Impacts of a reduction of seawater pH mimicking ocean acidification impacts on assemblage, structure and diversity of marine fungal communities

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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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Short title: Ocean acidification impacts fungal community structure and diversity

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, while information on marine fungi is lacking. Here, we analyze 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 based on 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.

Keywords: ocean acidification, marine fungi, phylogenetic signals, 18S rRNA gene sequence, ITS, microcosm, double-marker gene approach

1. Introduction

Oceans cover approximately 72% of the Earth's surface and are home to an uncountable number of organisms. However, over the last two centuries, environmental conditions in ocean systems have been changing due to an enormous increase in atmospheric carbon dioxide (CO_2) caused by anthropogenic 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. Dissolution of CO_2 in water results in the formation of carboxylic acid and therefore seawater acidification (Caldeira & Wickett 2003). This mechanism, called ocean acidification, has led to a drop in seawater pH by 0.1 units since the industrial revolution (Sabine et al. 2004). Future scenarios predict a further decrease by 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 highly understudied. Only in recent years, the use of next-generation sequencing (NGS) techniques has shed some light on fungal diversity (Richards et al. 2015, Rama et al. 2016, Taylor & Cunliffe 2016) and functioning (Gutierrez et al. 2011, Orsi et al. 2013, Orsi et al. 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

46 electrochemical gradient of H^+ ions across the plasma membrane (Bowman & Bowman 1986). The
47 activity and kinetics of excreted fungal exoenzymes are also mediated by the medium pH (Pritsch et
48 al. 2004, Courty et al. 2005). A further but indirect effect is the availability and accessibility of
49 substrates, such as the range and size of dissolved and particulate organic matter (Verdugo et al.
50 2004).

51 In a previous study, we tested the pH sensitivity of marine fungal communities in microcosms using
52 ITS fingerprinting (automated ribosomal intergenic spacer analysis; ARISA) (Krause et al. 2013).
53 The seawater pH was altered according to one of the possible near-future scenarios (Blackford &
54 Gilbert 2007). Interestingly, the structure of the marine fungal communities showed a significant pH-
55 dependent response. However, details on the taxonomic composition of the communities and the taxa
56 involved in these structural changes could not be provided due to the inherent limitations of the
57 applied fingerprinting technique. Thus, the purpose of the present study was to investigate the
58 underlying dynamics of the fungal community composition. We tested the modified double-marker
59 gene approach of Arfi *et al.* (2012) by applying a phylogenetic-based analysis using 18S rDNA in
60 addition to a hierarchical cluster-based approach for the ITS. We aimed to identify which parameters
61 of the communities change, how they change, whether specific fungal species benefit from changes
62 in pH, and whether the observed sensitivity of fungi toward lower pH values is clade-specific. We
63 hypothesize that the observed shift in the community structure is based on the response of a few
64 fungal species and that pH sensitivity is phylogenetically conserved.

2. Materials and Methods

2.1. Establishment of the microcosm experiments and sampling scheme

Surface seawater samples were collected at the Helgoland Roads station in the North Sea (Germany) on the 3rd of May, 2012. The sampling site is located off the island of Helgoland 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 weeks 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, Göttingen, Germany). Parameters of the carbonate system of every microcosm, including the pH (Suppl. Table S1), 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.

2.2. 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, Germany), 0.15 µM of the forward fungal-specific primer gITS7 (5'GTGARTCATCGARTCTTTG) (Ihrmark et al. 2012) and 0.25 µM of the

tagged eukaryotic ITS4 primer (5'TCCTCCGCTTATTGATATGC) (White et al. 1990). Cycling conditions followed Krause *et al.* (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'GTAGTCATATGCTTGTCTC) and the fungal-specific primer NS8 (5'TCCGCAGGTTCACCTACGGA) (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'TTAGCATGGAATAATRRAATAGGA)/nu-SSU-1536 (5'ATTGCAATGCYCTATCCCCA) (Borneman & Hartin 2000) was 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, Erlangen, Germany), the PCR products were sequenced on a Roche 454 GS-FLX Titanium platform (LGC, Berlin, Germany) 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 (http://www.ncbi.nlm.nih.gov/sra).

