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ORIGINAL ARTICLE

Study on the effects of near-future ocean acidification on marine yeasts: a microcosm approach

Evamaria Krause · Antje Wichels · René Erler · Gunnar Gerdts

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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 2 and 4 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 *Debaryomyces 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.

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E. Krause (✉) · A. Wichels · R. Erler · G. Gerdts
Alfred Wegener Institute for Polar and Marine Research, Biologische Anstalt Helgoland, Kurpromenade 201, 27498 Helgoland, Germany
e-mail: evamaria.krause@awi.de

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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 (Kutty and Philip 2008). 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 (Meyers and Ahearn 1974; Kutty and Philip 2008) and are frequently found on decaying algae (van Uden and Castelo Branco 1963; Seshadri and Sieburth 1971; Patel 1975). Additionally, yeasts occur associated with marine animals, for example, fish, shrimps, mussels, corals, and seabirds (van Uden and Castelo Branco 1963; Kawakita and van Uden 1965; Ravindran et al. 2001; Burgaud et al. 2010; Yang et al. 2011). Notably, pathogenic yeast species can also cause infections in animal hosts such as marine mammals and copepods (Seki and Fulton 1969; Higgins 2000). For marine fungi in general, the above-mentioned points have been reviewed by, for example, Hyde et al. (1998), Jones (2011), and Richards et al. (2012).

The optimum growth conditions for yeasts and filamentous fungi are at lower pH levels than those encountered in the seas (Davis 2009; 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, that is, below pH 7 (Norkrans 1966; Hoppe 1972). This preference for low pH levels leads to the question whether marine fungi 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). So far, microbial processes identified to be affected by ocean acidification are nitrogen fixation and cyanobacterial photosynthesis (Liu et al. 2010). 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). Yet, almost nothing is known concerning the direct acidification effects on marine occurring yeasts and filamentous fungi. In this study, we focus on marine yeasts, for which previous records are available at our study site (see below).

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, for example, estuarine, coastal, open-ocean, deep-sea, and various extreme habitats (Gadanho et al. 2003, 2006; Gadanho and Sampaio 2004, 2005; Chen et al. 2009; Burgaud et al. 2010; Coelho et al. 2010). 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 (Kurtzman and Robnett 1998; Fell et al. 2000). 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) analysis (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., Marklein et al. 2009; Stevenson et al. 2010; van Veen et al. 2010; Dhiman et al. 2011). 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; Oberbeckmann et al. 2011; Emami et al. 2012).

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 (Meyers et al. 1967; Gerdts et al. 2004) and additional information on yeast identities based on phenotypic identifications is available for certain periods (Meyers et al. 1967; Ahearn and Crow 1980). 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 2 and 4 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 setup

We sampled water at Helgoland Roads (54°11.3′N, 7°54.0′E) on April 14, 2011. The study site and microbiological sampling procedures have been described elsewhere (Wiltshire et al. 2010; Teeling et al. 2012). 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 1,000 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 airtight 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 2 and after 4 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 taken 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), that is, 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 airtight 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 made with the program CO2calc (Robbins et al. 2010), using the 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).

Yeast isolation and sampling of yeast biomass

To cultivate yeasts, samples from the initial seawater sample (10, 100, 500, and 1,000 ml) and from the experiment (10 and 100 ml) were filtered through sterile nitrocellulose filters (0.45-µm pore size, 47 mm diameter, gray 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, that is, 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 6 days. For MALDI-TOF MS, biomass was resuspended in 300 µl sterile deionized water and 900 µl pure ethanol was 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 (2,000–20,000 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, and values between 1.7 and 2.0 are considered 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. Furthermore, a reference spectrum dendrogram was created (MALDI BIOTYPER 3.0 software). In this dendrogram, the relationship between organisms is reflected by an arbitrary distance level, with distances normalized to a maximum value of 1,000. 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 gene 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). PCRs were performed in duplicate in volumes of 50 µl containing 50 ng of template DNA, 5 µl Taq Buffer (10×), 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 the comparison of the closest relatives in NCBI retrieved by BLAST. Sequences with more than 1,200 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 4 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 1,195 to 1,238 µatm. For the in situ treatment (pH_{NBS} 8.10), we calculated pCO_2 values from 459 to 493 µatm (Table 1).

