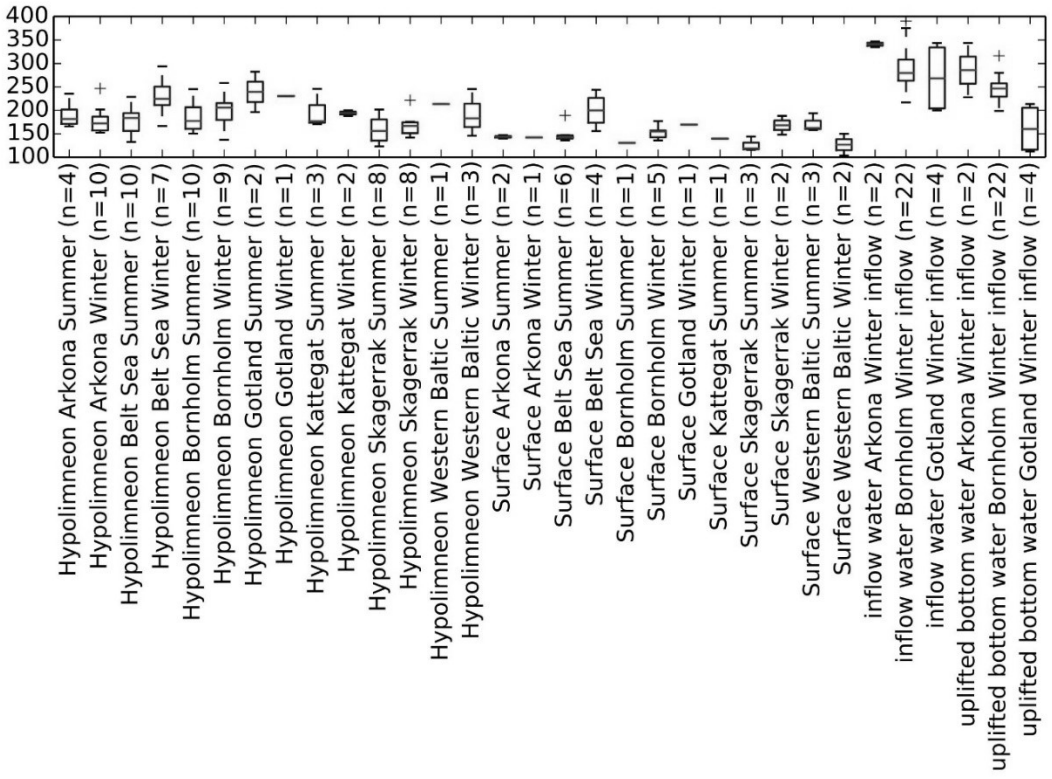

Figure 21: Chao1 alpha-diversity in the inflow water, the uplifted bottom water and surface and hypolimneon samples from a non-inflow situation in 2008 and 2009.

We found 38 OTUs from various phyla in significantly higher abundance in the Bornholm basin and the Arkona basin in this study compared to the reference winter samples from 2009 (Figure 19). Interestingly, one OTU related with *Deltaproteobacteria Nitrospina* and one related with *Deltaproteobacteria* OM27 clade were also significantly enriched in the inflow water compared to the uplifted bottom water and had a significantly higher abundance of 16S rRNA sequences than 16S rDNA sequences.

3.4. Discussion

Bacterial communities in estuaries and coastal margins have different compositions in space because of the prevalent gradients in salinity, nutrients and other properties (Kirchman *et al.*, 2005; Sharp *et al.*, 2009; Fortunato and Crump, 2011; Herlemann *et al.*, 2011; Fortunato *et al.*, 2013; Bergen *et al.*, 2015). Although several studies have shown that gradients lead to variation in community structure at a low phylogenetic resolution (Bouvier and Giorgio, 2002; Kirchman *et al.*, 2005), few studies have examined abundance of OTUs at an identity level of 97% (Kan *et al.*, 2008; Herlemann *et al.*, 2011; Fortunato *et al.*, 2012; Bergen *et al.*, 2015) and fewer have examined potential activity at the OTU level (Campbell *et al.*, 2011; Campbell and Kirchman, 2012). Here, we assessed for the first time changes in bacterial community composition and 16S rRNA:rDNA ratios as an index for potential activity along a waterbody of highly saline North Sea inflow water on its way from the Arkona basin into the central Baltic Sea. Our data indicate a high degree of mixing of the inflow water with the former bottom water, leading to an elevated bacterial diversity. We found a significant positive correlation of 16S rRNA:rDNA ratios with salinity, indicating that decreasing salinity controlled the potential activity of several taxa. In comparison to a non-inflow situation, our data suggest that some uncommon taxa are enriched in the inflow water and their 16S rRNA:rDNA ratios also indicate a high potential acitivity.

Characterization of the MBI water bodies

The highest impact of the inflow water was found in the Gotland basin where a typical anoxic bottom layer bacterial community (Labrenz *et al.*, 2007) was replaced by an oxic community, which was shifted from the Bornholm basin into the central Baltic Sea. This shows

that MBIs are not only important for the ventilation of the Baltic Sea deep basins (Omstedt *et al.*, 2004) but also directly alter the prevailing bacterial community composition.

The significant but weak difference between the inflow water and the former bottom water communities in the oxic basins suggests a high amount of mixing between these water bodies, which was also found in a modeling system of the inflow event (Gräwe *et al.*, 2015). Furthermore, Herlemann *et al*. (2011) found the critical salinity for community shifts on phylum level in the brackish area of the Baltic Sea (salinity 5 - 8), suggesting that the salinity change along the inflow water does not have impact on the broad community. Thus, a high phylogenetic resolution, which was used in this study, is necessary to identify MBI caused community shifts. On this level, we identified several taxa enriched in the inflow water compared to the former bottom water. Generally, these taxa were mainly found in *Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria*, most of which are related to known marine taxa (e.g., Brown et al., 2009).

However, an impact of environmental conditions on the potential activity was also indicated on phylum level by the strong variation of 16S rRNA:rDNA ratios, which may indicate that this potential activity is a valuable indicator in combination with abundance data. When analyzed individually, 16S rRNA:rDNA ratios of several taxa were enriched. These taxa were mainly found in *Firmicutes*, *Alphaproteobacteria* and *Gammaproteobacteria*, which are also related to known marine taxa and, in contrast to the general trend of decreasing potential activity with decreasing salinity, seem to benefit from the changing environmental conditions.

Both, enriched abundance and potential activity, might be caused by reduced resource competition for the respective taxa (Ferrera *et al.*, 2011) or by reduced grazing pressure (Jürgens and Matz, 2002).

Impact of the MBI on the natural Baltic Sea bacterial community

As the strong mixing of the inflow water with the former bottom water covers the impact of the MBI on the bacterial community to some extent when only these two water bodies are compared, we used reference samples from non-inflow situations for further clarification.

The observed mixing effects resulted in a significantly higher diversity in the inflow water and a high similarity to reference samples from the Bornholm basin. However, the inflow water community also had a high similarity with communities from the Western Baltic

and the Belt Sea, which is in accordance with the estimated origin of the inflow water assessed by backtracking results. This indicates that the inflow water community still comprised a part of the Western Baltic and Belt Sea community.