2.3. 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

113 >8 nt were discarded. After removing barcodes and primers from the remaining sequences, sequences
114 with a read length <200 nt were also discarded. In most sequence reads, quality values dropped
115 below a value of 25 at an nt position of ~ 420. Thus, all sequences were trimmed from the 3'-end to a
116 maximal length of 400 nt. Potential chimeric sequences were detected and removed using the
117 UCHIME program (Edgar et al. 2011). From this point on, the remaining ITS2 sequences were
118 subjected to hierarchical clustering analyses, while 18S rDNA sequences were further analyzed using
119 a phylogenetic-based approach.

120 **2.4. Fungal taxonomy**

121 Due to the primer attachment sites, the generated ITS reads contained sequence information from the
122 5.8S and 28S rDNA. As these fragments influence the sequence identification process, they were
123 removed from the generated sequence reads using the ITSx program (Bengtsson-Palme et al. 2013).
124 ITS2 sequences were clustered using a 97% sequence similarity threshold into operational taxonomic
125 units (OTUs) using the UCLUST algorithm (Edgar 2010). All these steps were processed using the
126 Quantitative Insights In Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010). Sequences were
127 blasted using the FHiTINGS program (Dannemiller et al. 2014). Finally, OTUs that were represented
128 by fewer than five reads and observed only in a single sample were removed. 18S rDNA reads
129 passing the above-described quality control requirements were clustered and classified by mapping
130 the sequences to our existing maximum likelihood reference tree (for details on the sequences,
131 alignment and methods see Panzer *et al.* (2015). The effects of pH and time on specific fungal groups
132 were tested using generalized linear modeling (Zuur et al. 2009), as our data followed Poisson or
133 negative binomial distributions. Model selection was carried out using the Akaike information
134 criterion (AIC); models were ordered according to the AIC, and those with lowest scores were
135 compared using likelihood ratio tests (LR). The best model was then evaluated for significance of the
136 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.

2.5. Alpha diversity analyses based on the ITS2 dataset

Diversity indices were calculated based on subsampled datasets using the PRIMER 6 software (Plymouth Marine Laboratories, UK). Next, the effects of the factors "pH" and "incubation time" on the diversity indices were analyzed. In the case for 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 in section 2.4. 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 (ML) 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 (see section 2.4).

2.6. 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 analyses (PCoAs). The similarity percentage (SIMPER) procedure was used to identify OTUs that contributed most to the observed dissimilarity between the two 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.

2.7. 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 implemented in the Galaxy platform (Giardine et al. 2005, Goecks et al. 2010). Unweighted and weighted distances were calculated.

2.8. 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.

3. Results

3.1. 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 number 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 (Suppl. Table S2). 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.

3.2. Taxonomic composition of the samples

The ITS2 dataset revealed Ascomycota to be the dominant phylum in all sample types, comprising between 72 to 96% of the relative (rel.) sequence abundance, from which 38 – 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 two 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 two weeks were also detected in the positive control (Suppl. Table 3).

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 – 98%) as in the ITS2 dataset, with Eurotiomycetes and Leotiomycetes as dominant groups in both sample types, followed by Dothideomycetes. The percentage of Basidiomycota sequences was relatively small (1 – 7%) and did not exceed more than 2% of the rel. sequence abundance in samples with a pH of 7.67. Using the 18S rDNA dataset, five 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% rel. sequence abundance. 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 (Suppl. Table 4).

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 five out of fifteen and eleven out of twenty-seven groups of the ITS2 and 18S rDNA datasets, respectively, were included in this analysis. Of those, the abundance of ten groups was significantly affected by the factor "pH". With the exceptions of Eurotiomycetes and ambiguous Ascomycota, the remaining eight 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).

