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Impacts of a reduction in seawater pH mimicking ocean acidification on the structure and diversity of mycoplankton communities

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ABSTRACT: Increases in atmospheric carbon dioxide (CO_2) change ocean chemistry, as dissolved CO_2 leads to a reduction in the seawater pH. Many marine taxa have been shown to be affected by ocean acidification; however, information on marine fungi is lacking. We analyzed the effect of pH on mycoplankton communities. The pH of microcosms was adjusted to a value mimicking the predicted ocean acidification in the near future. Fungal communities were analyzed using a double-marker gene approach, allowing a more detailed analysis of their response using 454 pyrosequencing. Mycoplankton communities in microcosms with *in situ* and adjusted water pH values differed significantly in terms of structure and diversity. The differences were mainly abundance shifts among the dominant taxa, rather than the exclusion of fungal groups. A sensitivity to lower pH values was reported for several groups across the fungal kingdom and was not phylogenetically conserved. Some of the fungal species that dominated the communities of microcosms with a lower pH were known pathogenic fungi. With the increasing awareness of the significant role fungi play in marine systems, including performing a diverse range of symbiotic activities, our results highlight the importance of including fungi in further research projects studying and modeling biotic responses to the predicted ocean acidification.

KEY WORDS: Ocean acidification · Marine fungi · Phylogenetic signals · 18S rRNA gene sequence · ITS · Microcosm · Double-marker gene approach

INTRODUCTION

Oceans cover approximately 72% of the Earth's surface and are home to an uncountable number of organisms. However, over the last 2 centuries, environmental conditions in ocean systems have been changing due to an enormous increase in atmospheric carbon dioxide (CO_2) caused by anthropo- genic activities, such as the burning of fossil fuels (Sabine et al. 2004). The oceans are known to act as a net sink for atmospheric CO_2 (Sabine et al. 2004). Dissolution of CO_2 in water results in the formation of carboxylic acid and thus increases the acidity of sea- water (Caldeira & Wickett 2003). This mechanism, called ocean acidification, has led to a drop in sea- water pH by 0.1 units since the industrial revolution

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(Sabine et al. 2004). Future scenarios predict a further decrease of 0.4 units within the current century (Caldeira & Wickett 2005).

Many organism groups seem to be sensitive to such a drop in pH, as reported for foraminifera (Webster et al. 2013), animals (Harvell et al. 2002, Hoegh-Guldberg et al. 2007), and bacteria (Witt et al. 2011, Krause et al. 2012). In contrast to other marine microorganisms, the importance of fungi for marine ecosystem functioning has long been underappreciated, which is why they have been greatly understudied. Only in recent years, the use of next-generation sequencing (NGS) techniques has shed some light on fungal diversity (Richards et al. 2015, Rämä et al. 2016, Taylor & Cunliffe 2016) and functioning (Gutiérrez et al. 2011, Orsi et al. 2013, 2015). Fungal diversity assessments are generally conducted using internal transcribed spacer (ITS) regions as marker genes (Schoch et al. 2012). However, the ITS is not the best marker for all fungal groups, and many environmental sequences can often only be classified to the phylum or kingdom level (Nilsson et al. 2016). To achieve a better resolution of fungal communities, Arfi et al. (2012) proposed to include a second phylogenetic marker (18S rDNA). High-resolution studies on marine fungal communities and their responses to ocean acidification are currently lacking, although fungi can be affected by changes in pH in diverse ways; one example is the uptake of nutrients and organic compounds driven by the electrochemical gradient of H^+ ions across the plasma membrane (Bowman & Bowman 1986). The activity and kinetics of excreted fungal exoenzymes are also mediated by the medium pH (Pritsch et al. 2004, Courty et al. 2005). A further but indirect effect is on the availability and accessibility of substrates, such as the range and size of dissolved and particulate organic matter (Verdugo et al. 2004).

In a previous study, we tested the pH sensitivity of marine fungal communities in microcosms by ITS fingerprinting using automated ribosomal intergenic spacer analysis (ARISA) (Krause et al. 2013). The seawater pH was altered according to one of the possible near-future scenarios (Blackford & Gilbert 2007). Interestingly, the structure of the marine fungal communities showed a significant pH-dependent response. However, details on the taxonomic composition of the communities and the taxa involved in these structural changes could not be provided due to the inherent limitations of the applied fingerprinting technique. Therefore the purpose of the present study was to investigate the underlying dynamics of the fungal community com-

position. We tested the modified double-marker gene approach of Arfi et al. (2012) by applying a phylogenetic-based analysis using 18S rDNA in addition to a hierarchical cluster-based approach for the ITS. We aimed to identify which parameters of the communities change, how they change, whether specific fungal species benefit from changes in pH, and whether the observed sensitivity of fungi toward lower pH values is clade-specific. We hypothesized that the observed shift in the community structure is based on the response of a few fungal species and that pH sensitivity is phylogenetically conserved.

MATERIALS AND METHODS

Establishment of the microcosm experiments and sampling scheme

Surface seawater samples were collected at the Helgoland Roads station in the North Sea on 3 May 2012. The sampling site is located off the island of Helgoland (Germany) in the southern North Sea (German Bight). Due to strong tidal currents, surface water samples are representative of the entire water body (Wiltshire et al. 2008). For the year 2100, a concentration of 1000 ppm of atmospheric CO_2 has been predicted (IPCC 2014), which would result in a mean surface seawater pH of 7.67 in the southern North Sea according to the model of Blackford & Gilbert (2007). Thus, after the sampled seawater was poured into 1.6 l glass jars, the microcosms were incubated either at the current *in situ* seawater pH (8.26) or at an adjusted pH of 7.67. The seawater pH was adjusted with 2 M HCl, which is a validated approach for acidifying microcosm-based experiments (Krause et al. 2012). For each pH treatment, 20 replicates were prepared and left in the dark over a maximum period of 4 wk at the *in situ* temperature on the day of sampling (8°C). The microcosms were mixed daily by manual inversion. Every week, the biomass in 5 replicates for each pH treatment was collected by filtration onto sterile nitrocellulose filters (0.45 µm pore size, 47 mm diameter, gray with a grid; Sartorius). Parameters of the carbonate system of every microcosm, including the pH (Table S1 in the Supplement at www.int-res.com/articles/suppl/a079p221_supp.pdf), were calculated based on a previously described method (Krause et al. 2013) and can be accessed at https://doi.pangaea.de/10.1594/PANGAEA.831726 (Pangaea ID 10.3354/ame01622). Biomass filters were stored at −20°C until further treatment.