Yeast isolation

The initial seawater sample yielded 48 (±1 SD) 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 2 weeks and 17 after 4 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

Table 1 pH_{NBS}, total alkalinity, and calculated pCO_2 at the start of the experiment and after 2 or 4 weeks of incubation

Treatment	pH_{NBS}	Total alkalinity (µmol kg^{-1})	pCO_2 (µatm)
pH 8.10			
Start	8.10	2,362	477
Week 2	8.12 ± 0.00	2,366 ± 5	459 ± 5
Week 4	8.09 ± 0.02	2,354 ± 21	493 ± 21
pH 7.81			
Start	7.81	2,257	931
Week 2	7.81 ± 0.01	2,235 ± 11	919 ± 35
Week 4	7.79 ± 0.01	2,242 ± 12	973 ± 26
pH 7.67			
Start	7.67	2,206	1,238
Week 2	7.70 ± 0.01	2,193 ± 2	1,195 ± 18
Week 4	7.69 ± 0.01	2,203 ± 9	1,236 ± 43

Given are the mean ± SD ($n = 5$)

pH_{NBS} pH measured on the National Bureau of Standards scale

their mass spectra to reference strains in the MALDI BIOTYPER database: Three isolates (EK61, EK62, EK82) displayed score values above 2.2 with *Debaryomyces 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, that is, 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 (Fig. 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 (Fig. 1). The phylogenetic placement of representative isolates is shown in Fig. 2 for species in the phylum Ascomycota and in Fig. 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 reference spectrum dendrogram (Fig. 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.

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) (Fig. 1). Considering only MALDI-TOF MS classes representing yeasts however (classes 3, 4, and 13 had filamentous fungi as closest relatives), only 8 ascomycete isolates were obtained, constituting less than 16 % of the yeasts isolated.

The majority of isolates were identified as the filamentous fungus *D. fagi* and as different members of the yeasts *Cryptococcus* sp. and *Candida sake*. Rare species were *Leucosporidium scottii*, *Cystofilobasidium infirmominiatum*, *G. pullulans*, *Kondoa malvinella*, and isolates related to the filamentous fungi *R. pratensis* var. *pratensis* and *T. setigerum* (Fig. 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) (Fig. 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 *C. sake* (class 1) and *L. scottii* (class 18), had also been obtained from the initial seawater sample. Additionally, one new class (class 16), identified as *G. 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 (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