A comparison of individual taxa in the inflow water and in reference samples revealed many taxa, mainly within the *Chloroflexi*, *Planctomycetes*, *Alpha-*, *Delta-* and *Gammaproteobacteria,* enriched in the inflow water. *Chloroflexi* are known to appear in the mesopelagic and deep ocean (Morris *et al.*, 2004) and were also found in high abundance in Baltic Sea sediments (Herlemann *et al*., submitted). This might indicate a potential input of *Chloroflexi* from sediments into the water column, driven by inflow water-caused turbulences. Such resuspension of sediments induced by inflowing highly saline bottom water is proved by turbidity measurements by the CTD probe, which show a rapid increase to values up to 2 ntu close to the seafloor. Above the layer of inflow water values of 0.1-0.2 ntu are typical. This situation of resuspended sediments by inflowing water can be seen in a video sequence taken at the Gotland Deep in the central Baltic (Naumann *et al*., submitted – supplematary online material). The video shows the arrival of the MBI of December 2014 in this central part in April 2015 and macro organisms like fish and polychaete were flushed into to the former "dead zone". High speed drifting sediment particles can be noticed bottom near.

Planctomycetes diversity was high in the inflow water and a variety of members of this group were found before in the Baltic Sea. Brettar *et al*.(2012) found two potential anaerobic ammonium oxidizing phylotypes at the suboxic zone of the central Baltic and Herlemann *et al.* (2011) observed several related OTUs in brackish and marine areas of the Baltic Sea. Interestingly, Rieck et al. (2015) recently analysed the particle-associated bacterial community in three salinity zones of the Baltic Sea and found a dominance of particle-associated *Planctomycetes* in the central Baltic. These *Planctomycetes* mainly consisted of CL500-3, which were also enriched in the inflow water and, controversially, this group is typically found in freshwater (Urbach *et al.*, 2001; Jackson *et al.*, 2014). Thus, origin and reason for enrichment of this group in the inflow water remain unclear and might indicate a broader distribution than previously expected.

Inflow water enriched *Alphaproteobacteria* mainly comprised *Rhodobacteraceae* and *Rhodospirillalceae* and enriched *Gammaproteobacteria* were exclusively represented by *Oceanospirillales.* These groups were also observed in the marine areas of the Baltic Sea

(Herlemann *et al.*, 2011) and therefore an immigration with the inflow water can be speculated.

Several members of *Deltaproteobacteria* were enriched in the inflow water, including two OTUs related to the OM27 clade and Nitrospina, respectively, which were also found enriched in the inflow water compared to the former bottom water and with an enriched 16S rRNA:rDNA ratio. The OM27 clade is known to be geographically wide distributed and was observed to be mainly particle attached (Orsi *et al.*, 2016). There is few knowledge about the potential ecological role of this group, despite hints for a potential predatory lifestyle (Orsi *et al.*, 2016), which might be an advantage in a dynamic system like the inflow water and therefore explain the high potential activity. In contrast, *Nitrospina* is well known to be a marine nitrite oxidizer with highest abundance below the euphotic zone (Ngugi *et al.*, 2015). Consequently, we hypothesize an immigration from marine sites with the inflow water and an enhanced potential activity because of a stimulation of nitrite oxidation by the inflow water nutrient conditions. Concentrations of inorganic nitrogen compounds in the inflow water were different from concentrations usually found in the study area. Low concentrations of ammonia and nitrite and high concentrations of nitrate might indicate an enhanced nitrification in the inflow water. Although we found no abundant ammonia oxidizing bacteria, only *Nitrosococcus* in low abundance, an enhanced nitrification is still explainable by the undetected presence of ammonia oxidizing archaea, which were not covered by the primer set used in this study. Ammonia oxidizing archaea are known to be globally distributed and high abundant (Francis *et al.*, 2005) and were also found to significantly contribute to nitrification in hypoxic waters of the Baltic Sea (Berg *et al.*, 2015).

Concluding remarks

This study describes for the first time the impact of a MBI on the Baltic Sea bacterial community. Mixing had a high impact on the community composition and resulted in an assemblage with highly similar signatures to both, the former bottom water community and the community from the inflow origin. Although the impact on phylum level was weak, significant community changes compared to normal conditions were detectable on OTU level. These findings demonstrate that some taxa are able to cope with the dynamic conditions in the inflow water and are also able to sustain a high level of potential activity. We found hints for a pronounced nitrification in the inflow water, potentially mediated by immigrated taxa.

Future studies on the impact of MBIs on the bacterial community need to focus on microbial community functioning, to determine if our observations lead to changes in bacteria-related ecosystem services.

Conclusions and outlook

Three studies were carried out as part of this thesis, in which the influence of changing environmental conditions on the abundance, activity, and community composition of bacterial assemblages was investigated. The first study addressed the influence of different levels of primary production and the fate of bacterial communities in aging upwelled water. In the second study, the combined impact of warming and acidification on bacterial assemblages was evaluated; direct and indirect effects were distinguished by monitoring the changes in organic matter dynamics. The third study addressed the influence of changing salinity conditions and the mixing of bacterial communities during a highly saline inflow of North Sea water into the Baltic Sea. Although all three studies focused on specific structuring factors, during each one efforts were made to differentiate and weight the influence of as many other potential factors as experimentally possible. The results highlight the enormous overall impact of environmental conditions on bacterial community composition and activity. They also provide the first tentative identifications of key taxa likely to be affected by changes in those conditions, which would have important implications for a variety of ecosystem services. The findings of this thesis can be summarized as follows:

i) Influence of primary production on bacterial communities

The research described in this thesis supports previous findings that bacteria and phytoplankton are strongly coupled (Cole *et al.*, 1988; Mou *et al.*, 2008; Teira *et al.*, 2009). Evidence of this coupling was obtained both in the Benguela upwelling and in the mesocosms experiments with Baltic Sea water, where bacterial abundance and production correlated significantly with phytoplankton development. In both experiments, the temporal/spatial succession of bacterial communities could be related

to phytoplankton bloom development, which followed known bloom dynamics (Teeling *et al.*, 2012). A dominance of *Bacteroidetes* and *Gammaproteobacteria* was observed during algal blooming and high abundance of "*Pelagibacterales*" was found in regions with low algal abundance. The novel results describing spatial bacterial succession in the Benguela upwelled water body provide the basis for future investigations of the mechanisms linking bacteria and phytoplankton in the ocean.

ii) Influence of ocean acidification and warming on bacterial communities

This thesis also supports previous work showing a strong effect of changing temperatures on bacterial activity and abundance (Hoppe *et al.*, 2008; Piontek *et al.*, 2009; Wohlers *et al.*, 2009; von Scheibner *et al.*, 2014) and on bacterial community composition. These effects can be separated into direct effects on bacteria and indirect, phytoplankton-mediated effects.