DNA extraction, PCR and pyrosequencing

DNA was extracted as described by Krause et al. (2013). The PCR on the ITS2 region was conducted in a reaction of 50 µl containing 100 ng of template DNA, 5 µl *Taq* buffer (10×), 2.5 µM MgCl$_2$, 250 µM of each dNTP, 2.5 U *Taq* polymerase (5 Prime), 0.15 µM of the forward fungal-specific primer gITS7 (5′GTG ART CAT CGA RTC TTT G) (Ihrmark et al. 2012) and 0.25 µM of the tagged eukaryotic ITS4 primer (5′TCC TCC GCT TAT TGA TAT GC) (White et al. 1990). Cycling conditions followed Krause et al. (2013). A second amplification round was conducted in 2 µl of a 1:10 dilution of the first PCR products and using the primers described above. The 18S rDNA was amplified in reactions of 50 µl containing 50 ng of the template DNA, 5 µl *Taq* buffer (10×) (5 Prime), 1.25 µM MgCl$_2$, 200 µM of each dNTP, 0.3 µM each of the eukaryotic primer NS1 (5′GTA GTC ATA TGC TTG TCT C) and the fungal-specific primer NS8 (5′TCC GCA GGT TCA CCT ACG GA) (White et al. 1990) and 1.25 U *Taq* polymerase. Cycling conditions started with a denaturation step of 94°C for 15 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 68°C for 2 min, with a final extension at 68°C for 10 min. For the second round, the fungal-specific primer pair nu-SSU-0817 (5′TTA GCA TGG AAT AAT RRA ATA GGA) and nu-SSU-1536 (5′ATT GCA ATG CYC TAT CCC CA) (Borneman & Hartin 2000) were used, with the first primer carrying the barcode. Amplifications were conducted in reactions of 50 µl, each containing 2 µl of the first PCR products, 5 µl *Taq* buffer (10×), 1.25 mM MgCl$_2$, 250 µM of each dNTP, 0.48 µM of each primer and 2.5 U *Taq* polymerase (5 Prime). Cycling conditions were the same as for the first PCR but with an annealing temperature of 68°C. The PCR steps included a positive control, which was a seawater sample taken at the time of sampling. After purification using the PeqGold Gel Extraction kit (PeqLab), the PCR products were sequenced on a Roche 454 GS-FLX Titanium platform (LGC) as single reads. Sequencing data are stored under the study accession number SRP065054 and can be downloaded from the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/sra).

Bioinformatics and OTU designation

For quality control, ITS2 and 18S rDNA sequences without a valid primer or DNA tag sequence and with average quality values of <25 Phred score, >2% ambiguous symbols or homopolymer chains of >8 nt were discarded. After removing barcodes and primers from the remaining sequences, sequences with a read length <200 nt were also discarded. In most sequence reads, quality values dropped below a value of 25 at a nt position of ~420. Thus, all sequences were trimmed from the 3′-end to a maximal length of 400 nt. Potential chimeric sequences were detected and removed using the UCHIME program (Edgar et al. 2011). From this point on, the remaining ITS2 sequences were subjected to hierarchical clustering analyses, while 18S rDNA sequences were further analyzed using a phylogenetic-based approach.

Fungal taxonomy

Due to the primer attachment sites, the generated ITS reads contained sequence information from the 5.8S and 28S rDNA. As these fragments influence the sequence identification process, they were removed from the generated sequence reads using the ITSx program (Bengtsson-Palme et al. 2013). ITS2 sequences were clustered using a 97% sequence similarity threshold into operational taxonomic units (OTUs) using the UCLUST algorithm (Edgar 2010). All these steps were processed using the Quantitative Insights In Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010). Sequences were blasted using the FHiTINGS program (Quast et al. 2013, Dannemiller et al. 2014). Finally, OTUs that were represented by fewer than 5 reads and observed only in a single sample were removed. 18S rDNA reads passing the above-described quality control requirements were clustered and classified by mapping the sequences to our existing maximum likelihood reference tree (for details on the sequences, alignment and methods see Panzer et al. 2015). The effects of pH and time on specific fungal groups were tested using generalized linear modeling (Zuur et al. 2009), as our data followed Poisson or negative binomial distributions. Model selection was carried out using the Akaike information criterion (AIC); models were ordered according to the AIC, and those with lowest scores were compared using likelihood ratio tests (LR). The best model was then evaluated for significance of the interaction term using LR. Tests were run in R (R Core Team 2015) using the 'glm' and 'glm.nb' functions of the Mass library (Venables & Ripley 2002). The values from the 'summary' table are reported.