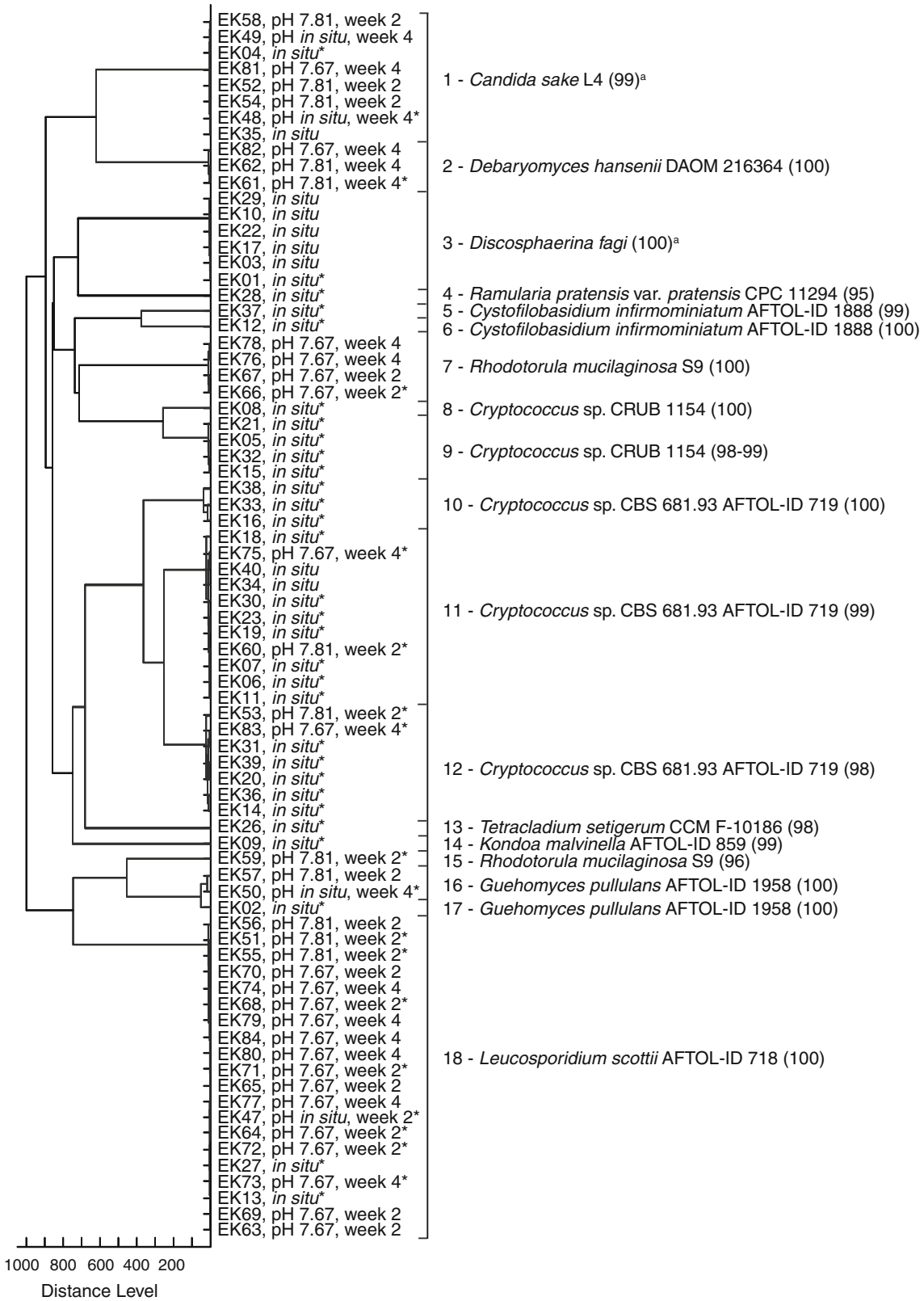

Fig. 1 Reference spectrum dendrogram of isolates, based on MALDI-TOF mass spectra. Isolates with score values above 2.0 among each other were grouped into classes. Isolates from the original seawater sample are designated as in situ; for isolates obtained after incubation, the pH level and the time of sampling are indicated. For each MALDI-TOF MS class, the closest relative and similarity (%) of representative isolates, as determined by partial LSU rRNA gene 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 gene 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	*Debaryomyces 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