3.3. 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

228 term (Table 2). The diversity of the abundant species declined at a pH of 7.67 (Suppl. Table S5,
229 Table 2). In contrast, the richness was higher at a pH of 8.26.

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

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

239 **3.4. Phylogenetic analysis**

240 Phylogenetic community structure: The unweighted UniFrac-based PCoA did not clearly separate the
241 samples with different pH treatments (Fig. 3A). Similar results were found when using the weighted
242 UniFrac as a metric, but the separation was slightly more pronounced than for the first analysis (Fig.
243 3B), while incubation time only showed an effect on samples from the first week. This indicates that
244 most of the samples are relatively similar from a phylogenetic perspective and that observed
245 community shifts must be explained based on shifts in taxa abundance, which is in accordance with
246 the non-phylogenetically based PCoA analyses.

247 Phylogenetic signal analysis: The Phylocom analysis showed significant phylogenetic signals in both
248 community types. The signals always indicated phylogenetic clustering with values of 2.2 for the
249 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
250 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).

4. Discussion

4.1. Assessing the double-marker gene approach for community analysis

In our approach, we used two 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 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 two 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.

4.2. Community responses

The mycoplankton community of the microcosms showed a significant response to the pH treatment. However, the communities in the two 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 the given environmental conditions (Horner-Devine & Bohannan 2006). In the communities in the two 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 (Penalva & 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

302 germination time and outgrowth can be affected by the environmental pH (Magan & Lacey 1984,
303 Porter et al. 1987). For pathogenic fungi with host-independent life stages (e.g., some Cryptomycota
304 or Chytridiomycetes species), responses to pH may depend on the life stage.

305 **4.3. Relevance for food webs**

306 Microbial communities highly influence resource use, disease dynamics and the stability of
307 ecosystem processes (Duffy & Stachowicz 2006, Stachowicz & Byrnes 2006). For example, a pH-
308 dependent loss of fungal diversity in freshwater systems (Wood-Eggenschwiler & Bärlocher 1983,
309 Tolkkinen et al. 2015) can lead to a drastic reduction in fungal leaf breakdown (Baudoin et al. 2008).
310 So far, comparable data for marine fungi are lacking, but recent studies on marine mycoplankton
311 have elucidated their importance in the breakdown of organic matter (Gutierrez et al. 2011) and the
312 control of phytoplankton populations (Gleason et al. 2008, Gutierrez et al. 2016). Thus, zoosporic
313 fungi are part of the pelagic food web, forming a "mycoloop"; herein, they (i) act as a food source for
314 zooplankton grazers, (ii) decompose particulate organic matter, (iii) convert inorganic compounds
315 into organic compounds, and (iv) parasite/hyperparasite on diverse organisms (Kagami et al. 2007,
316 Kagami et al. 2014). In our study, players in the "mycoloop", namely, Cryptomycota and
317 Chytridiomycetes, showed significant reductions under a reduced pH (Table 1). According to
318 dynamical model predictions, a reduction in zoosporic fungi in pelagic systems would affect the
319 overall food web structure: fewer fungal parasites lead to a higher abundance of large phytoplankton
320 species (>40 µm), which in turn lowers the numbers of smaller phytoplankton species due to resource
321 competition. As zooplankton species only graze on smaller phytoplankton, a reduction in zoosporic
322 fungi has an indirect and negative effect on zooplankton grazers (Miki et al. 2011). The involvement
323 of filamentous fungi in the pelagic food web has so far not been modeled due to missing data. In our
324 study, two of the most prominent groups, Eurotiomycetes and Dothideomycetes, both showed a
325 significant pH effect (Table 1). Gutierrez *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 seven 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 commercially 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.

4.4. Conclusion

Although the marine fungal communities in the two 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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6. Author contributions

Experimental design: EK, AW, GG. Sampling and setup of the experiment: EK. Data analyses: MR, KP. Statistical analyses: GG, LG. Writing of the paper: MR.

