ANNEXURE

List of publications from this study:


Cathrine Sumathi Jebaraj and Chandralata Raghukumar. Fungal activity in a marine oxygen-depleted laboratory microcosm. (MS submitted)


Presentation in workshop:

Anaerobic denitrification in fungi from the coastal marine sediments off Goa, India

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ABSTRACT

Denitrification is a microbial process during which nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen. The Arabian Sea contains one of the major pelagic denitrification zones and in addition to this, denitrification also takes places along the continental shelf. Prokaryotic microorganisms were considered to be the only players in this process. However recent studies have shown that higher microeukaryotes such as fungi can also adapt to anaerobic mode of respiration and reduce nitrate to harmful greenhouse gases such as NO and N₂O. In this study we examined the distribution and biomass of fungi in the sediments of the seasonal anoxic region off Goa from two stations. The sampling was carried out in five different periods from October 2005, when dissolved oxygen levels were near zero in bottom waters to March 2006. We isolated mycelial fungi, thraustochytrids and yeasts. Species of Aspergillus and thraustochytrids were dominant. Fungi were isolated under aerobic, as well as anaerobic conditions from different seasons. Four isolates were examined for their denitrification activity. Two cultures obtained from the anoxic sediments showed better growth under anaerobic condition than the other two cultures that were isolated from oxic sediments. Our preliminary results suggest that several species of fungi can grow under oxygen deficient conditions and participate in denitrification processes.

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Introduction

Anaerobic denitrification is an alternate respiratory process in prokaryotes that enables them to thrive under oxygen-depleted conditions. Denitrifying bacteria utilize nitrate and (or) nitrite as the final electron acceptor in their respiratory cycle and release nitrogen gas to the atmosphere (Zumft 1997). During this process, they successively reduce nitrate to nitrite, nitric oxide, nitrous oxide and nitrogen with the help of the enzymes dissimilatory nitrate reductase (nar), nitrite reductase (nir), nitric oxide reductase (nor) and nitrous oxide reductase (nos). In a marine nitrogen cycle this is an important pathway through which the fixed nitrogen is lost and leads to an imbalance in the total nitrogen budget (Naqvi et al. 2006). Nitric oxide (NO) and nitrous oxide (N₂O) are produced as intermediates during the denitrification process. These are among the harmful greenhouse gases that influence the earth’s climate by the destruction of the ozone in the stratosphere.

The Arabian Sea is characterized by a perennial, open ocean oxygen minimum zone (OMZ) and a seasonal, coastal anoxic region along the western continental shelf of India. The anoxic condition develops during the southwest monsoon, following the upwelling and intensifies during September and October each year. The coastal anoxic region is a hot spot for N₂O emission, a greenhouse gas that influences the
earth’s climate by the destruction of the ozone in the stratosphere (Naqvi et al. 2000). Microbial communities of the oxygen-depleted environment have often been assumed to have low species richness (Levin 2003). Culture independent studies in the oxygen-depleted environments have shown that these regions harbor a vast microbial diversity (Behnke et al. 2006; Dawson & Pace 2002; Massana et al. 2004; Stoeck & Epstein 2003). These microbes have unique physiological adaptations to survive in the adverse conditions. Recently reported group of anaerobic ammonia oxidizing bacteria is one of the examples (Dalsgaard et al. 2003). Molecular ecological studies have also shown a vast diversity of microeukaryotes in the anoxic regions of Cariaco Basin off the Venezuelan coast in the Caribbean (Stoeck et al. 2006) and in anaerobic sulfide and sulfur-rich spring in Oklahoma (Qingwei et al. 2005). The sequences of small subunit rDNA have revealed presence of deep novel branches within green algae, fungi, cercozoa, stramenopiles, alveolates, euglenozoa, unclassified flagellate and a number of novel lineages that has no similarity with any of the known sequences (Massana et al. 2004; Zuendorf et al. 2006). This suggests that oxygen-depleted environments harbor diverse communities of novel organisms, each of which might have an interesting role in the ecosystem.

The involvement of fungi as denitrifiers has been recently shown in the grassland ecosystem based on substrate-induced respiratory inhibition studies, which showed that they account for nearly 80% of the nitrous oxide production (Ronald & Laughlin 2002). Screening of fungal isolates has shown that all the major groups of fungi are capable of denitrification process, though they predominantly form only nitrous oxide (Shoun et al. 1992).

The presence, diversity and role of fungi in denitrification processes in the marine nitrogen cycle have not been studied. We have attempted in this study to survey the presence of fungi in the seasonal oxygen minimum zone off Goa. We also screened a few fungi for nitrate reduction and ammonia formation under aerobic and anaerobic conditions.

**Materials and methods**

**Sampling site and collection of sediments**

Two field stations Station-I (St-I), 15° 31' 080" N, 73° 39' 00" E and Station-II (St-II), 15° 30' 522' N, 73° 39' 00" E within the coastal anoxic zone off Goa (Fig 1) were sampled from October 2005 to March 2006. Sediment samples were collected with a gravity corer (66 cm length and 7 cm diam.) from these two locations. The overlying water was siphoned out and the cores were cut at 2 cm intervals down to 8 cm and extruded into alcohol sterilized clean plastic containers. They were processed in the laboratory on the same day for isolation of fungi and fixed in formalin for direct detection of fungi and bacteria. The remaining sediments were stored at -20°C for estimation of total organic carbon. Samples for dissolved oxygen in the near bottom water were fixed in Winkler’s reagents on board and stored in an icebox. Nitrite and DO were estimated in the laboratory immediately on return (Strickland & Parsons 1968).

**Distribution of fungi**

Isolation by particle plating technique

A portion of the sediment from the middle of each sub-section was removed with a flame-sterilized spatula and placed in sterile vials for isolation of fungi (Raghukumar et al. 2004). The media used for isolations were malt extract agar (MEA), malt extract broth (MEB), corn meal agar (CMA) and Czapek Dox agar (CDA). All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi. They were prepared in seawater and fortified with streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) to inhibit bacterial growth. Fungi were isolated by modified particle plating technique (Bills & Polishook 1994). For this approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 μm and 100 μm screens. The particles that passed through 200 μm mesh but were retained on the 100 μm mesh were spread-plated (Damare et al. 2006). Culturable colony forming units (CFU) of fungi were expressed as numbers g⁻¹ dry sediment of 100-200 μm size particles. Fungi isolated from the sediments were sub cultured and maintained on MEA slants at 5°C. Sporulating cultures were identified using the morphological keys (Domsch et al. 1980).
Fig 2 – Nitrite levels in micromole (bar) and dissolved oxygen concentration (line) of the near bottom water at St-I (A) & St-II (B).