Previously obtained results on the impact of ocean acidification on the broad bacterial community have been conflicting (e.g., Allgaier et al., 2008; Krause et al., 2012; Newbold et al., 2012; Teira et al., 2012; Oliver et al., 2014; Hartmann et al., 2015). A major breakthrough of the work presented in this thesis was the finding that the conflicting results reflect the overall weak effect of ocean acidification on the entire bacterial community. In fact, an effect is hardly detectable without high-resolution community analyses and sophisticated statistical analyses. However, we were able to show that several abundant and potentially important organic-matter-degrading taxa, e.g. *Loktanella* and *Bacteroidetes "*NS3a marine group", are sensitive to pCO_2 and that pCO_2 seemed to have a direct effect on these taxa. The effects on these taxa might in turn also influence specific organic matter degradation processes.

iii) Influence of a saline intrusion on bacterial communities

For the first time, this thesis describes the response of bacteria to a MBI event and supports the previously described strong influence of salinity on the bacteria community composition (Riemann *et al.*, 2008; Herlemann *et al.*, 2011, 2014; Bergen *et al.*, 2014).

Although mixing and changing environmental conditions in the inflow water had a strong selecting effect on the bacterial community, a major new finding of the work presented in this thesis was the ability of some emigrated taxa, e.g. *Nitrospina*, to cope with the dynamic conditions and to sustain potential activity. The novel results suggest a potential impact of MBIs on biogeochemical processes in the Baltic Sea.

Limitations of 16S rRNA based diversity studies

Analyses of microbial community structure based on 16S rRNA sequencing are considered semi-quantitative because methodological and biological biases can skew estimations of relative species abundance in a community. For example, the choice of DNA extraction method and PCR primers significantly affects operational taxonomic unit (OTU) representation in amplicon community profiles (Morgan *et al.*, 2010; Pinto and Raskin, 2012; Yuan *et al.*, 2012).

The most well-known biological bias in such profiles is the variation in gene copy number between species (Acinas *et al.*, 2004). This variation spans over an order of magnitude, from 1- to 15-fold in Bacteria (Acinas *et al.*, 2004), and biases amplicon-based bacterial diversity estimates. While in some cases a correction can be made by weighting read counts for a given species by the inverse of its gene copy number (Rastogi *et al.*, 2009; Yuan *et al.*, 2012), for most microbial species information on gene copy numbers is lacking. Moreover, the use of a correction is not an actual solution to the problem and it does not yield an unbiased result. An additional problem is that the reference databases for the known or inferred 16S rRNA gene copy numbers are currently not sufficient to cover the relevant groups in this study, such that for many species a correction could not be made. The alternative, applied in the upwelling study, was to use a cellular CARD-FISH approach to validate the amplicon sequencing data of selected groups.

Should we focus on abundant taxa?

One of the important contributions of the studies comprising this thesis was the high-resolution sequence approach used to determine affected taxa and taxonomic groups. However, all three studies focused on those that were dominant and only casually described taxa with low abundances, which in fact make up the majority.

In nearly all of the bacterial communities examined to date, the species abundance distributions has been such that the majority of taxa are present in low relative abundances and only a few are more abundant (Curtis *et al.*, 2002; Magurran, 2004). Although the nature of these low-abundance organisms is controversial (e.g., chimeras generated by PCR and overestimations of diversity with certain OTU-clustering algorithms), it is clear that microbial communities tend to harbor a great number of low-abundance taxa, many of which may be inactive. For example, in their analysis of cold deep-sea sediment samples, Hubert *et al.* (2009) detected hyperthermophilic microorganisms that became active in laboratory experiments after temperatures were raised to 50°C.

However, statistical approaches have shown that the removal of data on low-abundance taxa can result in better correlations between community composition and environmental parameters (Gobet *et al.*, 2010). Whether only abundant organisms directly alter the environment, while the activity of low-abundance taxa has much less impact, is unclear. Recent work suggests that low-abundance organisms are indeed important for the response to disturbances in aquatic environments (Szabó *et al.*, 2007), including their role as seed banks that bloom when conditions are again favorable. Other studies suggest that low-abundance organisms are disproportionately more active than taxa with higher abundances (Jones and Lennon, 2010).

Microbial assemblage structure and its functional implications

Consistent with the results of this thesis, taxon distribution patterns along spatial and temporal gradients have been identified by several other authors (e.g., Schauer et al., 2005; Jones et al., 2009; Herlemann et al., 2011; Eiler et al., 2012). For example, repeatable compositional responses to predation (Pernthaler, 2005), interactions with phytoplankton (Pinhassi *et al.*, 2004; Kent *et al.*, 2007; Teeling *et al.*, 2012) and environmental gradients (Kirchman *et al.*, 2005; Herlemann *et al.*, 2011; Bergen *et al.*, 2014) have been observed. Differences in the distribution of bacterial taxa among local communities derive primarily from the fact that species differ in their niche requirements and are sorted among sites according to these requirements and conditions.

Several studies focusing on traditional biogeographical concepts (Reche *et al.*, 2005; Lindström and Östman, 2011; Lindström and Langenheder, 2012; Andersson *et al.*, 2014) have shown that dispersal limitations in bacterial communities (e.g., Declerck et al., 2013) cause spatial differences in community composition, because dispersal rates are too low for a taxon to spread sufficiently from one site to another. Consequently, communities in locations with similar environmental conditions may differ in their species composition. Spatial separation may also reflect the importance of historical processes, whether evolutionary or ecological, that occurred at some point in the past, such as dispersal limitation, drift, or adaption to past environmental conditions. However, little is known about the impact of these processes (Martiny *et al.*, 2006; Leibold *et al.*, 2010; Hanson *et al.*, 2012).

An additional confounding factor is that many microorganisms can become dormant, entering a reversible state of reduced activity in response to environmental stressors (Jones and Lennon, 2010; Lennon and Jones, 2011). It has been estimated that <10% of a typical microbial community is active at any one time (Locey, 2010). Thus, the dormant component potentially represents a vast reservoir of genetic diversity.

The challenge remains to identify the relative importance of the different processes with respect to the observed and experimental biogeographic patterns. Hanson et al. (2012) described a two-step approach to determine whether processes other than those driving current selection affect spatial variation in microbial composition. In the first, correlations between microbial composition and measured environmental variables across many sampled locations are determined. These correlations serve as evidence of the influence of the current environment on the current distribution of microbial diversity. After controlling for this environmental effect, the second step tests whether geographic distance explains any of the remaining variation in microbial composition. Such a distance effect indicates that processes other than current selection affect spatial variation in microbial composition. In this case, historical effects have shaped the present-day microbial composition, either through past selection or through drift.

It also remains to gain deeper insights into the functioning of microbial communities under the influence of different environmental factors. The results obtained in this study only deliver first indications for potential functional changes from shifts in potentially biogeochemical relevant taxa. As functional analyses on single-cell and/or

community level advance (e.g. Microautoradiography and Fluorescence in situ Hybridization (MAR-FISH), transcriptome analysis), future studies will need to determine whether functional redundancy can compensate for shifts in important taxa or whether these shifts result in the disruption of organic matter and other biogeochemical cycling.

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Supplemental material

Chapter 1

Supplementary methods:

For the phylogenetic overview, representative sequences (400 bp) were aligned and next neighbors searched in ARB SINA (Pruesse et al., 2012). The results were imported into ARB (Ludwig et al., 2004) using LTP 119 as basis (Munoz et al 2011). Neighboring sequences and close related cultivated representative sequences (>1000 bp) were used to calculate a base tree using PHyML (pos_var_ssuref_bacteria as filter). Short sequences from this study were added using the quick add parsimony tool provided in ARB.