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561

562 **Table 1: Generalized modeling framework test of the effects of pH and week on the 13 most abundant fungal groups.** For several
563 groups, the test was not applicable due to low abundance (n/a). Displayed are the average abundances of the tested fungal groups at a pH of
564 8.26 and 7.67 as observed in the two different datasets, ITS and 18S rDNA. The p-value indicates whether the pH effect had a significant
565 effect on the occurrence of the fungal group and whether this was dependent on incubation time. Finally, the trend in abundance within the
566 two pH treatments is given. Av. Abund., average abundance; SD, standard deviation; P, p-value; L, pH 7.67; H, pH 8.26; ns, not significant.

Fungal group	**Av. Abund. ± (SD) at pH 8.26 (ITS)**	**Av. Abund. ± (SD) at pH 7.67 (ITS)**	**p (ITS)**	**Av. Abund. ± (SD) at pH 8.26 (18S rDNA)**	**Av. Abund. ± (SD) at pH 7.67 (18S rDNA)**	**P (18S rDNA)**	**Trend in abund. over the two pH treatments**
Cryptomycota	0	0	n/a	350±25.1	23±1.1	$4.3x10^{-14}$	L<H
Chytridiomycetes	0	0	n/a	184±5.1	61±2.8	$1.47x10^{-08}$	L<H
Microbotryomycetes	16 ±1.3	58±2.5	10^{-4}	44±2.4	35±1.4	$8.8x10^{-3}$ (for week 4) $4.2x10^{-2}$ (for week 1)	L>H L<H
Agaricomycetes	92±3.6	1±0	10^{-8}	66±5.7	14±1.3	$8x10^{-4}$	L<H
Ambiguous Ascomycota	371±8.1	582±4.9	$2x10^{-4}$	16±1.2	0	n/a	L>H
Ascomycota *Incertae Sedis*	2±0	0	n/a	42±2.1	16±1	$2x10^{-3}$	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	$3.57x10^{-05}$	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	$2.25x10^{-05}$	L>H

567

Table 2: Results of generalized linear model analysis testing the effects of incubation time and pH on the richness. Test included the examination of negative binomial and Poisson error structure. Likelihood ratio tests (LR) and associated (Chi squared-based) p-values compare the top two 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)=15.46. AIC: Akaike information criterion; LR: likelihood ratio test; p: p-value (Chi squared-based).

Fixed structure	**Error structure**	**AIC**	**LR**	**p**
Time + pH + TxpH	Negative binomial	341	15.46	0.0014
Time + pH	Negative binomial	351		
Time + pH + TxpH	Poisson	401		
Time + pH	Poisson	467		

574 **Table 3: PERMANOVA main test of fungal community composition based on ITS2-based Bray-Curtis dissimilarities of operational**
575 **taxonomic units.** Displayed are tests for the factors “pH” and “incubation time”, their interaction and the partitioning of multivariate
576 variation. p-values were obtained using type III sums of squares and 9999 permutations under the full model. *, statistical significance ($p < 0.05$); d.f.: degrees of freedom; SS: sums of squares; Sq. root: square root of the component of variation attributable to that factor in the
578 model in units of Bray-Curtis dissimilarity.

Sources of variation	**d.f.**	**SS**	**Pseudo-F**	**Sq.root**
pH*	1	28308	15,177	36,925
Time	3	8301	14,835	96,348
pH × time	3	7791	13,924	12,275
Residuals	31	57821	43,188	
Total	38	1025200000		

579

580 **Table 4: SIMPER analysis of the ITS2 dataset.** Operational taxonomic units (OTUs) contributing most to the observed community
581 dissimilarity among the two pH sample types. Only OTUs with more than 1% contribution to the observed community dissimilarities are
582 listed. Av. Abund., average abundance; Av. Diss, average dissimilarity.