Isolation by enrichment culturing
Isolation of cultures by enrichment method was carried out as follows. Approximately, 5 g of sediment samples were incubated in airtight glass bottles in 80 ml of enrichment media that consisted only of 10 mM sodium nitrate solution prepared in artificial seawater and were supplemented with antibiotics to inhibit growth of bacteria. The medium was then flushed with nitrogen gas and incubated for 30 d. At the end of the

Table 1 – Fungi isolated by particle plating of sediments from St-I & II in different seasons

<table>
<thead>
<tr>
<th>Field Trip</th>
<th>St-I</th>
<th>St-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture #</td>
<td>Identification</td>
<td>Percentage frequency</td>
</tr>
<tr>
<td>Oct 05</td>
<td>#1</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#11</td>
<td>Trichothecium sp.</td>
</tr>
<tr>
<td></td>
<td>#12</td>
<td>Humicola sp.</td>
</tr>
<tr>
<td></td>
<td>#21</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># An-1</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># An-2</td>
<td>Fusarium sp.</td>
</tr>
<tr>
<td></td>
<td># An-4</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
<tr>
<td>Nov 05</td>
<td>#1</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># T-77</td>
<td>Thrustochytrid</td>
</tr>
<tr>
<td></td>
<td>#30</td>
<td>Myceliophora sp.</td>
</tr>
<tr>
<td></td>
<td>#31</td>
<td>Byssochlamys sp.</td>
</tr>
<tr>
<td></td>
<td>#32</td>
<td>Cleistothecial form</td>
</tr>
<tr>
<td></td>
<td>#21</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Jan 06</td>
<td># 42-y</td>
<td>Yeast</td>
</tr>
<tr>
<td></td>
<td># 43-y</td>
<td>Yeast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thrustochytrids</td>
</tr>
<tr>
<td>Mar 06</td>
<td>#1</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># F0</td>
<td>Cladosporium sp.</td>
</tr>
<tr>
<td></td>
<td>#21</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># 57-aY</td>
<td>Yeast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
<tr>
<td>Apr 06</td>
<td>#1</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td># Th</td>
<td>Thrustochytrid</td>
</tr>
<tr>
<td></td>
<td># 60-Y</td>
<td>Yeast</td>
</tr>
<tr>
<td></td>
<td>#11</td>
<td>Trichothecium sp.</td>
</tr>
<tr>
<td></td>
<td>#21</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
</tbody>
</table>

a Fungi obtained by enrichment culturing; ND: no data.
incubation period, fungal colonies were isolated from these sediments by particle plating as described above.

**Bacterial and fungal abundance**

For direct counts of total bacteria, 1 g wet sediment was suspended in 10 ml sterile seawater with formalin as a fixative (5% final concentration) and was stored in the dark at 4°C. The formalin-fixed samples were sonicated (3 x 30 sec) in a water bath sonicator (Biosystems Ltd, India) and allowed to settle for 5 min on ice. The overlying clear water sample was filtered over 0.22 µm black polycarbonate nuclepore filters (Millipore, USA) and stained with an aqueous solution of acridine orange (0.01%) for 3 min. Bacterial cells were counted from 10-20 microscope fields with an epifluorescence microscope (Olympus BX60, Japan). The final numbers were expressed as total counts g⁻¹ dry sediment. The bacterial numbers were converted to fg carbon using the conversion factor of 20 (Peduzzi & Hendle 1991). The final values were expressed as pgC g⁻¹ dry sediment.

To estimate fungal biomass, formalin-fixed sediment was treated with 10% EDTA, stained with 0.01% of filter sterilized calcoflour (Sigma Chemicals, USA). Microscopic mounts of the sediment were then examined under ultraviolet light filter (excitation wave length 330 to 385 nm and barrier filter BA 420) of an epifluorescence microscope (Olympus BX60, Japan) to detect fluorescing fungal hyphae. The hyphal lengths were measured using an ocular micrometer. Considering the hyphae as a cylinder, length (h), the hyphal diameter as 2µm and applying the formula $3.14 \times \frac{h^2}{4}$, the total hyphal lengths were expressed as biovolume g⁻¹ dry sediment. The biovolume was converted to biomass using the conversion factor 0.2 g cm⁻³ (Newell et al. 1986). The C biomass was calculated by considering that 50% of the biomass content was C (Bittman et al. 2005). The results of fungal C biomass were expressed as pg C g⁻¹ sediment. The values are average of 2 replicate sediment samples examined. Bacterial cells, fungal hyphae and spores were photographed with a digital camera (Olympus 4.1 Mp, Japan).

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**Fig 3** - Fungal CFU g⁻¹ sediment at St-I (A) & St-II (B) (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, ● April 2006).

**Fig 4** - (A) fungal hyphae in sediments stained with the fluorescent brightner, Calcofluor. Bar = 10 µm, (B) Acridine orange-stained bacteria from the sediments, Bar = 10 µm.
**Estimation of organic carbon (OC)**

The OC content of the samples was determined by the difference between total carbon (TC) and inorganic carbon (IC). TC was analyzed by combustion of the samples at 1200 °C in an oxygen atmosphere and detection of CO₂ by coulometry (Prakash Babu et al. 1999). Inorganic C was analyzed by coulometry (UIC Coulometric®), after liberation of CO₂ in an acidification module (Engleman et al. 1985). An in-house reference standard (TW-TUC) was used for testing reproducibility and accuracy. The values are expressed as % OC and are average of 2 replicates.