Alpha diversity analyses based on the ITS2 dataset

Diversity indices were calculated based on sub-sampled datasets using the PRIMER 6 software (Plymouth Marine Laboratories). Next, the effects of the factors 'pH' and 'incubation time' on the diversity indices were analyzed. In the case of the Simpson and Shannon diversity data, the statistical general least squares (GLS) model (Galecki & Burzykowski 2013) was chosen, which can deal with data with heterogeneous variances. The details of the statistical procedure are given in Zuur et al. (2009) and Galecki & Burzykowski (2013), and here we only describe them in brief. In the first stage, we fitted models with different variance structure (i.e. different patterns of variance heterogeneity) using restricted maximum likelihood (REML) and selected the model with the best variance structure using the AIC and LR as explained above. In the second step, we used the model with the best variance structure to test for the effects of 'pH' and 'incubation time' on the average diversity. In this step, models are fitted using the maximum likelihood method and are then compared using the AIC, and significant effects are tested using LR. When significant effects are found, the parameters in the model with the significant terms are refitted using REML in order to express the appropriate estimates of variances and average trends. GLS analysis was carried out using the package 'nlme' (Pinheiro et al. 2015) implemented in R (R Core Team 2015). The effects of pH and incubation time on OTU richness were evaluated using generalized linear modeling (Zuur et al. 2009) with Poisson and negative binomial error structure as in the case of species counts .

Beta diversity analyses based on the ITS2 dataset

Beta diversity was analyzed based on subsampled datasets using permutational multivariate ANOVA (PERMANOVA) of square root-transformed OTU numbers (as Bray-Curtis similarities). To visualize the influence of the factors 'pH' and 'incubation time' on the fungal community structure, we performed principal coordinate analysis (PCoA). The similarity percentage (SIMPER) procedure was used to identify OTUs that contributed most to the observed dissimilarity between the 2 pH treatments. We examined only OTUs with a threshold of >1% of the total contribution to differences. All analyses were performed using the PRIMER 6 software.

Phylogenetic diversity of the 18S rDNA dataset

To evaluate the evolutionary relationships among taxa, we ran further phylogenetic metric-based PCoAs using the 18S rDNA dataset. Phylogenetic separation patterns were identified using the FastUniFrac suite (Hamady et al. 2010) implemented in the Galaxy platform (Giardine et al. 2005, Goecks et al. 2010). Unweighted and weighted distances were calculated.

Phylogenetic signals in the 18S rDNA dataset

To test for possible genetic conservatism among fungi to acclimate to the factor 'pH', phylogenetic signal analysis was performed using the software PHYLOCOM v 4.2 (Webb et al. 2008) with the Comstruct function. Evolutionary conservatism was analyzed based on the net relatedness index (NRI) and the nearest taxon index (NTI) using the 18S rDNA dataset. The NRI indicates how many taxa in a community are dispersed (negative value) or clustered (positive value) over the whole phylogenetic tree, while the NTI indicates dispersal/clustering at lower taxonomic levels. As a null model, the '−m 2' function was chosen, while the number of randomizations was set to 9999. A p-value of $p < 0.05$ against the random distributions was considered to indicate a significant phylogenetic signal. In a last step, Nodesig analyses were run to identify the clades/taxa in the phylogenetic tree that caused the phylogenetic signals.

RESULTS

Dataset description

In total, 237 967 and 532 304 sequence reads were generated from 42 samples when targeting the ITS2 and the 18S rDNA, respectively. For Sample 28, only five 18S rDNA reads were generated, and it was thus excluded from further analysis of the 18S rDNA dataset. Sequence trimming and processing resulted in 171 872 ITS2 and 404 281 18S rDNA sequence reads (Table S2 in the Supplement). Of these, 116 056 ITS2 sequences and 126 051 18S rDNA sequences were of fungal origin. The 18S rDNA sequences generated by co-amplification of the primer pair clustered mainly within the groups of *Telonema*, Alveolata and Stramenopiles.

Taxonomic composition of the samples

The ITS2 dataset revealed Ascomycota to be the dominant phylum in all sample types, comprising between 72 and 96% of the relative sequence abundance, from which 38 to 78% could not be further taxonomically assigned. Dothideomycetes and Eurotiomycetes were the most abundant ascomycete classes under a pH of 8.26 and 7.67, respectively. Basidiomycota were found to be present in all samples but with varying abundance between the 2 treatments. Up to 22% of the sequences were assigned to Agaricomycetes as the dominant group in samples with a pH of 8.26. In contrast, Microbotryomycetes was the most represented basidiomycete class in

samples with a pH of 7.67. No basal fungal lineages were described in the ITS2 dataset, and up to 10% of the sequences could not be assigned beyond the kingdom level (Fig. 1A). All abundant fungal groups in the present-day pH samples with incubation times of up to 2 wk were also detected in the positive control (Table S3 in the Supplement).

