Ocean acidification effects on marine microbial communities

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hiermit bestätige ich dass die vorliegende Arbeit von Frau Krause in korrektem Englisch verfasst wurde.

Mit freundlichen Grüßen

Prof. Myron A. Peck

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GENERAL INTRODUCTION

Ocean acidification

Since the beginning of the industrial period, atmospheric CO_2 has increased from approximately 280 ppm to 390 ppm (Schulz et al 2009) and 50% of this increase took place over the last three decades (Feely et al 2009). The main causes are fossil fuel burning and cement production. However, not all CO_2 released remains in the atmosphere, but part of it dissolves in the oceans. It was estimated that over the last 200 years, the oceans have taken up one quarter to almost half of anthropogenic CO_2 emissions (Feely et al 2009, Sabine et al 2004). Although part of this excess carbon is converted to phytoplankton biomass (Riebesell et al 2007), this has already led to a reduction in mean surface ocean pH from 8.2 to 8.1, corresponding to a 30% increase in proton concentration (Blackford and Gilbert 2007, Caldeira and Wickett 2003). By the year 2100, atmospheric CO_2 may reach or even surpass 1000 ppm and up to 1900 ppm have been predicted for the year 2300 with the depletion of all fossil fuel reserves (Caldeira and Wickett 2003, Raven et al 2005). The resulting decrease in seawater pH will be most pronounced in surface waters, where it may amount up to 0.7 units (Caldeira and Wickett 2003). In contrast, atmospheric CO_2 has not exceeded 500 ppm (pH >8.0) for the last 24 million years (Pearson and Palmer 2000).

To fully understand the effects of ocean acidification on marine organisms, it has to be taken into account that not only pH, but also the marine carbonate chemistry will change. The four parameters that describe marine carbonate chemistry are pH, partial pressure of carbon dioxide (pCO_2), dissolved inorganic carbon (DIC) and total alkalinity (TA). An uptake of CO_2 by the oceans leads to an increase in pCO_2 and DIC and a drop in pH (Schulz et al 2009). In contrast, there will be no change in TA, which describes the charge balance of seawater (Schulz et al 2009, Wolf-Gladrow et al 2007). In today's oceans, DIC is typically composed of 91% bicarbonate (HCO_3^-), 8% carbonate (CO_3^{2-}) and a small fraction of carbonic acid (H_2CO_3) and dissolved carbon dioxide ($CO_{2(aq)}$) (Hurd et al 2009). Assuming a pH of 7.65 however, H_2CO_3 and $CO_{2(aq)}$ will increase by 300% and HCO_3^- by 9%, whereas CO_3^{2-} will decrease by 56% (Hurd et al 2009, Schulz et al 2009). The reason for this imbalanced change in the components of DIC is that when excess H_2CO_3 dissolves, a fraction of the released protons combine with CO_3^{2-} to form more HCO_3^- (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^-.

It is important to note that the values for pH and carbonate system parameters given above are global means. At smaller regional and temporal scales, seawater pH and the carbonate system are more variable, due to e.g. phytoplankton blooms, stratification and upwelling events (Feely et al 2008, Hofmann et al 2011, Joint et al 2011). For instance in Kiel Fjord in the western Baltic Sea, pH can range from 7.49 to 8.23 over a yearly cycle, with lowest values during summer and autumn caused by the upwelling of oxygen poor, CO_2 enriched waters (Thomsen et al 2010). Thus to ultimately draw conclusions on ocean acidification effects on marine organisms, it is essential to characterize the carbonate system and its natural variability in their habitat (Hofmann et al 2011).

In the past years, numerous national and international research initiatives have been launched to investigate the effects of ocean acidification. Up to now, results suggest that the process most sensitive to low pH and decreased carbonate availability is calcification (Dupont et al 2010, Hendriks et al 2010). Furthermore, various studies focused on eukaryotic phytoplankton, some suggesting that increased carbon availability due to ocean acidification will have a fertilizer effect on primary production (Joint et al 2011, Liu et al 2010, Riebesell et al 2007). 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 and virtually nothing regarding other heterotrophic marine microbes such as archaea, protists and marine fungi.

Marine microbial food webs

Marine microbes are highly abundant and metabolically versatile and take part in nearly all marine geochemical reactions (Kirchman 2008). They comprise autotrophic and heterotrophic bacteria, archaea, protists (single-celled eukaryotes) and marine fungi. The latter can be divided into two ecologically distinct groups, the filamentous fungi and the unicellular yeasts. This thesis will focus on heterotrophic bacteria and marine fungi, which are both important players in nutrient cycling.

In marine food webs, heterotrophic bacteria decompose organic material and remineralize inorganic nutrients (Pomeroy et al 2007). They live on carbon sources such as phytoplankton exudates, detritus and organic material released by zooplankton or protozoan grazing (Pomeroy and Wiebe 1993). Due to their high abundance, heterotrophic bacteria dominate the assimilation of dissolved organic matter (DOM), which is the largest fraction of organic carbon in the ocean (Kirchman 2008). Through the microbial loop

(Azam et al 1983, Pomeroy 1974), marine bacteria are closely linked to higher trophic levels, because protozoan grazing channels back carbon and energy from bacteria to larger zooplankton. In addition to grazing, a second cause of bacterial mortality is viral lysis (Kirchman 2008).

Contrary to heterotrophic bacteria, marine fungi have traditionally been regarded as most important in the decomposition of vascular plant detritus in coastal regions (Kirchman 2008). Especially during productive periods however, marine fungi can also play major roles in plankton (Gao et al 2010, Gutiérrez et al 2011, Kutty and Philip 2008). Their role in biogeochemical cycles results from their ability to depolymerize complex substrates, most notably lignocellulose, a heteropolymer consisting of cellulose, hemicellulose and lignin (Hyde et al 1998). Furthermore, marine fungi decompose calcareous substrates, hydrocarbons and probably also chitin, keratin and tunicin (Bugni and Ireland 2004, Hyde et al 1998).

Although both marine bacteria and marine fungi are capable of salvaging nutrients from recalcitrant material and convert particulate to dissolved organic matter, they occupy different niches in these processes (Raghukumar 2004). Bacteria can colonize small detrital particles and utilize extremely low nutrient concentrations, given their high surface-to-volume ratio (Kirchman 2008, Wang et al 2012). In contrast, highest fungal numbers have been reported from productive areas and were found to be correlated to phytoplankton biomass (Fell 2001, Gao et al 2010, Gutiérrez et al 2011). This preference for higher substrate availability is most probably due to the lower surface-to-volume ratio of marine fungi (Kirchman 2008). While marine bacteria typically measure 0.2–0.6 µm (Pomeroy et al 2007), fungal mycelia can range from 1 to 4 µm in diameter and 10 to >400 µm in length (Gutiérrez et al 2011). Furthermore, filamentous fungi grow more slowly than bacteria (Wang et al 2012), but can thoroughly penetrate particulate detritus due to their hyphal growth (Raghukumar 2004). In contrast, the niche of yeasts has been proposed to be more similar to that of bacteria, due to their unicellular growth (Wang et al 2012). Yeasts colonize the surface of particles or may live in high DOM concentrations (Raghukumar 2004).

Investigating the diversity of marine bacteria and fungi

To characterize the effects of ocean acidification on heterotrophic bacteria and marine fungi, important first steps are to analyze whether their abundance or community structure

are affected. Especially community structure can give valuable hints on functional aspects and ultimately biogeochemical processes (Fuhrman and Hagström 2008). While for bacteria, adequate counting and community analysis methods are readily available, tools for the investigation of marine fungal communities are not as advanced yet (Jobard et al 2010).

Marine bacteria

The use of culture-independent direct counting and molecular phylogenetic methods has greatly altered the understanding of marine bacteria (Sherr and Sherr 2008). Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or automated ribosomal intergenic spacer analysis (ARISA) were widely applied to characterize marine bacterial community structure, its seasonal fluctuations and its dependence on environmental factors (Brown et al 2005, Fuhrman et al 2008, Sapp et al 2007). Furthermore, an unprecedented amount of sequence data has been generated, especially with pyrosequencing methods (Caporaso et al 2012, Fortunato et al 2012, Kirchman et al 2010, Sogin et al 2006). Both approaches revealed that in most ecosystems, bacterial community structure is characterized by seasonally recurring patterns and a large number of rare species (Andersson et al 2010, Fuhrman et al 2006, Fuhrman 2009, Gilbert et al 2012). These rare species are thought not to have a large impact on major biogeochemical processes, but may reach higher importance under favorable environmental conditions, thus representing a "seed bank" (Pedrós-Alió 2006). In fact, various rare taxa were already found to be undetectable in some months, but to constitute several percent of the community at other times (Brown et al 2005, Pernthaler and Amann 2005).

An in depth ecological investigation of important bacterioplankton groups is still difficult, as the majority of marine bacteria has only been identified by rRNA gene sequencing but remains uncultured (Giovannoni and Stingl 2005). Notable exceptions are the genus *Roseobacter* and the SAR11 clade of the *Alphaproteobacteria*, which can constitute up to 30% of natural bacterioplankton and have cultured representatives (Fuhrman and Hagström 2008). Members of the *Bacteroidetes* are culturable as well, but their contribution to marine bacterioplankton is not clear, as FISH counts and 16S rRNA clone libraries yield different results (Fuhrman and Hagström 2008). However, cultivation on rich media often yields bacteria that are rare *in situ*, especially *Gammaproteobacteria*, such as the genera *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Shewanella*, *Glaciola*, *Oceanospirillum* and *Colwellia* (Fuhrman and Hagström 2008, Fuhrman 2009, Giovannoni

and Rappé 2000). Yet this bias also reveals that there are two general ecological strategies among marine bacteria, which are efficient competition at ambient, i.e. low nutrient levels and efficient exploitation of environmental patchiness, i.e. high nutrient concentrations (Giovannoni and Stingl 2005). Certain members of both groups can be cultivated in unamended seawater, using different dilution cultivation methods (Ammerman et al 1984, Button et al 1993, Fuchs et al 2000, Pinhassi and Berman 2003). An example for the application of this technique is the determination of grazing pressure on different bacterial groups (Beardsley et al 2003).

Marine fungi

In 1979, Kohlmeyer and Kohlmeyer defined that "obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater and terrestrial milieus able to grow and possibly also sporulate in the marine environment". To include both groups, the terms "marine-derived fungi" or "marine fungi" have been used (Bugni and Ireland 2004, Richards et al 2012), which will be adhered to in this thesis as well.

Marine fungi comprise the four major groups *Ascomycota, Basidiomycota,* zygomycetes and chytrids (Richards et al 2012). Estimates of the total number of marine fungi range from 7000 to >10,000 species, with probably 1500 of them yeasts (Jones 2011). However, only 537 marine fungi have been described. The large discrepancy may result from a restriction of research efforts to certain habitats and substrates, the presence of unculturable and cryptic species and the question which species are considered marine fungi (Jones 2011). Although a frequently cited study reports 10^3 to 10^4 fungal cells per mL of seawater (Kubanek et al 2003), adequate methods for the quantification of marine fungi are lacking. The reason for this is that neither of the two widely used culture-independent methods, calcofluor white staining and ergosterol measurements, targets all fungal groups (Jobard et al 2010). Therefore, fungal abundance is often determined by cultivation, enumerating viable cells per mL of seawater or gram of substrate (Sadaba and Sarinas 2010, Wang et al 2012). A promising future option may be real-time quantitative PCR (Chemidlin Prévost-Bouré et al 2011). Likewise, there is no consensus on a single phylogenetic marker gene for marine fungi. Instead, both the ITS region and different regions of the 18S rRNA gene are used for analyses of total fungal communities (Kurtzman 2006, Richards et al 2012, Schoch et al 2012) and the D1/D2 region of the large

subunit (LSU) rRNA gene is frequently used for yeasts (Fell et al 2000, Kurtzman and Robnett 1998).

In recent years, DGGE analyses of planktonic fungi have revealed unexpected diversity and a majority of phylotypes not known from culture collections (Gao et al 2010, Gutiérrez et al 2010). Additionally, these studies detected a correlation of planktonic fungal diversity to phytoplankton biomass (Gao et al 2010, Gutiérrez et al 2011). Fungal ARISA (F-ARISA) has been applied to soil and salt marsh communities (Ranjard et al 2001, Torzilli et al 2006), but to the best of our knowledge not to planktonic fungal communities. Similarly, pyrosequencing was so far only used to characterize fungi associated with corals or mangroves (Amend et al 2012, Arfi et al 2012).

Interestingly, fungal 18S sequence libraries from marine environments predominantly contain yeast species (Richards et al 2012). Furthermore, many studies have investigated culturable marine yeasts in diverse marine environments (Burgaud et al 2010, Chen et al 2009, Gadanho et al 2003, Gadanho and Sampaio 2005). These investigations often use PCR-based pre-screening methods to pre-group isolates and limit sequencing efforts. Strategies used up to now are micro/ minisatellite-primed PCR (MSP-PCR) (Gadanho and Sampaio 2002, Gadanho et al 2003) and restriction fragment length polymorphism (RFLP) (Chen et al 2009). Another promising tool to rapidly classify yeasts is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This method generates proteomic mass spectra and has already been successfully applied to the identification of clinical yeast isolates (Dhiman et al 2011, Marklein et al 2009, Stevenson et al 2010, van Veen et al 2010). Therefore it may also be suitable for environmental or more specifically marine yeast isolates.

Effects of ocean acidification on heterotrophic marine bacteria and fungi

Direct vs. indirect effects

Both direct and indirect effects of ocean acidification on marine microbes can be expected. Indirect effects are mediated through the responses of non-microbial groups to ocean acidification, such as changes in primary production, grazing or viral lysis (Liu et al 2010). Furthermore, symbionts or parasites can be indirectly affected through changes in a host organism's physiology (Meron et al 2011, Vega Thurber et al 2009). In contrast, direct effects result from responses of microbes to decreasing pH and changes in seawater carbonate chemistry. Concerning direct pH effects, it has been pointed out that both

bacteria and fungi occur over a wide range of natural pH values, including extremely acidic environments (Davis 2009, Gadanho et al 2006, Joint et al 2011, López-Archilla et al 2004, Orij et al 2011, Rothschild and Mancinelli 2001). Nevertheless, small pH changes may favor distinct groups at the community level, possibly leading to compositional shifts (Liu et al 2010).

In general the alkaline marine milieu is considered to be a stress factor for both bacteria and fungi (Davis 2009, Orij et al 2011, Padan et al 2005). Their intracellular pH values are below mean seawater pH and therefore, they have to invest energy into their pH homeostasis (Orij et al 2009, Padan et al 2005, Teira et al 2012). For bacteria, mechanisms to cope with alkaline pH stress include upregulation of ATP synthase, cation/proton antiporters, metabolic acid production and modifications of cell surface layers to promote cytoplasmic proton retention (Padan et al 2005). In fact, a study investigating two bacterial isolates at high CO_2 (1000 ppm) found increased growth efficiency for a *Cytophaga*, but not for a *Roseobacter* strain (Teira et al 2012), indicating that certain groups may indeed profit energetically from ocean acidification. For fungi, knowledge concerning intracellular pH and its homeostasis in environmental and especially marine strains is scarce. Most knowledge derives from research related to strains pathogenic to humans or plants, because during infection, fungi are faced with different pH extremes (Davis 2009, Selvig and Alspaugh 2011). While fungi have efficient mechanisms to remove excess protons from their cytoplasm (Carmelo et al 1997, Diakov and Kane 2010), they have difficulties to sustain a functional plasma membrane proton gradient in alkaline environments (Orij et al 2011). This gradient is crucial however, as most fungi take up low molecular weight compounds over the plasma membrane (Richards et al 2012), primarily driven by this gradient (Davis 2009, Orij et al 2011). Concerning the growth of marine yeast strains, earlier studies suggested growth optima at below present-day seawater pH (Hoppe 1972, Norkrans 1966).

Yet care has to be taken not to oversimplify the underlying mechanisms. For instance, a study investigating the meta-transcriptome of bacterial communities in a mesocosm experiment reported higher numbers of transcripts related to bacterial proton pumps at high pCO_2 (Gilbert et al 2008). This is contrary to the suggested reduced energetic cost of pH homeostasis. Furthermore, elevated bacterial extracellular enzyme activities (Grossart et al 2006, Piontek et al 2010) and increased heterotrophic CO_2 assimilation (Teira et al 2012) were reported at reduced pH. Arguably, direct ocean acidification effects may also include

altered substrate availability, as changes in the speciation of nutrients such as phosphate, silicate and ammonia will occur with changing pH (Zeebe and Wolf-Gladrow 2001).

Results from mesocosms and coral-associated communities

So far, ocean acidification effects on marine bacterial communities have mainly been studied in mesocosms and in coral microbial communities. Mesocosms closely mimic natural conditions and typically enclose several cubic meters of water (Riebesell et al 2012). In these systems, both direct and indirect effects due to induced phytoplankton blooms and the resulting food-web interactions can be studied. However, results on bacterial processes and especially diversity are often contrasting or at best vague (Liu et al 2010). Allgaier et al (2008) found effects on free-living bacterial community structure by DGGE, but subsequent sequencing did not confirm these differences. In contrast, attached bacteria were only dependent on phytoplankton bloom development but not on pCO_2 (Allgaier et al 2008). Likewise, Newbold et al (2012) reported only minor evidence of changes in bacterial community structure. Concerning bacterial abundance, a single study found an increase in bacterial numbers with pCO_2 in the post-bloom phase of a mesocosm experiment (Grossart et al 2006), but other studies found no effect (Allgaier et al 2008, Newbold et al 2012). Unlike marine bacteria, marine fungi have so far been neglected in mesocosm studies.

In corals experimentally exposed to low pH, microbial communities were similar to those found in stressed or diseased hosts (Meron et al 2011, Vega Thurber et al 2009). This was not confirmed at natural CO_2 enriched sites though (Meron et al 2012). Additionally, in natural biofilms from the Great Barrier Reef, a decrease in *Alphaproteobacteria* and an increase in *Flavobacteriales* were reported at reduced pH (Witt et al 2011). To the best of our knowledge, the only record of fungi in the context of ocean acidification is a study by Vega Thurber et al (2009), which reported higher fungal abundance in corals at reduced pH. The authors interpreted this as an indirect effect of ocean acidification, as pH–stressed corals may be more susceptible to fungal colonization (Vega Thurber et al 2009).

Microcosm approaches: a highly replicated alternative

Considering the variety of different factors that influence microbial communities in mesocosm studies, detecting effects above this "background noise" would require a high replication. However, the studies of Allgaier et al (2008) and Newbold et al (2012) relied

only on one or two replicates per pCO_2 treatment, respectively. Another study analyzed bacterial community structure of one replicate and subsequently randomly split sequences obtained to carry out statistical calculations (Ray et al 2012). Furthermore, it is a common practice to sum up results from "similar" levels of pCO_2 to pCO_2 categories. Last but not least, previous studies have not looked at seasonal or year-to-year variability in community responses. These practices result from logistic and financial constraints, which highlight the need for cost-efficient, highly-replicated alternatives.

In this thesis, the effects of ocean acidification on marine bacterial and fungal communities are investigated in small-scale microcosms (1.1-1.6 L). The advantages are that experiments can be carried out at high replication and can be repeated at different time points. Furthermore, factors such as temperature and light can be controlled. This approach predominantly addresses direct effects of ocean acidification, but can yield valuable hypotheses to test in more complex experimental designs. Concerning bacterial community structure, a downside of this approach is that the confinement to experimental containers causes shifts (Ferguson et al 1984). This is also observed in large-scale mesocosms though (Ray et al 2012). To alleviate bias and take advantage of this effect, different dilution cultivation methods were applied to select for different bacterial groups. For bacterial abundance, the impact of confinement is still under debate; a recent study found no evidence for a volumetric bottle effect (Hammes et al 2010).

The study site Helgoland Roads

The study site Helgoland Roads (54°11.3′N, 7°54.0′E) is situated in the German Bight of the North Sea. For this site, long term records of temperature, salinity, nutrients, light penetration, phytoplankton and zooplankton exist since 1962 (Wiltshire et al 2008). Furthermore, different microbiological parameters have been recorded and seasonal differences in bacterial community structure were revealed (Gerdts et al 2004, Oberbeckmann et al 2011b, Sapp et al 2007, Teeling et al 2012). Additionally, cultivation experiments of the natural bacterial community at this site have been carried out before (Beardsley et al 2003, Eilers et al 2000a). Concerning marine yeasts, viable cell counts were determined from 1964 to 1966 and from 1980 to 1992 and sporadic information on yeast identities is available as well (Ahearn and Crow 1980, Gerdts et al 2004, Meyers et al 1967). In contrast, there is no description of the total marine fungal community at Helgoland Roads.

The carbonate system at Helgoland Roads has not been characterized in depth. Kempe (1996) presented and critically evaluated pH measurements at Helgoland Roads, which cover the period from 1962 to 1978. According to these records, fluctuations in pH range from <7.9 to >8.4, with most values between 8.0 and 8.2. These measurements are not very reliable though, as electrode drifting and unrealistically low pH records during summer occurred, the latter hinting at respiratory processes when samples were not directly processed (Kempe 1996). Nevertheless, Kempe (1996) used these records to estimate pCO_2 by the empirical relationship $pCO_2=1.58.86x10^9xe^{(-2.4420xpH)}$, yielding a range from <200 to >900 ppm, with an average of 480 ppm. Furthermore, a seasonal trend was apparent, with highest pCO_2 values in winter, followed by a rapid decline in spring and a slow recovery to higher pCO_2 values during autumn and winter (Kempe 1996). Despite the methodological constraints, this trend corresponds well to more recent descriptions of the carbonate system in the North Sea. In winter, the southeastern coast of the North Sea was described to be slightly CO_2 supersaturated with respect to the atmosphere, followed by a strong undersaturation with the spring bloom (Thomas et al 2004). Subsequently, primary production decreases and respiration dominates, leading to rising pCO_2 again (Thomas et al 2004). Notably, different patterns occur in the central and northern part of the North Sea (Thomas et al 2004).

Concerning future projections, it has been suggested that the mean surface pH in the southern North Sea (below 56°N) may decrease to 7.82 or 7.67 by the year 2100, assuming atmospheric CO_2 of 700 or 1000 ppm, respectively (Blackford and Gilbert 2007). These pH levels were consequently used for all ocean acidification experiments in this thesis.

RESEARCH AIMS

The aim of this study was to investigate direct effects of ocean acidification on marine microbial communities. The groups in focus were heterotrophic bacteria, marine fungi and marine yeasts. For each group, the questions whether moderate reductions in seawater pH alter community structure and abundance were addressed. As previous large-scale studies suffered from low replication, the experiments were conducted using microcosms. This allowed for high replication, robust statistical analyses and the consideration of seasonal and year-to-year differences in community assembly. To interpret results comprehensively, the natural variability of the carbonate system at Helgoland Roads was determined over a yearly cycle.

Description of the carbonate system at Helgoland Roads over a yearly cycle

To draw conclusions from ocean acidification studies, it is important to characterize the present-day variability of the carbonate system at the study site. Previous records of pH at Helgoland Roads are not reliable though. Therefore, pH and total alkalinity (TA) were determined daily over a yearly cycle from September 2010 to September 2011. Both parameters were measured according to standard procedures and were corrected with standard reference materials to ensure an accurate description. Together with oceanographic data, this allowed to calculate seawater pCO_2. The yearly pH cycle is described in Chapter II and measured TA and calculated pCO_2 values are considered in the general discussion.

Ocean acidification effects on marine bacterial communities

Previous ocean acidification studies found only minor effects on bacterial community structure and abundance. These studies were very complex though, involving direct and indirect ocean acidification effects, but lacked appropriate replication. To thoroughly investigate direct pH effects on marine bacterial communities, highly-replicated microcosm experiments were conducted at four time points (spring, summer and autumn, 2010, and in winter 2011). Natural seawater was incubated at different pH levels for four weeks. Distinct bacterial groups were investigated using different dilution culture strategies. The aim was to find out whether pH-dependent community shifts occur and which bacterial groups are involved in these shifts. This was determined by a combination

of ARISA and 16S ribosomal amplicon pyrosequencing. Effects on bacterial abundance were investigated by flow cytometry. This experiment is presented in Chapter I.

Ocean acidification effects on marine fungal communities

The only previous report on fungi in the context of ocean acidification is a study on corals, which were colonized by more fungi at reduced pH. However, no studies on direct ocean acidification effects on marine fungi exist, although they are known to prefer low pH values. To characterize direct pH effects on marine fungal abundance and community structure, a microcosm experiment was carried out in 2011 and in 2012. Natural seawater was incubated at different pH levels for four weeks. Fungal community structure was examined with the cultivation independent method F-ARISA and fungal abundance was determined by colony forming unit counts. The experiments are described in Chapter II.

Ocean acidification effects on marine yeasts

Marine yeasts are the most frequently found group in marine fungal 18S sequence libraries and occupy a different ecological niche than filamentous fungi. Therefore the direct pH effects on this fungal group were investigated in more detail. After incubation of natural seawater for two or four weeks at different pH levels in 2011, yeast isolates were obtained to determine pH effects on yeast community structure. Furthermore, MALDI-TOF MS was evaluated as a tool to identify and classify marine yeast isolates. The identification was complemented by partial sequencing of the LSU rRNA gene and results were compared to previous reports on yeasts at Helgoland Roads. This experiment is presented in Chapter III.

OUTLINE

This cumulative thesis consists of three chapters, each representing a stand-alone publishable manuscript.

Chapter I (published in *PLoS ONE* **7** (10): e47035; PDF on the supplementary CD-ROM)
Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M.B. and Gerdts, G. (2012)
Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach.
The planning, experiments, analyses and manuscript writing were carried out by Evamaria Krause under the guidance of Antje Wichels and Gunnar Gerdts. Luis Giménez assisted with the statistical analyses and Mirko Lunau with the analyses of flow cytometry counts. Additionally, both provided fruitful discussions. Markus B. Schilhabel was in charge of the 16S ribosomal amplicon pyrosequencing of tagged PCR products.

Chapter II (to be submitted to *Nature Climate Change*)
Krause, E., Wichels, A., Giménez, L. and Gerdts, G.
Marine fungi may benefit from ocean acidification
The planning, experiments, analyses and manuscript writing were carried out by Evamaria Krause under the guidance of Antje Wichels and Gunnar Gerdts. The bachelor student Diana Höhlig assisted with the experiment and F-ARISA fingerprints in 2011. Luis Giménez assisted with the statistical analyses and provided fruitful discussions.

Chapter III (submitted to *FEMS Microbiology Ecology*)
Krause, E., Wichels, A., Erler, R. and Gerdts, G.
Do marine yeasts benefit from ocean acidification?
The planning, experiments, analyses and manuscript writing were carried out by Evamaria Krause under the guidance of Antje Wichels and Gunnar Gerdts. The bachelor student Diana Höhlig assisted with the experiment and the intern Jonathan Herlet with the sequence analysis of yeast isolates. René Erler assisted with MALDI-TOF MS analyses and provided fruitful discussions.

CHAPTER I

Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach

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Abstract

As the atmospheric CO_2 concentration rises, more CO_2 will dissolve in the oceans, leading to a reduction in pH. Effects of ocean acidification on bacterial communities have mainly been studied in biologically complex systems, in which indirect effects, mediated through food web interactions, come into play. These approaches come close to nature but suffer from low replication and neglect seasonality. To comprehensively investigate direct pH effects, we conducted highly-replicated laboratory acidification experiments with the natural bacterial community from Helgoland Roads (North Sea). Seasonal variability was accounted for by repeating the experiment four times (spring, summer, autumn, winter). Three dilution approaches were used to select for different ecological strategies, i.e. fast-growing or low-nutrient adapted bacteria. The pH levels investigated were *in situ* seawater pH (8.15-8.22), pH 7.82 and pH 7.67, representing the present-day situation and two acidification scenarios projected for the North Sea for the year 2100. In all seasons, both automated ribosomal intergenic spacer analysis and 16S ribosomal amplicon pyrosequencing revealed pH-dependent community shifts for two of the dilution approaches. Bacteria susceptible to changes in pH were different members of *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Campylobacteraceae* and further less abundant groups. Their specific response to reduced pH was often context-dependent. Bacterial abundance was not influenced by pH. Our findings suggest that already moderate changes in pH have the potential to cause compositional shifts, depending on the community assembly and environmental factors. By identifying pH-susceptible groups, this study provides insights for more directed, in-depth community analyses in large-scale and long-term experiments.

Introduction

Since the beginning of the industrial period, the oceans have taken up one-quarter to one-third of anthropogenic CO_2 emissions (Feely et al 2009, Sabine et al 2004). This has already led to a reduction in surface ocean pH of 0.1 units, which may reach up to 0.7 units assuming the depletion of all fossil fuel reserves during the next three centuries (Caldeira and Wickett 2003). In contrast, pH has constantly remained above 8.1 for the last 23 million years (Pearson and Palmer 2000). By the year 2100, atmospheric pCO_2 values of 700 or 1000 ppm may lower mean surface pH in the southern North Sea to 7.82 or 7.67, respectively (Blackford and Gilbert 2007).

The effects of the anticipated rapid reduction in pH on marine organisms, and their ability to adapt, will determine future marine biodiversity and ecosystem functions. Yet the impact of ocean acidification on different groups of marine organisms remains under debate (Dupont et al 2010, Hendriks et al 2010), especially regarding heterotrophic bacteria as important players in marine biogeochemical cycles. 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, and even greater pH ranges are observed in freshwater lakes. This view was challenged by a meta-analysis on microbe-related ocean acidification research, which identified nitrogen fixation, cyanobacterial photosynthesis and elemental ratios as affected by changes in seawater carbonate chemistry (Liu et al 2010). Concerning other microbial processes and especially heterotrophic bacteria however, results have often been inconsistent and Liu et al (2010) concluded that "more research is needed at multi-species and community scales".

What we know about ocean acidification effects on bacterial communities predominantly stems from complex systems such as symbiotic microbial communities of corals or large-scale mesocosm experiments. At reduced pH, coral microbial communities were found to shift to bacteria associated with stressed or diseased hosts (Meron et al 2011, Vega Thurber et al 2009), which could however not be confirmed at natural CO_2 vent sites (Meron et al 2012). Furthermore, a decrease in the relative abundance of *Alphaproteobacteria* and an increase in the relative abundance of *Flavobacteriales* were observed in natural biofilms from the Great Barrier Reef (Witt et al 2011).

Knowledge on the seawater bacterial community has remained scarce though. In mesocosm experiments, only minor indications of bacterial community shifts with pH were found (Allgaier et al 2008, Newbold et al 2012). Notably, these findings relied on only one

or two replicates per pCO_2 treatment, which is a common problem in mesocosm studies. Although these experiments are biologically highly complex, involving indirect pH effects through food web interactions, they are usually carried out in low replication, due to logistical challenges and high costs. As a consequence, these experimental designs preclude a robust statistical interpretation. Furthermore, the natural variability of bacterial communities, which is characterized by seasonally recurring patterns (Andersson et al 2010, Fuhrman et al 2006, Gilbert et al 2012), is not taken into account.

Therefore, a straightforward small-scale approach is needed to allow for high replication and the consideration of differently assembled communities. The problem of limited culturability of marine bacteria can be addressed by dilution experiments. A certain fraction of natural bacterioplankton is able to grow in particle-free, unenriched seawater (Ammerman et al 1984), although the confinement to experimental containers causes shifts in community structure often described as the "bottle effect" (Ferguson et al 1984). To alleviate this bias and select for typical marine bacteria, the concept of dilution culture was developed (Button et al 1993). Originally, this approach followed a dilution to near extinction strategy to isolate previously uncultured marine strains (Schut et al 1993). The idea was developed further to study diluted cultures of entire bacterioplankton assemblages, in which depending on the dilution strategy, different parts of the bacterial community are selected for (Fuchs et al 2000, Pinhassi and Berman 2003). Dilution experiments were successful in e.g. identifying selective grazing mortality of bacterial groups (Beardsley et al 2003).

Here we present a highly replicated, culture-dependent investigation of direct pH effects on bacterial communities from the North Sea. The sampling station Helgoland Roads is well suited for this approach, as seasonal and long-term microbiological data are available (Eilers et al 2000b, Eilers et al 2001, Gerdts et al 2004, Sapp et al 2007) and dilution or enrichment experiments have been carried out before (Beardsley et al 2003, Eilers et al 2000a). We used three dilution approaches to study different bacterial groups and repeated the experiment in different seasons. We show that small changes in pH have direct effects on bacterial community composition and identify *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae* and *Campylobacteraceae* as phylogenetic groups responding most notably to differences in pH.

Materials and methods

Ethics statement

No specific permits were required for the sampling and activities performed in the study.

Experimental set-up and sampling

We conducted the experiment in spring, summer and autumn, 2010, and in winter 2011 (Table 1) with surface water sampled at 1 m depth at sampling station Helgoland Roads (54°11.3′N, 7°54.0′E), North Sea. Water samples were subjected to three different dilution protocols and three different pH levels (see sections below), yielding a total of nine different treatments. Each treatment was replicated five times in autoclaved one liter borosilicate glass bottles. Bottles were filled with 1100 mL to keep the headspace small (volume 50-60 mL) to prevent excessive CO_2 exchange at the air-seawater interface observed in systems open to the atmosphere (Gattuso and Lavigne 2009). Bottles were incubated in the dark at approximately *in situ* temperature (Table 1) and were mixed daily by inversion. Samples were taken after four weeks of incubation.

We used the following three dilution protocols: 'no dilution', 'serial dilution' (diluted weekly 1:1000 starting after one week), and 'initial dilution' (initially diluted 1:1000). Larger organisms were removed by filtration through 10 µm Isopore™ Membrane Filters (TCTP-type, Millipore, Eschborn, Germany). For the dilutions, we used seawater collected on the respective initial sampling date, which had been repeatedly sterilized by filtration (0.2 µm Isopore™ Membrane Filters, GTTP-type, Millipore).

The pH levels were the current *in situ* seawater pH (8.15-8.26, Table 1), pH 7.82 and 7.67. We acidified the initial seawater samples (fraction <10 µm) and the sterile-filtered seawater used for the dilutions with 2 M HCl. To determine pH at the beginning and after the four weeks of incubation, we used a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MF-DIN-N), calibrated with standard buffer solutions (pH 4.01, 6.87, 9.18) (all materials: SI Analytics, Mainz, Germany). All pH measurements were carried out at the respective incubation temperature and are reported on the National Bureau of Standards (NBS) scale.