**Screening of fungi for their nitrate utilization capacity under aerobic and anaerobic conditions**

Four different fungi were studied for their growth and denitrifying capacity. They were, # An-2 (*Fusarium* sp.) isolated after anaerobic incubation of the sediment, # 11 (*Tritirachium* sp.) which was isolated from the sediment during anoxic condition and # 31 (*Byssochlamys* sp.) and # 31a (*Paeclomycetes* sp.) were isolated from the sediments when the conditions were oxic. These cultures were compared with a well studied denitrifier of terrestrial origin, *Fusarium oxysporum* # MT-811 (Shoun & Tanimoto 1991), a gift from Dr. Shoun, Tokyo University, Japan. Starter cultures of these fungi were grown in mineral medium supplemented with 10 mM of sodium nitrate for 3 to 5 d. Approximately 10-15 mg (dry weight) of the mycelial suspension was used as an inoculum. The cultures were maintained under aerobic conditions in 100 ml conical flasks plugged with cotton containing 20 ml of medium and under anaerobic conditions in 100 ml serum bottles sealed air tight with butyl rubber stoppers and steel crimps after flushing with nitrogen gas.
through the medium for 2 min. The dissolved oxygen (DO) was determined by spectrophotometric method (Pai et al. 1993) at 0 h and at the end of the experiment on the day 10 and on days when there was significant nitrite formation. Replicate bottles were used exclusively for DO measurement. The cultures were harvested every 48 h up to 10 d and nitrite and ammonia formed were determined by spectrophotometrically (Strickland & Parsons 1968). The growth of the cultures was also measured on day 10 and biomass in mg dry weight was determined. All chemicals used were of analytical grade.

Statistical analyses were carried out using Excel (Microsoft) programme. The data were transformed and tested for normality before analysis by Cochran Q test.

### Results

The physico-chemical characteristics of the near bottom water at the two stations showed typical denitrifying conditions during October 2005, when the levels of DO were near zero and nitrite accumulation was seen and oxic conditions were restored in the same site by January 2006 (Fig 2).

#### Distribution of fungi

Isolations using both aerobic and anaerobic incubations yielded a total of 54 fungi from sediments of both the stations during the 5 sampling periods between October 2005 and April 2006 by the particle plating technique (Table 1). Among the mycelial fungi that formed CFUs, Aspergillus species showed the highest frequency of occurrence during most of the sampling period at both the stations. Humicola sp. was also frequent during the anoxic period of October 2005. The straminipilan fungi, thraustochytrids were the next most abundant fungi. The number of CFUs obtained by particle plating technique from each section of the sediment core ranged between 64 to 2622 g⁻¹ dry sediment of 100-200 μm size particles (Fig 3A and B). Enrichment culturing was carried out with samples collected from the two stations during the

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**Table 2 - ANOVA: two factor to show the significance of distribution between different parameters at spatial and temporal levels**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Df</th>
<th>F value</th>
<th>F-critical value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial C</td>
<td>7</td>
<td>1.1</td>
<td>2.5</td>
<td>0.41</td>
</tr>
<tr>
<td>(between depths)</td>
<td></td>
<td>8.2</td>
<td>3.1</td>
<td>0.001***</td>
</tr>
<tr>
<td>(between seasons)</td>
<td>3</td>
<td>1.0</td>
<td>1.7</td>
<td>0.44</td>
</tr>
<tr>
<td>Fungal C</td>
<td>7</td>
<td>1.0</td>
<td>2.5</td>
<td>0.44</td>
</tr>
<tr>
<td>(between depths)</td>
<td>3</td>
<td>1.7</td>
<td>3.1</td>
<td>0.001***</td>
</tr>
<tr>
<td>(between seasons)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal CFU</td>
<td>7</td>
<td>1.2</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>(between depths)</td>
<td>3</td>
<td>17.5</td>
<td>3.1</td>
<td>0.001***</td>
</tr>
<tr>
<td>(between seasons)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOC</td>
<td>5</td>
<td>2.9</td>
<td>2.7</td>
<td>0.04*</td>
</tr>
<tr>
<td>(between depths)</td>
<td>4</td>
<td>3.9</td>
<td>2.9</td>
<td>0.02*</td>
</tr>
<tr>
<td>(between seasons)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance. **significant at 0.1 %, *significant at 5 % level.)

---

**Table 3 - Correlation coefficient (r) between the biological parameters with DO as a dependent variable**

<table>
<thead>
<tr>
<th>DO</th>
<th>Bacterial C</th>
<th>Fungal C</th>
<th>Fungal CFU</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.34</td>
<td>-0.19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bacterial C</td>
<td>0.30</td>
<td>-0.19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fungal C</td>
<td>0.52</td>
<td>-0.08</td>
<td>-0.10</td>
<td></td>
</tr>
<tr>
<td>Fungal CFU</td>
<td>0.10</td>
<td>0.36</td>
<td>-0.19</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>0.17</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
The Nitrate utilization capacity of fungi under aerobic was found in the 2-4 cm section of the April 2006 sampling at St- 
but an isolate (# An-2) identified as Fusarium sp. was also 
sediments), # 11, from anoxic sediments and # 31 and # 
analyzed by 2-way ANOVA (Table 2). Bacterial and fungal bio-
nificantly different between the various sampling periods as 
ence, (Table 2). On the other hand, fungal CFUs and OC were sig-
sampling periods and showed no statistically significant differ-
from 2.5 to 3.5 % at the two stations (Fig 6A and B). The bacterial,
II (Fig 5B) and that of fungi at St-I in 4-6 cm sediment section 
ment cores at the two stations ranged between 1.2 to 
with acridine orange were measured by epifluorescence mi-
Fungal hyphae stained with Calcoflour, an optical brightener 
obtained (Table 1).

Bacterial, fungal and organic carbon

Fungal hyphae stained with Calcoflour, an optical brightener (Fig 4A) and bacterial cells (Fig 4B) from the sediments stained with acridine orange were measured by epifluorescence microscopy. Bacterial and fungal biomass for four depths of sediment cores at the two stations ranged between 1.2 to 500 x 10^3 pg C g^-1 sediment and 0.01 to 0.206 x 10^3 pg C g^-1 sediment respectively (Fig 5A-D). The maximal bacterial C biomass was found in the 2-4 cm section of the April 2006 sampling at St-II (Fig 5B) and that of fungi at St-I in 4-6 cm sediment section during January 2006 (Fig 5C). Total organic carbon ranged from 2.5 to 3.5 % at the two stations (Fig 6A and B). The bacterial, fungal biomass C and the organic C were more or less uniformly distributed in the sediment core from 0-8 cm during most of the sampling periods and showed no statistically significant difference, (Table 2). On the other hand, fungal CFUs and OC were significantly different between the various sampling periods as analyzed by 2-way ANOVA (Table 2). Bacterial and fungal biomass was not statistically related to dissolved oxygen content of the near bottom water and OC (Table 3).