Species	**Av. Abund. at pH 8.26**	**Av. Abund. at pH 7.67**	**Av. Diss.**	**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*	Incertae sedis	Incertae sedis
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*	Incertae sedis
Pezizomycotina sp.	2.13	6.27	1.82	2.22	*Ascomycota*	*Pezizomycotina*	Incertae sedis	Incertae sedis
Eurotiomycetes sp.	0.11	3.63	1.47	1.8	*Ascomycota*	*Pezizomycotina*	*Eurotiomycetes*	Incertae sedis
Pezizomycotina sp.	1.37	4.35	1.29	1.58	*Ascomycota*	*Pezizomycotina*	Incertae sedis	Incertae sedis
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*	Incertae sedis	Incertae sedis
Pezizomycotina sp.	1.22	3.89	1.21	1.48	*Ascomycota*	*Pezizomycotina*	Incertae sedis	Incertae sedis
Pezizomycotina sp.	0.77	2.82	0.91	1.11	*Ascomycota*	*Pezizomycotina*	Incertae sedis	Incertae sedis
Eurotiomycetes sp.	0	2.2	0.9	1.1	*Ascomycota*	*Pezizomycotina*	*Eurotiomycetes*	Incertae sedis
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*	Incertae sedis

583

584 **Figure legends**

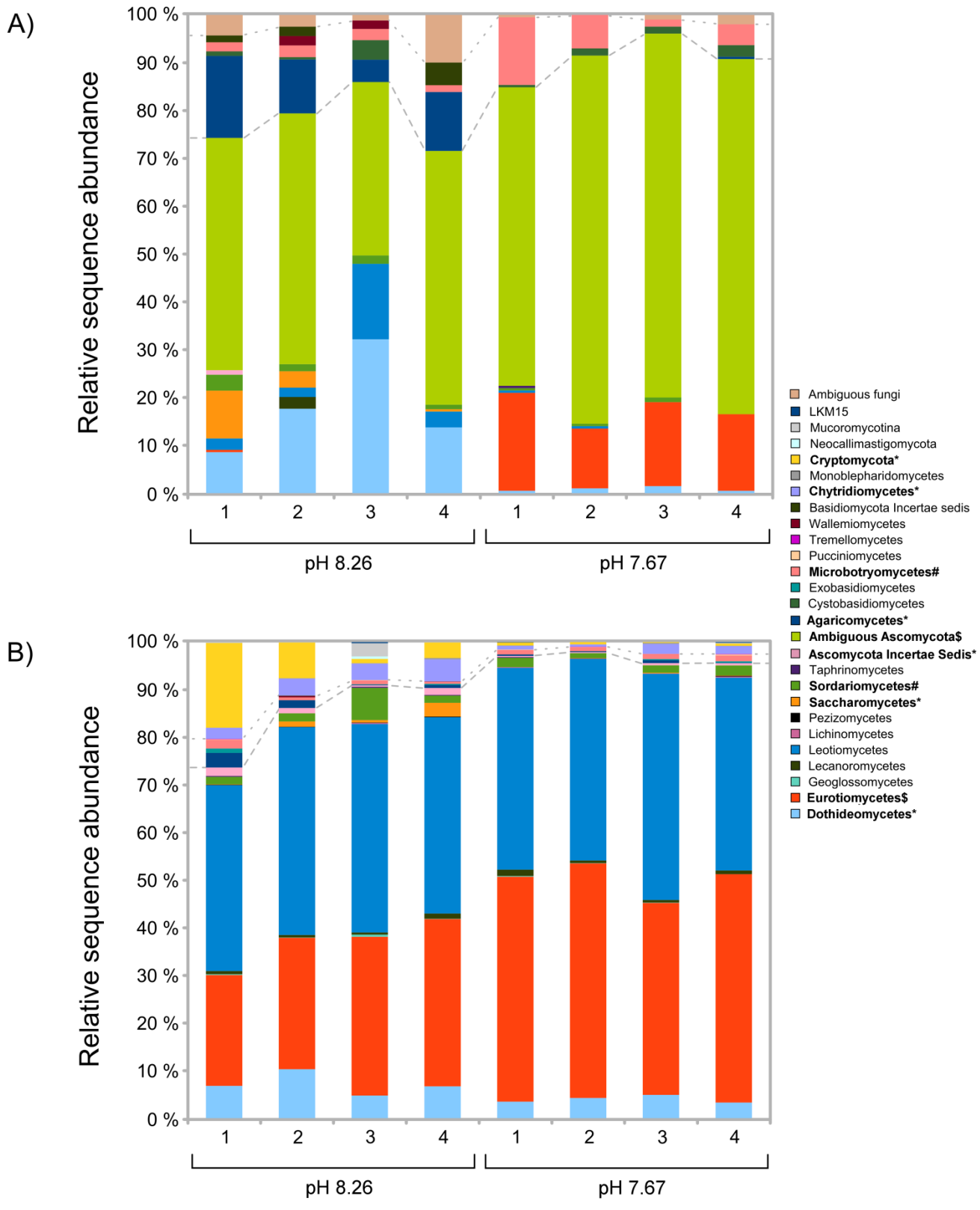
585 **Figure 1: Taxonomic assemblage of fungal communities in the two tested pH sample types over**
586 **four weeks of incubation.** Taxonomic composition based on the (A) ITS and (B) 18S rDNA dataset.
587 Fungal groups in bold showed a significant pH response: *, reduced abundance at a pH of 7.67; $,
588 increased abundance at a pH of 7.67; #, not all time points showed consistent effects. For more detail,
589 see Table 2. 1-4, weeks of incubation. Bottom to dashed lines: *Ascomycota*; dashed to dotted lines:
590 *Basidiomycota*; dashed lines to top: basal fungal lineages *sensu lato*.