For cell counts, samples of 4 mL were fixed with formaldehyde (1% [w/v] final concentration) for 1 h at room temperature and were subsequently stored at -80°C until further analysis. Aliquots of 500 µL were stained for 10 min with 10 µL of a freshly prepared 400x SYBR Green (invitrogen™, Life Technologies, Paisley, UK) solution in

sterile filtered dimethyl sulfoxide (DMSO). Directly prior to staining the cells, we added 10 µL of a diluted solution of Fluoresbrite® Polychromatic Red Microspheres 1.0 µm (Polysciences Europe, Eppelheim, Germany) as an internal counting standard (final concentration of about 10% of the expected number of cells). We analyzed samples with an Accuri C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, MI, USA) with the fluidics setting "slow" for 1.5 min. To reduce noise, we set a threshold on FL1-H of 550 for winter and spring and of 600 for summer and autumn samples, respectively. The actual flow through was calibrated with BD Trucount™ Controls (BD Biosciences, San Jose, CA, USA).

Biomass was collected on 0.2 µm Isopore™ Membrane Filters (GTTP-type, 47 mm diameter, Millipore) and DNA extraction was performed as previously described (Sapp et al 2007). DNA concentration and purity were determined by photometry using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland).

ARISA

For ARISA fingerprints, we used forward primer L-D-Bact-132-a-A-18 (5'-CCGGGTTTCCCCATTCGG-3') and reverse primer S-D-Bact-1522-b-S-20 (5'-TGCGGCTGGATCCCCTCCTT-3') (Ranjard et al 2000), the latter labeled with an infrared dye. PCR reactions were performed in volumes of 25 µL containing 10 ng template DNA, 2.5 µL Taq Buffer (10x), 5 µL TaqMaster PCR Enhancer (5x), 0.7 µL of each primer (20 µM), 0.75 µL dNTPs (2.5 mM each), and 1.4 U Taq DNA polymerase (5 Prime, Hamburg, Germany). Cycling conditions were: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 68°C for 1 min, with a final step at 68°C for 5 min. Aliquots of PCR products were verified on agarose gels and depending on agarose gel band intensities, original or diluted PCR products were mixed with an equal volume of formamide containing stop mix. Samples were heated to 95°C for 2 min, subsequently kept on ice for 10 min, and 0.25 to 0.5 µL were separated in 5.5% polyacrilamide gels. Running conditions were 1500 V for 14 h on a LI-COR 4300 DNA Analyzer. A pre-run of 15 min at 45°C was carried out to precondition the gel and sequencer prior to loading the samples. As a size reference, we used a 50-1500 bp standard (all materials: LI-COR Biosciences, Lincoln, NE, USA).

ARISA gels were analyzed with the Bionumerics 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium). Bands with intensities lower than 4% of the maximum value of the respective lane and bands smaller than 300 bp were neglected. Binning to band classes

was performed according to Brown et al (2005). Beta-diversity was calculated with the Jaccard coefficient.

16S ribosomal amplicon pyrosequencing

Based on ARISA results, samples were selected for pyrosequencing of the bacterial 16S rDNA (pH *in situ* and 7.67 of all 'no dilution' and 'serial dilution' treatments and of the 'initial dilution' in summer; additionally starting communities). Primers used to construct the amplicon library were of the structure 5'-[Roche's adaptor for long reads (Lib-L)] - [template-specific sequence]-3'. As template specific sequences we used forward primer GM3 (5'-AGAGTTTGATCMTGGC-3') and reverse primer 907 (5'-CCGTCAATTCMTTTGAGTTT-3') (Teeling et al 2012). To pool all 93 samples in a single run, 93 different forward primers were constructed, each containing a different 10 bp long Multiplex Identifier (Roche) between the adaptor sequence and the template specific sequence. Amplifications were conducted in two duplicate reactions of 50 µL, each containing 20 ng of template DNA, 5 µL Taq Buffer (10x), 10 µL TaqMaster PCR Enhancer (5x), 1.25 µL of each primer (20 µM), 5 µL dNTPs (2.5 mM each), and 2.5 U of Taq DNA polymerase (5 Prime). Cycling conditions were: 94°C for 10 min, followed by 20 cycles of 94°C for 1 min, 44°C for 1.5 min and 68°C for 2 min, with a final step at 68°C for 15 min. The duplicate reactions were combined and purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Amplicons were pooled equimolarly, including 2 µL of a negative control with a separate Multiplex Identifier to check for contaminations, and sequenced on 1/2 plate on a Roche 454 GS-FLX Titanium platform at the Institute of Clinical Molecular Biology (IKMB), University of Kiel, Germany. Sequence data were deposited in the NCBI Sequence Read Archive (accession number SRP014019).

Sequences were processed with the MOTHUR software (version 1.23.0, (Schloss et al 2009)). Flowgrams were trimmed to 360 to 800 flows and the shhh.flows command with standard settings was used to remove sequencing noise. After the removal of primer and barcode sequences, unique sequences were aligned to the SILVA reference database. After removing chimeras (chimera.uchime routine) and chloroplast sequences, 163,029 sequences remained (20,419 of them unique). Sequences started and ended at the same alignment position, 95% being of a length between 381 and 420 bp. A distance matrix was created and sequences were grouped into 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 (n=494). This resulted in a subset containing 2133 OTUs which we used for all further analyses. Sample coverage and richness (observed number of OTUs) were obtained as implemented in MOTHUR. OTU abundance data were square root transformed and beta-diversity was calculated with the Bray-Curtis coefficient.

Statistical analysis

The experiment was based on a crossed factorial design, consisting of the three factors 'season' (four levels, fixed), 'dilution' (three levels, fixed), and 'pH' (three levels, fixed). For pyrosequencing, the factors 'dilution' and 'pH' consisted of only two levels and the summer 'initial dilution' was analyzed separately. In case of a significant influence of 'pH' or of an interaction term involving 'pH', *post-hoc* comparisons for the factor 'pH' within the highest ranking interaction were performed.

Bacterial abundance and richness were analyzed through factorial ANOVAs (Underwood 1996) with Statistica 7.1 (StatSoft, Tulsa, OK, USA), using Tukey's HSD test for *post-hoc* comparisons. Beta-diversity was analyzed by permutational multivariate ANOVA (PERMANOVA, (Anderson 2001)) and similarity percentage analysis (SIMPER). SIMPER allowed us to calculate the total similarity within and dissimilarity between treatments, and to determine characteristic and discriminatory OTUs. To visualize patterns of the influence of 'pH' within 'season'-'dilution' combinations, we performed principal co-ordinate analysis (PCO, (Legendre and Legendre 1998)). For all multivariate analyses, we used Primer 6 with the add-on package PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK).

Results

Environmental data, pH manipulations, initial community composition and bacterial abundance

In situ pH varied from 8.15 to 8.22 and seawater temperature from 3.2 to 15.8°C (Table 1). At the end of the experiment, the mean absolute deviations from the initial pH values were 0.10±0.06 ('no dilution', n=60), 0.07±0.05 ('serial dilution', n=59) and 0.62±0.28 ('initial dilution', n=60) (see Table S1 for pH in all 'season'-'dilution' combinations). Initial bacterial abundance ranged from $3.3x10^5$ to $1.9x10^6$ cells mL^{-1} (Table 1). The starting community was more diverse in autumn and winter (134 and 181 observed OTUs) compared to spring and summer (69 and 54 observed OTUs). Starting communities were dominated by sequences of *Alphaproteobacteria* (especially in summer), *Flavobacteria* (especially in spring) and *Gammaproteobacteria*. Additionally, up to 20% *Bacteria* that could not be assigned to classes (autumn and winter) and 10% *Betaproteobacteria* (winter) occurred (Figure 1). After the four weeks of incubation, bacterial abundance ranged between $1.1x10^5$ and $6.1x10^6$ cells mL^{-1} and was significantly influenced by 'season' ($F_{3,143}$=21.451, p<0.001), 'dilution' ($F_{2,143}$=111.279, p<0.001), and their interaction ($F_{6,143}$=14.399, p<0.001), but not by 'pH' ($F_{2,143}$=1.6163, p=0.202) (Figure S1).

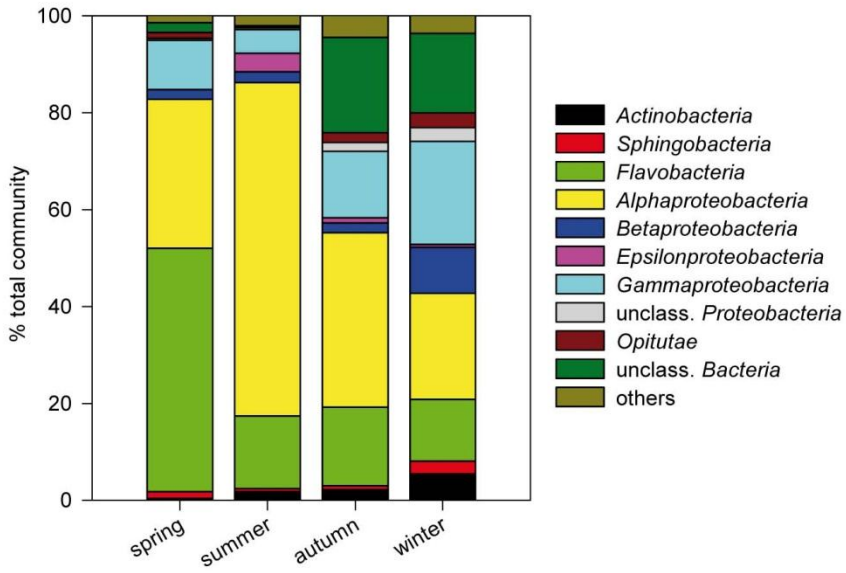

FIGURE 1. Relative abundance of bacterial classes in the starting communities (seawater fraction <10 µm). The chart was constructed based on the relative abundances of OTUs (16S ribosomal amplicon pyrosequencing) in the standardized subsample (n=494 sequences). Classes represented by less than 2% of sequences are summarized under "others".

TABLE 1. Sampling dates, initial seawater characteristics and incubation temperatures.

Season	Sampling day	pH_{NBS} *in situ*	Temperature [°C][1]	Bacteria mL^{-1} fraction < 10 µm	Incubation temperature [°C]
spring	April 8, 2010	8.26	4.7	$5.0x10^5$	5.0
summer	July 1, 2010	8.22	15.8	$1.9x10^6$	15.0
autumn	October 14, 2010	8.15	14.2	$6.5x10^5$	14.0
winter	January 20, 2011	8.19	3.2	$3.3x10^5$	3.0

[1]Seawater temperatures were obtained from the Helgoland Roads time-series and were directly measured in a seawater sample on the vessel on station (Wiltshire et al 2008).

Bacterial community composition

Based on ARISA fingerprints, all three experimental factors and their interactions significantly influenced bacterial community structure (PERMANOVA, Table 2). The highest amount of variation was explained by 'season', 'dilution' and their interaction term (Sq. root, Table 2). Communities in both 'no dilution' and 'serial dilution' were significantly influenced by 'pH' in all seasons (Table 3), predominantly already at pH 7.82. Additionally, communities from both lowered pH treatments differed from each other, except in summer. 'Initial dilution'-communities in contrast were influenced by 'pH' only in spring and summer.

For 16S ribosomal amplicon pyrosequencing, we selected all 'season'-'dilution' combinations for which significant differences between communities at pH *in situ* and 7.67 had been observed (Table 3). Sequencing data confirmed that community structure in both 'no dilution' and 'serial dilution' was significantly influenced by all experimental factors and interactions (PERMANOVA, Table 4). In accordance with ARISA results, 'season', 'dilution' and their interaction term were the highest sources of variation (Sq. root, Table 4). Except for the 'serial dilution' in autumn, all 'pH'-dependent differences were confirmed (Table 5 and Table 6). PCO plots of 'season'-'dilution' combinations are shown in Figure S2 (based on ARISA) and Figure S3 (based on 16S ribosomal amplicon pyrosequencing).

Sample coverage obtained by pyrosequencing ranged from 72.1 to 99.8% (Table S2), yielding bacterial richness estimates of 8 to 218 OTUs per sample (Figure S4). In both 'no dilution' and 'serial dilution', richness was significantly influenced by 'season' ($F_{3,63}$=122.201, p<0.0001), 'dilution' ($F_{1,63}$=1901.215, p<0.0001) and 'pH' ($F_{1,63}$=29.608, p<0.0001), and the interactions 'season'x'dilution' ($F_{3,63}$=138.125, p<0.0001), 'season'x'pH' ($F_{3,63}$=9.317, p<0.0001), 'dilution'x'pH' ($F_{1,63}$=28.395, p<0.0001), and 'season'x'dilution'x'pH' ($F_{3,63}$=2.880, p<0.05). For the 'serial dilution', no 'pH'-

dependent differences were found, whereas for the 'no dilution', richness was significantly higher at pH 7.67 than at pH *in situ* in spring ($p<0.001$) and autumn ($p<0.001$, Tukey's HSD test, 63 d.f.). In the 'initial dilution' in summer, richness was not significantly influenced by 'pH' ($F_{1,7}=1.093$, $p=0.331$).

TABLE 2. PERMANOVA main tests of bacterial community composition based on Jaccard dissimilarities of ARISA profiles.

Sources of variation	d.f.	SS	*pseudo F*	*p (perm)*[1]	Sq. root
Season	3	104240	24.066	**0.001**	27.297
Dilution	2	99744	34.543	**0.001**	28.508
pH	2	8222.3	2.8475	**0.001**	6.6905
Season x Dilution	6	162610	18.772	**0.001**	41.498
Season x pH	6	20929	2.416	**0.001**	11.713
Dilution x pH	4	15976	2.7663	**0.001**	11.33
Season x Dilution x pH	12	48537	2.8015	**0.001**	22.881
Residuals	143	206460			37.997
Total	178	667400			

[1]Significant results (*p (perm)*<0.05) are highlighted in bold.
Displayed are tests for the factors 'season', 'dilution', 'pH' and their interactions and the partitioning of multivariate variation. *p*-values were obtained using type III sums of squares and 999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Jaccard dissimilarities.

TABLE 3. PERMANOVA pair-wise comparisons of bacterial community composition based on Jaccard dissimilarities of ARISA profiles.

		no dilution		serial dilution		initial dilution	
Season	**Comparison**	***t (perm)***	***p (perm)*[1]**	***t (perm)***	***p (perm)*[1]**	***t (perm)***	***p (perm)*[1]**
spring	pH *in situ* vs. 7.82	1.7578	**0.009**	2.0402	**0.008**	1.4123	**0.022**
	pH *in situ* vs. 7.67	3.4604	**0.014**	1.4949	**0.032**	1.187	0.059
	pH 7.82 vs. 7.67	2.1161	**0.014**	1.7501	**0.017**	1.8286	**0.008**
summer	pH *in situ* vs. 7.82	1.38	**0.043**	1.7313	**0.011**	1.8089	**0.006**
	pH *in situ* vs. 7.67	1.7055	**0.008**	2.0209	**0.011**	1.5411	**0.01**
	pH 7.82 vs. 7.67	0.79417	0.836	1.2412	0.123	1.138	0.148
autumn	pH *in situ* vs. 7.82	1.7541	**0.006**	1.2752	0.12	1.179	0.083
	pH *in situ* vs. 7.67	2.0978	**0.011**	2.2008	**0.008**	1.1401	0.148
	pH 7.82 vs. 7.67	1.619	**0.005**	1.7405	**0.012**	0.86025	0.916
winter	pH *in situ* vs. 7.82	1.2116	0.163	3.3652	**0.007**	1.3223	0.051
	pH *in situ* vs. 7.67	1.3323	**0.046**	3.4765	**0.01**	1.3268	0.095
	pH 7.82 vs. 7.67	1.4745	**0.009**	3.3868	**0.008**	1.0803	0.259

[1]Significant results (*p (perm)*<0.05) are highlighted in bold.
Displayed are pair-wise *a posteriori* comparisons of the factor 'pH' within 'season'-'dilution' combinations, with at least 125 unique permutations per comparison.

TABLE 4. PERMANOVA main tests of bacterial community composition based on Bray-Curtis dissimilarities of OTUs (16S ribosomal amplicon pyrosequencing; 'no dilution' and 'serial dilution').

Sources of variation	d.f.	SS	*pseudo F*	*p (perm)*[1]	Sq. root
Season	3	74205	22.705	**0.001**	34.644
Dilution	1	71118	65.279	**0.001**	42.167
pH	1	3637.6	3.339	**0.005**	8.0436
Season x Dilution	3	62133	19.011	**0.001**	44.63
Season x pH	3	10129	3.0993	**0.001**	15.237
Dilution x pH	1	3431.6	3.1499	**0.002**	10.906
Season x Dilution x pH	3	10272	3.1429	**0.001**	21.771
Residuals	63	68634			33.007
Total	78	303900			

[1] Significant results (*p (perm)*<0.05) are highlighted in bold.
Displayed are tests for the factors 'season', 'dilution', 'pH' and their interactions and the partitioning of multivariate variation for 'no dilution' and 'serial dilution' treatments. *p*-values were obtained using type III sums of squares and 999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Bray-Curtis dissimilarities.

TABLE 5. PERMANOVA pair-wise comparisons of bacterial community composition based on Bray-Curtis dissimilarities of OTUs (16S ribosomal amplicon pyrosequencing; 'no dilution' and 'serial dilution').

Comparison	no dilution		serial dilution	
pH *in situ* vs. 7.67	***t (perm)***	***p (perm)*[1]**	***t (perm)*[1]**	***p (perm)*[1]**
spring	2.3732	**0.009**	1.5667	**0.039**
summer	1.1759	**0.042**	1.9808	**0.013**
autumn	1.5233	**0.005**	1.2019	0.146
winter	1.3372	**0.008**	3.7336	**0.013**

[1] Significant results (*p (perm)*<0.05) are highlighted in bold.
Displayed are pair-wise *a posteriori* comparisons of the factor 'pH' within 'season'-'dilution' combinations for 'no dilution' and 'serial dilution' treatments, with at least 125 unique permutations per comparison.

TABLE 6. PERMANOVA main tests of bacterial community composition based on Bray-Curtis dissimilarities of OTUs (16S ribosomal amplicon pyrosequencing; 'initial dilution' summer).

Sources of variation	d.f.	SS	*pseudo F*	*p (perm)* [1]	Sq. root
pH	1	1922.8	1.9165	**0.011**	14.384
Residuals	7	7023.1			31.675
Total	8	8945.9			

[1] Significant results (*p (perm)*<0.05) are highlighted in bold.
Displayed are the test for the factor 'pH' and the partitioning of multivariate variation for the 'initial dilution' (only summer experiment). *p*-values were obtained using type III sums of squares and 999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Bray-Curtis dissimilarities.

Bacterial groups in the 'season'-'dilution' combinations

Considering only pH *in situ* treatments, i.e. disregarding pH effects, communities in different dilutions within a given season differed between 86 and 97% from each other. Classes predominantly discriminating between 'no dilution' and 'serial dilution' were *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*. (Figure 2; see Table S3 for similarity and Table S4 for dissimilarity analyses).

'No dilution'-communities were characterized mainly by *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* which could not be assigned to families. *Flavobacteria* were the most important group in spring and winter, while in summer, *Alphaproteobacteria*, especially of the SAR11-clade, dominated. In autumn, the community was more diverse. Less abundant groups characteristic for 'no dilution'-communities included *Planctomycetacia*, *Betaproteobacteria*, and *Bacteria* that could not be assigned to classes.

In contrast, 'serial dilution'-communities were dominated by known representatives of the *Gammaproteobacteria*. Important groups were *Oceanospirillaceae* (all seasons), unclassified *Alteromonadales* (summer, autumn, winter), *Colwelliaceae* (spring, winter) and *Pseudoalteromonadaceae* (spring). In certain 'serial dilution'-communities, *Flavobacteriaceae* and *Rhodobacteraceae* additionally accounted for high contributions to total similarities. The 'initial dilution'-community in summer was dominated by *Gammaproteobacteria*, *Flavobacteriaceae* and *Rhodobacteraceae* as well. Within the *Gammaproteobacteria* however, *Alteromonadaceae* dominated.

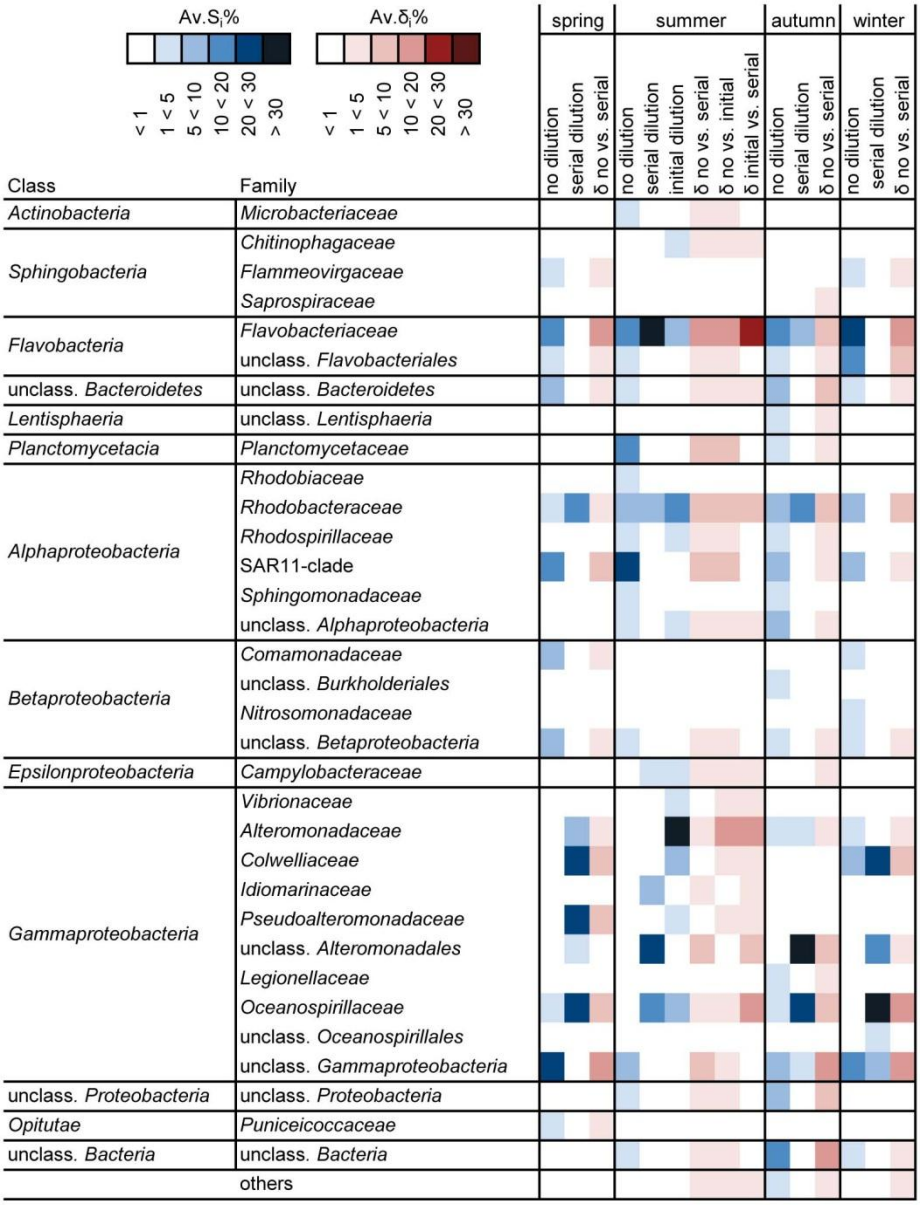

FIGURE 2. Bacterial groups in the 'season'-'dilution' combinations, based on SIMPER analysis of pH *in situ* levels.
Displayed are phylogenetic groups jointly contributing to 90% of the total similarity within and dissimilarity between pH *in situ* levels of the different dilution treatments, separately for each season (no: 'no dilution', serial: 'serial dilution', initial: 'initial dilution'). The heat map summarizes the contributions of single OTUs (16S ribosomal amplicon pyrosequencing) on the family level. Av.S_i%: average percentage contribution of the i th species to the total similarity, Av.δ_i%: average percentage contribution of the i th species to the total dissimilarity. Bacterial families contributing less than 1% to both totals are summarized under "others". The amount of contribution is indicated by the color of cells, darker colors represent higher contributions.

Bacterial groups responding differently to pH

In 'season'-'dilution' combinations with significant 'pH' effects (Table 5 and Table 6), communities of the two pH treatments differed between 49 and 63% from each other. The most important discriminatory classes were *Gammaproteobacteria*, *Flavobacteria*,

Alphaproteobacteria and *Epsilonproteobacteria* (Figure 3; see Table S3 for similarity and Table S5 for dissimilarity analyses).

Gammaproteobacterial families contributing to total dissimilarities were *Oceanospirillaceae*, *Colwelliaceae*, *Alteromonadaceae*, *Pseudoalteromonadaceae*, and to a lesser extend *Idiomarinaceae*, *Shewanellaceae* and *Vibrionaceae*. In spring and winter, *Oceanospirillaceae* were more characteristic for pH *in situ*, while in summer, they were more characteristic for pH 7.67. *Colwelliaceae* contributed more to total similarities at pH *in situ* in the 'serial dilution' in spring and winter and the 'initial dilution' in summer. In the 'no dilution' however, the opposite occurred. *Alteromonadaceae* dominated in the summer 'initial dilution', were they contributed more to total similarity at pH *in situ*. The same was found for the 'no dilution' in autumn, but in spring, they were more characteristic for pH 7.67. *Pseudoalteromonadaceae* contributed to total dissimilarity in spring and summer, and were always more characteristic for pH 7.67. Concerning *Gammaproteobacterial* families occurring in only one 'season'-'dilution' combination, *Idiomarinaceae* contributed more to total similarity at pH *in situ*, while *Shewanellaceae* and *Vibrionaceae* contributed more to total similarity at pH 7.67.

The *Flavobacterial* family *Flavobacteriaceae* was the group with the overall highest contributions to total dissimilarities. In spring, *Flavobacteriaceae* were more characteristic for pH 7.67, but in autumn and winter, they were more characteristic for pH *in situ*. In summer, no consistent trend was found.

Within the *Alphaproteobacteria*, high contributions to total dissimilarity were attributed to *Rhodobacteraceae*, and to lesser extends to SAR11-clade, *Rhodospirillaceae* and *Sphingomonadaceae*. For *Rhodobacteraceae*, higher contributions to total similarity were generally observed at pH *in situ*. SAR11-clade contributed more to total similarity at pH *in situ* in spring, but no important differences were found in the other seasons. The other *Alphaproteobacterial* families did not show consistent trends.

Within the *Epsilonproteobacteria*, *Campylobacteraceae*, represented exclusively by the genus *Arcobacter*, contributed a third to total similarity at pH 7.67 in the 'serial dilution' in winter but were absent at pH *in situ*. Similar but less pronounced tendencies were found in spring and autumn.

Discriminatory groups that were less abundant or present only in one or few 'season'-'dilution' combinations predominantly belonged to the classes *Sphingobacteria*, *Planctomycetacia*, *Actinobacteria*, *Betaproteobacteria* and *Opitutae*. Notably, also various groups that could not be assigned to families contributed to 'pH'-dependent dissimilarities.

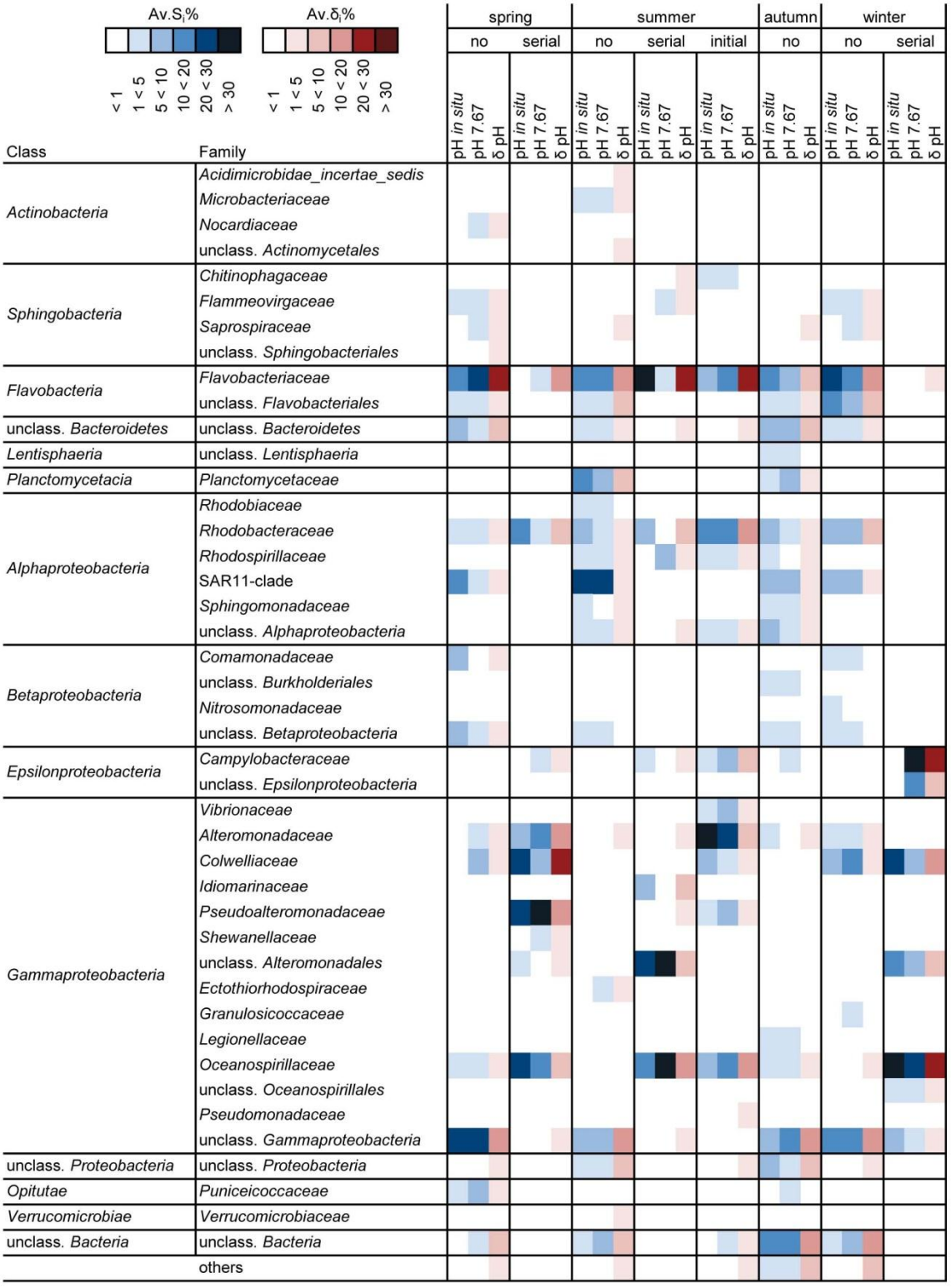

FIGURE 3. Bacterial groups responding differently to pH, based on SIMPER analysis.
Displayed are phylogenetic groups jointly contributing to 90% of the total similarity within and dissimilarity between pH levels, separately for each 'season'-'dilution' combination (no: 'no dilution', serial: 'serial dilution', initial: 'initial dilution'). The heat map summarizes the contributions of single OTUs (16S ribosomal amplicon pyrosequencing) on the family level. $Av.S_i\%$: average percentage contribution of the i th species to the total similarity, $Av.\delta_i\%$: average percentage contribution of the i th species to the total dissimilarity. Bacterial families contributing less than 1% to both totals are summarized under "others". The amount of contribution is indicated by the color of cells, darker colors represent higher contributions.

Discussion

The impact of ocean acidification on bacterial communities remains under debate as studies conducted so far are highly complex but lack sufficient replication. Here we present a thorough analysis of diversely assembled bacterial communities, shaped by the experimental factors season and dilution. Season takes into account phenology, i.e. climate-driven annually recurring patterns of pelagic bacterial community composition (Andersson et al 2010). Dilution on the other hand acts as a selective factor for the two major general ecological strategies observed among bacterioplankton: Groups efficiently competing at ambient nutrient levels and groups efficiently exploiting environmental patchiness, i.e. nutrient availability (Giovannoni and Stingl 2005). The first group was selected for in the undiluted incubations while different members of the second type were selected for by the dilution strategies. Despite the differently assembled communities, we observed community shifts in the undiluted incubations and in one of the dilution strategies in all seasons. We identified bacterial groups susceptible to changes in pH, highlighting that their specific response is often context-dependent.

Considerations on perturbations of the carbonate system using HCl

We used HCl to adjust pH to values expected for the North Sea for the year 2100. Values remained relatively stable for 'no dilution' and 'serial dilution' treatments, but larger deviations were observed for the 'initial dilution'. Interestingly, pH-dependent community shifts were least frequently found in initially diluted communities, probably at least partly due to these pH instabilities. Thus our 16S ribosomal amplicon pyrosequencing analyses and consequently our main findings and conclusions predominantly rely on 'no dilution' and 'serial dilution' treatments. Despite the relatively high pH deviations in 'initial dilution' treatments, we decided to report these results as well to present the complete experiment.

Although acidifying seawater with HCl has been extensively applied in ocean acidification studies (Gattuso and Lavigne 2009, Schulz et al 2009), it is not the most accurate approach in mimicking future changes in carbonate chemistry. In the course of ocean acidification, seawater CO_2 uptake will cause a reduction in pH accompanied by an increase in dissolved inorganic carbon (DIC, composed of the species H_2CO_3, $CO_2(aq)$, HCO_3^- and CO_3^{2-}) but no change in total alkalinity (TA). In contrast, additions of HCl lead to a decrease in pH at constant DIC and decreasing TA (Hurd et al 2009, Schulz et al 2009). Still both methods

lead to similar results, i.e. a decrease in pH and [CO_3^{2-}] and an increase in [CO_2] and [HCO_3^-] (Schulz et al 2009). For pCO_2 values not considerably exceeding 700 ppm, all carbonate system parameters change in similar magnitude with both perturbation methods (Schulz et al 2009). Higher acid perturbations lead to larger differences however, e.g. acidification by HCl to a pH of 7.5 leads to [HCO_3^-] values being about 22% lower compared to values reached by acidification to this pH by CO_2 uptake (Hurd et al 2009). Whether the small differences in carbonate system parameters between the two methods are problematic thus depends on whether the processes investigated are sensitive to small differences in [HCO_3^-] (Schulz et al 2009).