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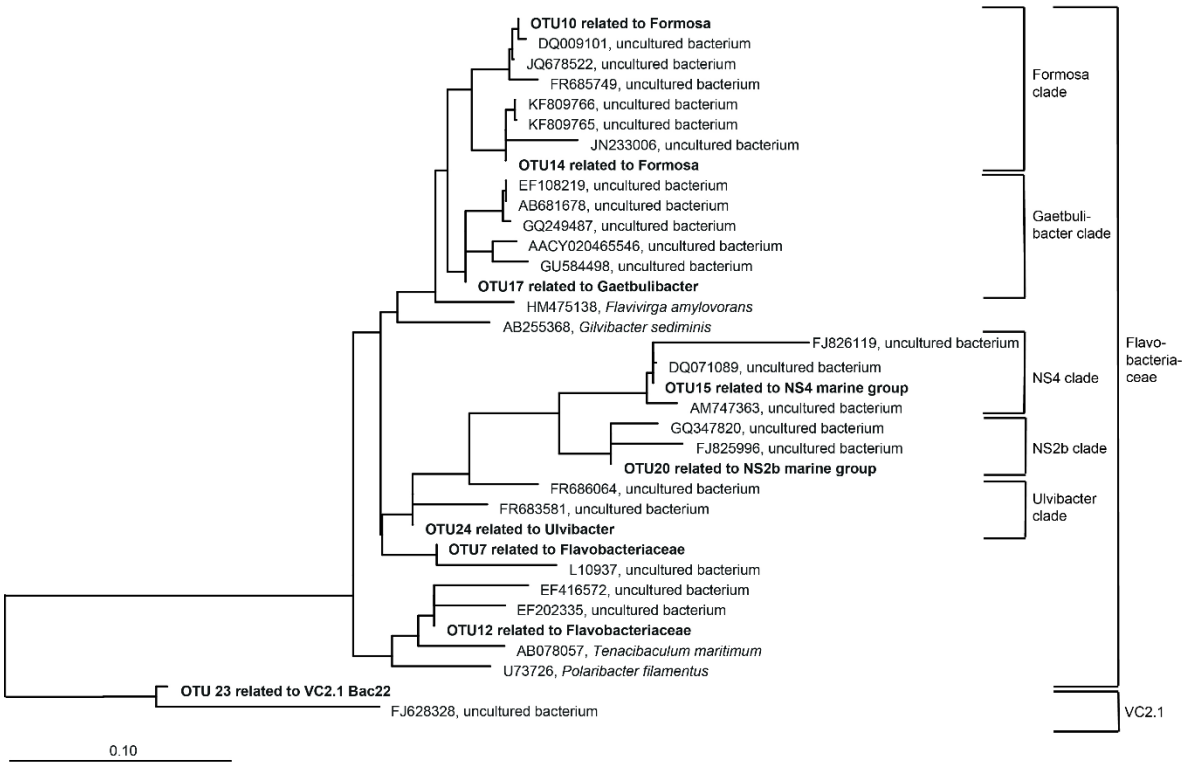

Figure S1: Phylogenetic tree of *Bacteroidetes* related operational taxonomic units (OTUs) from the 25 most-abundant OTUs.

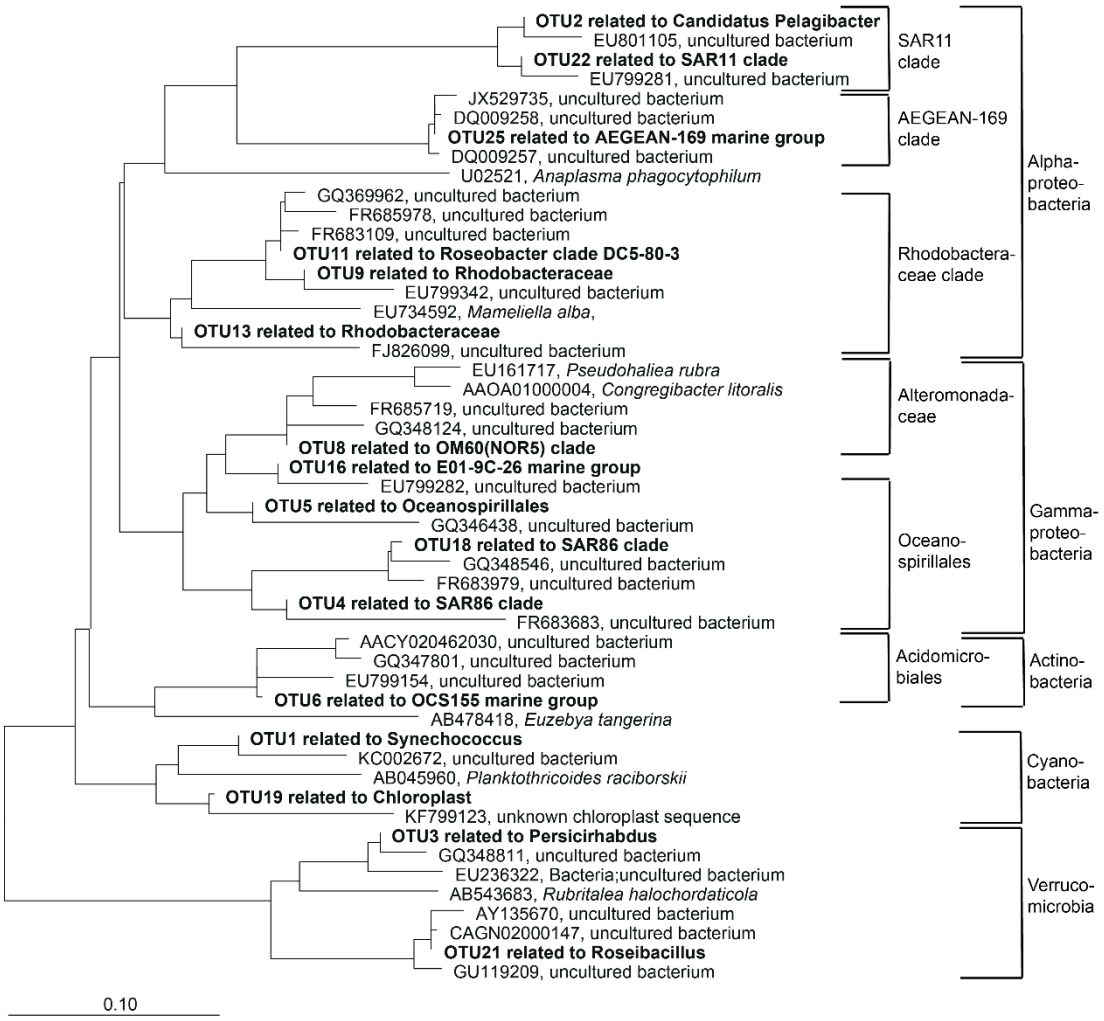

Figure S2: Phylogenetic tree of operational taxonomic units (OTUs) related to *Alpha-*, *Gammaproteobacteria*, *Actinobacteria*, *Cyanobacteria* and *Verrucomicrobia* from the 25 most-abundant OTUs.