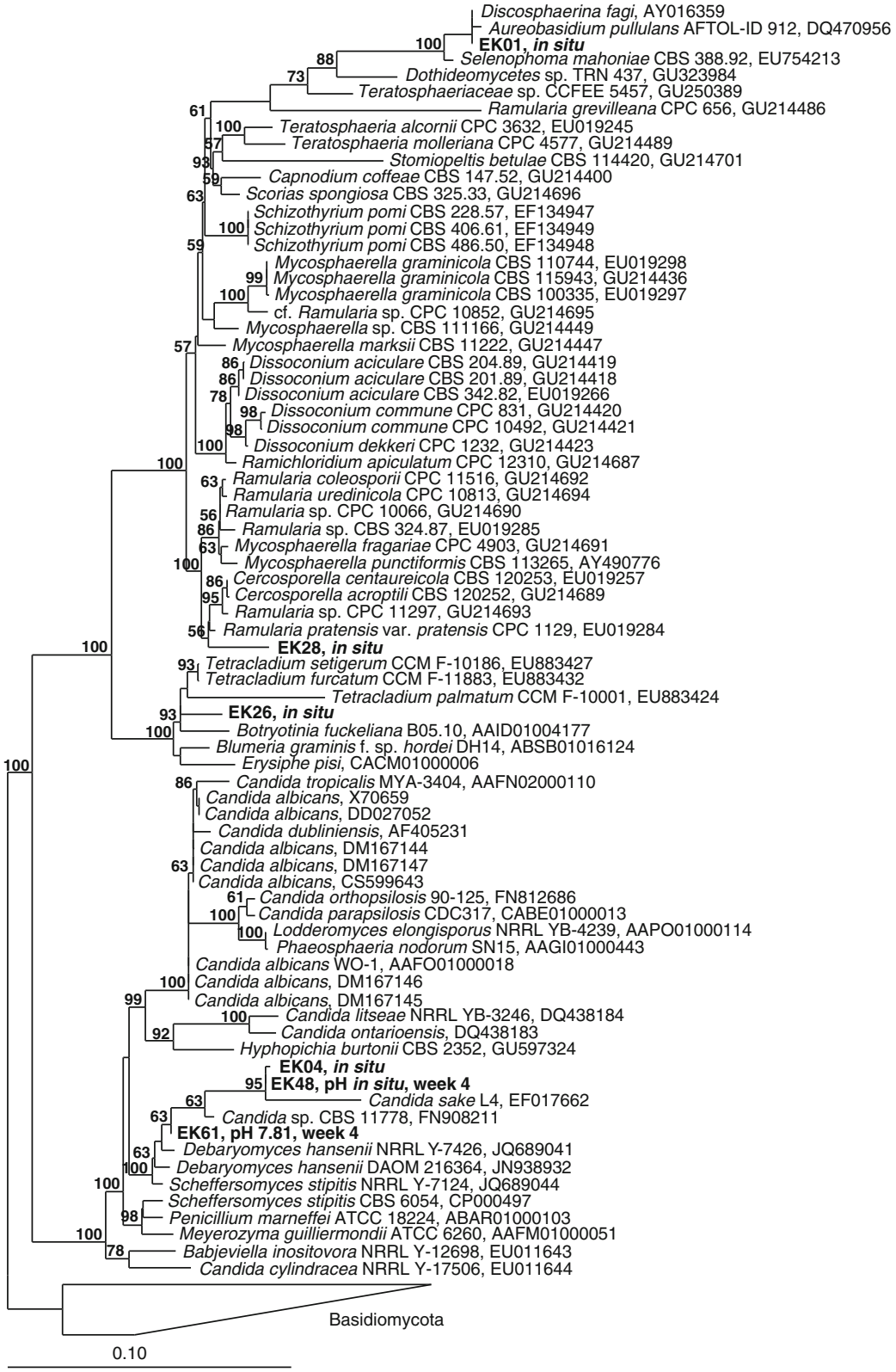

Fig. 2 Phylogenetic tree of members of the phylum Ascomycota, based on partial LSU rRNA gene sequences. Isolates obtained in this study (EK) are highlighted in *bold*; isolates from the initial seawater sample are designated as in situ; for isolates obtained after incubation, the pH level and the time 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