Concerning bacterial communities, the ability of certain groups to compete at different pH levels may depend both on changes in pH per se and on processes involving HCO_3^- availability. Changes in pH are likely to have a physiological effect, as bacteria living in the alkaline marine milieu (with pH values generally above 8.0) have to invert energy into the homeostasis of their cytoplasmic pH (7.4-7.8) (Padan et al 2005). Consequently, if the difference between external and internal pH becomes smaller due to ocean acidification, bacteria may profit energetically, depending on their pH homoeostasis mechanisms (Teira et al 2012). In a study investigating two marine bacterial strains, a *Cytophaga* strain exhibited a decreased respiration rate and thus enhanced growth efficiency at higher pCO_2, which was not found for a *Roseobacter* strain though (Teira et al 2012). In addition, both strains exhibited higher rates of heterotrophic CO_2 fixation, subsequently released as DOM, at higher pCO_2 levels (Teira et al 2012), which demonstrates that [HCO_3^-] plays an important role as well. With our perturbation approach using HCl, pH-dependent changes were likely to be detected, while the consequences of rising DIC availability may have been underestimated. Yet as all carbonate system species ([CO_2], [HCO_3^-], [CO_3^{2-}], [H^+]) will change in the same direction with both HCl and CO_2 perturbations, biological responses are unlikely to differ substantially and results should be directly comparable (Gattuso and Lavigne 2009, Schulz et al 2009). This has already been demonstrated for other organisms in ocean acidification studies systematically comparing both methods for pCO_2 values of up to 1200 ppm, namely for corals (Schneider and Erez 2006) and calcifying algae (Hoppe et al 2011).

Influence of pH on bacterial community structure and abundance

We combined ARISA and extensive 16S ribosomal amplicon pyrosequencing to permit a robust statistical analysis. The strategy of simultaneously pyrosequencing a large number

of samples, accepting fewer sequences instead of deep sampling, was recently applied to analyze bacterioplankton communities (Fortunato et al 2012) and was highly suitable for our study regarding the comparatively low bacterial diversity in laboratory incubations.

Not surprisingly, both methods revealed that the greatest influence on bacterial community composition could be attributed to season and dilution. Within the differently assembled communities, pH had a significant effect on all 'no dilution' and 'serial dilution' incubations (excluding pyrosequencing results for the 'serial dilution' in autumn). This indicates that even at moderate pH differences, community shifts can be observed. Furthermore, analysis of the complete data set by ARISA revealed that differences in community structure were predominantly observed already at pH 7.82, suggesting that a tipping point for bacterial community shifts may be reached even earlier. Communities from pH 7.82 and 7.67 also differed from each other (except in summer) indicating that slight differences in the degree of pH reduction are crucial. For 'initial dilution'-communities however, we observed pH-dependent differences only in spring and summer. Possibly, in addition to the pH instabilities discussed above, the variability between replicates introduced by the 1000-fold dilution at the beginning masked pH effects in autumn and winter, where initial diversity was higher.

In agreement with community structure, richness was predominantly influenced by season and dilution. Differences between pH levels were only observed in 'no dilution'-communities in spring and autumn, with higher richness at pH 7.67. Increased bacterial diversity at reduced pH has been reported for coastal microbial biofilms exposed to natural CO_2 vents under low, but not under ambient light conditions (Lidbury et al 2012) and for coral microbial communities (Meron et al 2011), where it was assumed to be the outcome of a temporary disturbance of community equilibrium (Connell 1978). Although our results provide only minor evidence to support this hypothesis, a moderate reduction in pH seems at least not to reduce bacterial diversity, as observed for other environmental stressors such as chemical compounds (Atlas et al 1991). Despite the good agreement concerning community structure, bacterial richness estimates were not consistent between 16S ribosomal amplicon pyrosequencing and ARISA (data not shown). Limitations of ARISA in estimating bacterial richness have previously been reported (Crosby and Criddle 2003, Kovacs et al 2010), but similarly, pyrosequencing can lead to an overestimation of richness due to sequencing errors and the presence of chimeric sequences (Kunin et al 2010). Using a subsample of equal number of sequences for all analyses however, we at least

"standardized" these error sources (Schloss et al 2011) and therefore consider pyrosequencing richness estimates comparable between samples of our study.

Concerning bacterial abundance, a reduction in pH down to 7.67 did not have an effect, which is in agreement with previous findings from mesocosm studies (Allgaier et al 2008, Grossart et al 2006, Newbold et al 2012). In this context, possible pH effects on nutrient availability and utilization should be considered. It has been reported that with decreasing seawater pH, bioavailability of dissolved iron decreases (Shi et al 2010). Additionally, significant changes in phosphate, silicate and ammonia have been predicted for the seawater pH reductions expected due to ocean acidification (Raven et al 2005, Zeebe and Wolf-Gladrow 2001). For hydrolysis reactions of dissolved organic matter such as organic acids, proteins and humic materials, a strong pH influence was predicted as well (Doney et al 2009), but no detailed investigations exist. Furthermore, lower pH was shown to lead to an increased activity of total protease in mesocosms (Grossart et al 2006) and extracellular α- and β-glucosidase in laboratory experiments investigating direct pH effects (Piontek et al 2010). In contrast, different responses of these enzymes were found after short-term incubation (3h) at reduced pH (Yamada and Suzumura 2010). In our experiment, the lack of pH-dependent differences in bacterial abundances suggests no change in overall nutrient availability or utilization at reduced pH.

Bacterial groups in the 'season'-'dilution' combinations

The specific members of bacterial groups establishing in the dilutions differed with the initial seasonal starting communities, but the following general trends were observed: Both dilution strategies resulted in the selection of known representatives of culturable *Gammaproteobacteria*, which has previously been reported for initially diluted seawater incubations (Beardsley et al 2003, Fuchs et al 2000). Dilution and unavoidable disruption of some cells during filtration result in more nutrients per individual cell than in the original sample, which *Gammaproteobacteria* exploit most efficiently (Eilers et al 2000b, Ferguson et al 1984, Fuchs et al 2000, Goldman and Dennett 1985). Apparently, when an incubation is diluted only after one week (and then repeatedly), other groups of *Gammaproteobacteria* establish than in an initial dilution. The comparatively high diversity maintained in the undiluted treatment may in turn be explained by the lack of nutrient input, preventing the dominance of fast-growing bacteria. The aspect of grazing may also have played a role in structuring the communities, as neither prefiltration nor dilution can completely prevent the development of grazers (Beardsley et al 2003).

Likewise, lysis of bacterial cells due to bacteriophages cannot be excluded. Whether viral pressure differed between the pH levels cannot be determined as direct pH effects on marine planktonic bacteriophages have not been investigated so far (Danovaro et al 2011).

Bacterial groups responding differently to pH

Various bacterial groups contributed to pH-dependent dissimilarities, predominantly different members of *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae* and *Campylobacteraceae*. Further discriminatory groups included *Sphingobacteria*, SAR11-clade, *Planctomycetacia, Betaproteobacteria*, *Actinobacteria*, *Opitutae* and bacteria that could not be assigned to families. Responses of bacterial groups were generally context-dependent, i.e. different in particular season and growth condition combinations.

Concerning *Gammaproteobacteria*, mostly the *Oceanospirillaceae* and families of the order *Alteromonadales* (*Colwelliaceae, Alteromonadaceae, Pseudoalteromonadaceae*, *Idiomarinaceae*, *Shewanellaceae* and others) were affected by pH. Among these groups, *Pseudoalteromonadaceae* were consistently found more characteristic for pH 7.67. In contrast, the response of *Oceanospirillaceae* and *Alteromonadaceae* was dependent on season, as *Oceanospirillaceae* were more characteristic for pH *in situ* in spring and winter, but more characteristic for pH 7.67 in summer; the opposite occurred for *Alteromonadaceae*. *Colwelliaceae* were more dependent on dilution, being more characteristic for pH *in situ* in undiluted treatments and more characteristic for pH 7.67 in diluted treatments. The dependence of the pH-response of *Gammaproteobacteria* on different environmental factors is supported by contrasting results obtained for two coral species transferred to high CO_2 vent sites (Meron et al 2012). Although they occurred only in one 'season'-'dilution' combination and did not reach high abundances, it is interesting to note that *Vibrionaceae* were more characteristic for pH 7.67, as an increase in *Vibrionaceae* and other disease-associated bacteria at reduced pH has previously been reported in corals (Meron et al 2011, Vega Thurber et al 2009). It remains to be elucidated whether this is a general trend, as the family contains human and animal pathogens (Thompson et al 2004), and is of increasing importance in temperate waters due to climate change (Baker-Austin et al 2010, Colwell 1996, Oberbeckmann et al 2011b, Paz et al 2007).

Flavobacteriaceae were the family with overall highest contributions to pH-dependent dissimilarities, but no season or dilution specific trends were found. The *Alphaproteobacterial* groups *Rhodobacteraceae* and SAR11-clade were generally more

characteristic for pH *in situ*. A change in the relative abundances of *Flavobacteriaceae* and *Rhodobacteracea*e in response to pH has previously been reported in biofilms from the Great Barrier Reef (Witt et al 2011), with *Rhodobacteraceae* decreasing and *Flavobacteriaeae* increasing with decreasing pH. In contrast, in a laboratory experiment with coral microbial communities, an increase of the relative abundance of *Rhodobacteraceae* at low pH was reported (Meron et al 2011). Thus within both of these complex groups, pH responses differ.

A striking growth of *Campylobacteraceae* was observed in the winter 'serial dilution' at pH 7.67, but not at pH *in situ*. Also in the other seasons, *Campylobacteraceae* were generally more characteristic for pH 7.67. This family was only represented by members of the genus *Arcobacter*, which has previously been reported at the study site (Eilers et al 2000b). Members of this genus are considered emerging human pathogens, often associated with fecal contamination of waters (Collado et al 2008, Snelling et al 2006). *Arcobacter* have also been described in terrestrial and marine animals, with shellfish being a potential source of human infection (Collado and Figueras 2011). It may seem surprising that this group was most important in the winter experiment, but in context of food preservation, *Arcobacter* have previously been reported to grow at refrigeration temperatures (Collado and Figueras 2011). Regarding pH tolerance, *Arcobacter* spp. were reported to grow at pH 5.5 to 8.0 with a growth optimum at pH 6.0 to 7.5 (D'Sa and Harrison 2005). Moreover, members of the *Campylobacteraceae* generally grow within broad pH ranges, and growth especially at low pH values was often reported regarding the survival in contaminated foods and the stomach transit (D'Sa and Harrison 2005, Murphy et al 2006, Reid et al 2008).

Concerning the less abundant groups, *Opitutae* (*Verrucomicrobia*) are noteworthy as *Verrucomicrobia* have only recently been reported to be nearly ubiquitously distributed and are especially abundant in waters around Helgoland (Freitas et al 2012). They were represented by the family *Puniceicoccaceae* and were more characteristic for pH 7.67 in spring and autumn. Similarly in corals, members of *Verrucomicrobiae* were only present at reduced pH (Meron et al 2011).

In summary, no consistent trend throughout all 'season'-'dilution' combinations was found for the majority of bacterial groups. Notable exceptions (neglecting groups that were only present in one 'season'-'dilution' combination) were *Pseudoalteromonadaceae* and *Opitutae*, which were always more characteristic for pH 7.67, suggesting that they may profit from pH reductions. The same tendency was found for *Arcobacter*. In contrast,

groups that were generally more characteristic for pH *in situ* were *Rhodobacteraceae* and SAR11-clade, hinting at difficulties of these groups to cope with reductions in pH.

Regarding the lack of additional trends, the following aspects have to be considered: First, bacterial families such as the *Rhodobacteraceae* are highly diverse and different members occur at certain time points, while being of very low importance in other seasons (Eilers et al 2001, Pernthaler and Amann 2005). Thus, supposing that different responses to pH occur at the genus, species, or even ecotype level, an analysis on the family level can only depict very rough patterns. Second, different environmental factors are likely to influence the response of bacterial groups to pH. In our experiment, a dependence on season could - besides the identity aspect mentioned above - hint at temperature effects, while a dependence on dilution could be associated with nutrient effects. Additionally, the co-occurring species must be taken into account. The high contribution to total similarity of *Arcobacter* at pH 7.67 in the winter 'serial dilution'-community for instance, may in turn have influenced the comparatively lower contribution of *Oceanospirillaceae* at this pH and vice versa.

In conclusion already moderate changes in pH have the potential to cause shifts in bacterial communities, depending on the pool of bacterial groups constituting this community and on the prevailing environmental conditions. We identified members of the *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae* and *Campylobacteraceae* as susceptible to changes in pH and confirmed some results obtained in previous studies. To ultimately draw conclusions about functional implications, the question remains whether a change in species composition observed in *in vitro* incubations starting with an abrupt change in pH is extendable to natural bacterioplankton communities and how adaptation will affect long-term consequences. Therefore, to test whether the patterns observed also exist in more complex environments, we suggest that the groups identified in this study deserve special attention in future long-term and mesocosm experiments.

Acknowledgements

We thank the crew of the RV Aade from the Alfred Wegener Institute of Polar and Marine Research Helgoland for the sampling support.

Supplement

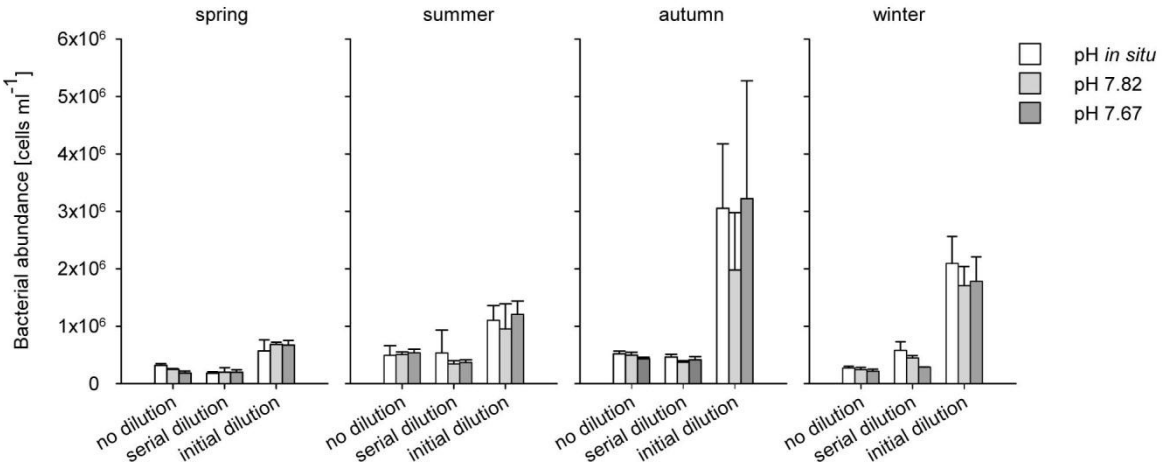

FIGURE S1. Bacterial abundance in the different treatments.
No significant pH effect on bacterial abundance was found (ANOVA).

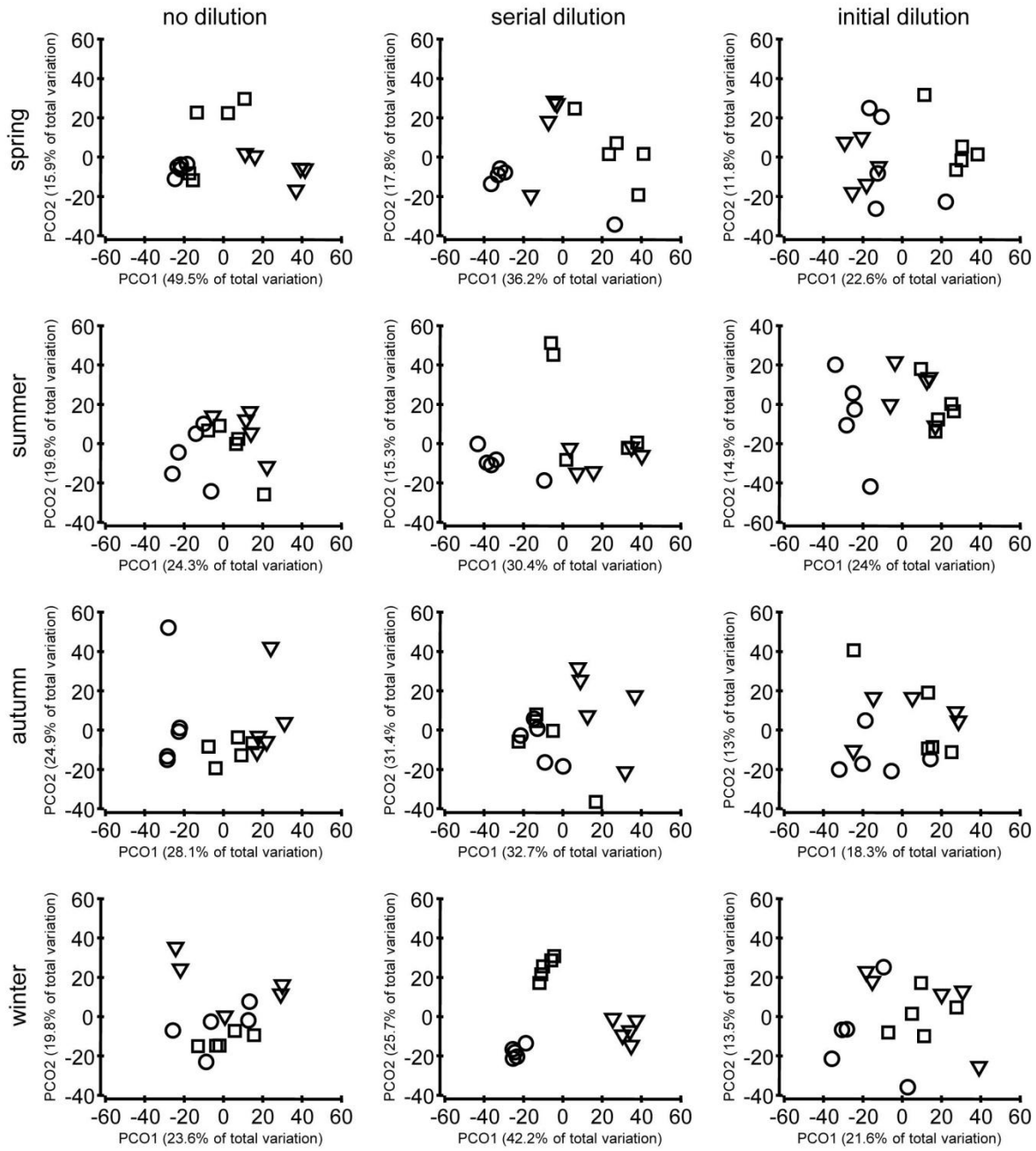

FIGURE S2. Influence of the factor 'pH' on bacterial community composition (ARISA).
Displayed are principal co-ordinate analysis plots (PCOs) for each 'season'-'dilution' combination based on Jaccard dissimilarities of ARISA profiles. Symbol shape represents the pH level (circles: pH *in situ*, squares: pH 7.82, triangles: pH 7.67).

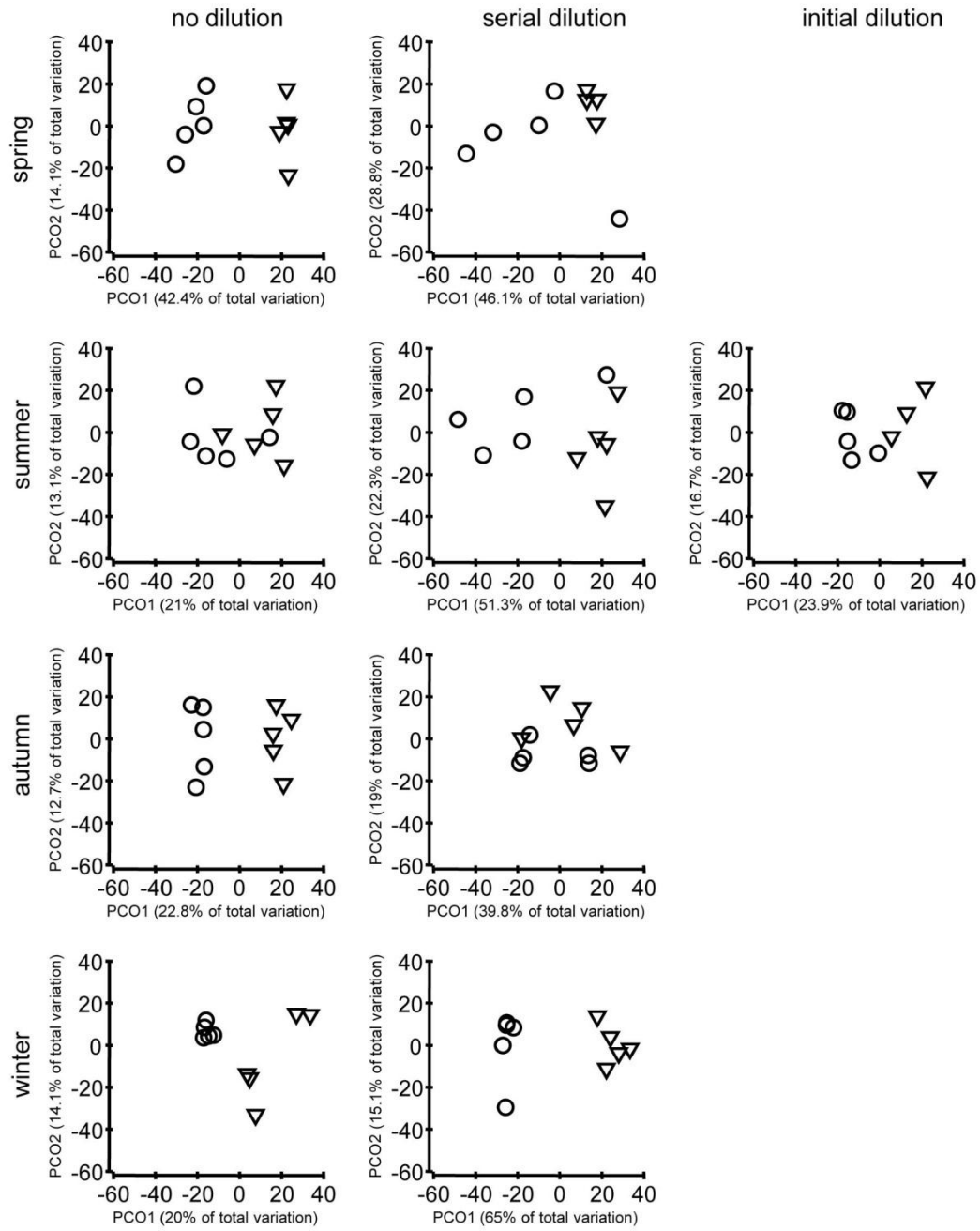

FIGURE S3. Influence of the factor 'pH' on bacterial community composition (16S ribosomal amplicon pyrosequencing).
Displayed are principal co-ordinate analysis plots (PCOs) for each 'season'-'dilution' combination based on Bray-Curtis dissimilarities of OTUs (16S ribosomal amplicon pyrosequencing). Symbol shape represents the pH level (circles: pH *in situ*, triangles: pH 7.67).

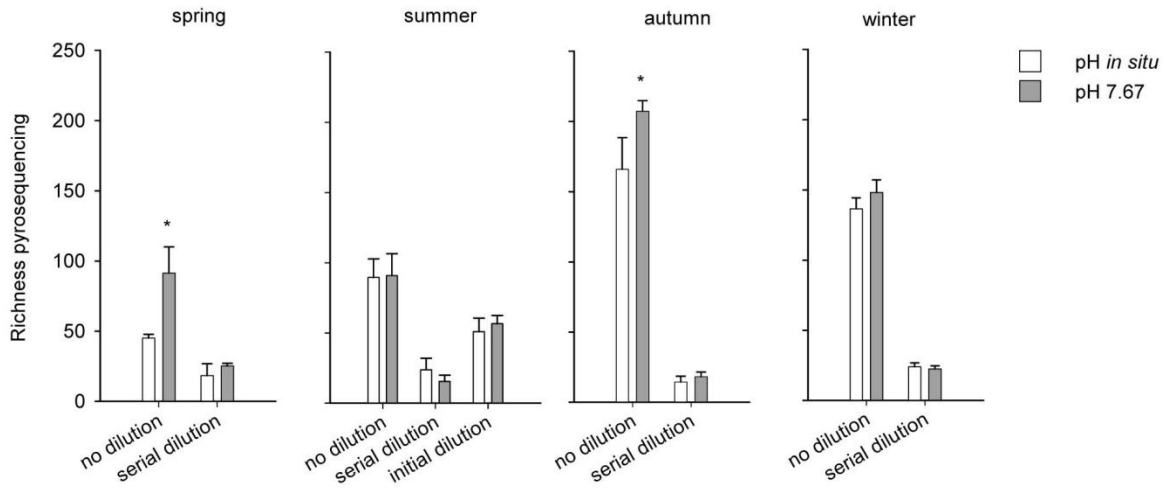

FIGURE S4. Bacterial richness in the different treatments.
Richness was determined based on the number of OTUs (16S ribosomal amplicon pyrosequencing) in the standardized subsample (n=494 sequences) and was compared by ANOVA, with Tukey's HSD test for *post-hoc* comparisons. Asterisks (*) represent significant differences ($p<0.05$) between pH levels within a 'season'-'dilution' combination.

TABLE S1. pH after the four weeks of incubation.

season	dilution	pH *in situ*	pH 7.82	pH 7.67
spring	no dilution	8.20 ± 0.00	7.79 ± 0.00	7.64 ± 0.01
	serial dilution	8.04 ± 0.01	7.90 ± 0.01	7.75 ± 0.03
	initial dilution	8.08 ± 0.01	7.56 ± 0.02	7.44 ± 0.03
summer	no dilution	8.05 ± 0.00	7.61 ± 0.01	7.49 ± 0.00
	serial dilution	8.21 ± 0.01	7.94 ± 0.03	7.84 ± 0.01
	initial dilution	7.27 ± 0.03	7.06 ± 0.05	6.93 ± 0.03
autumn	no dilution	8.06 ± 0.00	7.71 ± 0.01	7.59 ± 0.00
	serial dilution	8.08 ± 0.01	7.79 ± 0.01	7.72 ± 0.02
	initial dilution	7.22 ± 0.05	6.96 ± 0.03	6.95 ± 0.04
winter	no dilution	8.10 ± 0.01	7.75 ± 0.01	7.63 ± 0.01
	serial dilution	8.16 ± 0.01	7.89 ± 0.02	7.78 ± 0.02
	initial dilution	7.33 ± 0.10	7.24 ± 0.02	7.24 ± 0.01

Given are the means of five replicate incubations ± standard deviation. Starting values for the pH *in situ* treatment were 8.26 (spring), 8.22 (summer), 8.15 (autumn) and 8.19 (winter), respectively.

TABLE S2. Sample coverage obtained by 16S ribosomal amplicon pyrosequencing, based on the standardized subsample (n=494 sequences).

Sample		**spring**	**summer**	**autumn**	**winter**
d0		92.5%	95.1%	83.2%	76.5%
'no dilution'	pH *in situ*	94.9%	90.3%	76.5%	82.6%
		95.3%	90.7%	79.4%	81.2%
		95.7%	90.5%	84.2%	84.6%
		96.2%	88.3%	83.0%	81.4%
		97.0%	85.2%	76.5%	82.0%
	pH 7.67	94.5%	87.9%	72.3%	80.4%
		88.1%	92.5%	72.1%	77.5%
		89.7%	89.9%	72.7%	82.8%
		90.1%	87.7%	76.3%	80.8%
		89.5%	90.7%	74.7%	80.0%
'serial dilution'	pH *in situ*	99.6%	98.0%	99.2%	97.6%
		98.4%	99.2%	99.8%	98.2%
		96.8%	97.6%	98.4%	98.4%
		99.4%	97.8%	98.2%	97.8%
		98.4%	98.4%	98.4%	96.8%
	pH 7.67	98.6%	98.0%	98.2%	98.6%
		98.6%	97.6%	99.4%	99.2%
		99.0%	98.2%	98.8%	98.8%
		98.6%	99.4%	98.4%	98.4%
		-	98.8%	98.6%	97.6%
'initial dilution'	pH *in situ*	-	95.3%	-	-
		-	96.0%	-	-
		-	94.7%	-	-
		-	93.7%	-	-
		-	92.5%	-	-
	pH 7.67	-	93.9%	-	-
		-	94.1%	-	-
		-	-	-	-
		-	95.5%	-	-
		-	95.3%	-	-

Supplementary Tables S3, S4 and S5 can be found on the supplementary CD-ROM.

TABLE S3. Results of the SIMPER analysis giving the similarities within 'season'-'dilution'-'pH' combinations.
Displayed are the OTUs (16S ribosomal amplicon pyrosequencing) that predominantly contributed to 90% of to the total similarity. $Av.A_i$: average abundance of the i th species over all samples of the treatment, $Av.S_i$: average contribution of the i th species to the total similarity, $Av.S_i/SD$: the average value of the i th species as a typifying species, $Av.S_i\%$: average percentage contribution of the i th species to the total similarity, $\sum Av.S_i\%$: average cumulative contribution to the total similarity.

TABLE S4. Results of the SIMPER analysis giving the dissimilarities between pH *in situ* levels of the different dilution treatments, separately for each season.
Displayed are the OTUs (16S ribosomal amplicon pyrosequencing) that predominantly contributed to 90% of to the total dissimilarity. $Av.A_i$: average abundance of the i th species over all samples of the treatment (no: 'no dilution', serial: 'serial dilution', initial: 'initial dilution'), $Av.\delta_i$: average contribution of the i th species to the total dissimilarity, $Av.\delta_i/SD$: the average value of the i th species as a discriminating species, $Av.\delta_i\%$: average percentage contribution of the i th species to the total dissimilarity, $\sum Av.\delta_i\%$: average cumulative contribution to the total dissimilarity.

TABLE S5. Results of the SIMPER analysis giving the dissimilarities between the pH levels *in situ* and 7.67 of 'season'-'dilution' combinations significantly influenced by 'pH' according to PERMANOVA.
Displayed are the OTUs (16S ribosomal amplicon pyrosequencing) that predominantly contributed to 90% of to the total dissimilarity. $Av.A_i$: average abundance of the i th species over all samples of the treatment (no: 'no dilution', serial: 'serial dilution', initial: 'initial dilution'), $Av.\delta_i$: average contribution of the i th species to the total dissimilarity, $Av.\delta_i/SD$: the average value of the i th species as a discriminating species, $Av.\delta_i\%$: average percentage contribution of the i th species to the total dissimilarity, $\sum Av.\delta_i\%$: average cumulative contribution to the total dissimilarity.

Chapter II

Marine fungi may benefit from ocean acidification

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Since the industrial revolution, seawater pH has dropped by 0.1 units, and may decrease further 0.7 units within the next three centuries (Caldeira and Wickett 2003). Concerning heterotrophic microbes, the consequences of ocean acidification have mainly been discussed for bacterial processes and diversity (Joint et al 2011, Liu et al 2010). While bacterial numbers seem to be unaffected (Allgaier et al 2008, Krause et al 2012), experiments predict changes in bacterial growth efficiency, carbon assimilation and extracellular enzyme activity (Grossart et al 2006, Liu et al 2010, Piontek et al 2010, Teira et al 2012). However, the decomposition of complex substrates in marine environments, a key part of the flow of energy in ecosystems, is largely mediated by marine fungi. Despite their preference for low pH levels (Davis 2009, Orij et al 2011), marine fungi have been neglected in previous ocean acidification studies. Here we show that fungal abundance strongly increases with small reductions in seawater pH. In microcosm experiments, we incubated natural North Sea water at three different pH levels for four weeks. Compared to *in situ* seawater pH (8.10-8.26), fungal colony forming units were on average 9 times higher at pH 7.82 and 34 times higher at pH 7.67. In contrast, we observed fungal community shifts predominantly only at pH 7.67. Our results suggest that under realistic levels of ocean acidification, marine fungi will reach higher importance in marine biogeochemical cycles. At present, this is only observed periodically in upwelling areas (Gutiérrez et al 2011). The rise of this group of organisms will affect a variety of biotic interactions in the sea.

Both filamentous fungi and unicellular yeasts are widely distributed in marine environments. They have been reported from coastal, open ocean and deep-sea waters, as well as from salt marshes and mangroves, and colonize a variety of substrates (Gao et al 2010, Jones 2011). Due to their ability to degrade complex substrates such as lignocellulose and calcareous structures, they are important decomposers of herbaceous and woody remains and animal carcasses in the seas (Hyde et al 1998, Richards et al 2012). Furthermore, a variety of symbiotic and pathogenic interactions of fungi with algae and the marine fauna have been reported (Hyde et al 1998, Richards et al 2012). Yet fungi do not only colonize macroscopic substrates, but large populations of planktonic fungi, the mycoplankton, exist as well (Gao et al 2010, Wang et al 2012). Mycoplankton is particularly abundant in coastal surface waters, with diversity and abundance being positively correlated to phytoplankton biomass (Gao et al 2010, Gutiérrez et al 2011). Together with prokaryotes, planktonic fungi mediate the mineralization of particulate to dissolved organic matter and are capable of releasing nutrients from recalcitrant material (Wang et al 2012). Still their role in marine biogeochemical cycles is only incompletely understood and it has recently been suggested that their importance as degraders of organic matter in marine ecosystems has been greatly underestimated (Gao et al 2010, Gutiérrez et al 2011, Wang et al 2012). Likewise, marine fungal diversity is only beginning to be discovered (Amend et al 2012, Gao et al 2010, Jones 2011).

The majority of fungi cannot take up particles by phagocytosis and rely on osmotrophic feeding, i.e. they release extracellular enzymes to break down complex polymers and take up monomers by transport over the plasma membrane (Richards et al 2012, Wang et al 2012). This transport is driven by their plasma membrane potential, which primarily depends on the H^+-electrochemical gradient (Davis 2009, Orij et al 2011). In neutral-alkaline environments, establishing this gradient is challenging and limits fungal growth (Davis 2009, Orij et al 2011). A reduction in pH may consequently relieve pH stress on marine fungi. A similar benefit from moderate pH reductions was recently discussed for marine bacteria, as a strain of the genus *Cytophaga* exhibited enhanced growth efficiency at reduced pH (Teira et al 2012). However, to the best of our knowledge, direct ocean acidification effects on marine fungi have not been investigated. The only report on fungi in the context of ocean acidification is a study on coral microbial communities, which were found to contain more fungi at reduced pH (Vega Thurber et al 2009). This was most probably an indirect effect though, as corals weakened by pH stress may be less resistant to opportunistic fungal pathogens (Vega Thurber et al 2009).