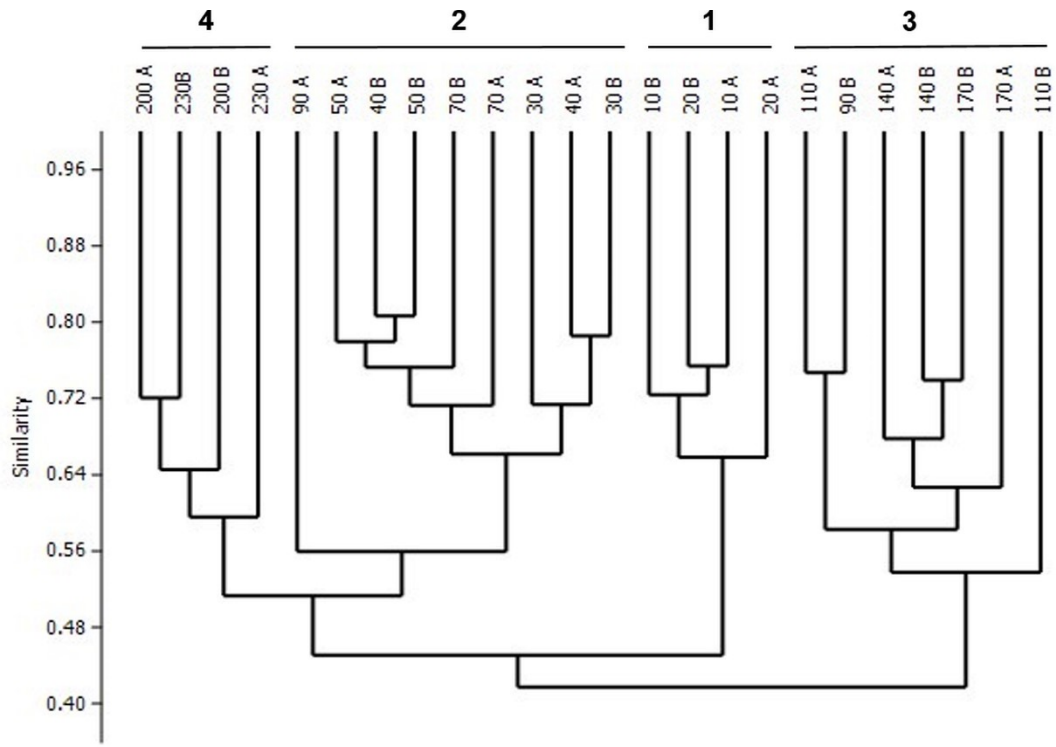

Figure S3: Cluster Analysis of the normalized abundance of all OTUs based on Bray-Curtis dissimilarity. Each number corresponds to the distance to shore in km of the respective sample from transect 1 (A) and transect 2 (B).

Chapter 2

Table S1: Discriminative OTUs determined by LEfSe in the autumn experiment with enrichment treatments.

	All mesocosms	Warm mesocosms	Cold mesocosms
OTU	**Temperature**	**CO2**	**CO2**
Otu0048_Actinobacteria_Ilumatobacter	Warm		
Otu0022_Actinobacteria_unclassified	Warm		
Otu0045_Actinobacteria_unclassified	Warm		
Otu0010_Bacteroidetes_Owenweeksia	Warm		
Otu0012_Bacteroidetes_NS5_marine_group	Warm	Low	
Otu0041_Bacteroidetes_NS5_marine_group	Warm		
Otu0002_Bacteroidetes_Polaribacter	Warm		
Otu0097_Bacteroidetes_unclassified	Warm		
Otu0113_Bacteroidetes_Fabibacter		Low	
Otu0011_Bacteroidetes_unclassified		Low	
Otu0062_Bacteroidetes_Owenweeksia			Low
Otu0029_Cyanobacteria_unclassified		Low	
Otu0055_Cyanobacteria_Synechococcus		Low	
Otu0122_Planctomycetes_unclassified	Cold		
Otu0005_Alphaproteobacteria_Loktanella	Cold	High	
Otu0003_Alphaproteobacteria_unclassified	Cold		
Otu0024_Betaproteobacteria_BAL58_marine_group	Cold	Low	
Otu0039_Gammaproteobacteria_OM60_NOR5__clade	Warm		
Otu0056_Gammaproteobacteria_SAR92_clade	Cold		
Otu0085_Gammaproteobacteria_Thalassomonas	Warm		
Otu0014_Gammaproteobacteria_unclassified			High

Table S2: Discriminative OTUs determined by LEfSe in the summer experiment with enrichment treatments.

	All mesocosms	Warm mesocosms	Cold mesocosms
OTU rank_Phylum_Genus	**Temperature**	**CO_2**	**CO_2**
Otu0042_Actinobacteria_unclassified	warm		high
Otu0079_Actinobacteria_unclassified	cold		
Otu0027_Bacteroidetes_unclassified	warm		
Otu0143_Bacteroidetes_Kordia	cold		medium
Otu0001_Bacteroidetes_NS3a_marine_group	cold	medium	
Otu0160_Bacteroidetes_NS3a_marine_group			low
Otu0075_Bacteroidetes_NS4_marine_group			low
Otu0023_Bacteroidetes_NS5_marine_group	warm		
Otu0017_Bacteroidetes_Robiginitalea	cold		
Otu0014_Bacteroidetes_unclassified	warm		
Otu0015_Bacteroidetes_unclassified		low	
Otu0099_Bacteroidetes_unclassified		medium	
Otu0107_Bacteroidetes_unclassified		low	
Otu0111_Bacteroidetes_unclassified			
Otu0028_Bacteroidetes_Tenacibaculum			medium
Otu0146_Bacteroidetes_unclassified			medium
Otu0178_Bacteroidetes_unclassified			medium
Otu0175_Candidate_division_TM7_unclassified		low	
Otu0011_Cyanobacteria_unclassified	warm		
Otu0026_Cyanobacteria_unclassified	warm		
Otu0034_Cyanobacteria_unclassified	cold		
Otu0121_Cyanobacteria_unclassified	warm		
Otu0141_Cyanobacteria_unclassified	cold		
Otu0002_Cyanobacteria_Synechococcus	warm		
Otu0003_Cyanobacteria_Synechococcus	warm		
Otu0124_Cyanobacteria_unclassified		low	
Otu0113_Cyanobacteria_unclassified		low	
Otu0101_Planctomycetes_CL500_3	warm		
Otu0142_Planctomycetes_Planctomyces	warm		
Otu0040_Planctomycetes_Rhodopirellula	warm		
Otu0062_Planctomycetes_Rhodopirellula	warm	low	
Otu0076_Planctomycetes_unclassified	warm		
Otu0057_Alphaproteobacteria_unclassified	warm		
Otu0153_Alphaproteobacteria_Hoeflea	warm		
Otu0007_Alphaproteobacteria_Candidatus_Planktomarina__DC5_80_3_lineage_	cold		
Otu0065_Alphaproteobacteria_Seohaeicola	warm		
Otu0037_Alphaproteobacteria_unclassified	warm		
Otu0055_Alphaproteobacteria_AEGEAN_169_marine_group	warm		high
Otu0046_Alphaproteobacteria_unclassified	warm		
Otu0235_Alphaproteobacteria_Loktanella			medium
Otu0033_Betaproteobacteria_MWH_UniP1_aquatic_group	warm		
Otu0044_Gammaproteobacteria_Halioglobus	warm		
Otu0060_Gammaproteobacteria_SAR92_clade	cold		
Otu0052_Gammaproteobacteria_unclassified	warm		
Otu0059_Gammaproteobacteria_unclassified	warm		
Otu0081_Gammaproteobacteria_Pseudoalteromonas	warm		
Otu0009_Gammaproteobacteria_Pseudospirillum	cold		
Otu0069_Gammaproteobacteria_unclassified	warm		
Otu0088_Gammaproteobacteria_unclassified	cold		
Otu0086_TM6_unclassified	warm		
Otu0137_unclassified_unclassified			medium
Otu0078_Verrucomicrobia_unclassified	warm		
Otu0091_Verrucomicrobia_unclassified	warm		
Otu0150_WCHB1_60_unclassified		low	