Nitrate utilization capacity of fungi under aerobic 
and anaerobic conditions

The culture # An-2 (isolated after anaerobic incubation of 
seeds), # 11, from anoxic sediments and # 31 and # 31a from oxic sediments were screened for their ability to reduce nitrate. The culture # MT-811 was included as positive culture. The cultures grew under anaerobic conditions as was evident from the increase in biomass measured on the last day of the experiment (Table 4). Two cultures, # An-2 and # 11 showed equally good growth under aerobic and anaerobic conditions, the cultures # 31 and # 31a showed seven and fivefold less growth respectively under anaerobic condition and # MT-811 showed a tenfold decrease in biomass. A distinct increase in nitrite accumulation was noticed intermittently in cultures grown in flasks (Table 5). The initial DO value in the flasks was 115.2 μM, as the cultures grew, DO was utilized and suboxic conditions developed (18-
97 μM) on day 4 or 6 depending on the increase in biomass. As the conditions became suboxic the cultures started to utilize nitrate for respiration and nitrite accumulation was seen in the flasks of the cultures (Table 5A). In the anaerobic cultures (after flushing with N2 gas) the initial DO was 9.5 μM and a significant difference in nitrite accumulation was observed under anaerobic conditions between cultures (Table 5B). Maximum nitrite accumulation was noticed in # 31 and this was statistically significant (P = 0.005) between aerobic and anaerobic cultures (Table 5C).

Ammonia formation was seen in cultures maintained under anaerobic condition. The positive control # MT-811 as well as all the other cultures showed ammonia formation to a varying degree during anaerobic incubation (Table 6A). A 2-way analysis of variance indicated significant difference between cultures in their capacity to accumulate ammonia

<table>
<thead>
<tr>
<th>Culture #</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td># MT-811</td>
<td>156.7 (96.4)</td>
<td>7.1 (12.3)</td>
<td>1852.1 (237.1)</td>
<td>199.4 (32.6)</td>
<td>56.9 (61.7)</td>
</tr>
<tr>
<td># An-2</td>
<td>85.5 (74.0)</td>
<td>270.7 (150.1)</td>
<td>213.7 (87.0)</td>
<td>455.9 (198.6)</td>
<td>394.8 (12.3)</td>
</tr>
<tr>
<td># 31</td>
<td>220.8 (105.4)</td>
<td>64.1 (0)</td>
<td>584.1 (203.8)</td>
<td>49.9 (32.6)</td>
<td>904.7 (163.2)</td>
</tr>
<tr>
<td># 31a</td>
<td>206.6 (32.0)</td>
<td>163.8 (65.9)</td>
<td>605.5 (184.2)</td>
<td>356.2 (117.7)</td>
<td>370.4 (89)</td>
</tr>
<tr>
<td># 31b</td>
<td>171.0 (42.7)</td>
<td>142.5 (49.4)</td>
<td>149.6 (21.4)</td>
<td>149.6 (354.0)</td>
<td>220.1 (89)</td>
</tr>
</tbody>
</table>

Values within brackets denote standard deviation.
**Discussion**

During this study except for thraustochytrids, all the other fungi obtained were common terrestrial fungi. Terrestrial run-off during monsoon might be the major source of input for such terrestrial fungi. Alternatively, many terrestrial species of fungi might have become adapted to marine conditions. This is evident from several recent reports of terrestrial species of fungi (geofungi) from the marine environment. The occurrence of endolithic fungi associated with molluscan shells as microborers (Golubic et al. 2005); Aspergillus sydowii as a pathogen of seafan in the Caribbean (Shinn et al. 2000); terrestrial fungi as putative pathogens in scleractinian corals (Raghukumar et al. 2006). Four cultures studied in the present study were capable of growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (#11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (Table 4). These results indicate that fungi isolated from the anoxic sediments might have adapted to a facultative anaerobic mode of life. Shoun & Tani-moto (1991) have shown that # MT-811 can grow under anaerobic conditions and utilize nitrate and nitrite for dissimilatory purpose. They have also shown that there is a substantial amount of cell growth during this process, which shows that the dissimilatory nitrate or nitrite reduction is an energy yielding reaction.

We observed denitrification activity by fungi under varying oxygen concentrations in our present study. Fungal denitrification process differs significantly from classical bacterial denitrification. Bacterial denitrification takes place only in the complete absence of oxygen and even a trace of oxygen could be toxic to the obligate anaerobes and inhibit denitrification. In fungi, reduction of nitrate takes place under suboxic conditions (300–900 μM O2) but excess oxygen (>900 μM O2) is growth of fungi therein. Occasionally fungal spores were also detected. Organic material in the sediments apparently supports growth of fungi. There was almost a uniform distribution of bacterial, fungal and organic carbon in the sediment core from 0–8 cm. Water mixing, high sedimentation rates and intense activity of the benthic meiofauna could have brought about this homogeneity of the sediments. Such a phenomenon has been also reported in deep-sea sediments of the Central Indian Ocean, where the conditions are more stable (Raghukumar et al. 2006).

**Table 6B – ANOVA: two factor to show the significance of ammonia accumulation by different cultures on different days**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Df</th>
<th>F value</th>
<th>F-critical value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(anaerobic)</td>
<td>4</td>
<td>0.8</td>
<td>3</td>
<td>0.53</td>
</tr>
<tr>
<td>(aerobic)</td>
<td>4</td>
<td>1.0</td>
<td>3</td>
<td>0.42</td>
</tr>
<tr>
<td>Different cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(anaerobic)</td>
<td>4</td>
<td>1.8</td>
<td>3</td>
<td>0.17</td>
</tr>
<tr>
<td>(aerobic)</td>
<td>4</td>
<td>11.5</td>
<td>3</td>
<td>0.00014**</td>
</tr>
</tbody>
</table>

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance, **significant at 1 %).
shown to inhibit the process (Zhou et al. 2001). It was observed in our studies that in the culture # 31, maximum nitrite accumulation occurred on day 6 when suboxic conditions set in (Table 5). As all fungi are not capable of nitrate reduction, but can use nitrite as an electron acceptor (Takaya 2002) experiments to screen isolates for their nitrite reducing capacity are to be carried out. Further, fungal denitrification is an incomplete process in comparison with the classical pathway. Fungi are known to stop with the formation of N_2O and fungal denitrifiers are not reported to produce N_2 as the final product (Bleakley & Tiedje 1982; Shoun et al. 1998). Because of this incomplete system, denitrification by fungi causes an increase in the green house gases and leads to detrimental effects on the global climate.