To test the hypothesis that reductions in seawater pH also directly affect fungal abundance and community structure, we carried out microcosm (1.6 L) experiments in spring 2011 and 2012 (see Methods). We incubated natural seawater from Helgoland Roads (North Sea) at three pH levels, representing the *in situ* seawater pH (8.10 in 2011 and 8.26 in 2012) and two near-future pH levels (7.82 and 7.67). These values were predicted for the mean surface pH in the southern North Sea at 700 or 1000 ppm of atmospheric CO_2, respectively (Blackford and Gilbert 2007). All microcosms were incubated at *in situ* temperature on the day of sampling (2011: 7°C, 2012: 8°C). After two and four weeks, we determined fungal abundance by colony forming unit counts at 7-8°C (*in situ* temperature) and at 18°C (average of the yearly maximum seawater temperature at Helgoland Roads, see Methods). We chose these temperatures to find out whether different fungal groups (cold-adapted vs. warm-adapted) were present and reacted differently to pH. Furthermore, in a cultivation independent approach, DNA from filter enriched biomass of the microcosms was directly extracted and subjected to community analysis by fungal automated ribosomal intergenic spacer analysis (F-ARISA (Ranjard et al 2001)).

In the seawater samples, we observed 88±2 colony forming units per liter (cfu L^{-1}) in spring 2011 and 34±5 cfu L^{-1} in spring 2012. Higher fungal abundance in 2011 may be explained by higher overall phytoplankton abundance on the sampling day in that year ($8.5x10^6$ cells L^{-1} in 2011 vs. $2.4x10^6$ cells L^{-1} in 2012, data obtained from the Helgoland Roads time series (Wiltshire et al 2008)). Quantitative estimates of marine fungi range from below 10 to more than 10^7 cfu L^{-1}, with highest numbers reported from productive coastal areas (Gao et al 2010, Wang et al 2012). Compared to these numbers, initial fungal abundance in our experiments was low, but a direct comparison to other sites is difficult as no standard counting methods exist (Gutiérrez et al 2011).

During the experiment, fungal numbers greatly increased and we observed a strong influence of pH (Figure 1). We counted up to $1.2x10^3$ cfu L^{-1} at pH *in situ*, up to $1.6x10^4$ cfu L^{-1} at pH 7.82 and up to $9.0x10^5$ cfu L^{-1} at pH 7.67 (Figure 2). After factorial ANOVAs (Table S1), the factor 'pH' significantly influenced cfu L^{-1} both in 2011 ($F_{2,118}$=3151, p<0.001) and 2012 ($F_{2,118}$=2480, p<0.001). 'Temperature' also had a significant effect on cfu L^{-1} in both years (2011: $F_{1,118}$=4094, p<0.001; 2012: $F_{1,118}$=11, p=0.001). To estimate whether the different incubation temperatures selected for different fungal populations, DNA from bulk cfu filters with mycel were extracted and analyzed by F-ARISA (Figure S1; this was only done in 2011). We found that 'Temperature' significantly influenced community structure (PERMANOVA, p (perm)<0.001, Table S2),

hinting at distinct cold- and warm-adapted groups. The pH effect on both groups was comparable though, as for both temperatures, cfu L^{-1} were always significantly higher at pH 7.82 and 7.67 than at pH *in situ* (Figure 2, Tukey's HSD test, 118 d.f.). On average, cfu L^{-1} increased by a factor of 8-9 between pH *in situ* and pH 7.82 and by a factor of 34 between pH *in situ* and pH 7.67. Results indicate that already a moderate acidification may lead to an increase in fungal abundance of almost an order of magnitude. A possible explanation is the lower energetic cost for establishing a functional plasma membrane H^+-gradient. Additionally, altered extracellular enzyme activity may have played a role. For bacteria, higher activities of both protease and extracellular glucosidases were reported at low pH (Grossart et al 2006, Piontek et al 2010). In future studies, size-fractionated hydrolysis experiments (Gutiérrez et al 2011) could yield insights on whether fungal enzyme activities are also affected.

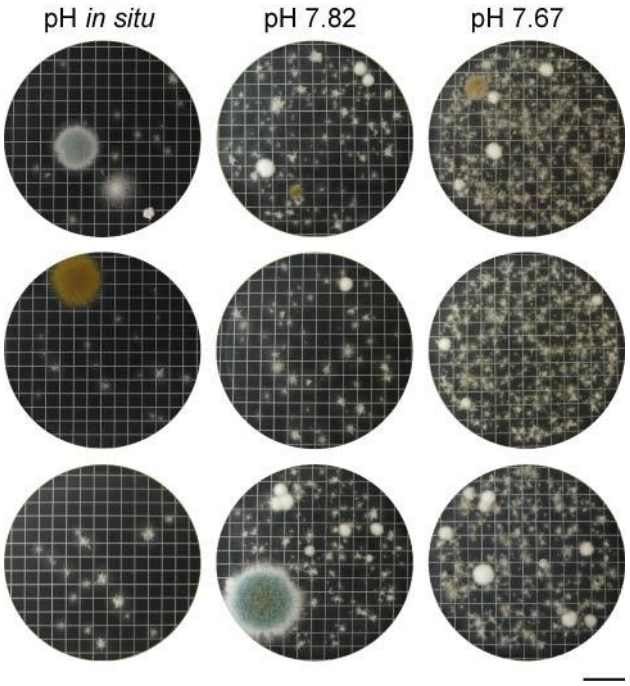

FIGURE 1. Fungal colonies obtained after incubation at different pH levels.
Displayed are filters (100 ml) from the 2012 experiment, week 4, incubated for five days at 18°C. Scale bar=1 cm.

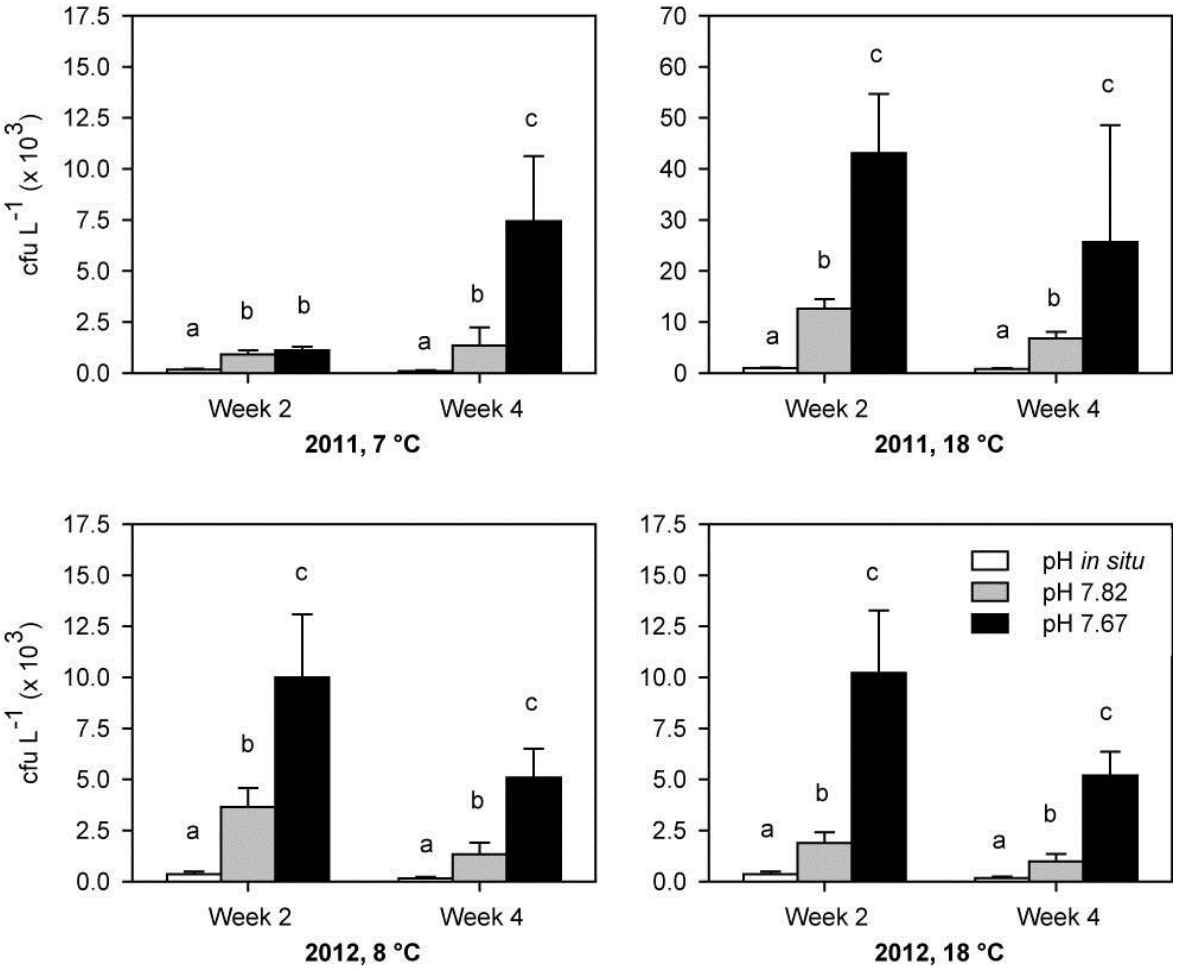

FIGURE 2. Colony forming units per liter (cfu L^{-1}) in response to pH.
Displayed are the mean ± standard deviation. Letters indicate significant differences after Tukey's HSD test, 118 d.f.. For ANOVA test statistics see Table S1. *N*=12-15 plates per pH. Note the different axis labeling for 2011, 18°C.

The cultivation-independent community analysis of filter enriched biomass of the microcosms revealed that 'Year' significantly influenced community structure, i.e. that different communities were present in the two years of the experiment (PERMANOVA, p (perm)<0.001, Table S3 and Figure S2). Furthermore, in both 2011 and 2012, community structure was significantly influenced by 'pH' (both years: p (perm)<0.001, Table S4a). Looking at 'pH' effects separately for each week, we predominantly observed significant differences between pH *in situ* and pH 7.67, but not between pH *in situ* and pH 7.82 (Figure 3, Table S4b). Thus in comparison to changes in fungal abundance, a tipping point for community shifts was reached at a lower pH. A moderate pH reduction (7.82) may be generally beneficial for the fungal community, while at pH 7.67 inter-specific differences in pH homeostasis mechanisms may become more pronounced, leading to compositional shifts.

Concerning richness, we observed 26 different F-ARISA band classes in the initial seawater sample in 2011 and 25 in 2012. During the experiment, richness ranged from 5 to 68 F-ARISA band classes per sample and was not influenced by 'pH' (Table S5 and Figure S3). There are however some limitations of F-ARISA estimates of fungal richness (Avis et al 2010).

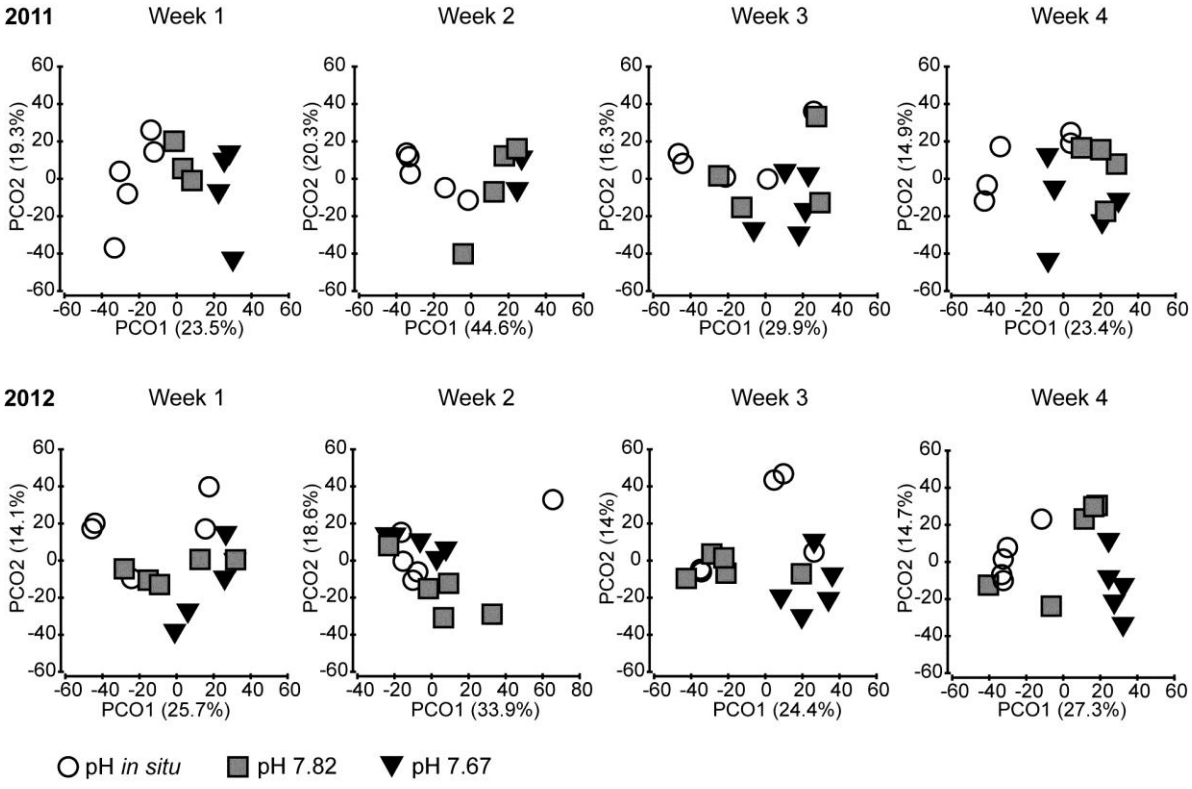

FIGURE 3. Fungal communities after incubation at different pH levels.
Principal coordinate ordination (PCO) plots of community structure for each 'Year'-'Week' combination, based on Jaccard dissimilarities of F-ARISA profiles.

Microbes already experience large natural differences in pH, due to e.g. seasonality, seawater depth or phytoplankton blooms (Joint et al 2011). Thus to draw conclusions from ocean acidification experiments, it is important to take into account the natural variability at the respective study site (Hofmann et al 2011). As no previous high resolution pH data were available for Helgoland Roads, we determined surface seawater pH over a yearly cycle from September 2010 to September 2011 on a work-daily basis. We found that pH_{NBS} was predominantly above 8.1 (Figure 4). Higher values of up to 8.4 were observed from mid-April until the end of May, presumably due to carbon uptake during the spring bloom (maximum chlorophyll a on April 26 2011: 15.4 µg L^{-1}; data obtained from the Helgoland Roads time-series (Wiltshire et al 2008)). Afterwards, pH decreased to

approximately 8.1 again, possibly because of decreasing primary production and dominance of respiratory processes in summer, as previously described for the southern North Sea (Thomas et al 2004). Thus although there are temporal pH variations at Helgoland Roads, values below 8.0 do presently not occur. Hence we cannot exclude that fungal responses may differ in regions regularly experiencing lower pH values. However, our results indicate that the pH effects observed are of a general nature. Different fungal communities developed in the two years of our study and furthermore, cultivation at different temperatures hinted at the presence of fungal groups occupying different fundamental niches. Still the direct pH effects on both abundance and community structure were consistent, indicating that they are not restricted to specific fungal groups. To scrutinize this assumption, we are currently planning to resolve fungal identities by a 454 pyrosequencing approach.

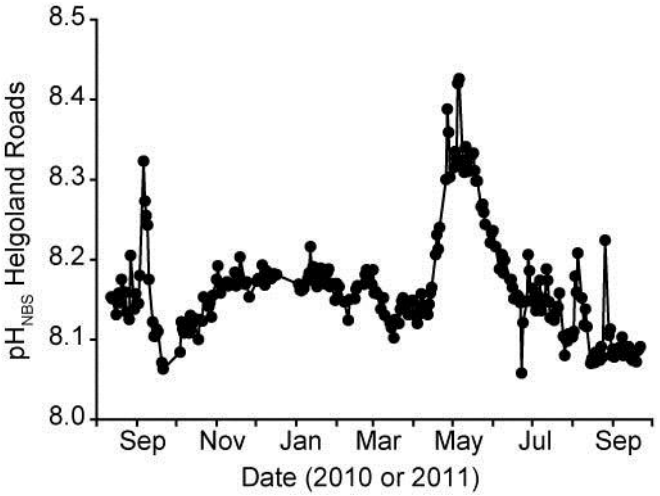

FIGURE 4. pH over a yearly cycle at Helgoland Roads.
pH was measured at *in situ* temperature on the National Bureau of Standards (NBS) scale.

An important implication of our study is the potential change in the relationship between bacterial and fungal abundance. Previous studies found no change in bacterial abundance with ocean acidification (Allgaier et al 2008, Krause et al 2012). Together with our results, this indicates that acidification may lead to an increased importance of fungi in microbial food webs. As fungi and bacteria occupy different niches in organic material degradation (Wang et al 2012), biogeochemical cycles may be affected. Possibly, this may lead to increased nutrient availability, as fungi decompose complex substrates and mediate the re-entry of organic matter such as essential amino acids, vitamins and polyunsaturated fatty acids to the food web (Richards et al 2012). Recently, high seasonal contributions of fungi

to microbial biomass and enzymatic activity have been reported for the Humboldt upwelling system off the coast of Chile (Gutiérrez et al 2011). In periods of high productivity during upwelling events, fungal biomass equaled that of prokaryotes (Gutiérrez et al 2011). Furthermore, over 90% of the hydrolysis of proteinaceous substrates was attributed to fungi, presumably accounting for up to 30% of the turnover of photosynthetic carbon (Gutiérrez et al 2011). This demonstrates that planktonic fungi can play a major role in microbial food webs, but further research is needed to elucidate their role in other marine environments as well.

A second important implication of our results concerns fungal interactions with other organisms. Many fungi are opportunistic pathogens. Especially in coastal areas with recreational beaches, high concentrations of fungi in seawater may pose a health hazard to humans (Vogel et al 2007). In addition, marine fungi comprise parasites of various commercially important fish, crustaceans and shellfish, which affects both natural populations and aquaculture (Hyde et al 1998, Ramaiah 2006, Schaumann and Priebe 1994). Therefore, our study points to the necessity to evaluate the risk of increasing fungal infections with ocean acidification. Furthermore, not only pH reductions, but also indirect effects of ocean acidification may affect marine fungi. First, higher primary production that may occur in response to higher CO_2 availability (Joint et al 2011, Liu et al 2010, Riebesell et al 2007) should be exploited by fungi. Secondly, fungal interactions with other marine organisms may be affected because pH stressed organisms are more susceptible to fungal colonization (Vega Thurber et al 2009). The fragile equilibrium between endolithic, i.e. calcareous substrate colonizing, fungi and their coral hosts is rapidly destroyed by environmental stressors (Golubic et al 2005), which has so far mainly been discussed under the aspect of climate warming. Other marine organisms prone to fungal infection such as shellfish and crustaceans (Hyde et al 1998, Wang et al 2012) are also sensitive to ocean acidification. Thus in a more acidic ocean, less-resistant hosts may be faced with a higher number of opportunistic fungal pathogens. These possible indirect effects of ocean acidification on marine fungi and their interactions urgently need to be addressed in future studies.

Methods

Experimental set-up. We sampled water at Helgoland Roads (54°11.3′N, 7°54.0′E) on April 14, 2011 and on May 3, 2012. The experimental design consisted of three pH levels: the current seawater *in situ* pH (8.10 in 2011 and 8.26 in 2012) and two near-future pH levels (7.82 and 7.67), which were adjusted with 2 M HCl. Per pH treatment, we incubated water samples in twenty replicate 1.6 L glass jars (acid-washed, autoclaved) with air-tight rubber seals. Jars were incubated at *in situ* temperature (7°C in 2011 and 8°C in 2012) for four weeks in the dark and were mixed daily by inversion. Each week, we analyzed five replicate jars per treatment.

Determination of pH. We used a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MF-DIN-N). The electrode was calibrated with standard buffer solutions from freshly opened glass ampoules (pH 4.01, 6.87, 9.18) (all materials: SI Analytics, Mainz, Germany). During the experiments, all pH measurements were carried out at incubation temperature (7°C in 2011 and 8°C in 2012). See Supplementary Methods for total alkalinity measurements, calculations of pCO_2 and determination of the pH cycle at Helgoland Roads. Carbonate chemistry parameters during the experiment are given in Table S5.

Colony forming units (cfu). Samples from the initial seawater sample (10, 100, and 500 mL) and from the experiment (10 and 100 mL), were filtered through sterile nitrocellulose filters (0.45 µm pore-size, 47 mm diameter, grey with grid, Sartorius, Göttingen, Germany) applying low pressure (100 mbar). Filters were placed onto Wickerham's YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% dextrose and 2.0% agar, pH 6.2), prepared with sterile-filtered seawater from the sampling site and supplemented with 250 mg L^{-1} chloramphenicol (Sigma, Saint Louis, MO, USA) to prevent bacterial growth. Furthermore, samples of 500 µL were directly spread onto the same medium using glass beads. Triplicate samples of each volume were incubated at 7°C (2011) or 8°C (2012), respectively, and at 18°C in the dark. After 4-5 days (18°C) or 12-16 days (7 or 8°C), all filamentous and yeast-like colonies were counted by eye. The average of the yearly maximum seawater temperature at Helgoland Roads (18°C) was calculated as the mean of the upper 10% of temperatures recorded for the study site from 2000 to 2010 (data obtained from the Helgoland Roads time-series (Wiltshire et al 2008)).

Community DNA extraction. Volumes of 800 mL for the experiment or 2000 mL for the seawater samples, respectively, were filtered through sterile nitrocellulose filters (0.45 µm pore-size, 47 mm diameter, grey with grid, Sartorius, Göttingen, Germany). A quarter of

each filter was suspended in 700 µL STE buffer (6.7% sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8) with 200 µL of a mixture of 0.1 mm zirconia/silica and 1.0 mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA). Mechanical lysis was performed using a FastPrep® FP120A Instrument (Qbiogene, Carlsbad, CA, USA) for 40 s at 6.0 m s^{-1}. DNA extraction was performed as previously described (Sapp et al 2007), omitting the lysozyme step and using an increased cell lysis temperature of 65°C. DNA concentration and purity were determined by photometry using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland).

Automated ribosomal intergenic spacer analysis (F-ARISA). We amplified the ITS1-5.8S-ITS2 region using forward primer 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and reverse primer 3126T (5'-ATATGCTTAAGTTCAGCGGGT -3') (Ranjard et al 2001), the latter labeled with an infrared dye. PCR reactions (25 µL) contained 50 ng template DNA, 2.5 µL Taq Buffer (10x), 0.56 µM of each primer, 300 µM dNTPs, and 1.4 U Taq DNA polymerase (5 Prime, Hamburg, Germany). Cycling conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 30 s and 68°C for 1 min, with a final step at 68°C for 5 min. Separation of PCR products using a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, USA) and analysis of gel images with the Bionumerics 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium) were carried out as previously described (Krause et al 2012). Richness was calculated as number of F-ARISA band classes per sample and community structure was analyzed based on the Jaccard coefficient.

Statistical analyses. The experiment was based on a crossed factorial design. To prevent bias by the lack of some data in 2011, we analyzed the two years separately. For community structure and richness analyses, the experimental factors were 'Week' (fixed) and 'pH' (fixed). For analysis of cfu L^{-1}, additional factors were 'Temperature' (fixed) and 'Jar' , nested in the interaction 'Week x pH' and considered as a random factor. Richness and cfu L^{-1} were analyzed through factorial ANOVAs with Statistica 7.1 (StatSoft, Tulsa, OK, USA), using Tukey's HSD test for *post-hoc* comparisons. Community structure was analyzed by permutational multivariate ANOVA (PERMANOVA). To visualize patterns of the influence of 'pH', we performed principal co-ordinate analysis (PCO). For all multivariate analyses, we used Primer 6 with the add-on package PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK). Details of the statistical analyses are given in the supplementary material.

Acknowledgements

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Supplement

TABLE S1. ANOVA main tests of fungal abundance, estimated as colony forming units per liter.

Year	Factor	d.f.	SS	MS	*F*	*p*
2011	Week	1	0.36	0.36	7.1	**0.009**
	pH	2	319.87	159.93	3150.7	**<0.001**
	Temperature	1	207.84	207.84	4094.4	**<0.001**
	Week x pH	2	6.59	3.29	64.9	**<0.001**
	Week x Temperature	1	11.03	11.03	217.2	**<0.001**
	pH x Temperature	2	1.90	0.95	18.7	**<0.001**
	Jar (Week x pH)	24	9.48	0.39	7.8	**<0.001**
	Week x pH x Temperature	2	17.58	8.79	173.1	**<0.001**
	Temperature x Jar	23	4.30	0.19	3.7	**<0.001**
	Error	118	5.99	0.05		
2012	Week	1	26.457	26.457	378.2	**<0.001**
	pH	2	346.973	173.486	2480.3	**<0.001**
	Temperature	1	0.775	0.775	11.1	**0.001**
	Week x pH	2	0.400	0.200	2.9	0.061
	Week x Temperature	1	0.314	0.314	4.5	**0.036**
	pH x Temperature	2	2.945	1.472	21.1	**<0.001**
	Jar (Week x pH)	24	11.802	0.492	7.0	**<0.001**
	Week x pH x Temperature	2	0.239	0.119	1.7	0.186
	Temperature x Jar	24	2.529	0.105	1.5	0.078
	Error	118	8.254	0.070		

Displayed are tests for the factors 'Week', 'pH', 'Temperature', 'Jar' and their interactions. *p*-values were obtained using type III sums of squares. d.f.: degrees of freedom, SS: sums of squares, MS: mean squares, *F*: F-values, *p*: significance level, significant results ($p<0.05$) are highlighted in bold. To improve normality, cfu L^{-1} data were natural log-transformed.

The factor 'Jar' is a random factor nested within 'Week x pH' because we analyzed each jar at only one time point but took more than one subsample per jar. This factor had a significant effect in both years, demonstrating that there was variation between replicate jars. Note that the total variation (*F*) explained by this factor was small though.

TABLE S2. PERMANOVA main tests comparing fungal community composition on colony forming unit filters (100 mL, 2011), based on Jaccard dissimilarities of F-ARISA profiles.

Sources of variation	d.f.	SS	*pseudo F*	*p (perm)*	Sq. root
Temperature	1	25469	12.034	**<0.001**	28.475
Week	1	6855.2	3.239	**<0.001**	12.827
pH	2	17843	4.216	**<0.001**	18.811
Temperature x Week	1	5456.5	2.578	**0.006**	15.230
Temperature x pH	2	17738	4.191	**<0.001**	26.501
Wek x pH	2	13039	3.081	**<0.001**	21.399
Temperature x Week x pH	2	10646	2.515	**<0.001**	25.826
Residuals	46	97351			46.004
Total	57	1.94E+05			

One replicate filter was analyzed per jar and temperature. Displayed are tests for the factors 'Temperature', 'Week', 'pH' and their interactions and the partitioning of multivariate variation. *p (perm)*-values were obtained using type III sums of squares and 9999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, *pseudo F*: F-value, *p (perm)*: significance level, significant results (*p (perm)*<0.05 are highlighted in bold, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Jaccard dissimilarities.

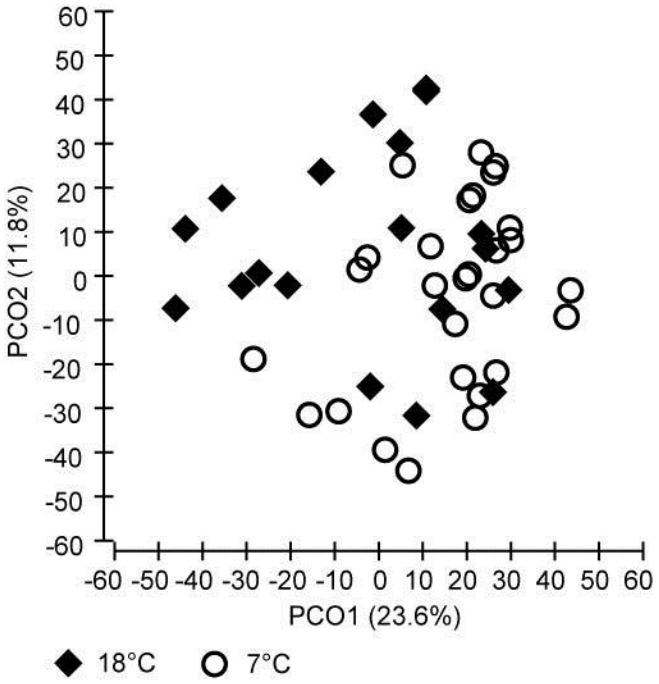

FIGURE S1. Fungal community composition on colony forming unit filters (100mL, 2011).
Principal coordinate ordination (PCO) plot based on Jaccard dissimilarities of F-ARISA profiles. One replicate filter was analyzed per jar and temperature.

TABLE S3. PERMANOVA main tests comparing fungal community composition in the experiments in 2011 and 2012, based on Jaccard dissimilarities of F-ARISA profiles.

Sources of variation	d.f.	SS	*pseudo F*	*p (perm)*	Sq. root
Year	1	32443	10.541	**<0.001**	23.078
Residuals	109	3.35E+05			55.477
Total	110	3.68E+05			

Displayed are tests for the factor 'Year' (random) and the partitioning of multivariate variation. *p (perm)*-values were obtained using type III sums of squares and 9999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, *pseudo F*: F-value, *p (perm)*: significance level, significant results (*p (perm)*<0.05 are highlighted in bold, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Jaccard dissimilarities.

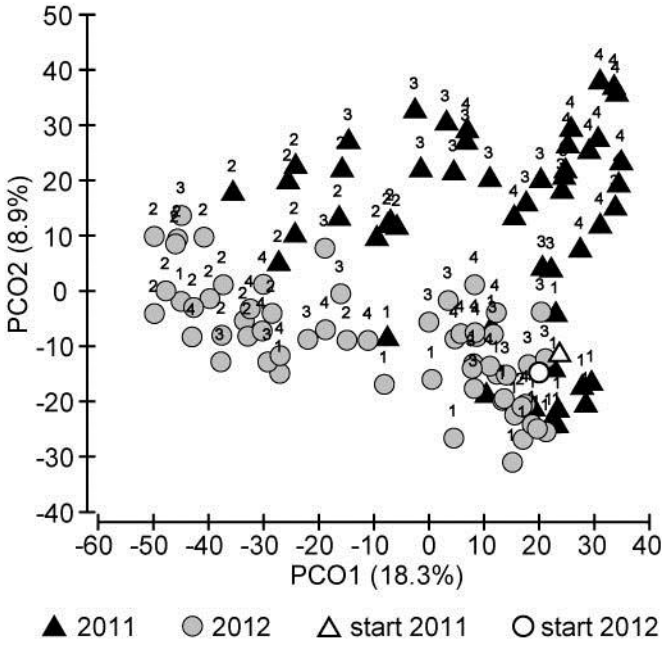

FIGURE S2. Fungal community composition in the experiments in 2011 and 2012.
Principal coordinate ordination (PCO) plot based on Jaccard dissimilarities of F-ARISA profiles. 'start' designates the community structure of the initial seawater samples. Numbers indicate the sampling week.

TABLE S4A. PERMANOVA main tests of fungal community composition separately for 2011 and 2012, based on Jaccard dissimilarities of F-ARISA profiles.

Year	Sources of variation	d.f.	SS	*pseudo F*	*p (perm)*	Sq. root
2011	Week	3	50647	8.824	**<0.001**	35.213
	pH	2	8686.2	2.270	**<0.001**	12.322
	Week x pH	6	18888	1.645	**<0.001**	17.310
	Residuals	39	74619			43.741
	Total	50	1.56E+05			
2012	Week	3	35056	5.086	**<0.001**	25.017
	pH	2	13966	3.039	**<0.001**	15.306
	Week x pH	6	20426	1.482	**0.001**	14.878
	Residuals	48	1.10E+05			47.932
	Total	59	1.80E+05			

Displayed are tests for the factors 'Week', 'pH' and their interactions and the partitioning of multivariate variation. *p (perm)*-values were obtained using type III sums of squares and 9999 permutations under the full model. d.f.: degrees of freedom, SS: sums of squares, *pseudo F*: F-value, *p (perm)*: significance level, significant results (*p (perm)*<0.05 are highlighted in bold, Sq. root: square root of the component of variation attributable to that factor in the model, in units of Jaccard dissimilarities.

TABLE S4B. PERMANOVA pair-wise comparisons of fungal community composition, based on Jaccard dissimilarities of F-ARISA profiles.

		2011		2012	
Week	**Comparison**	***t (perm)***	***p (perm)***	***t (perm)***	***p (perm)***
1	pH *in situ* vs. 7.82	1.366	0.136[a]	1.096	0.242
	pH *in situ* vs. 7.67	1.711	**0.008**	1.511	**0.016**
	pH 7.82 vs. 7.67	1.269	0.201[a]	1.284	**0.076**
2	pH *in situ* vs. 7.82	1.827	**0.018**	0.919	0.636
	pH *in situ* vs. 7.67	2.397	**0.018**[a]	1.114	0.199
	pH 7.82 vs. 7.67	0.964	0.453[a]	1.557	**0.034**
3	pH *in situ* vs. 7.82	1.012	0.461	1.105	0.229
	pH *in situ* vs. 7.67	1.518	**0.025**	1.380	**0.031**
	pH 7.82 vs. 7.67	0.956	0.543	1.561	**0.014**
4	pH *in situ* vs. 7.82	1.433	**0.029**	1.185	0.100
	pH *in situ* vs. 7.67	1.367	**0.040**	2.089	**0.009**
	pH 7.82 vs. 7.67	0.984	0.467	1.341	**0.018**

[a]Less than 100 unique perms, therefore Monte Carlo *p*-values are given. All other combinations had at least 126 unique permutations per comparison.
Displayed are pair-wise *a posteriori* comparisons of the factor 'pH' within each week. Significant results (*p (perm)*<0.05 are highlighted in bold.

TABLE S5. ANOVA main tests of fungal richness, based on the number of F-ARISA band classes.

Year	Factor	d.f.	SS	MS	*F*	*p*
2011	Week	3	2345.21	781.74	5.422	**0.003**
	pH	2	5.82	2.91	0.020	0.980
	Week x pH	6	706.92	117.82	0.817	0.563
	Error	39	5623.22	144.19		
2012	Week	3	4918.98	1639.66	9.448	**<0.001**
	pH	2	298.13	149.07	0.859	0.430
	Week x pH	6	974.67	162.44	0.936	0.478
	Error	48	8330.40	173.55		

Displayed are tests for the factors 'Week', 'pH' and their interactions. *p*-values were obtained using type III sums of squares. d.f.: degrees of freedom, SS: sums of squares, MS: mean squares, *F*: F-values, *p*: significance level, significant results ($p<0.05$) are highlighted in bold.