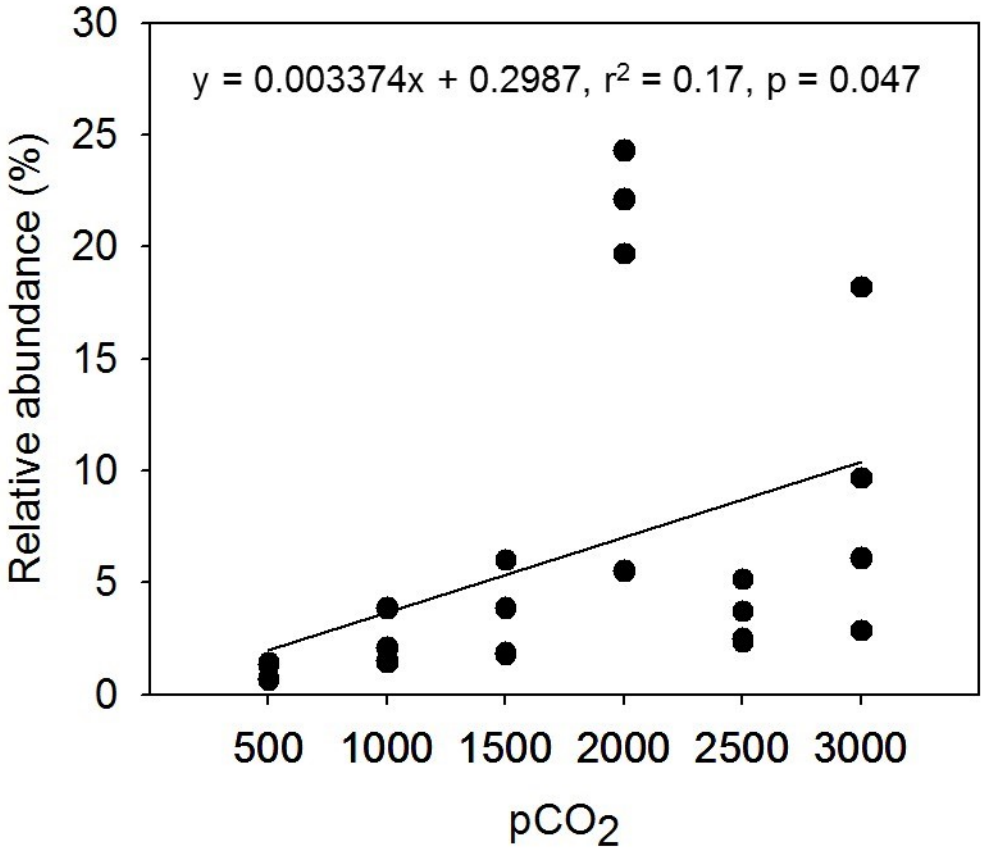

Figure S4: Relative abundance of OTU 0001 affiliated with the *Bacteroidetes "*NS3a marine group" from the summer experiment at the four sampled time points in the warm CO_2 treatments in relation to pCO_2. The regression line is shown.

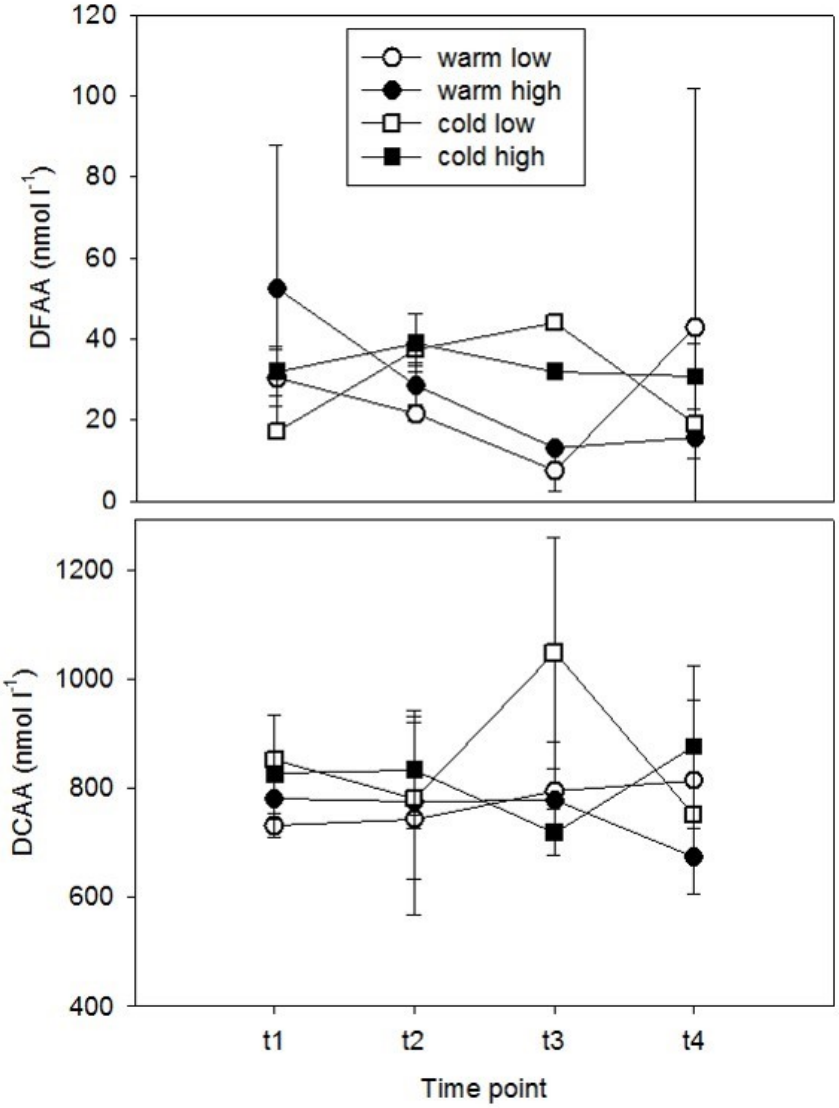

Figure S5: Dissolved free amino acid (DFAA) and dissolved combined amino acid (DCAA) concentrations at t_1–t_4 during the autumn experiment.