Fungi also follow another pathway to reduce nitrate under complete anaerobic conditions, which is referred as ammonia fermentation. This process was studied in the same four isolates under both aerobic and anaerobic conditions. There was ammonia formation by all the cultures under anaerobic conditions (Table 6A and B) and # An-2 showed a significant difference between aerobic and anaerobic culture conditions (Table 6C). This process in fungi appears to be widespread as 15 of 17 fungi tested by Zhou et al. (2002) showed ammonia formation under anaerobic condition.

Studies on the denitrifying activities of Fusarium oxysporum # MT-811 have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O_2 tension (Takaya 2002). Fungi show a multimodal type of respiration to rapidly adapt to changes in the oxygen supply, in anoxic conditions ammonia formation takes place, while denitrification process in suboxic and oxygen respiration under aerobic conditions (Takaya 2002). This may be a survival strategy for mycelial fungi to thrive in extreme and dynamic environments.

Advancements in the area of molecular ecology have seen an advent of discoveries of new microorganisms that partake in biogeochemical process. New groups are being added to the list of microorganisms that have an active role to produce harmful green house gases like NO and N_2O. Recently, a benthic foraminifer Globobulimina pseudospinosa has been demonstrated to show complete denitrification in marine sediments (Risgaard-Petersen et al. 2006). Apart from this study Straminipiles (hauostochytrids) have also been reported from anoxic habitats (Kolodziej & Stoeck 2007) but no studies have been attempted so far to understand their role in these habitats.

Our present study is the first report showing involvement of mycelial fungi in denitrification process in the marine anoxic sediments. Further studies on the presence of various enzymes that are responsible in denitrification and the genes responsible for them will shed more light on fungal processes in sedimentary denitrification in oxygen minimum zone of the Arabian Sea off Goa.

### Acknowledgements

The authors are thankful to Dr. Dileep Kumar M, the COM009 project leader and team members for their help during the field trips and for the chemical analyses data. We are extremely grateful to Dr. Shoun H. for lending us the culture # MT-811 for our studies. We are thankful to Dr Seshagiri Raghukumar for his critical review of the manuscript and for helping us in the identification of the fungi. This is NIO contribution number 4440.

### REFERENCES


Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation

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Abstract

In order to study fungal diversity in oxygen minimum zones of the Arabian Sea, we analyzed 1440 cloned small subunit rRNA gene (18S rRNA gene) sequences obtained from environmental samples using three different PCR primer sets. Restriction fragment length polymorphism (RFLP) analyses yielded 549 distinct RFLP patterns, 268 of which could be assigned to fungi (Dikarya and zygomycetes) after sequence analyses. The remaining 281 RFLP patterns represented a variety of nonfungal taxa, even when using putatively fungal-specific primers. A substantial number of fungal sequences were closely related to environmental sequences from a range of other anoxic marine habitats, but distantly related to known sequences of described fungi. Community similarity analyses suggested distinctively different structures of fungal communities from normoxic sites, seasonally anoxic sites and permanently anoxic sites, suggesting different adaptation strategies of fungal communities to prevailing oxygen conditions. Additionally, we obtained 26 fungal cultures from the study sites, most of which were closely related (> 97% sequence similarity) to well-described Dikarya. This indicates that standard cultivation mainly produces more of what is already known. However, two of these cultures were highly divergent to known sequences and seem to represent novel fungal groups on high taxonomic levels. Interestingly, none of the cultured isolates is identical to any of the environmental sequences obtained. Our study demonstrates the importance of a multiple-primer approach combined with cultivation to obtain deeper insights into the true fungal diversity in environmental samples and to enable adequate intersample comparisons of fungal communities.

Introduction

Fungi are primarily aerobic heterotrophs that play an essential role as decomposers of organic matter in a variety of environments. In marine ecosystems, fungi are the major decomposers of woody and herbaceous substrates and their importance lies in their ability to aggressively degrade lignocellulose (Newell, 1996). Marine fungi also contribute to the degradation of dead animals and animal parts (Kohlmeyer & Kohlmeyer, 1979), and are important pathogens of animals and plants or partners in mutualistic symbioses (Raghukumar, 1986; Alsumard et al., 1995; Pivkin, 2000).

Fungi were long thought to play only a minor role in the ecosystem processes of anoxic environments (Dighton, 2003). However, many fungal taxa were recently shown to possess metabolic adaptations to utilize nitrate and (or) nitrite as an alternative for oxygen (Shoun et al., 1992). This testifies to their potential to participate in anaerobic denitrification processes in biogeochemically highly active ecosystems such as the oxygen minimum zones (OMZs) of the Arabian Sea, which are characterized by high microbially mediated denitrification rates (Naqvi et al., 2006). Indeed, in a recent study, Jebaraj & Raghukumar (2009) showed that fungi isolated from anoxic marine waters of the Arabian Sea are capable of growth under...
oxygen-deficient conditions while performing anaerobic denitrification.

Such findings suggest that the abundance and ecological role of fungi in anoxic marine systems is probably underestimated. Support for this assumption comes from analyses of the small subunit rRNA locus (18S rRNA gene) amplified from genomic DNA isolated from environmental samples. While usually rare in open ocean surface waters, the majority of environmental 18S rRNA gene diversity surveys conducted in oxygen-depleted aquatic environments report large proportions of fungal 18S rRNA gene sequences (reviewed in Epstein & Lópe-Garcia, 2007). Many of these environmental sequences appear as unique phylogenetic branches that are highly divergent from previously described 18S rRNA gene fungal sequences (e.g. Takishita et al., 2005, 2007a, b; Lópe-Garcia et al., 2007; Stoek et al., 2007; Laurin et al., 2008). As a rule, such molecular diversity surveys targeting microbial eukaryotes have used domain-specific PCR primers to amplify 18S rRNA gene fragments from environmental samples. As such primers target most eukaryotic organisms across all major clades and only sample a small fraction of amplicon diversity present in a sample (Epstein & Lópe-Garcia, 2007), it is reasonable to assume that many fungi may have escaped these surveys. Thus, the full extent of fungal diversity may be orders of magnitude higher than these domain-targeted environmental diversity studies have shown. Indeed, PCR primers with specificity for fungal DNA, while reducing coamplification of DNA from nonfungal sources, were applied successfully for estimating the extent of fungal diversity in soil (e.g. Börneman & Hartin, 2000; Anderson et al., 2003; Malosso et al., 2006). To date, this targeted strategy that applies fungal-specific primers to analyze fungal diversity has gone untested for anoxic aquatic habitats.