In the 18S rDNA dataset, nearly all sequences could be assigned beyond the phylum level, leaving no sequences with an ambiguous taxonomic assignment at higher levels. Ascomycota showed similar abundance values (74 to 98%) as in the ITS2 dataset, with Eurotiomycetes and Leotiomycetes as dominant groups in both sample types, followed by Dothideomycetes. The percentage of Basidiomycota se-
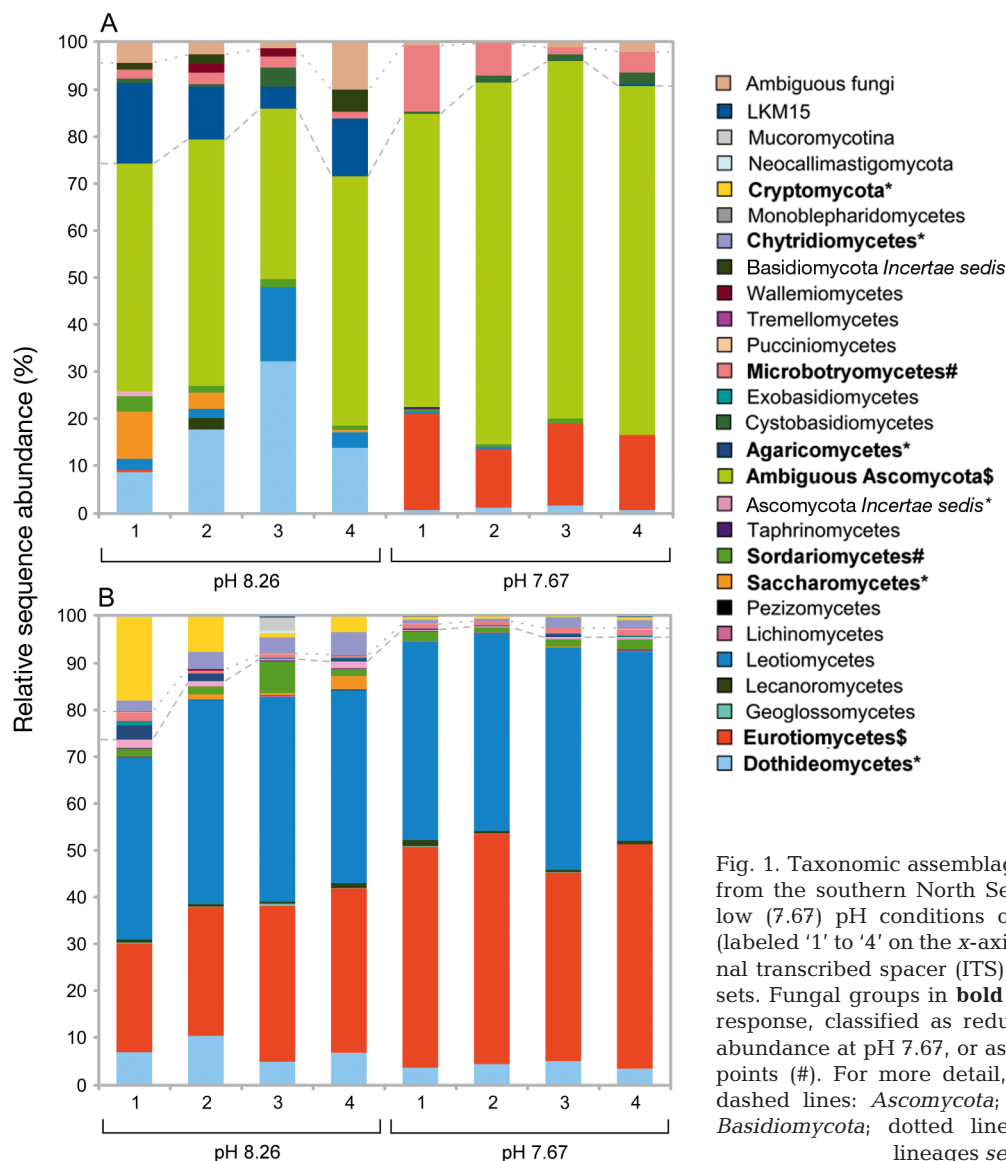

Fig. 1. Taxonomic assemblage of fungal communities from the southern North Sea under high (8.26) and low (7.67) pH conditions over 4 wk of incubation (labeled '1' to '4' on the *x*-axis), based on the (A) internal transcribed spacer (ITS) and (B) 18S rDNA datasets. Fungal groups in **bold** showed a significant pH response, classified as reduced (*) or increased ($) abundance at pH 7.67, or as inconsistent among time points (#). For more detail, see Table 2. Bottom to dashed lines: *Ascomycota*; dashed to dotted lines: *Basidiomycota*; dotted lines to top: basal fungal lineages *sensu lato*

quences was relatively small (1 to 7%) and did not exceed more than 2% of the relative sequence abundance in samples with a pH of 7.67. Using the 18S rDNA dataset, 5 basal fungal lineages sensu lato were detected, namely, Chytridiomycota, Cryptomycota, LKM15, Mucoromycotina and Neocallimastigomycota. In the *in situ* pH treatments, the fraction of sequences assigned to basal fungal lineages declined over time but started with a value of 20% relative sequence abundance (Fig. 1B). The positive control was more similar to the present-day pH samples than to the near-future pH samples but contained all abundant groups of both treatments (Table S4 in the Supplement).

Several fungal groups could not be tested for significant effects of pH and incubation time on their occurrence, as their abundances were too low for reliable results. Thus, only 5 out of 15 and 11 out of 27 groups of the ITS2 and 18S rDNA datasets, respectively, were included in this analysis. Of those, the abundance of 10 groups was significantly affected by the factor 'pH'. With the exceptions of Eurotiomycetes and ambiguous Ascomycota, the remaining 8 groups, namely, Cryptomycota, Chytridiomycetes, Microbotryomycetes, Agaricomycetes, Ascomycota *incertae sedis*, Sordariomycetes, Saccharomycetes and Dothideomycetes, had consistently lower abundances under the reduced pH of 7.67 (Table 1).

ITS-based diversity analyses

Alpha diversity. GLS analysis identified the interaction term of time and pH as significant for Shannon diversity ($F_{3,31}$ = 8.62, p = 0.0003). For Simpson diversity, the interaction term was marginally significant ($F_{3,31}$ = 2.66, p = 0.065), while time ($F_{3,34}$ = 6.1, p = 0.0022) and pH ($F_{1,34}$ = 10.59, p = 0.0022) had significant effects. For richness, the best model in terms of residuals included the interaction term (Table 2). The diversity of the abundant species declined at a pH of 7.67 (Tables 2 & S5). In contrast, the richness was higher at a pH of 8.26.

Beta diversity. Bray-Curtis similarity-based PCoA of the ITS2 dataset (Fig. 2) showed a clear separation of samples according to the pH treatment, which is supported by the PERMANOVA (Table 3). However, neither the factor 'incubation time' nor the interaction term was found to be significant.

The SIMPER analyses indicated that the OTUs of 15 individual taxa were largely responsible for the observed differences in fungal communities between the samples with different pH levels. The average dissimilarity between the 2 tested groups was 81.9%. Fourteen of the taxa were part of the core community of the microcosms with an adjusted pH of 7.67. In total, only 7 taxa could be identified to the species level, and of those, 3 were known potential pathogens (Table 4).