Chapter 3

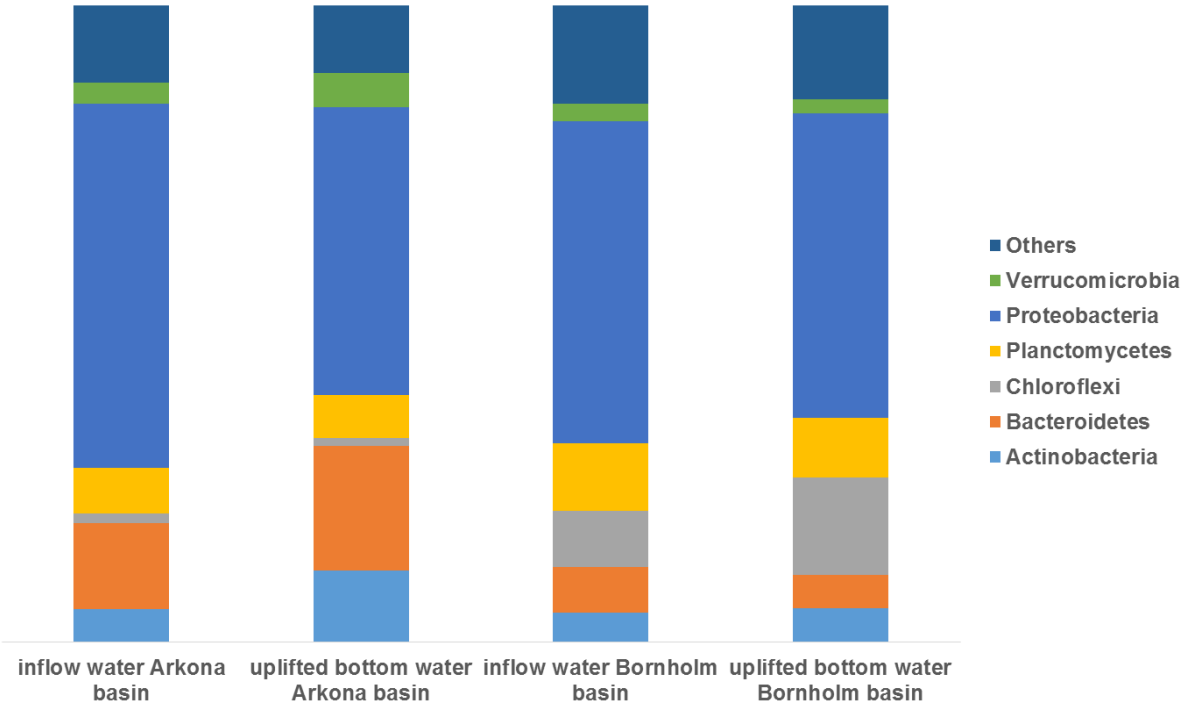

Figure S6: Bacterial community composition on phylum level in the Arkona and Bornholm basin.

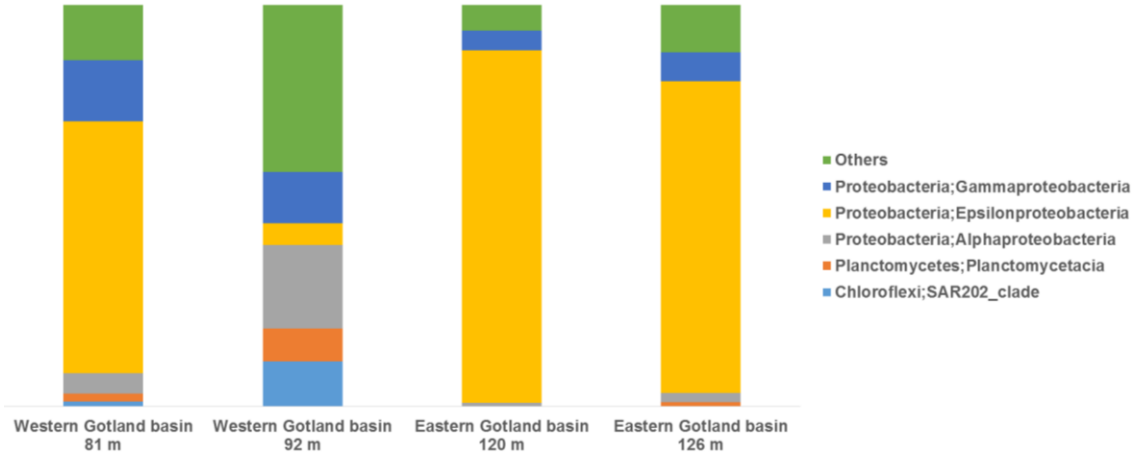

Figure S7: Bacterial community composition on classis level in the Gotland basin.

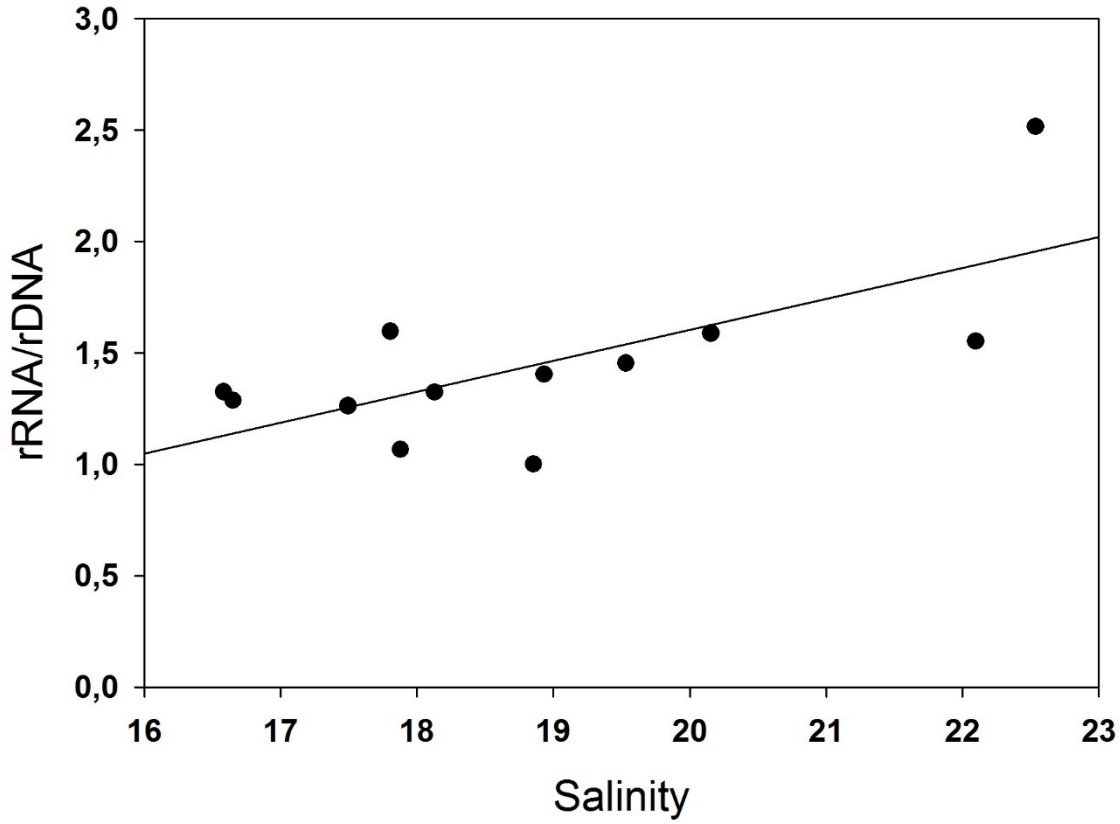

Figure S8: Average 16S rRNA:rDNA ratios vs salinity.