Using previously published primers designed for the specific amplification of fungal 18S rRNA gene from mixed-origin genomic DNA (Maivald et al., 1994; Kappe et al., 1996; Vannio & Hantula, 2000; Gomes et al., 2003), we analyzed fungal diversity in samples from the OMZ of the Arabian Sea. Therefore, we constructed clone libraries with two fungal-specific PCR primer sets and one domain-specific PCR primer set, routinely used in environmental diversity surveys. This strategy not only allowed an insight into fungal diversity in OMZs of the Arabian Sea but also revealed the substantial proportion of fungal diversity that is missed in a domain-specific PCR primer approach. Additionally, we used standard cultivation techniques to complement the molecular diversity surveys, unearthing a different subset of the fungal communities under study than the molecular approach. Finally, community structure analyses suggest that fungal assemblages thriving under different oxygen regimes are significantly distinct from each other, probably reflecting different adaptations to geochemically distinct environments.

**Materials and methods**

**Sampling sites**

Samples were collected from three sites in the Arabian Sea (Table 1). (1) Sampling of sediments at the coastal station (15° 29.951'N, 73° 40.827'E) off Goa at 25 m depth was carried out in October 2005 during the anoxic season (25_ANS), with no dissolved oxygen (DO) detectable, and in January 2006 during the oxic season (25_OXS), when oxygen saturation was restored. (2) Sampling in the perennial OMZ off Goa (15° 20.10'N, 72° 54.11'E) at a depth of 200 m was carried out during May 2007, when we collected anoxic sediment (200_ANS) and anoxic near-bottom water (200_ANW). (3) As a comparison, we sampled the permanently oxygenated sediment from a 3-m-depth site (03_CRS) in the coral reef region off Kavaratti (10° 34.588'N, 72° 38.019'E) in February 2007. Coral sand samples were collected by divers in sterile containers. All other sediment samples were collected using an ~60-cm-long gravity corer while water was collected in 5-L Niskin bottles. All samples were divided into aliquots for the isolation of fungi for cultivation and for total genomic DNA extraction. Samples for isolation were stored at 5°C and processed within 24 h. Water samples for molecular analyses (5 L of water) were drawn on Durapore filters (0.45 μm; Millipore (India) Pvt Ltd, Bangalore) under the

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Designation (color coding in Figs 1-3)</th>
<th>Sampling date</th>
<th>DO (μM)</th>
<th>Temp (°C)</th>
<th>Salinity (PSU)</th>
<th>Sampling material</th>
<th>Sample volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Near a shore off Goa (25 m)</td>
<td>25_ANS (brown)</td>
<td>October 2005</td>
<td>ND</td>
<td>22.5</td>
<td>35.4</td>
<td>Sediment</td>
<td>~500 mg</td>
</tr>
<tr>
<td>Near a shore off Goa (25 m)</td>
<td>25_OXS (orange)</td>
<td>January 2006</td>
<td>118.4</td>
<td>26.9</td>
<td>34.7</td>
<td>Sediment</td>
<td>~500 mg</td>
</tr>
<tr>
<td>OMZ off Goa (200 m)</td>
<td>200_ANS (blue)</td>
<td>May 2007</td>
<td>ND</td>
<td>14.8</td>
<td>35.6</td>
<td>Sediment</td>
<td>~500 mg</td>
</tr>
<tr>
<td>OMZ off Goa (200 m)</td>
<td>200_ANW (turquoise)</td>
<td>May 2007</td>
<td>ND</td>
<td>14.8</td>
<td>35.6</td>
<td>Water</td>
<td>~5 L</td>
</tr>
<tr>
<td>Coral reef off Kavaratti (3 m)</td>
<td>03_CRS (green)</td>
<td>February 2007</td>
<td>93.74</td>
<td>28.7</td>
<td>34.0</td>
<td>Sediment</td>
<td>~500 mg</td>
</tr>
</tbody>
</table>

ND, below detection limit; Temp, temperature.
Fungal diversity in the Arabian Sea near-bottom water was determined spectrophotometrically. In brief, approximately 1 g of sediment slurry was spread-plated on different fungal media such as malt extract agar, corn meal agar, and Czapek Dox agar (HiMedia Pvt Ltd, India) prepared in seawater and fortified with streptomycin (0.1 g in 100 mL medium) and penicillin (40 000 U in 100 mL medium) to inhibit bacterial growth. Incubation was carried out under anoxic conditions. The cultures isolated from the different sampling stations were identified based on their morphology and partial (~1600 bp) 18S rRNA gene sequence.

Isolation and identification of culturable fungi

Culturable fungi were isolated using the particle-plating technique of Bills & Polishook (1994), with slight modifications. In brief, approximately 1 g of sediment slurry was serially sieved through a 200-μm and a 100-μm mesh. The particles that passed through the 200-μm mesh, but were retained on the 100-μm mesh were spread-plated on different fungal media such as malt extract agar, corn meal agar and Czapek Dox agar (HiMedia Pvt Ltd, India) prepared in seawater and fortified with streptomycin (0.1 g in 100 mL medium) and penicillin (40 000 U in 100 mL medium) to inhibit bacterial growth. Incubation was carried out under anoxic conditions. The cultures isolated from the different sampling stations were identified based on their morphology and partial (~1600 bp) 18S rRNA gene sequence.