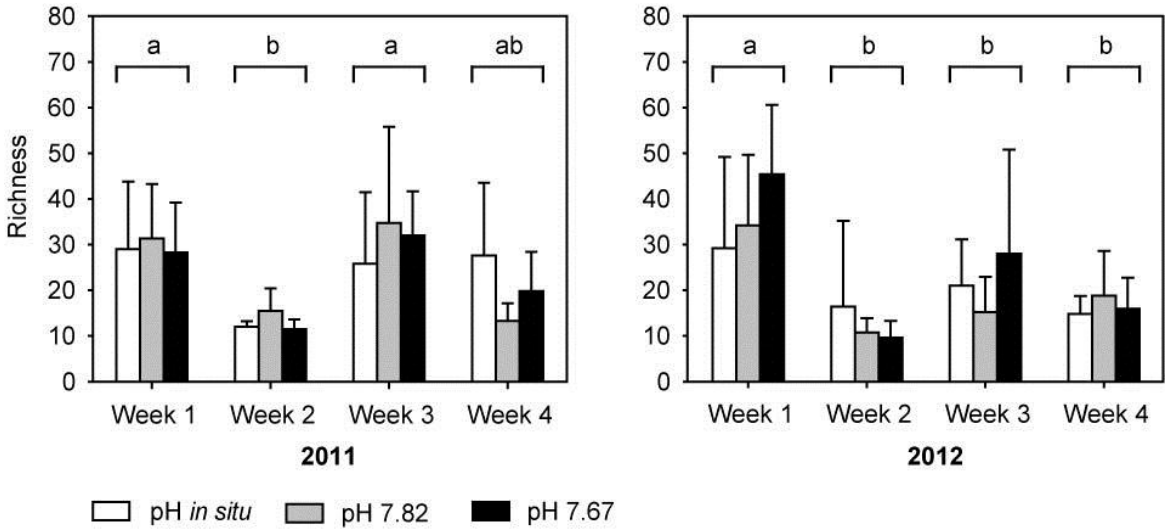

FIGURE S3. Fungal richness.
Richness was estimated based on the number of F-ARISA band classes per sample. Richness was significantly influenced by 'Week' but not by 'pH' (for ANOVA test statistics see Table S5). Letters indicate significant differences between weeks after Tukey's HSD test, 48 d.f., 'a' is significantly different from 'b', but both are not significantly different from 'ab'. *N*=2-5.

TABLE S6. pH_{NBS}, total alkalinity and calculated pCO_2 at the start of the experiment and after each week of incubation.

Year	pH	Week	pH_{NBS}	total alkalinity [µmol kg^{-1}]	pCO [µatm]
2011	pH *in situ*	start	8.10	2362	456
		1	8.13 ± 0.00	2361 ± 4	446 ± 5
		2	8.12 ± 0.00	2366 ± 5	465 ± 5
		3	8.10 ± 0.02	2344 ± 17	495 ± 23
		4	8.09 ± 0.02	2354 ± 21	498 ± 21
	pH 7.82	start	7.81	2257	892
		1	7.83 ± 0.01	2255 ± 2	905 ± 26
		2	7.81 ± 0.01	2235 ± 11	929 ± 36
		3	7.83 ± 0.01	2239 ± 12	936 ± 19
		4	7.79 ± 0.01	2242 ± 12	981 ± 26
	pH 7.67	start	7.67	2206	1230
		1	7.74 ± 0.05	2202 ± 14	1109 ± 134
		2	7.70 ± 0.01	2193 ± 2	1208 ± 18
		3	7.69 ± 0.02	2193 ± 4	1268 ± 48
		4	7.69 ± 0.01	2203 ± 9	1246 ± 43
2012	pH *in situ*	start	8.26	2332	355
		1	8.21 ± 0.01	2328 ± 10	382 ± 11
		2	8.18 ± 0.01	2328 ± 6	397 ± 10
		3	8.16 ± 0.04	2329 ± 6	400 ± 42
		4	8.14 ± 0.01	2327 ± 9	406 ± 14
	pH 7.82	start	7.82	2154	1000
		1	7.79 ± 0.01	2158 ± 7	999 ± 21
		2	7.76 ± 0.01	2163 ± 12	1052 ± 24
		3	7.74 ± 0.01	2168 ± 13	1060 ± 16
		4	7.70 ± 0.01	2158 ± 8	1139 ± 40
	pH 7.67	start	7.67	2117	1395
		1	7.65 ± 0.01	2119 ± 4	1390 ± 47
		2	7.62 ± 0.01	2099 ± 12	1442 ± 37
		3	7.61 ± 0.02	2138 ± 8	1453 ± 61
		4	7.58 ± 0.01	2113 ± 6	1482 ± 46

Given is the mean ± standard deviation. *N*=5.

Supplementary Materials and methods

Determination of Total Alkalinity (TA) and calculation pCO_2.

Samples for TA were 0.45 µm filtered, stored in 100 mL borosilicate bottles with Teflon-inlet at 5°C, and analyzed within a week. We estimated TA by open-cell duplicate potentiometric titration and calculation with modified Gran plots (Bradshaw et al 1981), using a TitroLine alpha plus titrator with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). The electrode was calibrated with technical buffer solutions from freshly opened glass ampoules (pH 4.0, 7.0) (all materials: SI Analytics, Mainz, Germany). Values were corrected with Certified Reference Material (CRM, Batch No. 104, Scripps Institution of Oceanography, USA).

For calculations of pCO_2, we converted pH values to the total scale, using Tris-based reference material (Batch No. 5, Scripps Institution of Oceanography, USA). Calculations were done with the program CO2calc (Robbins et al 2010), using dissociation constants of carbonic acid of Mehrbach et al (1973), refit by Dickson and Millero (1987) and dissociation constants for H_2SO_4 from Dickson (1990).

Determination of the yearly pH cycle at Helgoland Roads.

From September 2010 to September 2011, we daily (5 times a week) determined surface pH at sampling station Helgoland Roads (North Sea, 54°11.3′N, 7°54.0′E). Samples were taken between 6 and 10 a.m. Seawater temperature was measured on board the research vessel and was obtained from the Helgoland Roads time series (Wiltshire et al 2008). Samples were immediately brought to our laboratories at Helgoland Marine Biological Station and pH was measured at seawater temperature using a thermostat bath. At the sampling site Helgoland Roads, water depth ranges from 6-10 m and due to strong tidal currents, surface water samples are representative of the entire water column (Wiltshire et al 2010).

DNA extraction from colony forming unit filters

After 5 days (18°C) or 13 days (7°C), filters were removed from the agar plates and stored at -20°C until further analysis. For DNA extraction, filters were cut into quarters and each quarter was suspended in 700 µL STE buffer (6.7% sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8) with 200 µL of a mixture of 0.1 mm zirconia/silica and 1.0 mm zirconia

beads (BioSpec Products, Inc., Bartlesville, OK, USA). Mechanical lysis was performed using a FastPrep® FP120A Instrument for 40 s at 6.0 m s^{-1}. Lysates of the four quarters of each filter were combined and an aliquot of 500 µL was used for DNA extraction. DNA was purified with the GENECLEAN® *Turbo* Kit (Qbiogene, Carlsbad, CA, USA) and concentration and purity were determined by photometry using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland).

CHAPTER III

Do marine yeasts benefit from ocean acidification?

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Abstract

Marine yeasts play an important role in biodegradation and nutrient cycling and are often associated with marine flora and fauna. They show maximum growth at pH levels lower than present-day seawater pH. Thus contrary to many other marine organisms, they may actually profit from ocean acidification. Hence we conducted a microcosm study, incubating natural seawater from the North Sea at present-day pH (8.10) and two near-future pH levels (7.81 and 7.67). Yeasts were isolated from the initial seawater sample and after two and four weeks of incubation. Isolates were classified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and representative isolates were identified by partial sequencing of the large subunit rRNA gene. From the initial seawater sample, we predominantly isolated a yeast-like filamentous fungus related to *Aureobasidium pullulans*, *Cryptococcus* sp., *Candida sake* and various cold-adapted yeasts. After incubation, we found more different yeast species at near-future pH levels than at present-day pH. Yeasts reacting to low pH were related to *Leucosporidium scottii*, *Rhodotorula mucilaginosa*, *Cryptococcus* sp. and *Debaromyces hansenii*. Our results suggest that these yeasts will benefit from seawater pH reductions and give a first indication that the importance of yeasts will increase in a more acidic ocean.

Introduction

Marine environments are inhabited by a variety of yeast species, which are ecologically important due to their role in biodegradation and their associations with marine plants and animals. Yeasts are distinguished from other fungi by their unicellular growth and belong to the phyla *Ascomycota* or *Basidiomycota*, respectively. In seawater, their abundances vary from below ten to up to thousands of culturable cells per liter, with highest numbers reported from near-shore and nutrient rich areas (Fell 2001). Yeasts play an important role in the breakdown of plant and refractory material and the cycling of nutrients (Kutty and Philip 2008, Meyers and Ahearn 1974) and are frequently found on decaying algae (Patel 1975, Seshadri and Sieburth 1971, van Uden and Castelo Branco 1963). Additionally, yeasts occur associated with marine animals, e.g. fish, shrimps, mussels, corals, and seabirds (Burgaud et al 2010, Kawakita and van Uden 1965, Ravindran et al 2001, van Uden and Castelo Branco 1963, Yang et al 2011). Notably, pathogenic yeast species can also cause infections in animal hosts such as marine mammals and copepods (Higgins 2000, Seki and Fulton 1969).

A recurring research focus is dedicated to the origin of yeasts occurring in the marine environment and to the question which factors influence and limit their existence in seawater. Commonly, a distinction is drawn between truly marine species and species allochthonus to the marine environment. A common definition is that obligate marine yeasts have only been isolated from marine habitats, while facultative marine yeasts occur in terrestrial environments as well and are possibly introduced by terrestrial runoffs or winds (Hagler and Ahearn 1987, Kohlmeyer and Kohlmeyer 1979, Kutty and Philip 2008)). Yet high salinity levels, low temperatures and the alkaline pH have traditionally been regarded as factors inhibiting the propagation of yeasts in the marine environment (Norkrans 1966). Concerning pH, the optimum growth conditions for yeasts are at lower pH levels than those encountered in the seas (Orij et al 2011). In pure culture studies, marine yeasts exhibited maximum total growth, highest growth rate and shortest lag phase at acid pH levels, i.e. below pH 7 (Hoppe 1972, Norkrans 1966).

This preference for low pH levels leads to the question whether marine yeasts will benefit from ocean acidification. Rising CO_2 emissions and the resulting uptake of CO_2 by the oceans have already caused a reduction in surface seawater pH of 0.1 units and a further reduction of up to 0.7 units is anticipated with the depletion of all fossil fuel reserves during the next three centuries (Caldeira and Wickett 2003). Although anthropogenic ocean acidification will not cause seawater pH to drop below 7, pH levels will shift closer

towards yeast growth optima. So far, microbial processes identified to be affected by ocean acidification are nitrogen fixation and cyanobacterial photosynthesis (Liu et al 2010). Yet virtually nothing is known concerning marine occurring yeasts and filamentous fungi. A single study reported that fungal sequences increased by more than seven times in coral-associated microbial communities at reduced pH, which was attributed to an increased susceptibility of corals to fungal colonization at reduced pH (Vega Thurber et al 2009).

The investigation of yeasts in diverse aquatic environments has greatly advanced in recent years with the availability of molecular taxonomic methods (Kurtzman 2006). Yeast community composition was reported for e.g. estuarine, coastal, open-ocean, deep-sea, and various extreme habitats (Burgaud et al 2010, Chen et al 2009, Coelho et al 2010, Gadanho et al 2003, Gadanho and Sampaio 2004, Gadanho and Sampaio 2005, Gadanho et al 2006). For yeast identification, most of these studies sequenced the 600-650 bp D1/D2 region of the large subunit (LSU) rRNA gene, for which ample sequence information is available (Fell et al 2000, Kurtzman and Robnett 1998). To limit time-consuming and costly sequencing efforts to a small number of representative isolates, most studies employed pre-screening or classification methods. Besides phenotypic classifications, exclusively PCR-based approaches have been applied up to now, such as micro/ minisatellite-primed PCR (MSP-PCR) (Gadanho and Sampaio 2002, Gadanho et al 2003) and restriction fragment length polymorphism (RFLP) (Chen et al 2009).

An alternative approach to identify and classify yeast species is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This rapid and cost-effective method allows a comparison of species based on proteomic mass spectra. Mass spectra of unknown yeasts can be measured and aligned with reference spectra filed in a database, ideally leading to species-level identifications. Furthermore, reference spectra created from measurements of one isolate can be compared with those of other isolates to analyze phylogenetic relationships and thus classify unknown organisms. This mass spectrometric technique has been extensively applied to the study of clinically important yeasts, yielding high rates of correct species-level identifications (e.g. Dhiman et al 2011, Marklein et al 2009, Stevenson et al 2010, van Veen et al 2010). Yet, to our knowledge, it has not been applied to yeasts in the context of ecological studies. Still the approach is highly promising, as previously demonstrated for environmental bacterial strains (Dieckmann et al 2005, Emami et al 2012, Oberbeckmann et al 2011a).

Here we present the first investigation of ocean acidification effects on marine yeasts, employing MALDI-TOF MS and partial sequencing of the LSU rRNA gene. Our study site

Helgoland Roads is located near the island Helgoland approximately 50 km off the German Coast in the North Sea. At this site, yeast abundances (viable cell counts) have been recorded from 1964 to 1966 and from 1980 to 1992 (Gerdts et al 2004, Meyers et al 1967) and additional information on yeast identities based on phenotypic identifications is available for certain periods (Ahearn and Crow 1980, Meyers et al 1967). For our study, a water sample was taken in mid-April 2011 and was incubated in laboratory microcosms at different pH levels (pH *in situ* [8.10], 7.81 and 7.67). Yeasts were isolated from the initial seawater sample and after two and four weeks of incubation. The main objective of this study was thus to investigate whether moderate pH reductions, as anticipated in the course of ocean acidification, alter yeast community composition. At the same time, we present the first investigation of the yeast community at Helgoland Roads based on molecular taxonomic methods and evaluate the performance of MALDI-TOF MS in identifying and classifying environmental yeast isolates.

Materials and methods

Experimental set-up

We sampled water at Helgoland Roads (54°11.3′N, 7°54.0′E) on April 14, 2011. Water temperature was 6.8°C. The experimental design consisted of three pH levels, which were the current seawater *in situ* pH (8.10) and two near-future pH levels, pH 7.81 and pH 7.67. The mean surface pH in the southern North Sea may reach these values by the year 2100, assuming atmospheric CO_2 of 700 or 1000 ppm, respectively (Blackford and Gilbert 2007). We acidified water samples with 2 M HCl. For each pH level, we set up ten replicate 1.6 L glass jars (acid-washed, autoclaved) with an air-tight rubber seal. Jars were incubated at 7°C in the dark and were mixed daily by inversion. Samples from five replicate jars of each treatment were analyzed after two and after four weeks, respectively.

Determination of pH and Total Alkalinity (TA)

We used a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MF-DIN-N). All pH measurements were carried out at 7.0°C. The electrode was calibrated with standard buffer solutions from freshly opened glass ampoules (pH 4.01, 6.87, 9.18) (all materials: SI Analytics, Mainz, Germany), i.e. we measured pH on the National Bureau of Standards (NBS) scale. Samples for total alkalinity were filtered through 0.45 µm pore-size nitrocellulose filters (Sartorius, Göttingen, Germany) and were stored at 5°C in 100 mL bottles closed air-tight with screws with Teflon-inlet. Samples were analyzed within a week. Total alkalinity was estimated from open-cell duplicate potentiometric titration and calculation with modified Gran plots (Bradshaw et al 1981), using a TitroLine alpha plus titrator with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). The electrode was calibrated with technical buffer solutions from freshly opened glass ampoules (pH 4.0, 7.0) (all materials: SI Analytics, Mainz, Germany). Measurements were corrected using Certified Reference Material (CRM, Batch No. 104, Scripps Institution of Oceanography, USA). Calculations of carbon dioxide partial pressure were performed with the program CO2calc (Robbins et al 2010), using the dissociation constants of carbonic acid of Mehrbach et a. (1973), refit by Dickson and Millero (1987) and dissociation constants for H_2SO_4 from Dickson (1990).

Yeast isolation and sampling of yeast biomass

To cultivate yeasts, samples from the initial seawater sample (10, 100, 500 and 1000 mL) and from the experiment (10 and 100 mL), were filtered through sterile nitrocellulose filters (0.45 µm pore-size, 47 mm diameter, grey with grid, Sartorius, Göttingen, Germany) applying low pressure (100 mbar). Filters were placed onto Wickerham's YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% dextrose and 2.0% agar, pH 6.2), prepared with sterile-filtered seawater from the sampling site and supplemented with 250 mg L^{-1} chloramphenicol (Sigma, Saint Louis, MO, USA) to inhibit bacterial growth. Furthermore, samples of 500 µL were directly spread onto the same medium using glass beads. Triplicate samples of each volume were incubated at 7°C in the dark. After 13 days, all yeast-like colony forming units were counted and colonies were picked if accessible, i.e. not overgrown by filamentous fungi. Isolates were obtained by at least two successive differential streaks on Wickerham's YM agar without chloramphenicol.

For sampling of yeast biomass for DNA extraction and MALDI-TOF MS, single colonies were streaked out on Wickerham's YM agar without chloramphenicol and were incubated at 18°C for six days. For MALDI-TOF MS, biomass was resuspended in 300 µL sterile deionized water and 900 µL pure ethanol were added. Samples were mixed by inversion and stored at -20°C until further analysis. For DNA extraction, biomass was resuspended in 300 µL STE buffer (6.7% sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8) and stored at -20°C until further analysis.

MALDI-TOF MS

Cells were extracted using a standard formic acid/ acetonitrile method (Mellmann et al 2008). Extracts (1.2 µL) were spotted onto a sample target in duplicate, left to air-dry, and were overlaid with 1.2 µL of matrix, containing alpha-cyano-4-hydroxycinnamic acid (HCCA) as a saturated solution in 50% acetonitrile and 2.5% trifluoracetic acid. For measurements, we used a microflex™ benchtop instrument (Bruker Daltonics) in the positive linear mode (2000- 20,000 Da). Six spectra were recorded per isolate. To create one spectrum, 240 laser shots were generated at fixed optimal laser energy of 150 µJ and 3 ns of pulse width. Spectra were integrated into one reference spectrum for each isolate, which was subsequently compared to reference spectra in the MALDI BIOTYPER database (V3.2.1.0). Additionally, reference spectra of all isolates were compared among each other (MALDI BIOTYPER 3.0 software) and score values were assigned, describing

the degree of similarity between different spectra. Values above 2.0 are considered species level identifications, values between 1.7 and 2.0 genus level identifications (Wieser et al 2012). Consequently, isolates displaying score values above 2.0 among each other were assigned to a MALDI-TOF MS class. At least one representative isolate of each MALDI-TOF MS class was selected for sequencing. If applicable, we sequenced at least one isolate obtained from the initial seawater sample and one isolate obtained from the experiment, representing the pH level with the highest number of isolates in this class.

DNA extraction and partial large subunit (LSU) rRNA gene sequencing

DNA extraction of isolates was performed as previously described (Sapp et al 2007) omitting the lysozyme step and using an increased incubation temperature of 65°C instead of 50°C for the cell lysis step (Gadanho et al 2003). Briefly, cells were lysed by the addition of sodium dodecyl sulfate (1%) and DNA extraction was performed using phenol/chloroform. DNA was precipitated with isopropanol, dissolved in sterile water and stored at -20°C. DNA concentration and purity were determined by photometry using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland).

A ca. 950 bp fragment of the LSU rRNA was amplified using forward primer NL1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG -3‘) (White et al 1990) and reverse primer LR6 (5’- CGC CAG TTC TGC TTA CC -3’) (Fell 2001). PCR reactions were performed in duplicate in volumes of 50 µL containing 50 ng of template DNA, 5 µL Taq Buffer (10x), 1.4 µL of each primer (20 µM), 1.5 µL dNTPs (2.5 mM each), and 2.5 U of Taq DNA polymerase (5 Prime, Hamburg, Germany). Amplifications were carried out according to the following protocol: Initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 68°C for 1 min, with a final extension step at 68°C for 2 min. The duplicate reactions were combined and purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Sequencing was performed in both directions using primers NL1 and LR6 with an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The resulting sequences were aligned using ALIGNIR2.0® (LI-COR). In order to determine the closest relative of environmental isolates, sequences were compared with sequences in the GenBank database. The sequences obtained in this study are available from GenBank under the accession numbers JX679415-JX679461 (LSU rRNA).

Phylogenetic analysis

The sequences were phylogenetically analyzed using the ARB® software package (Ludwig et al 2004). After the addition of the partial LSU gene sequences to the ARB LSU reference database (LSUref_1900, release August 2011), the sequence alignment was carried out with the integrated Fast Aligner. Additionally, the alignment was refined by comparison of the closest relatives in NCBI retrieved by BLAST. Sequences with more than 1200 nucleotides were used to calculate the phylogenetic tree. Partial sequences were added using the ARB 'parsimony interactive' tool. The phylogenetic relationship of LSU rRNA sequences was deduced by the neighbor-joining method with the correction algorithm of Felsenstein (1993). Matrices were calculated via neighbor-joining based on the phylogenetic distances of the isolates using the 'similarity' correction.

Results

Carbonate chemistry manipulations

The pH_{NBS} of the initial seawater sample was 8.10, which was consequently taken as the *in situ* or present-day treatment. The corresponding calculated pCO_2 was 477 µatm. The pH of the incubations remained very constant during the four weeks of the experiment, the means of each treatment not deviating more than 0.03 units from the initially adjusted values (Table 1). The resulting calculated pCO_2 values of the intermediate acidification treatment (pH_{NBS} 7.81) ranged from 919 to 973 µatm and the pCO_2 of the high acidification treatment (pH_{NBS} 7.67) from 1195 to 1238 µatm. For the *in situ* treatment (pH_{NBS} 8.10), we calculated pCO_2 values from 459 to 493 µatm (Table 1).

TABLE 1. pH_{NBS}, total alkalinity and calculated pCO_2 at the start of the experiment and after two or four weeks of incubation

Treatment		pH_{NBS}	total alkalinity [µmol kg^{-1}]	pCO_2 [µatm]
pH 8.10	start	8.10	2362	477
	week 2	8.12 ± 0.00	2366 ± 5	459 ± 5
	week 4	8.09 ± 0.02	2354 ± 21	493 ± 21
pH 7.81	start	7.81	2257	931
	week 2	7.81 ± 0.01	2235 ± 11	919 ± 35
	week 4	7.79 ± 0.01	2242 ± 12	973 ± 26
pH 7.67	start	7.67	2206	1238
	week 2	7.70 ± 0.01	2193 ± 2	1195 ± 18
	week 4	7.69 ± 0.01	2203 ± 9	1236 ± 43

Given is the mean ± standard deviation (*N*=5); NBS, National Bureau of Standards.

Yeast isolation

The initial seawater sample yielded 48 (±1 s.d.) colony forming units per liter (cfu L^{-1}). For samples taken during the experiment, a robust estimation of cfu L^{-1} was not possible, due to low colony numbers and the overgrowth by filamentous fungi. The growth of filamentous fungi also frequently hindered the isolation of yeasts. Yet in total, we were able to pick 89 colonies with yeast appearance, 51 of the initial seawater sample and 38 during the experiment. Of the 38 colonies obtained during the experiment, 21 were isolated after two weeks and 17 after four weeks of incubation. Concerning the pH levels, four colonies were picked at pH *in situ*, 12 at pH 7.81 and 22 at pH 7.67.

Yeast identification and classification by MALDI-TOF MS and partial LSU rRNA gene sequence analysis

All 89 isolates (both from the initial seawater sample and from the experiment) were analyzed by MADLI-TOF MS. Seven isolates could be directly identified by comparing their mass spectra to reference strains in the MALDI BIOTYPER database: Three isolates (EK61, EK62, EK82) displayed score values above 2.2 with *Debaromyces hansenii* DSM 70590 and the remaining four isolates (EK66, EK67, EK76, EK78) were most similar to *Rhodotorula mucilaginosa* DSM 70403, with score values ranging from 1.9 to 2.2. Furthermore, the latter isolates had score values between 1.7 and 2.0 to *R. mucilaginosa* DSM 18184 and *R. mucilaginosa* DSM 70404. All other hits to strains in the reference database were ≤1.5, i.e. below probable genus-level identifications.

As the majority of isolates could not be identified with the reference strains available in the MALDI BIOTYPER database, mass spectra were compared among isolates and isolates displaying score values above 2.0 among each other were assigned to a MALDI-TOF MS class. This yielded 18 different MALDI-TOF MS classes (Figure 1), the five largest of them collectively containing 65 isolates. Eight classes consisted of only one isolate.

To resolve the identity of each MALDI-TOF MS class, partial sequences of the LSU rRNA gene were obtained for one or more representative isolates and were compared with reference sequences in the GenBank database (Table 2). Overall, sequencing consistently revealed the same closest relative for isolates within a MALDI-TOF MS class (Table 2). Consequently, we assigned one closest relative, including sequence similarity percentage, to all isolates within a class (Figure 1). The phylogenetic placement of representative isolates is shown in Figure 2 for species in the phylum *Ascomycota* and in Figure 3 for species in the phylum *Basidiomycota*.

Concerning the consensus of both methods, sequencing confirmed the MALDI BIOTYPER identification of isolates of class 7 as *R. mucilaginosa* and of isolates of class 2 as *D. hansenii*. Furthermore, partial LSU rRNA gene sequencing revealed different closest relatives for distinct MALDI-TOF MS classes. In one case however, the same closest relative and percentage similarity was found for two classes: for both class 16 and class 17, 100% sequence similarity was found to *Guehomyces pullulans* AFTOL-ID 718. The close relationship between the two classes was also seen in the mass spectrometry dendrogram (Figure 1). As MALDI-TOF MS score values among the two isolates in class 16 and the only isolate in class 17 were between 1.5 and 1.7 however, both classes were considered as distinct for further analyses.

In total, five out of the 18 MALDI-TOF MS classes were assigned to the *Ascomycota* (31 isolates) and 13 to the *Basidiomycota* (58 isolates). For three out of the five ascomycete MALDI-TOF MS classes, closest relatives were filamentous fungi of the subphylum *Pezizomycotina*: class 3 (13 isolates) showed 100% sequence similarity to the fungal strain *Discosphaerina fagi*, class 4 (one isolate) 95% sequence similarity to the fungal plant pathogen *Ramularia pratensis* var. *pratensis* and class 13 (one isolate) 98% sequence similarity to the aquatic hyphomycete *Tetracladium setigerum*. In contrast, all basidiomycete MALDI-TOF MS classes had yeast species as closest relatives.

Regarding species identifications, 100% sequence similarity to described species was found for nine of the MALDI-TOF MS classes, containing 48 isolates. For two of the classes (classes 4 and 5), each containing only one isolate, sequence similarity to the closest relative was below 97%, hinting at the isolation of new species.

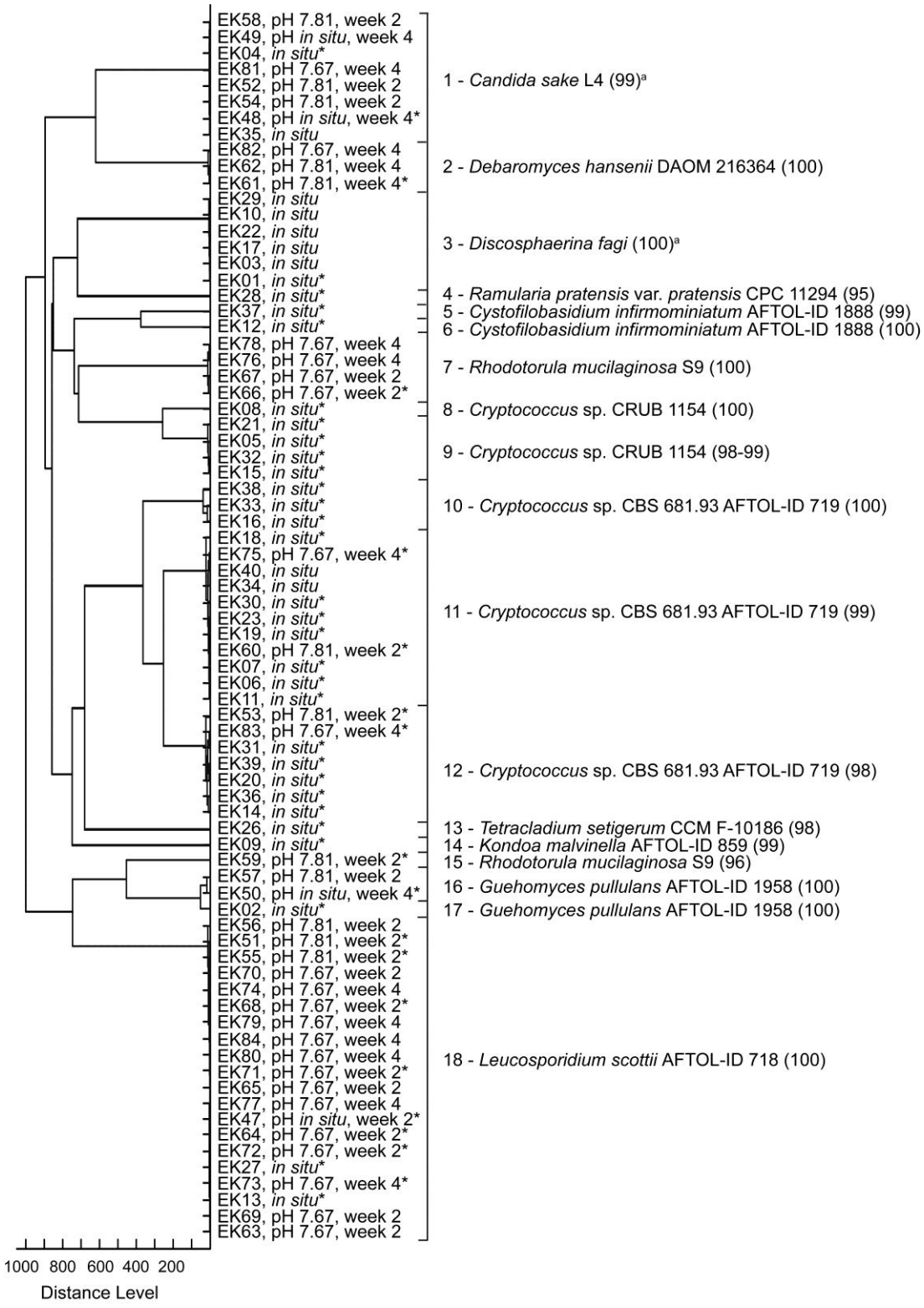

FIGURE 1. Mass spectrometry dendrogram of isolates, based on MALDI-TOF mass spectra.
Isolates with score values above 2.0 among each other were grouped into classes. Isolates from the original seawater sample are designated as *in situ*; for isolates obtained after incubation, the pH level and the time of sampling are indicated. For each MALDI-TOF MS class, the closest relative and similarity (%) of representative isolates, as determined by partial LSU rRNA sequence analysis, are given. Isolates selected for sequencing are marked with an asterisk (*).
[a], note that for MALDI-TOF MS class 1 and 3, additional isolates (not displayed) were obtained from the initial seawater sample: 6 isolates of class 1 and 7 isolates of class 3.