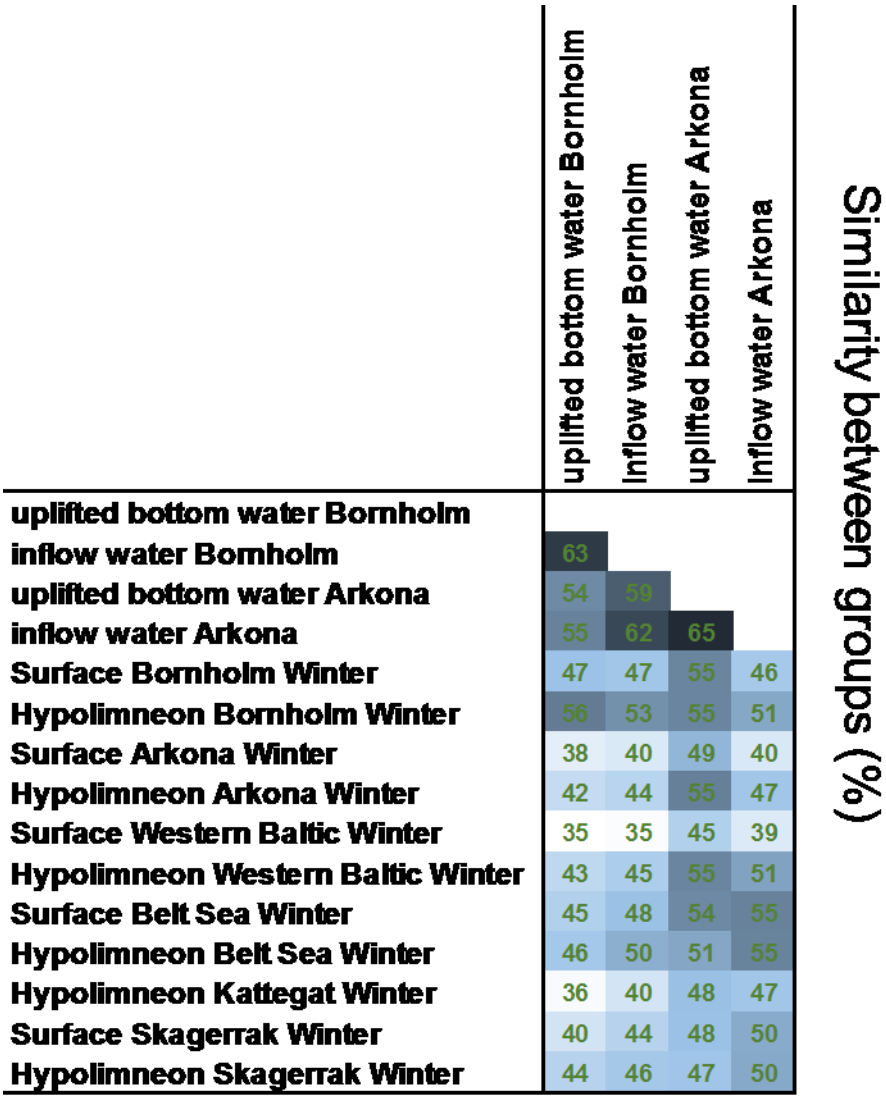

Figure S9: Similarity between bacterial communities on OTU level in the inflow water, the uplifted bottom water and surface and hypolimneon samples from a non-inflow situation in 2009.

Publications, manuscripts and conferences

The content of chapter 1 has already been published in a peer-reviewed journal. Chapter 2 is submitted to an international journal and chapter 3 is to be submitted in near future.

Publications in peer-reviewed journals

Bergen, Benjamin, Herlemann, D.P.R., Jürgens, K. (2015) Zonation of bacterioplankton communities along aging upwelled water in the northern Benguela upwelling. *Front. Microbiol.:* 6:621. doi: 10.3389/fmicb.2015.00621.

Contributions: BB and KJ designed the sampling scheme. BB took and processed the samples. BB and DH analyzed the sequence data. BB wrote the manuscript. DH and KJ did proof-reading of the manuscript.

Bergen, Benjamin, Herlemann, D.P.R., Labrenz, M., Jürgens, K. (2014) Distribution of the verrucomicrobial clade *Spartobacteria* along a salinity gradient in the Baltic Sea. *Environ. Microbiol. Rep.*: **6**, 625–630.

This publication is part of the diploma thesis of BB.

Bergen, Benjamin, Endres, S., Engel, A., Zark, M., Dittmar, T., Sommer, U., Jürgens, K. (submitted) Acidification and warming affect prominent bacteria in two seasonal phytoplankton bloom mesocosms. *Environ. Microbiol.,* doi: 10.1111/1462-2920.13361

Contributions: BB and KJ designed the sampling scheme. US provided the indoor mesocosms and the setup. BB, SE and MZ took and processed samples, BB, SE and MZ analyzed samples. BB, SE and MZ wrote the manuscript. SE, TD, US and KJ did proof reading of the manuscript.

Manuscripts submitted or in preparation

Bergen, Benjamin, Naumann, M., Gräwe, U., Herlemann, D.P.R., Labrenz, M., Jürgens, K. (in preparation) Response of Baltic Sea bacterial communities to a major saline inflow event.

Contributions: BB, MN, ML and KJ designed the sampling scheme. MN took the samples. BB processed the samples. UG provided model results. BB and DH analyzed the samples. BB, MN and UG wrote the manuscript. DH, ML and KJ did proof reading of the manuscript.

Talks at conferences

Bergen, Benjamin, Endres, S., Engel, A., Sommer, U., Jürgens, K. Effect of acidification and warming on planktonic bacterial communities during two seasonal phytoplankton bloom mesocosms. Aquatic Sciences Meeting (ASLO) 2015, Granada, Spanien, 23.02.2015

Poster presentations at conferences

Bergen, Benjamin, Endres, S., Engel, A., Zark, M., Dittmar, T., Sommer, U., Jürgens, K. Effect of acidification and warming on planktonic bacterial communities during two seasonal phytoplankton bloom mesocosms. Symposium on Aquatic Microbial Ecology (SAME14), Uppsala, Schweden, 26.08.2015

Bergen, Benjamin, Herlemann, D.P.R., Jürgens, K. Zonation of bacterioplankton communities along aging upwelled water in the northern Benguela region. Symposium on Aquatic Microbial Ecology (SAME13), Stresa, Italien, 10.09.2013

Bergen, Benjamin, Herlemann, D.P.R., Labrenz, M., Jürgens, K. Cellular quantification by CARD-FISH confirms that *Verrucomicrobia* are important members of the brackish bacterial community in the Baltic Sea. Wissenschaftlicher Beirat des IOW, 13.03.2013

Bergen, Benjamin, Herlemann, D.P.R., Labrenz, M., Jürgens, K. Cellular quantification by CARD-FISH confirms that *Verrucomicrobia* are important members of the brackish bacterial community in the Baltic Sea. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) 2013, Bremen, Deutschland, 11.3.2013

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The opportunity for this doctoral project has not been conveyed to me commercially. In particular, I did not engage an organization that, for a fee, seeks supervisors for the preparation of dissertations. Nor did I engage an organization to, in whole or in part, take care of incumbent obligations with regard to the examinations for me.

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