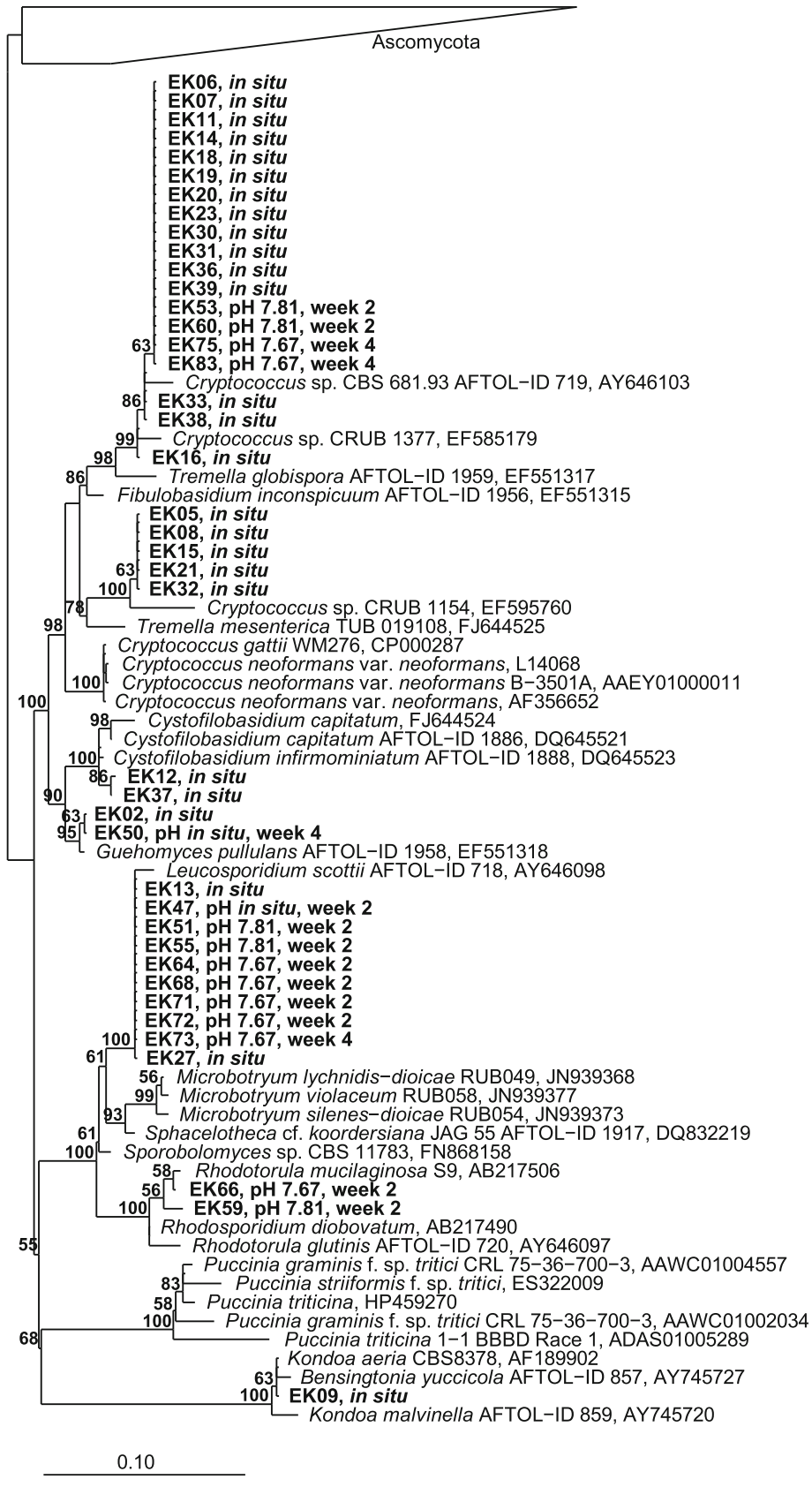

Fig. 3 Phylogenetic tree of members of the phylum Basidiomycota, based on partial LSU rRNA gene sequences. Isolates obtained in this study (EK) are highlighted in *bold*; isolates from the original seawater sample are designated as in situ; for isolates obtained after incubation, the pH level, and the 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

Table 3 Isolates obtained from the initial seawater sample (in situ) and after 2 and 4 weeks of incubation at different pH levels, based on MALDI-TOF MS classification

MALDI-TOF MS class[a]	in situ	pH in situ		pH 7.81		pH 7.67	
		Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
1. *Candida sake* L4 (99)	+	−	+	+	−	−	+
2. *Debaryomyces 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 gene sequence analysis

classes were found only after incubation at near-future pH levels and were identified as *R. mucilaginosa* (class 7), *D. 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 *L. scottii*. Remarkably, isolates of this class were obtained considerably more often after incubation at near-future pH levels, especially from pH 7.67 (Fig. 1).