TABLE 2. Taxonomic classification of representative isolates of MALDI-TOF MS classes, based on partial LSU rRNA sequence analysis

Isolate	MALDI-TOF MS class	bp	Closest relative (BLAST)	Accession Number	Similarity (%)	Fungal group
EK04	1	935	*Candida sake* L4	EF017662	99	A
EK48	1	927	*Candida sake* L4	EF017662	99	A
EK61	2	937	*Debaromyces hansenii* DAOM 216364	JN938932	100	A
EK01	3	897	*Discosphaerina fagi*	AY016359	100	A
EK28	4	931	*Ramularia pratensis* var. *pratensis* CPC 11294	EU019284	95	A
EK37	5	963	*Cystofilobasidium infirmominiatum* AFTOL-ID 1888	DQ645523	99	B
EK12	6	963	*Cystofilobasidium infirmominiatum* AFTOL-ID 1888	DQ645523	100	B
EK66	7	941	*Rhodotorula mucilaginosa* S9	AB217506	100	B
EK08	8	969	*Cryptococcus* sp. CRUB 1154	EF595760	100	B
EK21	9	970	*Cryptococcus* sp. CRUB 1154	EF595760	98	B
EK05	9	970	*Cryptococcus* sp. CRUB 1154	EF595760	98	B
EK32	9	964	*Cryptococcus* sp. CRUB 1154	EF595760	98	B
EK15	9	958	*Cryptococcus* sp. CRUB 1154	EF595760	99	B
EK38	10	945	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	100	B
EK33	10	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	100	B
EK16	10	750	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	100	B
EK18	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK75	11	755	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK30	11	961	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK23	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK19	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK60	11	946	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK07	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK06	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK11	11	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	99	B
EK53	12	871	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK83	12	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK31	12	961	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK39	12	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK20	12	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK36	12	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK14	12	960	*Cryptococcus* sp. CBS 681.93 AFTOL-ID 719	AY646103	98	B
EK26	13	937	*Tetracladium setigerum* CCM F-10186	EU883427	98	A
EK09	14	951	*Kondoa malvinella* AFTOL-ID 859	AY745720	99	B
EK59	15	924	*Rhodotorula mucilaginosa* S9	AB217506	96	B
EK50	16	966	*Guehomyces pullulans* AFTOL-ID 1958	EF551318	100	B
EK02	17	966	*Guehomyces pullulans* AFTOL-ID 1958	EF551318	100	B
EK51	18	960	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK55	18	920	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK68	18	953	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK71	18	953	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK47	18	959	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK64	18	953	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK72	18	910	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK27	18	780	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK73	18	953	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B
EK13	18	953	*Leucosporidium scottii* AFTOL-ID 718	AY646098	100	B

bp, base pair; A, *Ascomycota*; B, *Basidiomycota.*

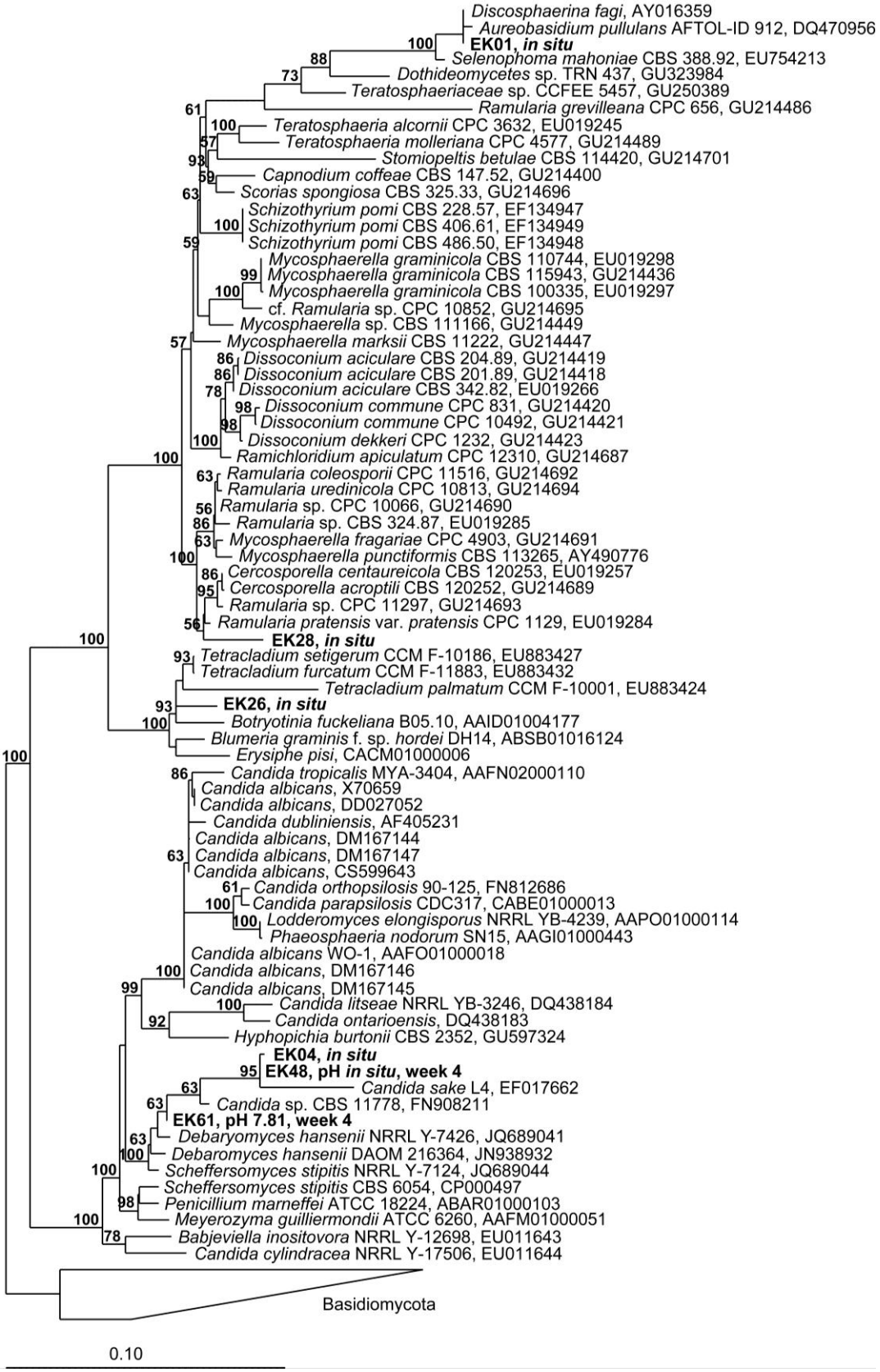

FIGURE 2. Phylogenetic tree of members of the phylum *Ascomycota*, based on partial LSU rRNA sequences. Isolates obtained in this study (EK) are highlighted in bold; isolates from the initial seawater sample are designated as *in situ*; for isolates obtained after incubation, the pH level and the time of sampling are indicated. GenBank accession numbers are given. Bootstrap values >50% are displayed. Scale bar represents 10 nucleotide substitutions per 100 nucleotides. Sequences of isolates obtained in this study are available from GenBank under the accession numbers JX679415-JX679461.

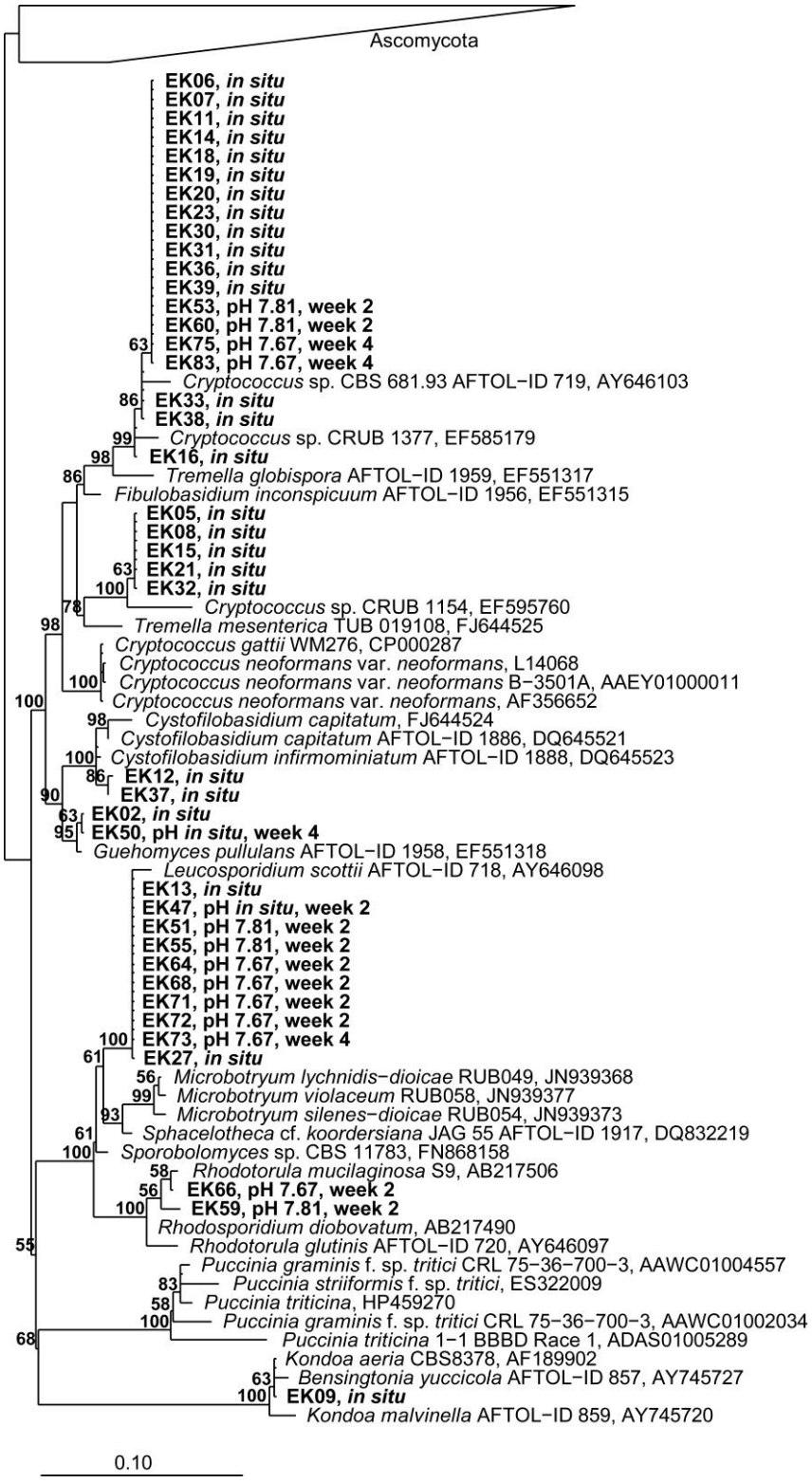

FIGURE 3. Phylogenetic tree of members of the phylum *Basidiomycota*, based on partial LSU rRNA sequences.

Isolates obtained in this study (EK) are highlighted in bold; isolates from the original seawater sample are designated as *in situ*; for isolates obtained after incubation, the pH level and the time of sampling are indicated. GenBank accession numbers are given. Bootstrap values >50% are displayed. Scale bar represents 10 nucleotide substitutions per 100 nucleotides. Sequences of isolates obtained in this study are available from GenBank under the accession numbers JX679415-JX679461.

Yeasts in the initial seawater sample

In the initial seawater sample, we found 14 MALDI-TOF MS classes (Table 3), with similar amounts of *Ascomycota* (22 isolates) and *Basidiomycota* (29 isolates) (Figure 1). Considering only MALDI-TOF MS classes representing yeasts however (classes 3, 4 and 13 had filamentous fungi as closest relatives), only eight ascomycete isolates were obtained, constituting less than 16% of the yeast community.

The majority of isolates were identified as the filamentous fungus *Discosphaerina fagi* and as different members of the yeasts *Cryptococcus* sp. and *Candida sake*. Rare species were *Leucosporidium scottii*, *Cystofilobasidium infirmominiatum, Guehomyces pullulans*, *Kondoa malvinella*, and isolates related to the filamentous fungi *Ramularia pratensis* var. *pratensis* and *Tetracladium setigerum* (Figure 1).

Yeasts after incubation at present-day pH and near-future pH levels

Taken together all pH levels, after incubation we found eight MALDI-TOF MS classes (Table 3) with higher amounts of *Basidiomycota* (29 isolates) than Ascomycota (9 isolates) (Figure 1). Notably, no non-yeast isolates were obtained after incubation

After incubation at *in situ* pH, we found three MALDI-TOF MS classes (Table 3). Two of these, related to *Candida sake* (class 1) and *Leucosporidium scottii* (class 18), had also been obtained from the initial seawater sample. Additionally, one new class (class 16), identified as *Guehomyces pullulans,* was found. Yet a closely related class (class 17), also identified as *G. pullulans*, was obtained from the initial seawater sample as well (Table 3).

After incubation at near-future pH levels, we found eight MALDI-TOF MS classes (seven classes at pH 7.81 and six classes at pH 7.67, Table 3). All three classes found after incubation at pH *in situ* were found after incubation at the near-future pH levels as well. Two of the additional classes (classes 11 and 12) were related to *Cryptococcus* sp. and had been previously detected in the initial seawater sample (Table 3). The remaining three classes were found only after incubation at near-future pH levels and were identified as *Rhodotorula mucilaginosa* (class 7), *Debaromyces hansenii* (class 2) and as related to *R. mucilaginosa* (class 15). The predominant MALDI-TOF MS class found after incubation was class 18, identified as *Leucosporidium scottii*. Remarkably, isolates of this class were obtained considerably more often after incubation at near-future pH levels, especially from pH 7.67 (Figure 1).

TABLE 3. Isolates obtained from the initial seawater sample (*in situ*) and after two and four weeks of incubation at different pH levels, based on MALDI-TOF MS classification

		pH *in situ*		pH 7.81		pH 7.67	
MALDI-TOF MS class[a]	***in situ***	**week 2**	**week 4**	**week 2**	**week 4**	**week 2**	**week 4**
1 - *Candida sake* L4 (99)	+	-	+	+	-	-	+
2 - *Debaromyces hansenii* DAOM 2163648 (100)	-	-	-	-	+	-	+
3 - Discosphaerina fagi (100)	+	-	-	-	-	-	-
4 - *Ramularia pratensis* var. *pratensis* CPC 11294 (95)	+	-	-	-	-	-	-
5 - *Cystofilobasidium infirmominiatum* AFTOL-ID 1888 (99)	+	-	-	-	-	-	-
6 - *Cystofilobasidium infirmominiatum* AFTOL-ID 1888 (100)	+	-	-	-	-	-	-
7 - *Rhodotorula mucilaginosa* S9 (100)	-	-	-	-	-	+	+
8 - *Cryptococcus sp.* CRUB 1154 (100)	+	-	-	-	-	-	-
9 - *Cryptococcus sp.* CRUB 1154 (98-99)	+	-	-	-	-	-	-
10 - *Cryptococcus* sp. CBS 681.93 AFTOL-ID 719 (100)	+	-	-	-	-	-	-
11 - *Cryptococcus* sp. CBS 681.93 AFTOL-ID 719 (99)	+	-	-	+	-	-	+
12 - *Cryptococcus* sp. CBS 681.93 AFTOL-ID 719 (98)	+	-	-	+	-	-	+
13 - *Tetracladium setigerum* CCM F-10186 (98)	+	-	-	-	-	-	-
14 - *Kondoa malvinella* AFTOL-ID 859 (99)	+	-	-	-	-	-	-
15 - *Rhodotorula mucilaginosa* S9 (96)	-	-	-	+	-	-	-
16 - *Guehomyces pullulans* AFTOL-ID 1958 (100)	-	-	+	+	-	-	-
17 - *Guehomyces pullulans* AFTOL-ID 1958 (100)	+	-	-	-	-	-	-
18 - *Leucosporidium scottii* AFTOL-ID 718 (100)	+	+	-	+	-	+	+

+, detected; -, not detected

[a] Given is the closest relative and similarity (%) of representative isolates of each MALDI-TOF MS class, as determined by partial LSU rRNA sequence analysis.

Discussion

As yeasts prefer low pH, their importance in marine ecosystems may increase with ocean acidification. Using a microcosm approach, we present the first investigation of the effects of moderate pH reductions on a marine yeast community. The possible implications are discussed along with a general consideration of the yeast community at Helgoland Roads and the strategy of identifying and classifying environmental yeast isolates by MALDI-TOF MS.

Carbonate system perturbations

The pH values of the microcosms remained very stable during incubation. The present-day pH we measured matched the value reported by Blackford and Gilbert (2007) for the southern North Sea (8.10 vs. 8.06±0.06 s.d.), thus pH gradations realized in our study closely mimicked near-future pH levels. However, considering the use of HCl, it has to be taken into account that there are differences in carbonate system changes depending on whether pH is altered by HCl or CO_2 additions. Although for CO_2 not considerably exceeding 700 ppm, all carbonate system parameters change in similar magnitude with both perturbation methods (Schulz et al 2009), higher acid perturbations lead to lower bicarbonate concentrations than CO_2 additions (Hurd et al 2009, Schulz et al 2009). Yet biological responses are not expected to differ substantially as all carbonate system species ($[CO_2]$, $[HCO_3^-]$, $[CO_3^{2-}]$, $[H^+]$) will change in the same direction with both methods (Gattuso and Lavigne 2009, Schulz et al 2009).

Yeast identification and classification by MALDI-TOF MS

Of the 89 environmental isolates obtained in this study, only seven could be directly identified with reference spectra available in the MALDI BIOTYPER database. They were identified as *Rhodotorula mucilaginosa* or *Debaromyces hansenii*, respectively, with score values between 1.9 and 2.2. Sequencing of a ca. 950 bp region of the LSU rDNA confirmed the MALDI-TOF MS identification. Thus MALDI-TOF MS yielded correct species-level identifications, but the percentage of isolates that could be directly identified was very small, due to the lack of environmental strains in the MALDI BIOTYPER database.

Our results indicate however, that MALDI-TOF MS can also be used as a rapid tool to classify environmental yeast isolates and thus limit sequencing efforts. Classification proved very reliable: within a MALDI-TOF MS class, partial sequencing of the LSU rRNA gene of representative isolates consistently revealed the same closest relative. Likewise, different classes were found to represent different closest relatives and percentage similarities. Yet one exception occurred, as *Guehomyces pullulans* was found as the closest relative with 100% sequence similarity for two classes. This may hint at differences at the sub-species level, as isolates in one group originated from the initial seawater sample and in the other group from the experiment. The resolution of sequencing the 950 bp region of the LSU rDNA may not be sufficient to resolve these differences. The problem of multiple groups representing one species is encountered with PCR based pre-screening methods as well. In a study employing MSP-PCR fingerprints, four different patterns were found among 22 isolates eventually identified as the species *Debaromyces hansenii* var. *hansenii* (Gadanho et al 2003). This was not seen as a methodological limitation however; instead it was hypothesized to indicate different populations within a species (Gadanho et al 2003).

In summary, the classification success achieved by MALDI-TOF MS was comparable to that achieved with PCR based pre-screening methods of environmental yeast isolates (Chen et al 2009, Gadanho and Sampaio 2002, Gadanho et al 2003). Yet direct MALDI-TOF MS identification of environmental yeast isolates is still limited. As identification successes obtained for clinical yeast isolates are very promising (Dhiman et al 2011, Marklein et al 2009, Stevenson et al 2010, van Veen et al 2010), an expansion of the database towards environmental isolates is desirable. With these data becoming available, it will be possible to fully exploit the capacity of this rapid and cost-effective method for environmental studies.

Yeast community at Helgoland Roads

At the sampling time in mid-April 2011, we observed 48 yeast colony forming units per liter (cfu L^{-1}). This is in the lower range of previous records: At Helgoland Roads, strong fluctuations from less than 10 to up to 600 cfu L^{-1} were reported, with highest abundances in the summer months (July-September) (Ahearn and Crow 1980, Crow et al 1977, Gerdts et al 2004, Meyers et al 1967).

The most frequently isolated species belonged to typical marine yeast genera and a yeast-like filamentous fungus. Isolates were predominantly identified as related to

Discosphaerina fagi, *Cryptococcus* sp. and *Candida sake*. *Discosphaerina* (formerly *Columnosphaeria*) *fagi* has been suggested as a possible teleomorph, i.e. sexual stage, of the black yeast-like fungus *Aureobasidium pullulans* (Zalar et al 2008), which occurs widespread in the marine environment (Meyers et al 1967). The genera *Cryptococcus* and *Candida* are known to contain true marine yeasts (Kutty and Philip 2008) and the species *Candida sake* is present in various aquatic environments including lakes, lagoons and antarctic seawater (Boguslawska-Was and Dabrowski 2001, Brandão et al 2011, Buzzini et al 2012). Furthermore, members of this species are pathogens of freshwater prawn, fish and marine invertebrates (Brilhante et al 2011, de Araujo et al 1995, Hatai and Egusa 1975).

Among the rare species, the high incidence of species known from cold environments can be pointed out, namely *Leucosporidium scottii*, *Cystofilobasidium infirmominiatum*, *Guehomyces pullulans* and *Kondoa malvinella*. These species occur in arctic or antarctic seawater, algae and glacial environments (Buzzini et al 2012, Fonseca et al 2000, Vaz et al 2011). However, most of them have also been reported from soil (Lim et al 2010, Maksimova and Chernov 2004) or associated with animals (Bruce and Morris 1973, Kobatake et al 1992, Zacchi and Vaughan-Martini 2002), including deep-sea corals and mussels (Burgaud et al 2010, Galkiewicz et al 2012). Additionally, we found two filamentous fungi of terrestrial or freshwater origin. These were related to the *Ramularia pratensis* var. *pratensis*, a necrotrophic plant pathogen described on *Rumex crispus* (Crous et al 2007) and the aquatic hyphomycete *Tetracladium setigerum*. Aquatic hyphomycetes are important decomposers of leaf litter in running waters and are dispersed as spores on substrates such as wood or in the digestive tract of aquatic detrivores (Anderson and Shearer 2011). These species may have been introduced by coastal water influx, which is known to recur at the study site (Wiltshire et al 2010).

Comparing the species composition observed to earlier reports from the North Sea and the study site (1964-1966 and 1980-1992) reveals both similarities and differences. *Cryptococcus* and *Candida* species, *A. pullulans* and *C. infirmominiatum* (under the previous designation *Rhodosporidium infirmio-miniata*) have previously been reported in the North Sea (Ahearn and Crow 1980, Crow et al 1977, Meyers et al 1967, Meyers and Ahearn 1974). However, we found *Debaromyces hansenii* and *Rhodotorula mucilaginosa* (formerly *R. rubra*) only after incubation, although they were the most abundant species in North Sea water samples in both 1964-66 and 1976 (Ahearn and Crow 1980, Meyers et al 1967). This could be related to seasonality, as especially *D. hansenii* was reported to occur

with algal blooms and is closely related to high nutrient situations (Ahearn and Crow 1980, Meyers et al 1967). In contrast, our study was carried out in mid-April, at the beginning of the spring bloom (chlorophyll a on April 14 2011: 4.5 µg L^{-1}, maximum on April 26 2011: 15.4 µg L^{-1}; data obtained from the Helgoland Roads time-series (Wiltshire et al 2008)). Furthermore, although year round sampling campaigns were previously conducted, no reports about cold-adapted yeast species exist. The differences may in part be explained as we used a different isolation medium and a lower incubation temperature (7 vs. 18°C). Additionally, our identifications are based on sequencing, whereas previous observations were based on morphology and biochemical capabilities.

In summary, the initial yeast community, taken as starting point for the experiment, displayed characteristics of a typical marine sample with coastal influences. It comprised mainly true marine species but also some species of terrestrial or freshwater origin. In addition, various cold-adapted yeasts were present, possibly due to the relatively high latitude of the study site and the time of sampling in mid-April. The differences to previous records suggest a more detailed reinvestigation of the North Sea yeast community using molecular methods.

Yeasts after incubation: present-day vs. near-future pH levels

At *in situ* pH, we only found yeasts related to *Candida sake*, *Leucosporidium scottii* and *Guehomyces pullulans*, which were all found in the initial seawater sample as well. At near-future pH levels, we additionally found yeasts related to *Cryptococcus* sp., *Rhodotorula mucilaginosa*, and *Debaromyces hansenii*. Thus after incubation at near-future pH levels, we both recovered a higher number of species and obtained yeasts not found in the initial seawater sample. This indicates that these species profited from reduced pH. In terms of total yeast abundances however, no calculations can be presented. Still it is remarkable that more isolates were obtained from near-future pH incubations. Especially for *Leucosporidium scottii*, the number of isolates was inversely related to pH, suggesting that a moderate reduction in pH may lead to higher yeast abundances.

Our results yield only first insights though, as we were not able to quantitatively isolate all yeasts due to the overgrowth by filamentous fungi. Furthermore, the maximum sampling volume was 100 ml for the incubations, compared to 1000 ml for the *in situ* sample, reducing the possibility of detection of rare species. Using a nutrient rich medium and a direct detection method (DGGE), Gadanho and Sampaio (2004) observed a higher number of yeast species, especially ascomycetes, after incubation. Thus the low number of species

we found after incubation may be explained by nutrient limitation. For bacteria, the phenomenon of community shifts in laboratory incubations has long been known as the "bottle effect" (Ferguson et al 1984). Yet, to our knowledge, no detailed investigations of the response of marine yeast communities to experimental confinement exist and estimates on the percentage of culturable marine yeasts are lacking (Fell 2001). Recently, a DGGE analysis of mycoplankton of Hawaiian coastal waters revealed many new fungal phylotypes but not a single match with fungi previously cultured from this environment (Gao et al 2010). Therefore, future studies on ocean acidification effects on marine yeasts should include direct detection methods.

Despite these limitations, our findings indicate that *Leucosporidium scottii*, *Rhodotorula mucilaginosa* and related species, *Cryptococcus* sp. and *Debaromyces hansenii* grow better at near-future seawater pH levels. In general, alkaline pH is considered to be a stress factor that yeasts have to cope with in the marine environment. The intracellular pH of yeasts was reported to be around 7 when growing on glucose in laboratory studies and to remain stable between extracellular pH values from 3.0 to 7.5 (Orij et al 2009). At lower external pH values, protons can be efficiently exported out of the cytoplasm, either to the exterior of the cell or into the vacuole, involving different ATPases (Carmelo et al 1997, Diakov and Kane 2010, Orij et al 2011). In contrast, alkaline external pH values exacerbate the establishment of a functional plasma membrane proton gradient and consequently the uptake of nutrients (Orij et al 2011). Thus a moderate reduction in seawater pH partially relieves the pH stress yeasts encounter in the marine environment. The extent to which different species will benefit may depend on their specific pH regulation mechanisms, as was recently proposed for marine bacteria (Teira et al 2012).

Taking into account that more fungi are found in corals exposed to pH stress (Vega Thurber et al 2009), our results indicate that with ocean acidification, both direct and indirect pH effects may favor fungi. Although our results on yeasts abundances remain very preliminary, this gives rise to some concern, as particularly the genera we isolated at near-future pH levels also contain pathogenic species. Members of *Cryptococcus* sp. are known to infect marine mammals (Higgins 2000) and also humans (Khawcharoenporn et al 2007). Likewise, *Rhodotorula mucilaginosa* and *Debaromyces hansenii* are considered emerging yeast pathogens (Hazen 1995). Notably, the importance of yeasts in surveillance programs at bathing beaches has already been pointed out (Shah et al 2011, Vogel et al 2007). Concerning microbial food webs, it is furthermore interesting to note that a moderate acidification does not seem to influence bacterial abundances (Allgaier et al

2008, Grossart et al 2006, Krause et al 2012, Newbold et al 2012). A possible consequence would be a relative increase in the importance of yeasts in biogeochemical cycles.

In conclusion, this study yields valuable first insights concerning the effects of ocean acidification on marine yeasts. We demonstrate that especially *Leucosporidium scottii*, *Rhodotorula mucilaginosa*, *Cryptococcus* sp. and *Debaromyces hansenii* benefit from moderate pH reductions. Furthermore, a moderate reduction in pH seems to be generally beneficial to the yeast community, suggesting a higher importance of yeasts in a more acidic ocean. The implications for biogeochemical cycles and pathogenic interactions deserve a thorough investigation in future ocean acidification studies.

Acknowledgements

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GENERAL DISCUSSION

Rising atmospheric CO_2 is causing an acidification of the world's oceans. The consequences for marine bacterial and fungal communities remain poorly understood. While bacterial communities were studied in complex systems but at low replication, marine fungal communities were not specifically addressed at all. Thus this thesis aimed to investigate direct ocean acidification effects on both groups in highly replicated microcosm experiments. Concerning marine fungi, both the response of the total community and more specifically the response of yeasts were studied. The study site was Helgoland Roads, located in the southern North Sea. For this region, atmospheric CO_2 of 700 or 1000 ppm by the year 2100 may cause reductions in seawater pH to values of 7.82 or 7.67, respectively (Blackford and Gilbert 2007). However, the present-day carbonate system at Helgoland Roads is not well characterized. Therefore the first prerequisite of this study was to determine the natural variability of pH and pCO_2 at this site.

Description of the carbonate system at Helgoland Roads over a yearly cycle

From September 2010 to September 2011, we determined pH and total alkalinity (TA) at Helgoland Roads on a work-daily basis. From these two parameters together with temperature, salinity and phosphate and silicate concentrations obtained from the Helgoland Roads time series (Wiltshire et al 2008), we calculated seawater carbon dioxide partial pressure (pCO_2). The pH values given below are reported on the National Bureau of Standards (NBS) scale.

Over the yearly cycle, pH ranged from 8.06 to 8.43 with a mean of 8.17±0.07, demonstrating that seasonal differences of almost 0.4 pH units occur at Helgoland Roads. TA ranged from 2300 to 2407 µmol kg^{-1}. The mean TA of 2345±20 µmol kg^{-1}, is in the range of previous records for the German Bight (Brasse et al 1999). The resulting calculated pCO_2 ranged from 215 to 526 µatm with a mean of 416±62 µatm. Concerning seasonal differences, it became apparent that the strongest drops in pCO_2 were related to peaks in chlorophyll a concentration (Figure 1). High phytoplankton abundance, indicated by high chlorophyll a concentrations, possibly lead to increased CO_2 uptake through photosynthesis. Especially during the spring bloom from mid-April to May, this may explain the observed decrease in pCO_2 and increase in pH. The carbonate system at

Helgoland Roads was thus dominated by biological processes, as reported previously for the German Bight (Brasse et al 1999, Lorkowski et al 2012).

In more detail, Thomas et al (2004) reported that in winter, almost the entire North Sea is in equilibrium with the atmosphere and only some parts of the southeastern coast are slightly supersaturated, which we observed for Helgoland Roads especially from January to April 2011. A strong reduction in pCO_2 was reported for the spring bloom (Thomas et al 2004), which in our record reduced pCO_2 to a minimum of 215 µatm on May 6, 2011, 10 days after the highest chlorophyll a concentration on April 26, 2011. In the southern nonstratified part of the North Sea, a slow recovery of pCO_2 and finally supersaturation due to decreasing primary production and increasing respiration occurs in summer (Thomas et al 2004). Regarding our measurements, this may correspond to the period from May to September 2011. The supersaturation decreases again in autumn, due to sinking temperatures and CO_2 outgassing (Thomas et al 2004), which we observed from mid-September 2010 to beginning of January 2011. Overall, Thomas et al (2004) suggested that regarding the yearly average, the part of the North Sea south of 54°N is a week source of atmospheric CO_2, which may be reflected in our average record of 416±62 µatm. In contrast, the stratified central and northern parts of the North Sea represent strong sinks and efficiently pump atmospheric CO_2 to the North Atlantic Ocean (Thomas et al 2004).

Previous records at Helgoland Roads (1962 to 1978) have suggested pCO_2 values from <200 to >900 ppm with an average of 480 ppm [1 ppm = 1 µatm in dry air at standard pressure, (Zeebe and Wolf-Gladrow 2001)]. Comparing these records to our results underscores the hypothesis of Kempke (1996) that high values reported in summer months are not realistic and are most probably due to respiration in samples that were not immediately processed. Therefore, it is impossible to deduce from comparison with these previous records whether an acidification trend is already visible at Helgoland Roads.

In conclusion, we found that pH differences at Helgoland Roads over a yearly cycle can reach 0.4 units, corresponding to differences in pCO_2 exceeding 300 µatm. However, these fluctuations occur in the opposite direction than ocean acidification. As organisms at Helgoland Roads are currently not exposed to pH values lower than 8.0, the projected acidification to pH 7.82 or 7.67 used in the experiments represents a strong perturbation. As we sampled only one year, this record presents a first insight. To gain more knowledge on inter-annual variability and long-term trends, the methods established in this thesis are now continued on a twice-a-week basis in the time-series program at Helgoland Roads.

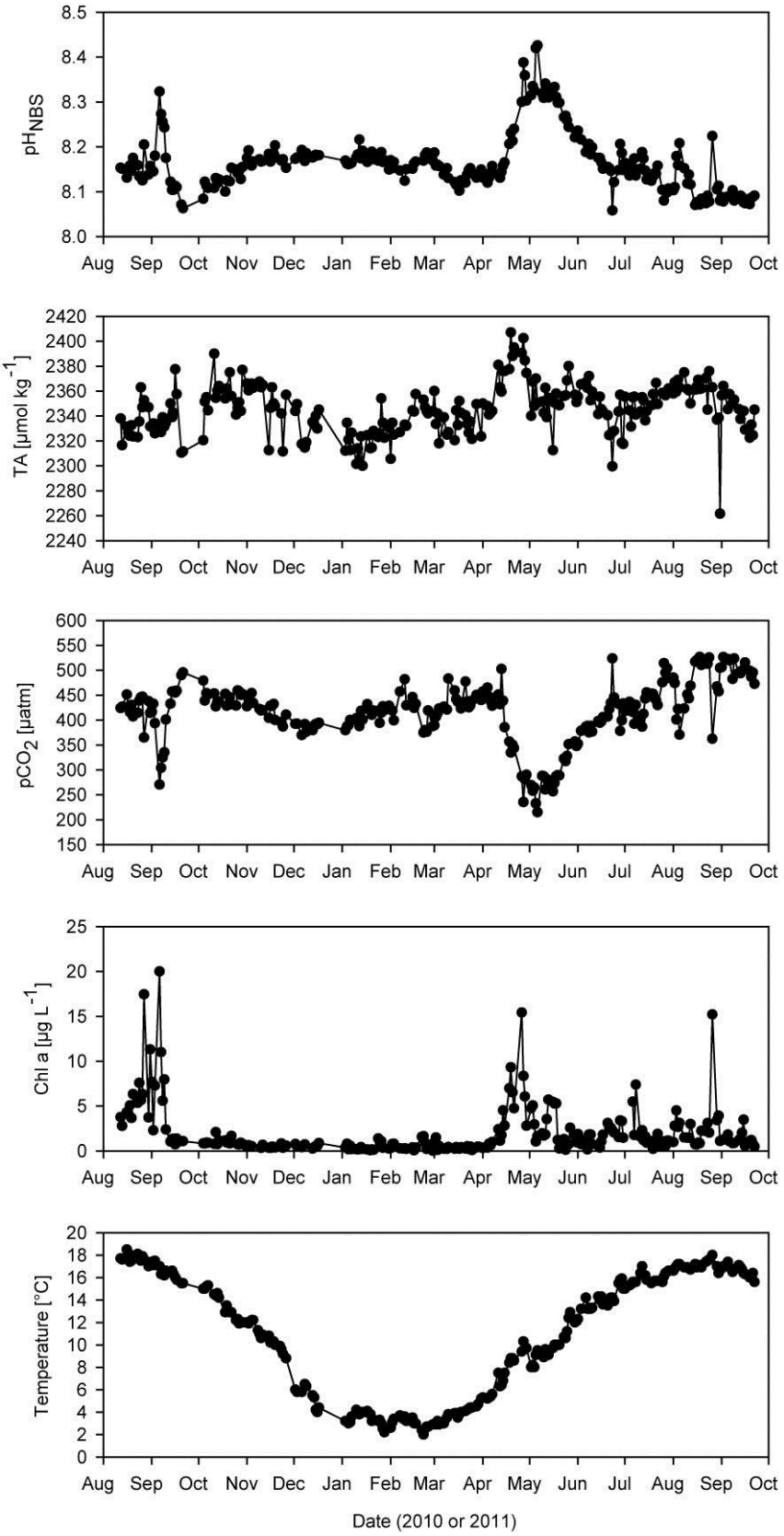

FIGURE 1. Carbonate system and contextual parameters at Helgoland Roads over a yearly cycle
pH_{NBS}: pH measured on the National Bureau of Standards scale, converted to the total scale for calculations of pCO_2; TA: total alkalinity; Chl a: chlorophyll a. For calculations of pCO_2, we used the program CO2calc (Robbins et al 2010).

Ocean acidification effects on marine bacterial communities

To investigate direct ocean acidification effects on bacterial communities, we carried out microcosm experiments, incubating the bacterial community from Helgoland Roads at different pH levels over a period of four weeks. Experiments were repeated in spring, summer, autumn 2010 and winter 2011, to take seasonal variation in bacterial community structure into account. Furthermore, we applied different dilution protocols to select for distinct ecological groups.

Combining different seasons and dilution strategies proved successful in selecting for differently assembled bacterial communities. Comparing only communities at pH *in situ*, we found that the undiluted microcosms were characterized by *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*. In detail, *Flavobacteria* were most important in spring and winter, *Alphaproteobacteria*, especially SAR11, in summer and a more diverse community occurred in autumn. *Gammaproteobacteria* in the undiluted treatments could not be assigned to families, indicating that they did not represent readily culturable groups. In contrast, the serially diluted microcosms were characterized by *Gammaproteobacteria* frequently reported from cultivation studies, such as *Oceanospirillaceae, Colwelliaceae* and *Pseudoalteromonadaceae*, and to a lesser extend *Flavobacteriaceae* and *Rhodobacteraceae*. As observed for the undiluted microcosms, the relative contribution of these groups differed with season. Dilution with sterile seawater multiplies the amount of nutrients available per cell and an additional nutrient input occurs due to cell breakage during filtration (Goldman and Dennett 1985). This probably fostered the development of fast-growing groups (Beardsley et al 2003, Eilers et al 2000b, Fuchs et al 2000). In undiluted treatments in contrast, oligotrophic bacteria growing efficiently at ambient nutrient concentrations developed, demonstrated for instance by the presence of members of the SAR11 clade, which can be cultivated only at low nutrient concentrations (Rappé et al 2002).