591 **Figure 2: Principal coordinate analysis (PCoA) of the ITS2 dataset.** The test is based on square
592 root-transformed data using Bray-Curtis dissimilarities of operational taxonomic unit counts.

593 **Figure 3: Ordination analysis of phylogenetic distances revealing phylogenetic relatedness**
594 **among communities.** PCoAs are based on (A) unweighted and (B) weighted UniFrac metrics using
595 the 18S rDNA data.

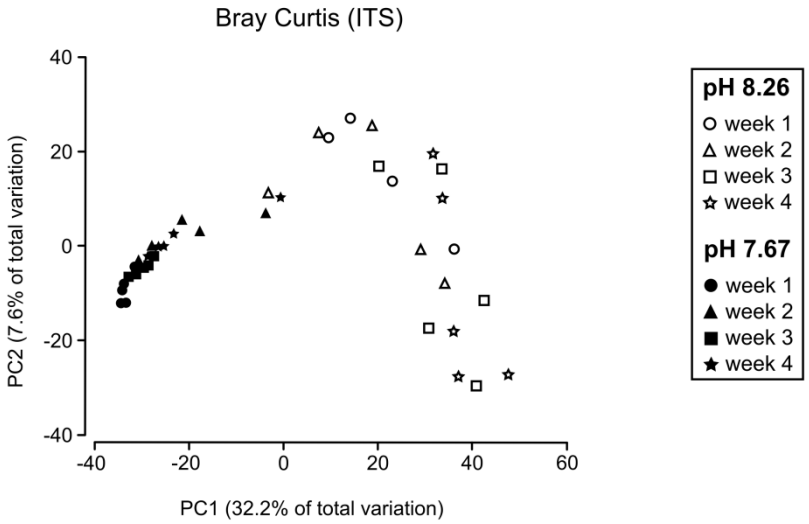
596 **Figure 4: Identification of phylogenetic signals via Phylocom analysis testing for clade-specific**
597 **adaptation to a pH of 8.26 and 7.67.** (A) Comstruct analysis revealed significant phylogenetic
598 clustering in samples for both pH treatments. (B) Nodesig analysis identified the fungal groups that
599 caused the observed phylogenetic signals. The schematic phylogenetic tree depicts only the fungal
600 clades that significantly contributed to the observed phylogenetic clustering. Identified significant
601 clustering was assigned to class/subphylum (left) and order levels (right). NRI, net relatedness index;
602 NTI, nearest taxon index; blue, clustering effect present in both pH sample types; red, clustering
603 effect observed only in the sample type with a pH of 8.26; green, effect only observed in sample type
604 with a pH of 7.67.