Table 1. Generalized modeling framework test of the effects of pH and time on the 12 most abundant fungal groups in seawater samples from the southern North Sea. Abundances (mean ± SD) of the tested fungal groups under high pH (8.26) and low pH (7.67) conditions are shown for 2 different datasets, derived from assessments using internal transcribed spacer (ITS) regions and 18S rDNA. p-values indicate whether the pH effect had a significant effect on the occurrence of the fungal group and whether this was dependent on incubation time. The final column shows the trend in abundance between the 2 pH treatments. n/a: p-value not shown due to low abundance; ns: not significant

Fungal group	ITS			18S rDNA			Trend
	Abundance pH 8.26	Abundance pH 7.67	p	Abundance pH 8.26	Abundance pH 7.67	p	
Cryptomycota	0	0	n/a	350 ± 25.1	23 ± 1.1	4.3×10^{-14}	L < H
Chytridiomycetes	0	0	n/a	184 ± 5.1	61 ± 2.8	1.47×10^{-08}	L < H
Microbotryomycetes	16 ± 1.3	58 ± 2.5	10^{-4}	44 ± 2.4	35 ± 1.4	8.8×10^{-3} (for Week 4)	L > H
						4.2×10^{-2} (for Week 1)	L < H
Agaricomycetes	92 ± 3.6	1 ± 0	10^{-8}	66 ± 5.7	14 ± 1.3	8×10^{-4}	L < H
Ambiguous Ascomycota	371 ± 8.1	582 ± 4.9	2×10^{-4}	16 ± 1.2	0	n/a	L > H
Ascomycota *incertae sedis*	2 ± 0	0	n/a	42 ± 2.1	16 ± 1	2×10^{-3}	L < H
Sordariomycetes	15 ± 1.2	4 ± 0	n/a	146 ± 14	80 ± 1.9	0.01 (for Week 3)	L < H
Saccharomycetes	29 ± 3.9	0	n/a	62 ± 6.2	4 ± 0	3.57×10^{-05}	L < H
Leotiomycetes	42 ± 5.9	2 ± 0	n/a	2163 ± 13.3	2174 ± 19.4	0.95	ns
Lecanoromycetes	5 ± 0.5	0	n/a	33 ± 1.2	38 ± 1.3	0.41	ns
Dothideomycetes	133 ± 10.2	7 ± 0.8	n/a	360 ± 14.8	201 ± 3.5	0.0	L < H
Eurotiomycetes	1 ± 0	133 ± 2.7	n/a	1520 ± 26.5	201 ± 3.5	2.25×10^{-05}	L > H

Table 2. Results of generalized linear model analysis testing the effects of incubation time and pH on the richness of mycoplankton communities in seawater samples from the southern North Sea. Tests included the examination of negative binomial and Poisson error structure. Likelihood ratio tests (LR) and associated (Chi squared-based) p-values compare the top 2 models, testing for the significance of the interaction term. The LR uses the degrees of freedom of the model with the interaction (df = 31) and the one without the interaction (df = 34) to calculate the difference (df = 3) and then the LR(3) value shown. AIC: Akaike information criterion

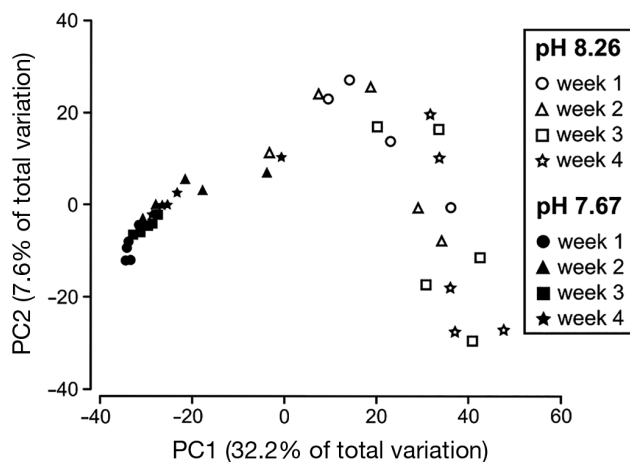| Fixed structure	Error structure	AIC	LR	p
Time + pH + (T × pH)	Negative binomial	341	15.46	0.0014
Time + pH	Negative binomial	351		
Time + pH + (T × pH)	Poisson	401		
Time + pH	Poisson	467		

Fig. 2. Principal coordinate analysis (PCoA) of the ITS2 dataset of fungal communities from the southern North Sea under high (8.26) and low (7.67) pH conditions over 4 wk of incubation. The test is based on square root-transformed data using Bray-Curtis dissimilarities of operational taxonomic unit counts

Table 3. Results of PERMANOVA tests of fungal community composition based on ITS2-based Bray-Curtis dissimilarities of operational taxonomic units (OTUs) for the factors 'pH' and 'incubation time', their interaction and the partitioning of multivariate variation. p-values were obtained using type III sums of squares (SS) and 9999 permutations under the full model. The final column shows values for the square root of the component of variation attributable to that factor in the model in units of Bray-Curtis dissimilarity. *statistically significant ($p < 0.05$)

Sources of variation	df	SS	Pseudo-F	Sq. root
pH*	1	28 308	15.177	36.925
Time	3	8301	1.4835	9.6348
pH × time	3	7791	1.3924	12.275
Residuals	31	57 821		43.188
Total	38	1.0252×10^5		

Phylogenetic analysis

Phylogenetic community structure. The unweighted UniFrac-based PCoA did not clearly separate the samples with different pH treatments (Fig. 3A). Similar results were found when using the weighted UniFrac as a metric, but the separation was slightly more pronounced than for the first analysis (Fig. 3B), while incubation time only showed an effect on samples from the first week. This indicates that most of the samples are relatively similar from a phylogenetic perspective and that observed community shifts must be explained based on shifts in taxa abundance, which is in accordance with the non-phylogenetically based PCoA.