Despite these differences in community assembly, we found consistent pH effects for both undiluted and serially diluted microcosms in all seasons. Community shifts were predominantly already found between pH *in situ* and pH 7.82, suggesting that a tipping point for community shifts may be reached even earlier. Focusing on differences between pH *in situ* and pH 7.67 revealed that different members of *Gammaproteobacteria*, *Flavobacteriaceae, Rhodobacteraceae, Campylobacteraceae* and further less abundant groups were involved in pH-dependent community shifts. Groups that were generally more characteristic for pH 7.67 and thus may profit from ocean acidification were

Pseudoalteromonadaceae, *Opitutae* and *Campylobacteraceae*. In contrast, members of *Rhodobacteraceae* and SAR11-clade were more characteristic for pH *in situ*, suggesting that they do not compete well at reduced pH. For most other groups however, pH responses differed in their direction, indicating that they are context-dependent.

Within the *Gammaproteobacteria*, the pH response of *Oceanospirillaceae* and *Alteromonadaceae* was different in different seasons, probably hinting at temperature effects. The response of *Colwelliaceae* on the other hand was dependent on dilution, possibly indicating nutrient effects. In contrast, no specific trends were found for members of the *Flavobacteriaceae*. This could hint at complex interactions of temperature and nutrient effects and directly competing species. For instance, Ray et al (2012) reported an interaction of DOM input and seawater acidification on bacterial community composition. The response of especially *Flavobacteriaceae* and *Gammaproteobacteria* to glucose additions differed with acidification (Ray et al 2012). For *Gammaproteobacteria*, different responses were also observed in two coral species transferred to natural CO_2 vent sites (Meron et al 2012).

The lower contribution of *Rhodobacteraceae* to communities at pH 7.67 is in agreement with findings in biofilms from the Great Barrier Reef (Witt et al 2011). Additionally, in a laboratory study a *Cytophaga* strain exhibited increased growth rate at reduced pH, which was not found for a *Roseobacter* strain (Teira et al 2012). This may imply that members of the family *Rhodobacteraceae* do not benefit energetically from acidification and may thus be outcompeted by other groups that will benefit. However, another study reported an increase of *Rhodobacteraceae* in coral microbial communities exposed to low pH (Meron et al 2011). Here it has to be taken into account that *Rhodobacteraceae* are a highly diverse family and different pH responses may occur at the genus, species or possibly even ecotype level.

An unexpected result was the strong reaction of *Campylobacteraceae*, represented only by the genus *Arcobacter*, which we observed at pH 7.67 in the winter experiment. This genus was previously found at the study site (Eilers et al 2000b) and has been reported from seawater and zooplankton samples from the Mediterranean Sea as well (Fera et al 2004). *Arcobacter* spp. were also reported as indicators for fecal pollution of waters (Collado et al 2008). The immense profit of *Arcobacter* in the winter experiment may hint at a seed bank effect (Pedrós-Alió 2006). Under favorable environmental conditions, rare species, i.e. bacterial phylotypes usually present in low numbers, may attain high importance, which has already been discussed in the context of ocean acidification (Liu et al 2010). This may

apply to *Campylobacteraceae*, as they are known to grow at low pH in contaminated food and are able to survive the stomach transit (D'Sa and Harrison 2005, Murphy et al 2006, Reid et al 2008). A possible profit of the genus *Arcobacter* from ocean acidification deserves attention in future studies, as members of this genus are considered emerging human pathogens and possible pathways of human infection are ingestion of contaminated waters and consumption of contaminated shellfish (Collado and Figueras 2011, Snelling et al 2006).

Groups that were not very abundant in our experiment, but have previously been reported to be sensitive to ocean acidification in coral microbial communities were *Opitutae* (*Verrucomicrobia*) and *Vibrionaceae* (Meron et al 2011, Vega Thurber et al 2009). Consistent with previous studies, both groups were more characteristic for low pH treatments. A more thorough investigation would be especially important for the family *Vibrionaceae*, as it comprises species pathogenic to humans and animals (Thompson et al 2004) and may become more important in temperate waters with climate change (Baker-Austin et al 2010, Baker-Austin et al 2012, Oberbeckmann et al 2011b). Although *Vibrio* spp. were reported to constitute more than 2% of the total bacterial community at Helgoland Roads during periods of warm water temperatures (Oberbeckmann et al 2011b), the low numbers of sequences of this group detected in our experiment prevents a more detailed conclusion. Probably, nutrient concentration in the microcosms was too low to favor the growth of *Vibrionaceae* over other groups, as previous studies reported growth of *Vibrio* spp. predominantly in substrate amended seawater (Eilers et al 2000a, Pinhassi and Berman 2003).

We found no pH effect on bacterial abundances, which is in agreement with previous mesocosm studies (Allgaier et al 2008, Newbold et al 2012). This may contradict an overall energetic profit from reduced pH, which has been proposed due to decreasing alkaline stress on marine bacteria with ocean acidification (Teira et al 2012). Furthermore, it may also suggest that there was no increase in overall nutrient availability and utilization, although changes in the speciation of dissolved elements (e.g. P, N, Mn, Se), bioavailability of dissolved iron and activities of total protease and extracellular α- and β-glucosidase have been reported at reduced pH (Grossart et al 2006, Pearson and Palmer 2000, Piontek et al 2010, Shi et al 2010, Zeebe and Wolf-Gladrow 2001). Others factors potentially influencing bacterial numbers are grazing and viral lysis, which cannot be excluded by filtration or dilution (Beardsley et al 2003). To the best of our knowledge,

differences in these processes on bacterioplankton with ocean acidification have not been studied (Danovaro et al 2011, Liu et al 2010).

In this context it can be mentioned that we initially aimed at a characterization of the entire microbial community developing in the microcosms. We analyzed all samples with primers specific for the three domains *Bacteria*, *Archaea*, and *Eukarya* (Table 1). PCR products were initially analyzed via denaturing high performance liquid chromatography (DHPLC). The idea was to separate and collect single peaks by denaturing runs. Unfortunately, signals were often very weak and furthermore, separation of PCR products was insufficient. However, although PCR products could not be resolved, our results at least yielded insights on the presence of *Archaea* and *Eukarya*. For *Archaea*, only two of 183 samples (179 from the experiment and the four starting communities) yielded PCR products. This indicates that *Archaea* did not grow in high numbers in the microcosms. For *Eukarya*, we found signals in 146 samples, with most negative results from the winter experiment. However, no dilution or pH specific trend was visible (data not shown).

In summary, the experimental design enabled us to investigate diversely assembled microbial communities and to detect pH effects based on robust statistical analyses. We found that already moderate pH reductions may cause shifts in bacterial community structure. The direction of the response was mostly context-dependent, hinting at interactions of pH effects with other factors and at differences in the response of bacterial groups below the family level. In future ocean acidification studies, an in-depth investigation with e.g. specific primers or FISH probes seems especially interesting for the diverse and naturally abundant *Rhodobacteraceae* and the commonly rare but potentially pathogenic genus *Arcobacter*.

Ocean acidification effects on marine fungal communities

As marine fungi have so far not been investigated in ocean acidification studies, we carried out microcosm experiments, incubating natural North Sea water at different pH levels for four weeks, in spring 2011 and 2012. Microcosms were incubated at *in situ* temperature (7-8°C), and fungal numbers were determined by colony forming unit (cfu) counts at *in situ* temperature and 18°C. Furthermore, we analyzed fungal community structure with a culture-independent fingerprint approach (F-ARISA).

Fungal numbers were strongly influenced by pH. We observed up to 1.2×10^3 cfu L^{-1} at pH *in situ*, up to 1.6×10^4 cfu L^{-1} at pH 7.82 and up to 9.0×10^5 cfu L^{-1} at pH 7.67. On average,

fungal numbers were nine times higher comparing pH *in situ* and pH 7.82 and 34 times higher comparing pH *in situ* and pH 7.67. The effect occurred regardless of incubation temperature, indicating that there was no difference in the response of cold- and warm-adapted groups. This implies that already a moderate pH reduction may cause a strong increase in fungal abundances. A possible explanation is that at reduced pH, fungi have to invest less energy into their pH homeostasis, as they have been reported to have difficulties to establish a functional plasma membrane proton gradient at alkaline pH (Davis 2009, Orij et al 2011). Additionally, the activity of fungal extracellular enzymes may be affected, which has already been shown for bacteria (Grossart et al 2006, Piontek et al 2010).

Previously, highest fungal abundance has been reported from highly productive areas (Ahearn and Crow 1980, Gao et al 2010, Gutiérrez et al 2011, Kutty and Philip 2008). The most elaborate account stems from the Humboldt upwelling system off Chile (Gutiérrez et al 2011), where during upwelling periods, fungal biomass can rival that of prokaryotes and fungi may mediate up to 30% of photosynthetic carbon turnover. The high increase in fungal numbers with ocean acidification indicates that fungi may reach higher importance in marine microbial food webs in other systems as well. Concerning possible consequences for marine biogeochemical cycles, higher fungal abundance could lead to higher decomposition rates of complex substrates. As fungi channel back nutrients such as essential amino acids, vitamins and polyunsaturated fatty acids to the food web (Richards et al 2012), this may lead to higher organic matter availability. Furthermore, it has recently been suggested that fungal zoospores, produced by chytrids, may represent a food source for grazers and filter-feeders in coastal environments, especially during algal blooms with high rates of infection (Gleason et al 2011). Thus there are various ways in which an increase in fungal abundances may affect marine food webs, which should be addressed by future studies.

As marine fungi comprise numerous pathogenic species, rising fungal abundance is also a reason for concern, as it may affect the diverse interactions of fungi with marine algae and animals. A first indication of this was given by the study of Vega Thurber et al (2009) which found more fungi in corals exposed to low pH (6.7). In coral microbial communities, this may be the consequence of disturbed equilibrium between fungi and their coral hosts due to environmental stressors (Golubic et al 2005, Vega Thurber et al 2009). Fungi also infect other marine organisms, such as fish, crustaceans and shellfish (Hyde et al 1998, Ramaiah 2006, Schaumann and Priebe 1994), many of which are commercially important. Notably, especially shellfish and crustacean as calcifying

organisms and also juvenile stages of fishes are sensitive to ocean acidification per se (Doney et al 2009, Frommel et al 2012). Thus hosts weakened by pH-stress may encounter a higher number of opportunistic fungal pathogens. Regarding recreational beach areas, high fungal loads were also reported to be potentially hazardous to humans (Vogel et al 2007).

Concerning fungal community structure, we found that different communities were present in the two years of the experiment. Nevertheless in both years, pH effects were predominantly observed only at pH 7.67. This implies that regardless of inter-annual variability in fungal community assembly, a tipping point for community shifts is reached only at pH 7.67. Up to now, we did not resolve fungal identities, but a 454 pyrosequencing approach is planned. Furthermore in 2012, we isolated fungi from the microcosms using a most probable number (MPN) approach. Ongoing investigations will hopefully elucidate, which fungal groups were involved in the community shifts observed in these experiment.

In conclusion, a strong pH effect on fungal abundance was detected already at pH 7.82, while fungal community structure was only affected at pH 7.67. Thus ocean acidification will primarily impact fungal abundances. At higher acidification, inter-specific differences in pH homeostasis mechanisms may additionally lead to community shifts.

Ocean acidification effects on marine yeasts

Based on marine fungal 18S libraries, yeasts appear to be the most abundant fungal group in the oceans. Furthermore, previous data on yeast numbers and identities are available for Helgoland Roads. Hence we investigated ocean acidification effects on this group in more detail. We obtained yeast isolates from the microcosm experiment in 2011 and the seawater sample used for this experiment. Isolates were classified and identified by MALDI-TOF MS and partial sequencing of the LSU rRNA gene of representative isolates.

Only seven out of 89 isolates could be directly identified to the species level by MALDI TOF MS, either as *Rhodotorula mucilaginosa* (four isolates) or as *Debaromyces hansenii* (three isolates). This indicates that direct identification is limited by the lack of reference spectra of environmental strains in the MALDI BIOTYPER database. We proceeded by assigning isolates to classes, based on the similarity of their proteomic mass spectra, which yielded 18 distinct MALDI-TOF MS classes. For each class, sequencing of representative isolates consistently revealed the same closest relative and, with one exception, different closest relatives were found for different MALDI-TOF MS classes.

Thus the reliability of MALDI-TOF MS is comparable to PCR-based pre-screening methods for marine yeast isolates (Chen et al 2009, Gadanho et al 2003). Consequently, MALDI-TOF MS can be used to limit sequencing efforts in a rapid and cost-effective way. As results for clinical yeast isolates (Dhiman et al 2011, Marklein et al 2009, Stevenson et al 2010, van Veen et al 2010) as well as for environmental bacterial strains (Dieckmann et al 2005, Emami et al 2012, Oberbeckmann et al 2011a) are promising, an extension of the available database towards environmental yeasts is desirable.

Concerning yeast identities in the seawater sample, we predominantly found *Discosphaerina fagi*, which is a sexual-stage of the ascomycete filamentous fungus *Aureobasidium pullulans* (Zalar et al 2008), and the basidiomycete yeasts *Cryptococcus* sp. and *Candida sake*. *A. pullulans* and the genera *Cryptococcus* and *Candida* are typically found in marine environments, including the North Sea (Ahearn and Crow 1980, Crow et al 1977, Kutty and Philip 2008, Meyers et al 1967, Meyers and Ahearn 1974). Furthermore, we found various cold-adapted species in low numbers, which were *Leucosporidium scottii*, *Cystofilobasidium infirmominiatum*, *Guehomyces pullulans* and *Kondoa malvinella*. These species have been reported both from glacial environments and polar seawater or algae (Buzzini et al 2012, Fonseca et al 2000, Vaz et al 2011), but also from soil (Lim et al 2010, Maksimova and Chernov 2004) and associated with animals (Bruce and Morris 1973, Burgaud et al 2010, Galkiewicz et al 2012, Kobatake et al 1992, Zacchi and Vaughan-Martini 2002). Additionally, we found two yeast-like filamentous fungi related to *Ramularia pratensis* var. *pratensis* and *Tetracladium setigerum*, which are most likely of terrestrial origin (Anderson and Shearer 2011, Crous et al 2007). Except for *C. infirmominiatum*, these cold-adapted species have previously not been reported at Helgoland Roads, possibly because of differences in cultivation techniques. Furthermore, we found *R. mucilaginosa* and *D. hansenii* only after incubation, although they were frequently reported as the most abundant species in North Sea waters (Ahearn and Crow 1980, Meyers et al 1967).

In summary, the yeast community at Helgoland Roads at the sampling time in mid-April 2010 comprised predominantly typical marine species, various individually rare cold-adapted species and two terrestrial species, hinting at a coastal influence.

Concerning yeast abundance, we found 48 yeast colony forming units per liter (cfu L^{-1}) in the initial seawater sample, which is relatively low compared to previous records of <10 to >600 cfu L^{-1} (Gerdts et al 2004, Meyers et al 1967). Colony numbers obtained after incubation were low as well, which together with the overgrowth by filamentous fungi

prevented a quantitative analysis. Nevertheless, more yeast isolates and a higher number of different species were obtained at both pH 7.82 and at 7.67 than at pH *in situ*. Results are preliminary, but indicate that yeasts may reach higher abundance with ocean acidification. The possible health hazard of high yeast numbers in bathing waters has already been pointed out (Shah et al 2011, Vogel et al 2007) and a possible influence of ocean acidification underscores the need for yeast surveillance programs in recreational areas.

No isolates were found to be more characteristic for pH *in situ*, which would have suggested a negative effect of pH reduction on certain groups. In contrast, we found that *L. scottii*, *R. mucilaginosa* and related species, as well as *Cryptococcus* sp. and *D. hansenii* reacted positively to low pH, indicating that these yeasts may benefit from ocean acidification. For *L. scottii*, higher numbers of isolates were found with decreasing pH. The other groups were only found after incubation at pH 7.82 or 7.67. Notably, these groups comprise pathogenic species, with *Cryptococcus* infecting both marine mammals (Higgins 2000) and humans (Khawcharoenporn et al 2007) and *R. mucilaginosa* and *D. hansenii* considered as emerging yeast pathogens (Hazen 1995).

Interestingly, the yeasts we found at reduced pH predominantly belong to genera also present in extremely acidic environments such as the Rio Tinto River. There, the dominance of basidiomycete yeasts such as *Cryptococcus* sp., *Debaromyces hansenii*, *Leucosporidium scottii* and *Rhodotorula* species, including *Rhodotorula mucilaginosa*, was attributed to their greater tolerance of unfavorable environmental conditions (Gadanho et al 2006, López-Archilla et al 2004). Although pH levels in these environments (pH 2-3) are considerably lower than those expected due to ocean acidification, and consequently different cellular processes should be involved, the similarities detected are intriguing.

Initially, we also planned to characterize the yeast community in the microcosms with a culture-independent approach. We tried to establish a nested PCR (see Table 1 for primers) used for temperature gradient gel electrophoresis (TGGE) of marine yeast communities (Gadanho and Sampaio 2004) and planned to separate PCR products via DHPLC. We were not able to obtain specific PCR products in the first PCR of this method though. However, the planned 454 pyrosequencing approach mentioned above will hopefully also yield insights on the total yeast community present in the microcosms.

In summary, this thesis presents the first investigation of the culturable yeast community at Helgoland Roads using molecular methods and indicates that MALDI-TOF MS is a promising tool for the identification and classification of marine yeasts. The positive response of *L. scottii*, *R. mucilaginosa* and related species, *Cryptococcus* sp. and

D. hansenii to low pH indicated that these yeasts will profit from ocean acidification. Although results on abundances are preliminary, a trend towards higher yeast abundance with ocean acidification was detected as well.

TABLE 1. Additional primers used in this thesis

Target	Primer	Sequence	Reference
Bacteria	341f-GC	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3'	(Muyzer et al 1993)
	907r	5'-CCG TCA ATT CCT TTR AGT T-3'	(Muyzer et al 1995)
Eukarya	Euk1A	5'-CTG GTT GAT CCT GCC AG-3'	(Díez et al 2001)
	516r-GC	5'-ACC AGA CTT GCC CTC C GGG GGG CAC GGG GGC GGG GCG GGC CCC GCG CGG GGC CCG C -3'	(Díez et al 2001)
Archaea	340F-GC	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCC CTA YGG GGY GCA SCA G-3′	(Gantner et al 2011)
	1000R	5′-GGC CAT GCA CYW CYT CTC-3′	(Gantner et al 2011)
Fungi	ITS1FD	5'-GGT CAT TTA GWG GAA STA A-3'	(Gadanho and Sampaio 2004)
	LR6	5'-CGC CAG TTC TGC TTA CC-3'	(Fell 2001)

Conclusion

In this thesis, the effects of ocean acidification on marine bacterial and fungal communities were studied in highly replicated laboratory microcosm experiments. The high replication, also taking seasonal and year-to-year variability into account, and robust statistical analyses allowed for the detection of pH effects on both groups. Furthermore, we characterized the natural variability of the carbonate system at the study site.

The pH system at Helgoland Roads was found to be predominantly influenced by biological processes, leading to a pH range of 8.06 to 8.43 over a yearly-cycle, corresponding to pCO_2 from 215 to 526 µatm. This is in agreement with previous records from the southern North Sea and demonstrates that organisms at Helgoland Roads are currently not exposed to pH values lower than 8.0. The pH levels predicted for the southern North Sea for the year 2100 (pH 7.82 or 7.67), thus must be considered strong perturbations of the system. Concerning the implications of changes in pH however, it has to be kept in mind that the time-scale of our experiment, allowed for acclimation to new pH levels, which is defined as "qualitative or quantitative changes in gene expression", but presumably not adaptation, i.e. genetic change (Hurd et al 2009).

For the bacterial community, we observed shifts predominantly already at pH 7.82 while for the total fungal community, shifts were only observed at pH 7.67. These effects were found regardless of seasonal and dilution-induced (bacteria) or inter-annual (fungi) variability in community assembly. This indicates that in general, a tipping point for shifts in bacterial community structure is reached at smaller pH differences than for fungi. Concerning marine yeasts though, differences were already apparent between pH *in situ* and pH 7.82, although we were not able to confirm this based on a statistical analysis.

For bacteria, different members of *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Campylobacteraceae* and further less abundant groups were found to be involved in pH-dependent community shifts. The direction of the pH response, i.e. whether groups were more characteristic for present-day or near-future pH levels, was mostly context-dependent. Influencing factors may include seasonal assembly of certain groups, temperature or nutrient effects or effects of directly competing species. Concerning yeasts, we found that *Leucosporidium scottii*, *Rhodotorula mucilaginosa* and related species, as well as *Cryptococcus* sp. and *Debaromyces hansenii* reacted positively to low pH. This implies that for both bacteria and yeasts, groups possibly affected by ocean acidification comprise typical inhabitants of marine waters, such as *Rhodobacteraceae* in case of bacteria or *Rhodotorula*, *Debaromyces* and *Cryptococcus* in case of yeasts.

Furthermore, rare species such as *Campylobacteraceae* or *L. scottii* may profit from ocean acidification under certain conditions, possibly hinting at seed bank effects. Notable is also that with *Arcobacter*, *Cryptococcus* sp., *R. mucilaginosa* and *D. hansenii*, species that are potentially pathogenic to human and animal hosts were favored by a decrease in pH, highlighting the need for focused research efforts on these groups. Concerning total marine fungal community structure, a 454 pyrosequencing approach is currently planned to resolve identities as well.

Consistent with previous studies, no pH effect was found on bacterial abundances. In contrast, compared to pH *in situ*, fungal numbers were nine times higher at pH 7.82 and 34 times higher at pH 7.67. For marine yeasts, a similar trend was visible but not quantifiable. This indicates that fungal abundance may considerably increase with ocean acidification. Furthermore, it raises the question which underlying cellular mechanisms cause the great difference in the responses of both groups to pH reductions. As bacteria and fungi occupy different niches in marine microbial food webs, higher fungal abundance may affect biogeochemical processes. Here, experiments targeting both bacterial and fungal abundance and possibly also enzyme activities at the same time would be desirable. Furthermore, increases in fungal abundance also give rise to concern, considering that fungi infect both humans and marine organisms such as corals, fish, shellfish and crustaceans. Since calcifying organisms and juvenile stages of fishes are sensitive to ocean acidification per se, hosts weakened by pH-stress may be faced with a higher number of opportunistic fungal pathogens in a more acidic ocean.

This thesis focused on direct ocean acidification effects on marine bacterial and fungal communities and pointed out possible affected groups. The microcosm approach was extremely useful to separate effects and to gain insights into trends concerning community structure and abundances. As ocean acidification will also entail complex indirect effects, the responses observed can now be scrutinized in more complex experiments. If these studies will be carried out at sufficient replication as well, this will allow detecting whether the observed trends will persist or will be superimposed by indirect effects such as changing phytoplankton composition, increased photosynthesis or different grazing pressures.

SUMMARY

Anthropogenic CO_2 emissions are causing an acidification of the world's oceans. The consequences for marine organisms and especially heterotrophic bacteria remain under debate, and almost nothing is known concerning marine fungi. Both microbial groups are important players in organic matter decomposition and nutrient cycling, and their pH tolerance is known to be broad in relation to the predicted acidification. So far, ocean acidification effects on marine bacterial communities have mainly been investigated in large-scale mesocosm studies. In these systems, indirect effects mediated through complex food web interactions come into play. Until now, these experiments were not carried out in sufficient replication.

In this thesis, we chose an alternative approach and investigated bacterial and fungal communities in highly replicated microcosm experiments (1-1.6 L). The duration of the experiments was four weeks. We incubated the natural microbial community from Helgoland Roads (North Sea) at *in situ* seawater pH, pH 7.82 and pH 7.67. These pH levels represent the present-day situation and acidification at atmospheric CO_2 of 700 or 1000 ppm, projected for the southern North Sea for the year 2100. For the bacterial community, different dilution approaches were used to select for different ecological groups. Seasonality was accounted for by repeating the experiment four times (spring, summer, autumn, winter). In a second experiment repeated in two consecutive years, we investigated direct pH effects on marine fungal communities. We additionally isolated marine yeasts and identified them by Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and partial sequencing of the large subunit (LSU) rRNA gene.

To reveal changes in community structure, we applied the culture-independent fingerprint method automated ribosomal intergenic spacer analysis (ARISA) for both bacteria and fungi. Bacterial communities were furthermore analyzed by 16S ribosomal amplicon pyrosequencing. Abundances were determined by flow cytometry (bacteria) and colony forming unit counts (fungi). To be able to interpret results comprehensively, we determined the natural variability of the carbonate system at Helgoland Roads over a yearly cycle.

We found that from September 2010 to September 2011, pH at Helgoland Roads ranged from 8.06 to 8.43, corresponding to partial pressures of carbon dioxide (pCO_2) of 215-

526 µatm. The acidification predicted for the year 2100 consequently represents a strong perturbation of the system.

Bacterial communities developing in the microcosms were primarily influenced by season and dilution, demonstrating that diverse communities had been generated. We predominantly found pH-dependent shifts in bacterial community structure already at pH 7.82. Groups involved in these shifts were different members of *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Campylobacteraceae* and further less abundant groups. While *Rhodobacteraceae* were consistently less characteristic for reduced pH, *Campylobacteraceae* profited from pH reduction. For most other bacterial groups however, pH effects were context-dependent, i.e. dependent on season, dilution or an interaction of effects. Regarding bacterial abundance, no pH effect was found.

Fungal community structure was significantly different between both years of the experiment, hinting at inter-annual variability. Shifts in response to pH occurred predominantly only at pH 7.67. In contrast, a strong pH effect was observed on fungal abundance. In comparison to *in situ* pH, fungal numbers were on average 9 times higher at pH 7.82 and 34 times higher at pH 7.67. Concerning marine yeasts, *Leucosporidium scottii*, *Rhodotorula mucilaginosa* and related species, as well as *Cryptococcus* sp. and *Debaromyces hansenii* reacted positively to low pH.

Our findings demonstrate that already small reductions in pH have direct effects on both bacterial and fungal communities. A tipping point for community shifts appears to be reached earlier for bacteria than for fungi. Regarding bacteria and yeasts, both naturally abundant groups and rare species were affected by pH reductions. The strong increase in fungal numbers at reduced pH suggests that with ocean acidification, marine fungi may reach higher importance in marine biogeochemical cycles and as infectious agents.

Using a microcosm approach, a robust analysis of direct ocean acidification effects on marine bacterial and fungal communities was accomplished. Results yield valuable hypotheses to test in future large-scale and long-term studies.

ZUSAMMENFASSUNG

CO_2-Emmissionen anthropogenen Ursprings sind Hauptverursacher für die Versauerung der Weltmeere. Ihre Auswirkungen auf marine Organismen und speziell auf heterotrophe Bakterien sind unklar und es gibt bisher kaum Untersuchungen im Bezug auf marine Pilze. Sowohl Bakterien als auch Pilze spielen eine wichtige Rolle beim Abbau von organischem Material sowie in Nährstoffkreisläufen, und beide Gruppen tolerieren weite pH-Bereiche. Bisher wurden die Effekte der Ozeanversauerung auf marine Bakteriengemeinschaften vor allem in Mesokosmos-Studien untersucht. In diesen komplexen Systemen kommen indirekte Effekte durch Nahrungsnetz-Interaktionen ins Spiel. Diese Experimente lassen sich außerdem aus logistischen Gründen bisher nur gering repliziert durchführen.

In der vorliegenden Arbeit wurde daher ein alternativer Ansatz verfolgt und Ozeanversauerungs-Effekte auf Bakterien- und Pilzgemeinschaften in hochreplizierten, vierwöchigen Mikrokosmos-Experimenten (1-1.6 L) untersucht. Der Fokus lag auf den natürlichen mikrobiellen Gemeinschaften der Helgoländer Reede (Nordsee), die bei *in situ* pH, pH 7.82 und pH 7.67 inkubiert wurden. Diese pH-Abstufungen entsprechen der heutigen Situation sowie der für die südliche Nordsee für das Jahr 2100 vorausgesagten Versauerung bei atmosphärischen CO_2-Werten von 700 bzw. 1000 ppm. Für Bakteriengemeinschaften wurden verschiedene Verdünnungsansätze gewählt, um unterschiedliche ökologische Gruppen zu selektieren. Um saisonale Unterschiede zu berücksichtigen wurde das Experiment außerdem an vier Zeitpunkten des Jahres wiederholt. In einem zweiten Experiment in zwei aufeinanderfolgenden Jahren wurden direkte pH-Effekte auf marine Pilzgemeinschaften untersucht. Neben der Betrachtung der gesamten Pilzgemeinschaft wurden zusätzlich Hefen isoliert und mittels „Matrix-assisted laser desorption/ionization-time-of-flight“ Massenspektrometrie (MALDI-TOF MS) und partieller Sequenzierung des LSU rRNA-Gens identifiziert.

Um Änderungen der Gemeinschaftsstruktur der jeweiligen Gruppen aufzuklären wurde mit jeweils bakterien- bzw. pilzspezifischen Primern die kultivierungsunabhängige Fingerprint-Methode „automated ribosomal intergenic spacer analysis“ (ARISA) angewandt. Bakterielle Gemeinschaften wurden darüber hinaus mittels 16S rRNA-Gen-Pyrosequenzierung analysiert. Die Abundanzen wurden durch Durchflusszytometrie (Bakterien) und Koloniezahlbestimmung (Pilze) ermittelt. Um die Ergebnisse umfassend interpretieren zu können wurde zudem die natürliche Variabilität des Karbonat-Systems an der Helgoländer Reede über einen Jahresverlauf bestimmt.

Von September 2010 bis September 2011 variierte der pH an der Helgoländer Reede von 8.06 bis 8.43, was einem CO_2-Partialdruck (pCO_2) von 215-526 µtm entspricht. Demnach stellt die vorausgesagte Versauerung eine starke Veränderung des Systems dar.

Die Bakteriengemeinschaften in den Mikrokosmen waren vor allem von der Jahreszeit und dem Verdünnungsansatz abhängig. Darüber hinaus gab es einen signifikanten pH-Einfluss, überwiegend schon bei pH 7.82. Repräsentativ für diese Änderungen waren Mitglieder der *Gammaproteobacteria*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Campylobacteraceae* und weitere weniger abundante Gruppen. Während *Rhodobacteraceae* generell weniger charakteristisch für einen niedrigen pH waren, profitierten *Campylobacteraceae* von einer pH-Erniedrigung. Für die meisten anderen bakteriellen Gruppen waren die pH-Effekte dagegen Kontext-abhängig, d.h. abhängig von der Jahreszeit, dem Verdünnungsansatz oder einer Wechselwirkung mehrerer Effekte. Unterschiedliche pH-Werte hatten keinen Effekt auf die Bakterienzahlen.

Die Struktur der Pilzgemeinschaft unterschied sich in beiden Jahren signifikant. pH-abhängige Änderungen traten in den meisten Fällen allerdings erst bei pH 7.67 auf. Im Gegensatz dazu wurde ein starker pH-Effekt auf die Abundanzen der Pilze beobachtet. Gegenüber dem *in situ* pH waren diese im Durchschnitt bei pH 7.82 um den Faktor neun und bei pH 7.67 um den Faktor 34 erhöht. Unter den marinen Hefen reagierten *Leucosporidium scottii*, *Rhodotorula mucilaginosa* und verwandte Arten, *Cryptococcus* sp. und *Debaromyces hansenii* positiv auf einen niedrigeren pH-Wert.

Die Ergebnisse zeigen dass schon eine geringe Erniedrigung des pH-Wertes einen direkten Effekt auf sowohl Bakterien- als auch Pilzgemeinschaften hat. Hierbei scheint eine Änderung der Bakteriengemeinschaft im Gegensatz zu Pilzen bereits bei geringeren pH-Unterschieden aufzutreten. Eine Erniedrigung des pH-Wertes hatte bei Bakterien und Hefen Auswirkungen sowohl auf natürlich abundante als auch auf seltene Arten. Die Zunahme von Pilzen weist darüber hinaus darauf hin, dass marine Pilze durch die Ozeanversauerung sowohl in marinen biogeochemischen Stoffkreisläufen an Bedeutung gewinnen könnten, als auch als Krankheitserreger eine wichtigere Rolle spielen könnten.

Im Rahmen eines hoch-replizierten Mikrokosmos-Ansatzes wurde eine robuste Analyse der direkten Ozeanversauerungs-Effekte auf marine Bakterien- und Pilzgemeinschaften erreicht. Die Resultate führen zu wertvollen Hypothesen, die in zukünftigen Mesokosmos und Langzeit-Experimenten getestet werden sollten.

REFERENCES

Ahearn DG, Crow SA (1980). Yeasts from the North Sea and Amoco Cadiz oil. *Bot Mar* **23:** 125-127.

Allgaier M, Riebesell U, Vogt M, Thyrhaug R, Grossart HP (2008). Coupling of heterotrophic bacteria to phytoplankton bloom development at different pCO_2 levels: a mesocosm study. *Biogeosciences* **5:** 1007-1022.

Amend AS, Barshis DJ, Oliver TA (2012). Coral-associated marine fungi form novel lineages and heterogeneous assemblages. *ISME J* **6:** 1291-1301.

Ammerman JW, Fuhrman JA, Hagstrom A, Azam F (1984). Bacterioplankton growth in seawater: I. growth kinetics and cellular characteristics in seawater cultures. *Mar Ecol Prog Ser* **18:** 31-39.

Anderson JL, Shearer CA (2011). Population genetics of the aquatic fungus *Tetracladium marchalianum* over space and time. *PLoS ONE* **6:** e15908.

Anderson MJ (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **26:** 32-46.

Andersson AF, Riemann L, Bertilsson S (2010). Pyrosequencing reveals contrasting seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. *ISME J* **4:** 171-181.

Arfi Y, Buée M, Marchand C, Levasseur A, Record E (2012). Multiple markers pyrosequencing reveals highly diverse and host-specific fungal communities on the mangrove trees *Avicennia marina* and *Rhizophora stylosa*. *FEMS Microbiol Ecol* **79:** 433-444.

Atlas RM, Horowitz A, Krichevsky M, Bej AK (1991). Response of microbial populations to environmental disturbance. *Microb Ecol* **22:** 249-256.