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 marine yeasts. 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 (Table 1). 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 SD), 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 (Schulz et al. 2009; Hurd 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 *R. mucilaginosa* or *D. 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 (Table 2). Likewise, different classes were found to represent different closest relatives and percentage similarities. Yet, one exception occurred, as *G. pullulans* was found as the closest relative with 100 % sequence similarity for two classes. This may hint at differences at the subspecies 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 *D. 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 (Gadanho and Sampaio 2002; Gadanho et al. 2003; Chen et al. 2009). Yet, direct identification of environmental yeast isolates by MALDI-TOF MS is still limited. As identification successes obtained for clinical yeast isolates are very promising (Marklein et al. 2009; Stevenson et al. 2010; van Veen et al. 2010; Dhiman et al. 2011), an expansion of the database toward 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) (Meyers et al. 1967; Crow et al. 1977; Ahearn and Crow 1980; Gerdts et al. 2004).

The most frequently isolated species belonged to typical marine yeast genera and a yeast-like filamentous fungus. Isolates were predominantly identified as related to *D. fagi*, *Cryptococcus* sp., and *C. sake* (Fig. 1). *Discosphaerina* (formerly *Columnosphaeria*) *fagi* has been suggested as a possible teleomorph, that is, 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 *C. 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 (Hatai and Egusa 1975; de Araujo et al. 1995; Brilhante et al. 2011).

Among the rare species, the high incidence of species known from cold environments can be pointed out, namely *L. scottii*, *C. infirmominiatum*, *G. pullulans,* and *Kondoa malvinella* (Fig. 1). These species occur in Arctic or Antarctic seawater, algae, and glacial environments (Fonseca et al. 2000; Vaz et al. 2011; Buzzini et al. 2012). However, most of them have also been reported from soil (Maksimova and Chernov 2004; Lim et al. 2010) 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 *R. pratensis* var. *pratensis*, a necrotrophic plant pathogen described on *Rumex crispus* (Crous et al. 2007), and the aquatic hyphomycete *T. 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 from the North Sea (Meyers et al. 1967; Meyers and Ahearn 1974; Ahearn and Crow 1980; Crow et al. 1977). However, we found *D. hansenii* and *R. mucilaginosa* (formerly *R. rubra*) only after incubation (Table 3), although they were the most abundant species in North Sea water samples in both 1964–1966 and 1976 (Meyers et al. 1967; Ahearn and Crow 1980). 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 (Meyers et al. 1967; Ahearn and Crow 1980). 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 versus 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 the 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 versus near-future pH levels

At in situ pH, we only found yeasts related to *C. sake*, *L. scottii,* and *G. pullulans*, which were all found in the initial seawater sample as well (Table 3). At near-future pH levels, we additionally found yeasts related to *Cryptococcus* sp., *R. mucilaginosa*, and *D. hansenii* (Table 3). Thus, after incubation at near-future pH levels, we recovered a higher number of species than after incubation at present-day pH. Furthermore, we obtained yeasts not detected in the initial seawater sample only after incubation at near-future pH levels. 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 *L. scottii*, the number of isolates was inversely related to pH (Fig. 1), 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 1,000 ml for the initial seawater 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 also 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 *L. scottii*, *R. mucilaginosa,* and related species, *Cryptococcus* sp. and *D. 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. This may hold true for fungi in general, as osmotrophic feeding driven by the plasma membrane proton gradient is a characteristic feature of this group (Davis 2009; Richards et al. 2012). 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 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, *R. mucilaginosa* and *D. hansenii* are considered emerging yeast pathogens (Hazen 1995). Notably, the importance of yeasts in surveillance programs at bathing beaches has already been pointed out (Vogel et al. 2007; Shah et al. 2011).

Concerning microbial food webs, it is furthermore interesting to note that a moderate acidification does not seem to influence bacterial abundances (Grossart et al. 2006; Allgaier et al. 2008; 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 *L. scottii*, *R. mucilaginosa*, *Cryptococcus* sp., and *D. 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.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments described comply with the current German laws.

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