Phylogenetic signal analysis. The Phylocom analysis showed significant phylogenetic signals in both community types. The signals always indicated phylogenetic clustering with values of 2.2 for the NRI and 1.2 for the NTI and solely with a value of 1.6 for the NRI in samples with a pH of 8.26 and 7.67, respectively (Fig. 4A). The Nodesig analysis indicated that the phylogenetic signals were mainly caused by the same clades under both pH conditions. The exceptions were significant sequence clustering within clades of Saccharomycetales and Pleosporales exclusively in samples with an adjusted pH (Fig. 4B).

DISCUSSION

Assessing the double-marker gene approach for community analysis

In our approach, we used 2 marker genes, namely, the ITS and 18S rDNA. The ITS resolves many fungal taxa to the species or genus level but performs less well on basal fungal lineages and lacks phylogenetic power (Schoch et al. 2012). Marine fungal communities are characterized by their (larger) fraction of basal fungi (Panzer et al. 2015, Richards et al. 2015) and many undescribed fungal taxa and groups (Manohar & Raghukumar 2013). Their classification depends on the respective phylogenetic marker applied (Le Calvez et al. 2009, Jones et al. 2011, Nagahama et al. 2011). Indeed, in our study, nearly all fungal sequences were classified to a lower taxonomic level with the 18S rDNA, also revealing the presence of basal fungi. In contrast, the taxonomy of

Table 4. SIMPER analysis of the ITS2 dataset of fungal taxa in samples collected from the southern North Sea, showing average abundances of operational taxonomic units (OTUs) under high pH (8.26) and low pH (7.67) conditions, average dissimilarities between the 2 treatments, and the contributions of each OTU to observed community dissimilarity (COD). Only OTUs with more than 1% contribution to the observed community dissimilarities are listed. *Inc. sed.: incertae sedis*

Species	Abundance pH 8.26	Abundance pH 7.67	Dissi-milarity	COD (%)	Phylum	Subphylum	Class	Order
Cyphellophora reptans	0.49	32.78	13.39	16.33	Ascomycota	Pezizomycotina	Eurotiomycetes	Chaetothyriales
Pezizomycotina sp.	7.85	22.94	6.6	8.06	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Sporobolomyces symmetricus	0.38	7.56	2.85	3.48	Basidiomycota	Pucciniomycotina	Cystobasidiomycetes	Sporidiobolales
Cadophora malorum	1.16	6.87	2.84	3.46	Ascomycota	Pezizomycotina	Leotiomycetes	Helotiales
Sakaguchia dacryoidea	1.04	4.87	2.14	2.6	Basidiomycota	Pucciniomycotina	Cystobasidiomycetes	*Inc. sed.*
Pezizomycotina sp.	2.13	6.27	1.82	2.22	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Eurotiomycetes sp.	0.11	3.63	1.47	1.8	Ascomycota	Pezizomycotina	Eurotiomycetes	*Inc. sed.*
Pezizomycotina sp.	1.37	4.35	1.29	1.58	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Neophaeosphaeria quadriseptata	2.28	1.2	1.26	1.54	Ascomycota	Pezizomycotina	Dothideomycetes	Pleosporales
Pezizomycotina sp.	1.15	3.95	1.25	1.53	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Pezizomycotina sp.	1.22	3.89	1.21	1.48	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Pezizomycotina sp.	0.77	2.82	0.91	1.11	Ascomycota	Pezizomycotina	*Inc. sed.*	*Inc. sed.*
Eurotiomycetes sp.	0	2.2	0.9	1.1	Ascomycota	Pezizomycotina	Eurotiomycetes	*Inc. sed.*
Acremonium butyri	0.76	2.57	0.88	1.07	Ascomycota	Pezizomycotina	Sordariomycetes	Hypocreales
Rhodotorula aurantiaca	0.21	2.3	0.85	1.04	Basidiomycota	Pucciniomycotina	Cystobasidiomycetes	*Inc. sed.*

several fungal sequences was only poorly resolved with the ITS, and no basal fungal lineages were detected (Fig. 1).

A phylogenetic-based community approach differs from a non-phylogenetic one with respect to the method used to classify the sequence reads and the metrics used for statistics. The former incorporates the phylogenetic relatedness among taxa into the analyses, which offers further insights into the evolutionary and ecological drivers shaping the community (Horner-Devine & Bohannan 2006, Emerson et al. 2011). In contrast, the standard approach for non-phylogenetic markers, such as the ITS, includes similarity threshold-based sequence clustering (e.g. Li & Godzik 2006, Edgar 2010), taxonomic OTU assignment by k-mer similarity search (e.g. Altschul et al. 1990) and, most critically, statistics that consider all OTUs as equivalent independently of whether they are phylogenetically highly divergent or not (Faith 1994, Martin 2002). In our approach, we combined the individual advantages of the 2 marker genes rather than using them in an additive manner as done by Arfi et al. (2012) and additionally minimized the primer bias (Hong et al. 2009). Finally, the ITS, possessing good resolution to the species/genus level, was used for the alpha and beta diversity analyses and for the identification of core species (by SIMPER). In contrast, the 18S rDNA was used to resolve the phylogeny of undescribed taxa/groups and basal fungal lineages to reveal differences in the phylogenetic structure among communities and to test for possible evolutionary conservatism of pH sensitivity in fungal clades.