Avis PG, Branco S, Tang Y, Mueller GM (2010). Pooled samples bias fungal community descriptions. *Mol Ecol Resour* **10:** 135-141.

Azam F, Fenchel T, Field JG, Gray JS, Meyer-Reil LA, Thingstad F (1983). The ecological role of water-column microbes in the sea. *Mar Ecol Prog Ser* **10:** 257-263.

Baker-Austin C, Stockley L, Rangdale R, Martinez-Urtaza J (2010). Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. *Environ Microbiol Rep* **2:** 7-18.

Baker-Austin C, Trinanes JA, Taylor NGH, Hartnell R, Siitonen A, Martinez-Urtaza J (2012). Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nat Clim Chang***:** in press.

Beardsley C, Pernthaler J, Wosniok W, Amann R (2003). Are readily culturable bacteria in coastal North Sea waters suppressed by selective grazing mortality? *Appl Environ Microbiol* **69:** 2624-2630.

Blackford JC, Gilbert FJ (2007). pH variability and CO_2 induced acidification in the North Sea. *J Mar Syst* **64:** 229-241.

Boguslawska-Was E, Dabrowski W (2001). The seasonal variability of yeasts and yeast-like organisms in water and bottom sediment of the Szczecin Lagoon. *Int J Hyg Envir Heal* **203:** 451-458.

Bradshaw AL, Brewer PG, Shafer DK, Williams RT (1981). Measurements of total carbon dioxide and alkalinity by potentiometric titration in the GEOSECS program. *Earth Planet Sci Lett* **55:** 99-115.

Brandão LR, Libkind D, Vaz ABM, Espírito Santo LC, Moliné M, de García V *et al* (2011). Yeasts from an oligotrophic lake in Patagonia (Argentina): diversity, distribution and synthesis of photoprotective compounds and extracellular enzymes. *FEMS Microbiol Ecol* **76:** 1-13.

Brasse S, Reimer A, Seifert R, Michaelis W (1999). The influence of intertidal mudflats on the dissolved inorganic carbon and total alkalinity distribution in the German Bight, southeastern North Sea. *J Sea Res* **42:** 93-103.

Brilhante RSN, Paiva MAN, Sampaio CMS, Teixeira CEC, Castelo-Branco DSCM, Leite JJG *et al* (2011). Yeasts from *Macrobrachium amazonicum*: a focus on antifungal susceptibility and virulence factors of *Candida* spp. *FEMS Microbiol Ecol* **76:** 268-277.

Brown MV, Schwalbach MS, Hewson I, Fuhrman JA (2005). Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7:** 1466-1479.

Bruce J, Morris EO (1973). Psychrophilic yeasts isolated from marine fish. *Antonie Van Leeuwenhoek* **39:** 331-339.

Bugni TS, Ireland CM (2004). Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat Prod Rep* **21:** 143-163.

Burgaud G, Arzur D, Durand L, Cambon-Bonavita MA, Barbier G (2010). Marine culturable yeasts in deep-sea hydrothermal vents: species richness and association with fauna. *FEMS Microbiol Ecol* **73:** 121-133.

Button DK, Schut F, Quang P, Martin R, Robertson BR (1993). Viability and isolation of marine bacteria by dilution culture: theory, procedures, and inital results. *Appl Environ Microbiol* **59:** 881-891.

Buzzini P, Branda E, Goretti M, Turchetti B (2012). Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. *FEMS Microbiol Ecol* **82:** 217-241.

Caldeira K, Wickett ME (2003). Anthropogenic carbon and ocean pH. *Nature* **425:** 365-365.

Caporaso JG, Paszkiewicz K, Field D, Knight R, Gilbert JA (2012). The Western English Channel contains a persistent microbial seed bank. *ISME J* **6:** 1089-1093.

Carmelo V, Santos H, Sá-Correia I (1997). Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1325:** 63-70.

Chemidlin Prévost-Bouré N, Christen R, Dequiedt S, Mougel C, Lelièvre M, Jolivet C *et al* (2011). Validation and application of a PCR primer set to quantify fungal communities in the soil environment by real-time quantitative PCR. *PLoS ONE* **6:** e24166.

Chen YS, Yanagida F, Chen LY (2009). Isolation of marine yeasts from coastal waters of northeastern Taiwan. *Aquat Biol* **8:** 55-60.

Coelho MA, Almeida JMF, Martins IM, da Silva AJ, Sampaio JP (2010). The dynamics of the yeast community of the Tagus river estuary: testing the hypothesis of the multiple origins of estuarine yeasts. *Antonie Van Leeuwenhoek* **98:** 331-342.

Collado L, Inza I, Guarro J, Figueras MJ (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ Microbiol* **10:** 1635-1640.

Collado L, Figueras MJ (2011). Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. *Clin Microbiol Rev* **24:** 174-192.

Colwell RR (1996). Global climate and infectious disease: the cholera paradigm. *Science* **274:** 2025-2031.

Connell JH (1978). Diversity in tropical rain forests and coral reefs. *Science* **199:** 1302-1310.

Crosby LD, Criddle CS (2003). Understanding bias in microbial community analysis techniques due to rrn operon copy number heterogeneity. *Biotechniques* **34:** 790-802.

Crous PW, Braun U, Groenewald JZ (2007). *Mycosphaerella* is polyphyletic. *Stud Mycol* **58:** 1-32.

Crow SA, Bowman PI, Ahearn DG (1977). Isolation of atypical *Candida albicans* from the North Sea. *Appl Environ Microbiol* **33:** 738-739.

D'Sa EM, Harrison MA (2005). Effect of pH, NaCl content, and temperature on growth and survival of *Arcobacter* spp. *J Food Protect* **68:** 18-25.

Danovaro R, Corinaldesi C, Dell'Anno A, Fuhrman JA, Middelburg JJ, Noble RT *et al* (2011). Marine viruses and global climate change. *FEMS Microbiol Rev* **35:** 993-1034.

Davis DA (2009). How human pathogenic fungi sense and adapt to pH: the link to virulence. *Curr Opin Microbiol* **12:** 365-370.

de Araujo FV, Soares CAG, Hagler AN, Mendonça-Hagler LC (1995). Ascomycetous yeast communities of marine invertebrates in a Southeast Brazilian mangrove ecosystem. *Antonie Van Leeuwenhoek* **68:** 91-99.

Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL (2011). Performance and cost analysis of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for routine identification of yeast. *J Clin Microbiol* **49:** 1614-1616.

Diakov TT, Kane PM (2010). Regulation of vacuolar proton-translocating ATPase activity and assembly by extracellular pH. *J Biol Chem* **285:** 23771-23778.

Dickson AG, Millero FJ (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Res A* **34:** 1733–1743.

Dickson AG (1990). Standard potential of the reaction: $AgCl(s)+1/2H_2(g)=Ag(s)+HCl(aq)$, and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *J Chem Thermodyn* **22:** 113-127.

Dieckmann R, Graeber I, Kaesler I, Szewzyk U, von Döhren H (2005). Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by Intact-Cell-MALDI-TOF mass spectrometry (ICM-MS). *Appl Microbiol Biotechnol* **67:** 539-548.

Díez B, Pedrós-Alió C, Marsh TL, Massana R (2001). Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. *Appl Environ Microbiol* **67:** 2942-2951.

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009). Ocean acidification: the other CO_2 problem. *Ann Rev Mar Sci* **1:** 169-192.

Dupont S, Dorey N, Thorndyke M (2010). What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? *Estuar Coast Shelf Sci* **89:** 182-185.

Eilers H, Pernthaler J, Amann R (2000a). Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. *Appl Environ Microbiol* **66:** 4634-4640.

Eilers H, Pernthaler J, Glöckner FO, Amann R (2000b). Culturability and in situ abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66:** 3044-3051.

Eilers H, Pernthaler J, Peplies J, Glöckner FO, Gerdts G, Amann R (2001). Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. *Appl Environ Microbiol* **67:** 5134-5142.

Emami K, Askari V, Ullrich M, Mohinudeen K, Anil AC, Khandeparker L *et al* (2012). Characterization of bacteria in ballast water using MALDI-TOF mass spectrometry. *PLoS ONE* **7:** e38515.

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008). Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science* **320:** 1490-1492.

Feely RA, Doney SC, Cooley SR (2009). Ocean acidification: present conditions and future changes in a high-CO_2 world. *Oceanography* **22:** 36-47.

Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50:** 1351-1371.

Fell JW (2001). Collection and identification of marine yeasts. In: Paul J (ed). *Methods in microbiology*, Vol. 30. Academic Press: New York. pp 347–356.

Felsenstein J (1993). PHYLIP (Phylogeny Inference Package). 3.5c ed.

Fera MT, Maugeri TL, Gugliandolo C, Beninati C, Giannone M, La Camera E *et al* (2004). Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean Sea. *Appl Environ Microbiol* **70:** 1271-1276.

Ferguson RL, Buckley EN, Palumbo AV (1984). Response of marine bacterioplankton to differential filtration and confinement. *Appl Environ Microbiol* **47:** 49-55.

Fonseca Á, Sampaio JP, Inácio J, Fell JW (2000). Emendation of the basidiomycetous yeast genus *Kondoa* and the description of *Kondoa aeria* sp. nov. *Antonie Van Leeuwenhoek* **77:** 293-302.

Fortunato CS, Herfort L, Zuber P, Baptista AM, Crump BC (2012). Spatial variability overwhelms seasonal patterns in bacterioplankton communities across a river to ocean gradient. *ISME J* **6:** 554-563.

Freitas S, Hatosy S, Fuhrman JA, Huse SM, Mark Welch DB, Sogin ML *et al* (2012). Global distribution and diversity of marine *Verrucomicrobia*. *ISME J* **6:** 1499-1505.

Frommel AY, Maneja R, Lowe D, Malzahn AM, Geffen AJ, Folkvord A *et al* (2012). Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. *Nat Clim Change* **2:** 42-46.

Fuchs BM, Zubkov MV, Sahm K, Burkill PH, Amann R (2000). Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2:** 191-201.

Fuhrman JA, Hewson I, Schwalbach MS, Steele JA, Brown MV, Naeem S (2006). Annually reoccurring bacterial communities are predictable from ocean conditions. *Proc Nat Acad Sci USA* **103:** 13104-13109.

Fuhrman JA, Hagström Å (2008). Bacterial and archaeal community structure and its patterns. In: Kirchman DL (ed). *Microbial ecology of the oceans*, 2 edn. John Wiley & Sons, Inc.: New York. pp 45-90.

Fuhrman JA, Steele JA, Hewson I, Schwalbach MS, Brown MV, Green JL *et al* (2008). A latitudinal diversity gradient in planktonic marine bacteria. *Proc Nat Acad Sci USA* **105:** 7774-7778.

Fuhrman JA (2009). Microbial community structure and its functional implications. *Nature* **459:** 193-199.

Gadanho M, Sampaio JP (2002). Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodotorula*: *Rh. glutinis* sensu stricto and *Rh. dairenensis* comb. nov. *FEMS Yeast Res* **2:** 47-58.

Gadanho M, Almeida JMGCF, Sampaio JP (2003). Assessment of yeast diversity in a marine environment in the south of Portugal by microsatellite-primed PCR. *Antonie Van Leeuwenhoek* **84:** 217-227.

Gadanho M, Sampaio JP (2004). Application of temperature gradient gel electrophoresis to the study of yeast diversity in the estuary of the Tagus river, Portugal. *FEMS Yeast Res* **5:** 253-261.

Gadanho M, Sampaio JP (2005). Occurrence and diversity of yeasts in the Mid-Atlantic ridge hydrothermal fields near the Azores Archipelago. *Microb Ecol* **50:** 408-417.

Gadanho M, Libkind D, Sampaio JP (2006). Yeast diversity in the extreme acidic environments of the Iberian Pyrite Belt. *Microb Ecol* **52:** 552-563.

Galkiewicz JP, Stellick SH, Gray MA, Kellogg CA (2012). Cultured fungal associates from the deep-sea coral *Lophelia pertusa*. *Deep Sea Res Part 1 Oceanogr Res Pap* **67:** 12-20.

Gantner S, Andersson AF, Alonso-Saéz L, Bertilsson S (2011). Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. *J Microbiol Meth* **84:** 12-18.

Gao Z, Johnson ZI, Wang G (2010). Molecular characterization of the spatial diversity and novel lineages of mycoplankton in Hawaiian coastal waters. *ISME J* **4:** 111-120.

Gattuso JP, Lavigne H (2009). Technical note: approaches and software tools to investigate the impact of ocean acidification. *Biogeosciences* **6:** 2121-2133.

Gerdts G, Wichels A, Döpke H, Klings KW, Gunkel W, Schütt C (2004). 40-year long-term study of microbial parameters near Helgoland (German Bight, North Sea): historical view and future perspectives. *Helgol Mar Res* **58:** 230-242.

Gilbert JA, Field D, Huang Y, Edwards R, Li WZ, Gilna P *et al* (2008). Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities. *PLoS ONE* **3:** e3042.

Gilbert JA, Steele JA, Caporaso JG, Steinbrueck L, Reeder J, Temperton B *et al* (2012). Defining seasonal marine microbial community dynamics. *ISME J* **6:** 298-308.

Giovannoni SJ, Rappé M (2000). Evolution, diversity, and molecular ecology of marine prokaryotes. In: Kirchman DL (ed). *Microbial ecology of the oceans*, 1 edn. John Wiley & Sons, Inc.: New York. pp 47-84.

Giovannoni SJ, Stingl U (2005). Molecular diversity and ecology of microbial plankton. *Nature* **437:** 343-348.

Gleason FH, Küpper FC, Amon JP, Picard K, Gachon CMM, Marano AV *et al* (2011). Zoosporic true fungi in marine ecosystems: a review. *Mar Freshw Res* **62:** 383-393.

Goldman JC, Dennett MR (1985). Susceptibility of some marine-phytoplankton species to cell breakage during filtration and post-filtration rinsing. *J Exp Mar Bio Ecol* **86:** 47-58.

Golubic S, Radtke G, Le Campion-Alsumard T (2005). Endolithic fungi in marine ecosystems. *Trends Microbiol* **13:** 229-235.

Grossart HP, Allgaier M, Passow U, Riebesell U (2006). Testing the effect of CO_2 concentration on the dynamics of marine heterotrophic bacterioplankton. *Limnol Oceanogr* **51:** 1-11.

Gutiérrez MH, Pantoja S, Quiñones RA, González RR (2010). First record of filamentous fungi in the coastal upwelling ecosystem off central Chile. *Gayana* **74:** 66-73.

Gutiérrez MH, Pantoja S, Tejos E, Quiñones RA (2011). The role of fungi in processing marine organic matter in the upwelling ecosystem off Chile. *Mar Biol* **158:** 205-219.

Hagler AN, Ahearn DG (1987). Ecology of aquatic yeasts. In: Rose AH, Harrison JS (eds). *The Yeasts*. Academic Press: London. pp 181-205.

Hammes F, Vital M, Egli T (2010). Critical evaluation of the volumetric "bottle effect" on microbial batch growth. *Appl Environ Microbiol* **76:** 1278-1281.

Hatai K, Egusa S (1975). *Candida sake* from gastro-tympanites of amago, *Onchorynchus rhodurus*. *Bull Jpn Soc Sci Fish* **41:** 993-993.

Hazen KC (1995). New and emerging yeast pathogens. *Clin Microbiol Rev* **8:** 462-478.

Hendriks IE, Duarte CM, Álvarez M (2010). Vulnerability of marine biodiversity to ocean acidification: a meta-analysis. *Estuar Coast Shelf Sci* **86:** 157-164.

Higgins R (2000). Bacteria and fungi of marine mammals: a review. *Can Vet J* **41:** 105-116.

Hofmann GE, Smith JE, Johnson KS, Send U, Levin LA, Micheli F *et al* (2011). High-frequency dynamics of ocean pH: a multi-ecosystem comparison. *PLoS ONE* **6:** e28983.

Hoppe CJM, Langer G, Rost B (2011). *Emiliania huxleyi* shows identical responses to elevated pCO_2 in TA and DIC manipulations. *J Exp Mar Biol Ecol* **406:** 54-62.

Hoppe HG (1972). Untersuchungen zur Ökologie der Hefen im Bereich der westlichen Ostsee. *Kiel Meeresforsch* **28:** 54-77.

Hurd CL, Hepburn CD, Currie KI, Raven JA, Hunter KA (2009). Testing the effects of ocean acidification on algal metabolism: considerations for experimental designs. *J Phycol* **45:** 1236-1251.

Hyde KD, Jones EBG, Leano E, Pointing SB, Poonyth AD, Vrijmoed LLP (1998). Role of fungi in marine ecosystems. *Biodivers Conserv* **7:** 1147-1161.

Jobard M, Rasconi S, Sime-Ngando T (2010). Diversity and functions of microscopic fungi: a missing component in pelagic food webs. *Aquat Sci* **72:** 255-268.

Joint I, Doney SC, Karl DM (2011). Will ocean acidification affect marine microbes? *ISME J* **5:** 1-7.

Jones EBG (2011). Are there more marine fungi to be described? *Bot Mar* **54:** 343-354.

Kawakita S, van Uden N (1965). Occurrence and population densities of yeast species in the digestive tracts of gulls and terns. *J Gen Microbiol* **39:** 125-129.

Kempe S (1996). Coastal seas: a net source or sink of atmospheric carbon dioxide? LOICZ/R&S/95-1. LOICZ: Texel.

Khawcharoenporn T, Apisarnthanarak A, Mundy LM (2007). Non-*neoformans* cryptococcal infections: a systematic review. *Infection* **35:** 51-58.

Kirchman DL (2008). Introduction and overview. In: Kirchman DL (ed). *Microbial ecology of the oceans*, 2 edn. John Wiley & Sons, Inc.: New York. pp 1-26.

Kirchman DL, Cottrell MT, Lovejoy C (2010). The structure of bacterial communities in the western Arctic Ocean as revealed by pyrosequencing of 16S rRNA genes. *Environ Microbiol* **12:** 1132-1143.

Kobatake M, Kreger van Rij NJW, Plácido MTLC, Van Uden N (1992). Isolation of proteolytic psychrotrophic yeasts from fresh raw seafoods. *Lett Appl Microbiol* **14:** 37-42.

Kohlmeyer J, Kohlmeyer E (1979). *Marine mycology: the higher fungi*. Academic Press: New York.

Kovacs A, Yacoby K, Gophna U (2010). A systematic assessment of automated ribosomal intergenic spacer analysis (ARISA) as a tool for estimating bacterial richness. *Res Microbiol* **161:** 192-197.

Krause E, Wichels A, Giménez L, Lunau M, Schilhabel MB, Gerdts G (2012). Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PLoS ONE* **7:** e47035.

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DO, Fenical W (2003). Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. *P Natl Acad Sci USA* **100:** 6916-6921.

Kunin V, Engelbrektson A, Ochman H, Hugenholtz P (2010). Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environ Microbiol* **12:** 118-123.

Kurtzman CP, Robnett CJ (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73:** 331-371.

Kurtzman CP (2006). Yeast species recognition from gene sequence analyses and other molecular methods. *Mycoscience* **47:** 65-71.

Kutty SN, Philip R (2008). Marine yeasts - a review. *Yeast* **25:** 465-483.

Legendre P, Legendre L (1998). *Numerical ecology*, 2 edn. Elsevier Science B.V.: Amsterdam.

Lidbury I, Johnson V, Hall-Spencer JM, Munn CB, Cunliffe M (2012). Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. *Mar Pollut Bull* **64:** 1063-1066.

Lim YW, Kim BK, Kim C, Jung HS, Kim BS, Lee JH *et al* (2010). Assessment of soil fungal communities using pyrosequencing. *J Microbiol* **48:** 284-289.

Liu JW, Weinbauer MG, Maier C, Dai M, Gattuso JP (2010). Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquat Microb Ecol* **61:** 291-305.

López-Archilla AI, González AE, Terrón MC, Amils R (2004). Ecological study of the fungal populations of the acidic Tinto River in southwestern Spain. *Can J Microbiol* **50:** 923-934.

Lorkowski I, Pätsch J, Moll A, Kühn W (2012). Interannual variability of carbon fluxes in the North Sea from 1970 to 2006 - Competing effects of abiotic and biotic drivers on the gas-exchange of CO_2. *Estuar Coast Shelf Sci* **100:** 38-57.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363-1371.

Maksimova IA, Chernov IY (2004). Community structure of yeast fungi in forest biogeocenoses. *Microbiology* **73:** 474-481.

Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T *et al* (2009). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for fast and reliable identification of clinical yeast isolates. *J Clin Microbiol* **47:** 2912-2917.

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol Oceanogr* **18:** 897-907.

Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P *et al* (2008). Evaluation of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass

Spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol* **46:** 1946-1954.

Meron D, Atias E, Kruh LI, Elifantz H, Minz D, Fine M *et al* (2011). The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. *ISME J* **5:** 51-60.

Meron D, Rodolfo-Metalpa R, Cunning R, Baker AC, Fine M, Banin E (2012). Changes in coral microbial communities in response to a natural pH gradient. *ISME J* **6:** 1775-1785.

Meyers SP, Ahearn DG, Gunkel W, Roth FJJ (1967). Yeasts from the North Sea. *Mar Biol* **1:** 118-123.

Meyers SP, Ahearn DG (1974). Implication of yeasts and yeast-like fungi in marine processes. *Veröff Inst Meeresforsch Bremerh* **Suppl. 5:** 321-338.

Murphy C, Carroll C, Jordan KN (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J Appl Microbiol* **100:** 623-632.

Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. *Appl Environ Microbiol* **59:** 695-700.

Muyzer G, Hottenträger S, Teske A, Wawer C (1995). Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA-a new molecular approach to analyze the genetic diversity of mixed microbial communities. In: Akkermans ADL, van Elsas JD, de Bruijn FD (eds.). *Molecular microbial ecology manual.* Kluwer Academic Publishers: Dordrecht. pp 1-22.

Newbold LK, Oliver AE, Booth T, Tiwari B, Desantis T, Maguire M *et al* (2012). The response of marine picoplankton to ocean acidification. *Environ Microbiol* **14:** 2293–2307.

Norkrans B (1966). Studies on marine occurring yeasts: growth related to pH, NaCl concentration and temperature. *Arch Mikrobiol* **54:** 374-392.

Oberbeckmann S, Wichels A, Maier T, Kostrzewa M, Raffelberg S, Gerdts G (2011a). A polyphasic approach for the differentiation of environmental *Vibrio* isolates from temperate waters. *FEMS Microbiol Ecol* **75:** 145-162.

Oberbeckmann S, Wichels A, Wiltshire KH, Gerdts G (2011b). Occurrence of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in the German Bight over a seasonal cycle. *Antonie Van Leeuwenhoek* **100:** 291-307.

Orij R, Postmus J, Ter Beek A, Brul S, Smits GJ (2009). *In vivo* measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth. *Microbiology* **155:** 268-278.

Orij R, Brul S, Smits GJ (2011). Intracellular pH is a tightly controlled signal in yeast. *Biochim Biophys Acta* **1810:** 933-944.

Padan E, Bibi E, Ito M, Krulwich TA (2005). Alkaline pH homeostasis in bacteria: new insights. *Biochim Biophys Acta* **1717:** 67-88.

Patel KS (1975). The relationship between yeasts and marine algae. *Proc Indian Acad Sci* **82 B:** 25-28.

Paz S, Bisharat N, Paz E, Kidar O, Cohen D (2007). Climate change and the emergence of *Vibrio vulnificus* disease in Israel. *Environ Res* **103:** 390-396.

Pearson PN, Palmer MR (2000). Atmospheric carbon dioxide concentrations over the past 60 million years. *Nature* **406:** 695-699.

Pedrós-Alió C (2006). Marine microbial diversity: can it be determined? *Trends Microbiol* **14:** 257-263.

Pernthaler J, Amann R (2005). Fate of heterotrophic microbes in pelagic habitats: focus on populations. *Microbiol Mol Biol Rev* **69:** 440-461.

Pinhassi J, Berman T (2003). Differential growth response of colony-forming α- and γ-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Appl Environ Microbiol* **69:** 199-211.

Piontek J, Lunau M, Händel N, Borchard C, Wurst M, Engel A (2010). Acidification increases microbial polysaccharide degradation in the ocean. *Biogeosciences* **7:** 1615-1624.

Pomeroy LR (1974). The ocean's food web: a changing paradigm. *Bioscience* **24:** 499-504.

Pomeroy LR, Wiebe WJ (1993). Energy sources for microbial food webs *Aquat Microb Ecol* **7:** 101-118.

Pomeroy LR, Williams PJI, Azam F, Hobbie JE (2007). The microbial loop. *Oceanography* **20:** 28-33.

Raghukumar S (2004). The role of fungi in marine detrital processes. In: Ramaiah N (ed). *Marine microbiology: facets & opportunities*. National Institute of Oceanography: Goa. pp 91-101.

Ramaiah N (2006). A review on fungal diseases of algae, marine fishes, shrimps and corals. *Indian J Mar Sci* **35:** 380-387.

Ranjard L, Brothier E, Nazaret S (2000). Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl Environ Microbiol* **66:** 5334-5339.

Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Appl Environ Microbiol* **67:** 4479-4487.

Rappé MS, Connon SA, Vergin KL, Giovannoni SJ (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418:** 630-633.

Raven J, Caldeira K, Elderfield H, Hoegh-Guldberg O, Liss P, Riebesell U (2005). Ocean acidification due to increasing atmospheric carbon dioxide. Policy Document 12/05. Roy. Soc. Rep. 12.

Ravindran J, Raghukumar C, Raghukumar S (2001). Fungi in *Porites lutea*: association with healthy and diseased corals. *Dis Aquat Org* **47:** 219-228.

Ray JL, Töpper B, An S, Silyakova A, Spindelböck J, Thyrhaug R *et al* (2012). Effect of increased pCO_2 on bacterial assemblage shifts in response to glucose addition in Fram Strait seawater mesocosms. *FEMS Microbiol Ecol***:** in press.

Reid AN, Pandey R, Palyada K, Naikare H, Stintzi A (2008). Identification of *Campylobacter jejuni* genes involved in the response to acidic pH and stomach transit. *Appl Environ Microbiol* **74:** 1583-1597.

Richards T, Jones M, Leonard G, Bass D (2012). Marine fungi: their ecology and molecular diversity. *Ann Rev Mar Sci* **4:** 495-522.

Riebesell U, Schulz KG, Bellerby RGJ, Botros M, Fritsche P, Meyerhöfer M *et al* (2007). Enhanced biological carbon consumption in a high CO_2 ocean. *Nature* **450:** 545-549.

Riebesell U, Czerny J, von Bröckel K, Boxhammer T, Büdenbender J, Deckelnick M *et al* (2012). Technical note: a mobile sea-going mesocosm system - new opportunities for ocean change research. *Biogeosci Discuss* **9:** 12985-13017.

Robbins LL, Hansen ME, Kleypas JA, Meylan SC (2010). CO2calc - A user-friendly seawater carbon calculator for Windows, Mac OS X, and iOS (iPhone). U.S. Geological Survey Open-File Report 2010–1280.

Rothschild LJ, Mancinelli RL (2001). Life in extreme environments. *Nature* **409:** 1092-1101.

Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL *et al* (2004). The oceanic sink for anthropogenic CO_2. *Science* **305:** 367-371.

Sadaba RB, Sarinas BGS (2010). Fungal communities in bunker C oil-impacted sites off southern Guimaras, Philippines: a post-spill assessment of Solar 1 oil spill. *Bot Mar* **53:** 565-575.

Sapp M, Wichels A, Wiltshire KH, Gerdts G (2007). Bacterial community dynamics during the winter-spring transition in the North Sea. *FEMS Microbiol Ecol* **59:** 622-637.

Schaumann K, Priebe K (1994). *Ochroconis humicola* causing muscular black spot disease of Atlantic Salmon (*Salmo Salar*). *Can J Bot* **72:** 1629-1634.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB *et al* (2009). Introducing mothur: open-source, platform-independent, community-supported software

for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537-7541.

Schloss PD, Gevers D, Westcott SL (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PloS ONE* **6:** e27310.

Schneider K, Erez J (2006). The effect of carbonate chemistry on calcification and photosynthesis in the hermatypic coral *Acropora eurystoma*. *Limnol Oceanogr* **51:** 1284-1293.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA *et al* (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *P Natl Acad Sci USA* **109:** 6241-6246.

Schulz KG, Barcelos e Ramos JBE, Zeebe RE, Riebesell U (2009). CO_2 perturbation experiments: similarities and differences between dissolved inorganic carbon and total alkalinity manipulations. *Biogeosciences* **6:** 2145-2153.

Schut F, Devries EJ, Gottschal JC, Robertson BR, Harder W, Prins RA *et al* (1993). Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. *Appl Environ Microbiol* **59:** 2150-2160.

Seki H, Fulton J (1969). Infection of marine copepods by *Metschnikowia* sp. *Mycopathologia* **38:** 61-70.

Selvig K, Alspaugh JA (2011). pH response pathways in fungi: adapting to host-derived and environmental signals. *Mycobiology* **39:** 249-256.

Seshadri R, Sieburth JM (1971). Cultural estimation of yeasts on seaweeds. *Appl Microbiol* **22:** 507-512.

Shah AH, Abdelzaher AM, Phillips M, Hernandez R, Solo-Gabriele HM, Kish J *et al* (2011). Indicator microbes correlate with pathogenic bacteria, yeasts and helminthes in sand at a subtropical recreational beach site. *J Appl Microbiol* **110:** 1571-1583.

Sherr E, Sherr B (2008). Understanding roles of microbes in marine pelagic food webs: a brief history. In: Kirchman DL (ed). *Microbial ecology of the oceans*, 2 edn. John Wiley & Sons, Inc.: New York. pp 27-44.

Shi D, Xu Y, Hopkinson BM, Morel FMM (2010). Effect of ocean acidification on iron availability to marine phytoplankton. *Science* **327:** 676-679.

Snelling WJ, Matsuda M, Moore JE, Dooley JSG (2006). Under the microscope: *Arcobacter*. *Lett Appl Microbiol* **42:** 7-14.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR *et al* (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *P Natl Acad Sci USA* **103:** 12115-12120.

Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR (2010). Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for identification of clinically important yeast species. *J Clin Microbiol* **48:** 3482-3486.

Teeling H, Fuchs BM, Becher D, Klockow C, Gardebrecht A, Bennke CM *et al* (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336:** 608-611.

Teira E, Fernández A, Álvarez-Salgado XA, García-Martín EE, Serret P, Sobrino C (2012). Response of two marine bacterial isolates to high CO_2 concentration. *Mar Ecol Prog Ser* **453:** 27-36.

Thomas H, Bozec Y, Elkalay K, de Baar HJW (2004). Enhanced open ocean storage of CO_2 from shelf sea pumping. *Science* **304:** 1005-1008.

Thompson FL, Iida T, Jean Swings J (2004). Biodiversity of Vibrios. *Microbiol Mol Biol Rev* **68:** 403-431.

Thomsen J, Gutowska MA, Saphörster J, Heinemann A, Trübenbach K, Fietzke J *et al* (2010). Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* **7:** 3879-3891.

Torzilli AP, Sikaroodi M, Chalkley D, Gillevet PM (2006). A comparison of fungal communities from four salt marsh plants using automated ribosomal intergenic spacer analysis (ARISA). *Mycologia* **98:** 690-698.

Underwood AJ (1996). *Experiments in ecology - their logical design and interpretation using analysis of variance*. Camebridge University Press: Camebridge.

van Uden N, Castelo Branco R (1963). Distribution and population densities of yeast species in Pacific water, air, animals, and kelp off southern California. *Limnol Oceanogr* **8:** 323-329.

van Veen SQ, Claas ECJ, Kuijper EJ (2010). High-throughput identification of bacteria and yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in conventional medical microbiology laboratories. *J Clin Microbiol* **48:** 900-907.

Vaz ABM, Rosa LH, Vieira MLA, de Garcia V, Brandão LR, Teixeira LCRS *et al* (2011). The diversity, extracellular enzymatic activities and photoprotective compounds of yeasts isolated in Antarctica. *Braz J Microbiol* **42:** 937-947.

Vega Thurber R, Willner-Hall D, Rodriguez-Mueller B, Desnues C, Edwards RA, Angly F *et al* (2009). Metagenomic analysis of stressed coral holobionts. *Environ Microbiol* **11:** 2148-2163.

Vogel C, Rogerson A, Schatz S, Laubach H, Tallman A, Fell J (2007). Prevalence of yeasts in beach sand at three bathing beaches in South Florida. *Water Res* **41:** 1915-1920.

Wang G, Wang X, Liu X, Li Q (2012). Diversity and biogeochemical function of planktonic fungi in the ocean. In: Raghukumar C (ed). *Biology of Marine Fungi*. Springer: Berlin. pp 71-88.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). *PCR protocols: a guide to methods and applications*. Academic Press: San Diego. pp 315–324.

Wieser A, Schneider L, Jung J, Schubert S (2012). MALDI-TOF MS in microbiological diagnostics - identification of microorganisms and beyond (mini review). *Appl Microbiol Biotechnol* **93:** 965-974.

Wiltshire KH, Malzahn AM, Wirtz K, Greve W, Janisch S, Mangelsdorf P *et al* (2008). Resilience of North Sea phytoplankton spring bloom dynamics: an analysis of long-term data at Helgoland Roads. *Limnol Oceanogr* **53:** 1294-1302.

Wiltshire KH, Kraberg A, Bartsch I, Boersma M, Franke HD, Freund J *et al* (2010). Helgoland Roads, North Sea: 45 years of change. *Estuar Coast* **33:** 295-310.

Witt V, Wild C, Anthony KRN, Diaz-Pulido G, Uthicke S (2011). Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef. *Environ Microbiol* **13:** 2976-2989.

Wolf-Gladrow DA, Zeebe RE, Klaas C, Kortzinger A, Dickson AG (2007). Total alkalinity: The explicit conservative expression and its application to biogeochemical processes. *Marine Chemistry* **106:** 287-300.

Yamada N, Suzumura M (2010). Effects of seawater acidification on hydrolytic enzyme activities. *J Oceanogr* **66:** 233-241.

Yang SP, Wu ZH, Jian JC (2011). Distribution of marine red yeasts in shrimps and the environments of shrimp culture. *Curr Microbiol* **62:** 1638-1642.

Zacchi L, Vaughan-Martini A (2002). Yeasts associated with insects in agricultural areas of Perugia, Italy. *Ann Microbiol* **52:** 237-244.

Zalar P, Gostinčar C, de Hoog GS, Uršič V, Sudhadham M, Gunde-Cimerman N (2008). Redefinition of *Aureobasidium pullulans* and its varieties. *Stud Mycol* **61:** 21-38.

Zeebe RE, Wolf-Gladrow D (2001). *CO_2 in seawater: equilibrium, kinetics, isotopes*. Elsevier: Amsterdam.

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