Genomic DNA was extracted from the freeze-dried mycelial mats of each culture using a high salt concentration extraction buffer (100 mM Tris-HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8) and 1% CTAB). One milliliter of buffer was added to approximately 500 mg of mycelia and the total genomic DNA was extracted using chloroform–phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). The 18S region of the rDNA was amplified using fungal-specific primers NS1 and FR1 (Table 2). The 50-μL PCR reactions included 0.5 U HotStart Taq DNA polymerase (Qiagen, Hildesheim, Germany) in 1 x HotStart Taq buffer, 200 μM of each dNTP and 0.5 μM of each oligonucleotide primer. PCR was performed using initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 2 min, with a final extension at 72°C for 7 min. The PCR products were cloned separately for each fungal culture using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmids were isolated from positive overnight cultures using the Fast Plasmid Mini Prep kit (Eppendorf, Hamburg, Germany). One representative clone of each culture was sequenced bidirectionally (M13 sequencing primers) by MWG-Biotech on an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. In order to evaluate the intrastrain variability of the 18S rRNA gene, for five strains (FCAS35, FCAS36, FCAS41, FCAS89 and FCAS125), we have sequenced four amplicons. Sequences were included in phylogenetic analyses as described below. GenBank accession numbers of sequences from cultured isolates are GQ120154–GQ120179 and GU072534–GU072548 for duplicate amplicons.

PCR primer selection, environmental 18S rRNA gene clone libraries construction and operational taxonomic unit (OTU) calling

Total DNA from the five environmental samples (25_ANS, 25_OXS, 200_ANS, 200_ANW and 03_CRS) was extracted using a high salt concentration extraction buffer, followed by chloroform–phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). Amplification of 18S rRNA gene was carried out using three different primer sets (Table 2). The primer sets were chosen based on their specificity and adequate length of amplified fragments in order to carry out robust phylogenetic analyses.

The first primer set (Fungi) consisted of the fungal-specific NS1 and FR1 primers resulting in ~1650-bp fragments. According to homology searches, this primer set has only moderate specificity and coamplifies a range of non-fungal eukaryote genes such as Metazoa, Cercozoa, Viridiplantae, Alveolata, Centrobheliozoa and Bangiophyceae (Pang & Mitchell, 2005). The second primer set (Fung2) included the fungal-specific UF1 and S3 primers amplifying ~900-bp fragments. This primer set has a relatively high fungal specificity and previously amplified only a few nontarget taxa (green algae and Pseudomonas) from Antarctic soil samples (Malosso et al., 2006). We also applied the universal

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primers</th>
<th>Primer sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fung1</td>
<td>NS1</td>
<td>GTA GTC ATA TGC TGG TCT C</td>
<td>Vainio &amp; Hanula (2000)</td>
</tr>
<tr>
<td></td>
<td>FR1</td>
<td>AGC CAT TCA ATC GGT AIT</td>
<td>Gomes et al. (2003)</td>
</tr>
<tr>
<td>Fung2</td>
<td>UF1</td>
<td>CGA ATC GCA TGG CCT TG</td>
<td>Kappe et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>AGT CAA ATT AAG CCG CAG</td>
<td>Maiwald et al. (1994)</td>
</tr>
<tr>
<td>EukAB</td>
<td>EukA</td>
<td>AAC CTG GTT GAT CCT GCC AGT</td>
<td>Medlin et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>EukB</td>
<td>TGA TCC TTC TGC AGG TIC ACC TAG</td>
<td>Medlin et al. (1988)</td>
</tr>
</tbody>
</table>

Table 2. Primer sets used in this study to amplify 18S rRNA gene sequences from genomic environmental DNA.
eukaryote primer pair EukA and EukB, routinely used in environmental eukaryote diversity surveys (Massana et al., 2004; Stoeck et al., 2006; Euringer & Luenders, 2008), which amplifies nearly the full length of the 18S rRNA gene (Table 2) of a wide range of higher eukaryote taxon groups (Medlin et al., 1988).

Amplicons were ligated into pGEM-T vector and transformed into Escherichia coli cells (TOP 10 strain) using Invitrogen's TA-cloning kit as described above. For each library and primer set, we selected nearly 100 positively screened colonies (blue-white screening) for overnight growth and plasmid extraction using Qiagen's 96-well Directprep Kit. The presence of 18S rRNA gene inserts was confirmed by a standard M13-PCR amplification of extracted plasmids. In sum, 1/440 positively screened plasmids were subjected to restriction fragment length polymorphism (RFLP) analyses. Therefore, between 200 and 400 ng of amplification products with expected sizes were digested with 7.5 U of the restriction endonuclease HaeIII (New England Biolabs, Beverly, MA) for 60 min at 37 °C, followed by an inactivation step for 20 min at 80 °C. The resulting bands were separated by electrophoresis in a 2.5% low-melting-point agarose gel at 80 V for 2–3 h. At least one representative clone of each RFLP pattern (n = 549) was partially sequenced (c. 600 nucleotides) at MWG-Biotech as described above.

Because partial fragments could only be adequately aligned within each primer set (the sequence overlap was too small for partial fragments to be aligned between sequences obtained by different primer sets), sequences of each individual primer set were grouped separately into OTUs based on 99.0% sequence similarity using three independent (one for each primer pair-derived sequence set) DOTUR analyses (Schloss & Handelsman, 2005). One representative of each OTU that was identified as a fungal sequence using a BLASTN search in the GenBank nr-database (Altschul et al., 1997) was chosen for full-fragment sequencing (n = 100). The obtained sequences were checked for chimeras using the CHECK_CHIMERA CHECK program and the CHECK_CHIMERA utility [Ribosomal Database Project (Cole et al., 2003), as well as partial treeing analysis (Robison-Cox et al., 1995)]. Nine potentially chimeric sequences were identified and removed before subsequent sequence analyses. This left us with 91 full-fragment sequences that were analyzed together in a final DOTUR analysis. Similarities between two sequences were calculated using a custom program (PARALIGN) provided by M. Nebel (University of Kaiserslautern), which uses IUB matrix-based pairwise alignments. In total, 549 partial and 91 full-fragment environmental sequences have been deposited in the GenBank database under accession numbers GU071985–GU072533, GU072549–GU072590 and GQ120105–GQ120153.