Community responses

The mycoplankton community of the microcosms showed a significant response to the pH treatment. However, the communities in the 2 treatments were phylogenetically highly related, and the observed effect was mainly based on a change in abundance among the dominant taxa rather than a change in the general community composition or extinction of several fungal groups (Fig. 2). Thus, the mycoplankton community can cope with a drop in pH within the tested range, but sensitivity to the pH change differs highly among fungal taxa. The phylogenetic signal analysis indicated no clade-specific sensitivity to one or the other pH (Fig. 4). A phylogenetic signal can occur when, for example, closely related taxa share a character or suite of characters allowing them to be adaptive to
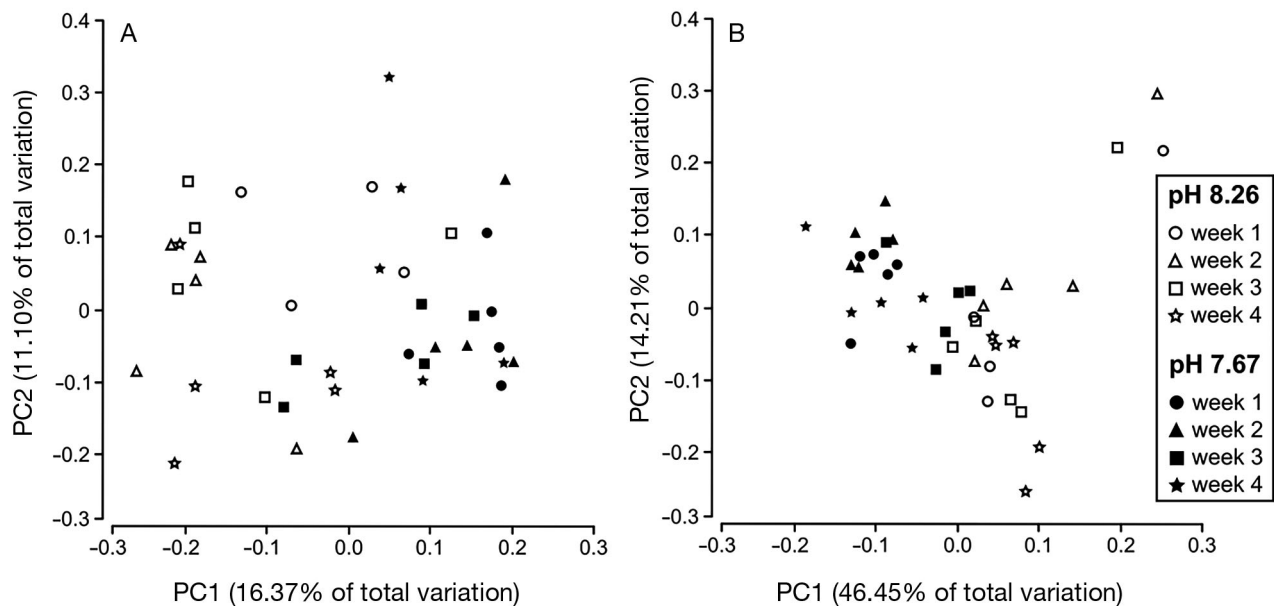
Fig. 3. Principal coordinate analysis (PCA) of phylogenetic distances showing phylogenetic relatedness among fungal communities from the southern North Sea under high (8.26) and low (7.67) pH conditions over 4 wk of incubation, for (A) unweighted and (B) weighted UniFrac metrics using the 18S rDNA data

the given environmental conditions (Horner-Devine & Bohannan 2006). In the communities in the 2 pH treatments, small significant clustering effects were observed, but phylogenetic signals were nearly exclusively caused by the same clades in both pH treatments (Fig. 4). This observation points to other factors than the pH causing the clustering effect. In our case, it can be assumed that the habitat-specific conditions of the pelagial at Helgoland Roads are the structuring factors. Panzer et al. (2015) recently demonstrated that aquatic fungal community structure is highly influenced by the source habitat, resulting in habitat-specific phylogenetic signals. Thus, the sensitivity to a pH shift observed in our study must be caused by different and (clade-)independent characters; it has, for example, been shown that diverse transcriptional regulatory systems control the response to the environmental pH (Peñalva & Arst 2004, Selvig & Alspaugh 2011). In addition, fungi seem to react quite differently to the environmental pH depending on their life stage; for example, the fungal germination time and outgrowth can be affected by the environmental pH (Magan & Lacey 1984, Porter et al. 1987). For pathogenic fungi with host-independent life stages (e.g. some Cryptomycota or Chytridiomycetes species), responses to pH may depend on the life stage.