Figure 1:


A)
B)
Relative sequence abundance
100 %
90 %
80 %
70 %
60 %
50 %
40 %
30 %
20 %
10 %
0 %
1
2
3
4
pH 8.26
pH 7.67
Ambiguous fungi
LKM15
Mucoromycotina
Neocallimastigomycota
Cryptomycota*
Monoblepharidomycetes
Chytridiomycetes*
Basidiomycota Incertae sedis
Wallemiomycetes
Tremellomycetes
Pucciniomycetes
Microbotryomycetes#
Exobasidiomycetes
Cystobasidiomycetes
Agaricomycetes*
Ambiguous Ascomycota$
Ascomycota Incertae Sedis*
Taphrinomycetes
Sordariomycetes#
Saccharomycetes*
Pezizomycetes
Lichinomycetes
Leotiomycetes
Lecanoromycetes
Geoglossomycetes
Eurotiomycetes$
Dothideomycetes*

607 **Figure 2:**

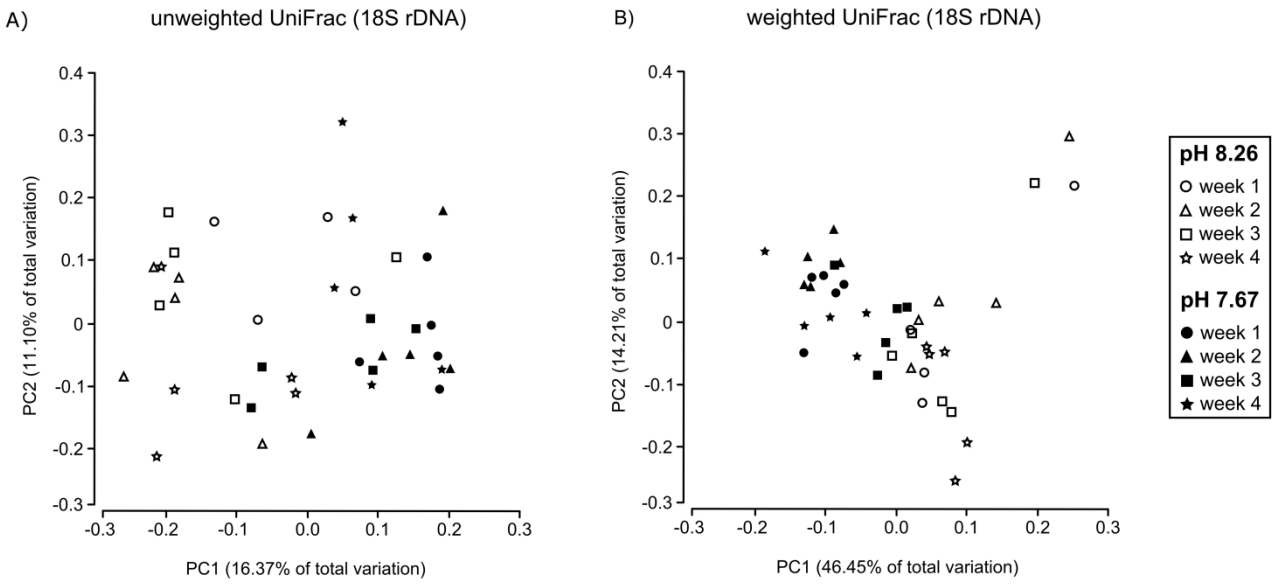
608



609

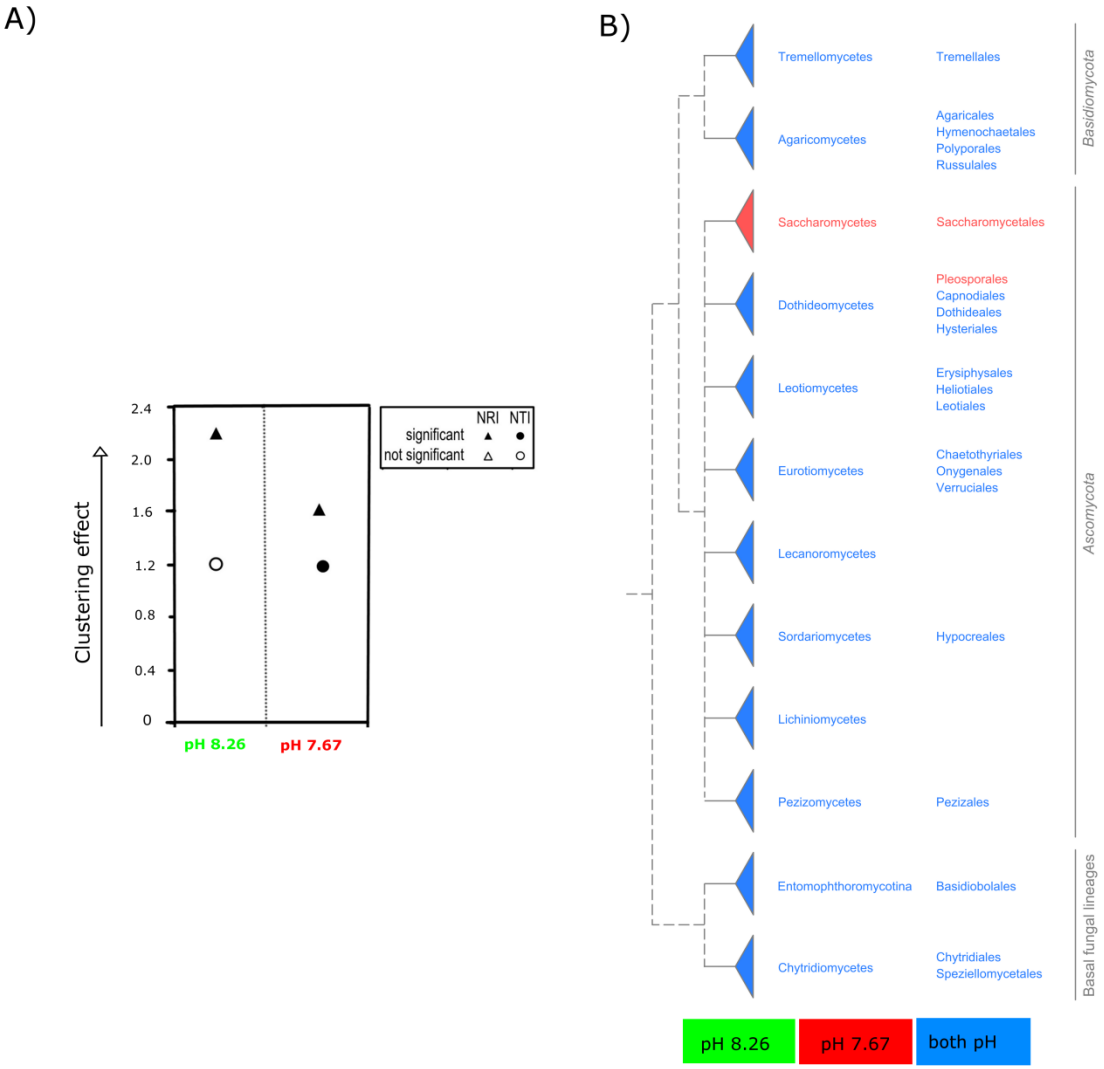
610 **Figure 3:**

611



612

613 **Figure 4:**



614