Relevance for food webs

Microbial communities highly influence resource use, disease dynamics and the stability of ecosystem processes (Duffy & Stachowicz 2006, Stachowicz & Byrnes 2006). For example, a pH-dependent loss of fungal diversity in freshwater systems (Wood-Eggenschwiler & Bärlocher 1983, Tolkkinen et al. 2015) can lead to a drastic reduction in fungal leaf breakdown (Baudoin et al. 2008). So far, comparable data for marine fungi are lacking, but recent studies on marine mycoplankton have elucidated their importance in the breakdown of organic matter (Gutiérrez et al. 2011) and the control of phytoplankton populations (Gleason et al. 2008, Gutiérrez et al. 2016). Thus, zoosporic fungi are part of the pelagic food web, forming a 'mycoloop'; herein, they (1) act as a food source for zooplankton grazers, (2) decompose particulate organic matter, (3) convert inorganic compounds into organic compounds, and (4) parasite/hyperparasite on diverse organisms (Kagami et al. 2007, 2014). In our study, players in the 'mycoloop', namely, Cryptomycota and Chytridiomycetes, showed significant reductions under a reduced pH (Table 1). According to dynamical model predictions, a reduction in zoosporic fungi in pelagic systems would affect the overall food web structure: fewer fungal parasites lead to a higher abundance of large phyto-
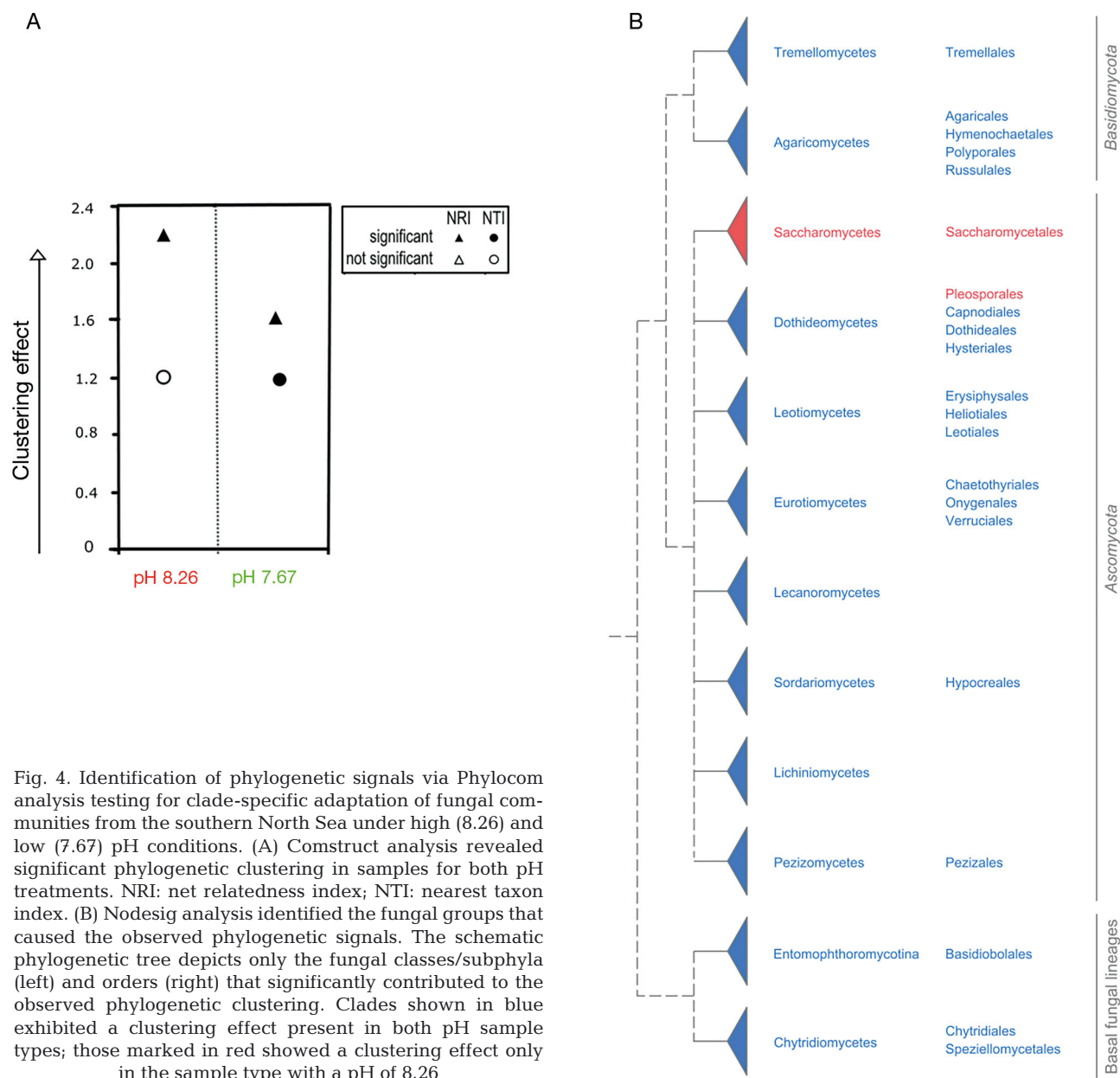
Fig. 4. Identification of phylogenetic signals via Phylocom analysis testing for clade-specific adaptation of fungal communities from the southern North Sea under high (8.26) and low (7.67) pH conditions. (A) Comstruct analysis revealed significant phylogenetic clustering in samples for both pH treatments. NRI: net relatedness index; NTI: nearest taxon index. (B) Nodesig analysis identified the fungal groups that caused the observed phylogenetic signals. The schematic phylogenetic tree depicts only the fungal classes/subphyla (left) and orders (right) that significantly contributed to the observed phylogenetic clustering. Clades shown in blue exhibited a clustering effect present in both pH sample types; those marked in red showed a clustering effect only in the sample type with a pH of 8.26

plankton species (>40 µm), which in turn lowers the numbers of smaller phytoplankton species due to resource competition. As zooplankton species only graze on smaller phytoplankton, a reduction in zoosporic fungi has an indirect and negative effect on zooplankton grazers (Miki et al. 2011). The involvement of filamentous fungi in the pelagic food web has so far not been modeled due to missing data. In our study, 2 of the most prominent groups, Eurotiomycetes and Dothideomycetes, both showed a significant pH effect (Table 1). Gutiérrez et al. (2011) suggest an important role in the degradation of

phytoplankton bloom-derived organic matter for these fungi. Thus, a pH-driven increase in filamentous fungal biomass might have a negative impact on organisms competing for the same food source.

Three of the 7 key taxa (described to the genus level) that significantly benefited from the pH reduction are pathogenic fungi that cause disease in marine algae (Almeida et al. 2010) and animals (Wirth & Goldani 2012) (Table 4). Pathogenic fungal activities in marine environments may lead to the breakdown of ecosystems (Raghukumar & Ravindran 2012) or cause significant economic losses when commer-

cially important organisms are infected (Gachon et al. 2010, Hatai 2012). Given that pathogenicity is regulated by several factors (e.g. host physiology, species interactions and environmental factors; Fuhrman 2009, Selvig & Alspaugh 2011), more detailed studies are urgently needed to examine the effects of ocean acidification on this important group.

Conclusion

Although the marine fungal communities in the 2 pH treatments were phylogenetically closely related, their taxonomic composition and diversity differed significantly. The observed changes were mainly caused by a restructuring of the abundant species. Sensitivity to the pH reduction was not phylogenetically conserved. The reported pH sensitivity contrasts with the wide pH tolerance observed in marine fungi under controlled conditions. We have only just begun to understand the ecological role of marine fungi, but given the newly discovered role mycoplankton play in organic matter breakdown and phytoplankton bloom dynamics, further studies are urgently needed to place the observed pH-dependent community shift and possible consequences for the marine ecosystem into context.

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