Phylogenetic analyses

Environmental 18S rRNA gene sequences were compared initially with those in GenBank using BLAST analysis to determine their approximate phylogenetic affiliation. Sequences of environmental clones, together with their closest GenBank cultured and uncultured matches, were aligned using the arb FASTAaligner utility. Alignments were manually refined using phylogenetically conserved secondary structures. The conserved and unambiguously aligned positions were used in subsequent phylogenetic analyses. Maximum-likelihood analyses were conducted using RAxML (Stamatakis et al., 2008) on the CIPRES Portal v. 1.15 (http://www.phylo.org). The relative stability of tree topologies was assessed using 1000 bootstrap replicates. Heuristic searches for bootstrap analyses used stepwise addition, starting trees with simple addition of sequences and tree bisection and reconnection branch swapping. Maximum-likelihood bootstrap analyses were carried out using RAxML with all free model parameters estimated by RAxML as described in Stamatakis et al. (2008). Details on model parameters for the individual alignments are given in the legends of Figs 1–3.

Community comparisons

The program package SPADE (Chao & Shen, 2003–2005) was used to calculate the jaccard index as a measure of similarity between two communities based on the abundance of environmental fungal OTUs (Jabundance). An unweighted pair group mean average (UPGMA) cluster analysis based on Jabundance data was performed using the Cluster analysis module of STATISTICA v. 7 (StatSoft, Tulsa, OK). A Venn diagram was constructed to display the overlap in OTU composition between the different primer sets using VENNY (Oliveros, 2007). Species accumulation curves and abundance-based coverage estimator of species richness for all clone libraries were calculated in ESTIMATES (Colwell, 2005).

Results and discussion

We subjected 1440 environmental clones obtained from five individual sampling events in the Arabian Sea (Table 1) to RFLP analyses. The clones grouped into 549 distinct RFLP patterns, which were subjected to 18S rRNA gene sequence analyses. Surprisingly, GenBank BLASTN analyses of one representative sequence from each RFLP pattern identified only 268 unique RFLP patterns as members of the kingdom Fungi. Considering the replicates of these unique patterns, in total, we could putatively identify 455 out of the initial 1440 clones as taxonomically affiliated to the kingdom Fungi. The remaining RFLP patterns (n = 281) represented sequences falling into a number of different taxonomic
Fig. 1. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum Ascomycota showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site distribution shape parameter at 0.645 and the proportion of invariable sites at 0.312, based on 925 unambiguously aligned positions. Full support from 1000 bootstrap replicates is indicated by a black-filled dot at the respective node. Other support values are only displayed when > 50. Numbers in parentheses following environmental OTU names indicate the number of sequences of this OTU that were found in the individual libraries. Sequences from cultured isolates are in green and designated as FCAS followed by the culture identification number. Color coding for environmental libraries is given in Table 1.

Fungi, 54 OTUs for primer set Fung1, 12 OTUs for primer set Fung2 and 12 OTUs for primer set EukAB. After we sequenced one complete fragment from at least one representative of each individual OTU, we were able to analyze all sequences in one individual group (Table 3, Supporting Information, Table S1). The partial sequences (n = 268) obtained from representatives of each unique RFLP pattern grouped into 91 distinct OTUs called at 99% sequence similarity: 25 OTUs for primer set

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Fig. 2. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum Basidiomycota showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.737 and the proportion of invariable sites at 0.336, based on 785 unambiguously aligned positions. Further legend as in Fig. 1.

DOTUR run, which resulted in 48 distinct OTUs overall, indicating that a number of OTUs obtained by the individual primer-set analyses were shared among two or three primer sets. Table 4 presents an overview of the numbers of clones, RFLP patterns, sequences and OTUs analyzed in this study.
Fungal diversity in the Arabian Sea

Archaeospora leptoticha
Paraglomus occultum
Paraglomus scintillans
Glomus coronatum
Scutellospora nodosa

65

Gamsiella multidivaricata
Mortierella woffii

CCW 35 (anoxic environment, Cariaco Basin)

Conidiobolus brefeldianus
Conidiobolus coronatus
Gilbertella persicaria
Mucor racemosus
Pilaira anomala

Zychaea mexicana

LKM15 (detritus-dependent community)
Clone WIM27 (agriculture filed)

IAFDv7 (methanol-fed denitrification system)

Furculomyces boomerangus
Smittium commune
Pseudoharpella arcolamylica
Cochlonema euryblastum
Kuzuhaea moniliformis
Piptocephalis corymbifera

Chytridiomycota

Chytriales sp. JEL187
Rhizoclosmatium sp. JEL347-h

Fig. 3. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phyla Zygomycota, Glomeromycota and Chytridiomycota showing the position of the environmental OTU obtained from the Arabian Sea branching as sister to Mortierellales. The tree was constructed using a GTR + I + G evolutionary model with the variable-site \(\gamma\) distribution shape parameter at 0.571 and the proportion of invariable sites at 0.39 based on 1436 unambiguously aligned positions. Further legend as in Fig. 1.
Selectivity of fungal-specific primers

More than half of the unique RFLP patterns obtained and analyzed by sequencing and BLASTN (n = 549) turned out to be of nonfungal origin (n = 281) (Table 4). As expected, the largest proportion (74.07%) of such nonfungal sequences was retrieved within the RFLPs obtained using the universal eukaryote primer set EukAB, but also putatively fungal-specific primer sets retrieved 48.18% (Fung1) and 35.05% (Fung2) unique nonfungal taxa (Table 3). These findings met our expectations, considering that primer set Fung1 has a lower specificity to fungi compared with primer set Fungi, while excluding the coamplification of other eukaryote sequences. As noted earlier (Anderson et al., 2003), this is particularly difficult using the highly conserved 18S rRNA gene region (Page & Holmes, 1998). Several authors claim that they have designed or applied truly specific fungal 18S rRNA gene primer pairs (Smit et al., 1999; Borneman & Hartin, 2000). However, there is conflicting evidence for this specificity as other authors reported amplification of nonfungal templates using the same primer pairs (Borneman & Hartin, 2000; Anderson et al., 2003). It seems that most, if not all, fungal 18S rRNA gene primers are prone to observable cross-kingdom amplifications. Further evidence for this comes from BLAST homology analyses for fungal 18S rRNA gene primers indicating a relatively low success rate for most primer pairs in recovering fungal sequences, but coamplification of nonfungal targets (Pang & Mitchell, 2005). The degree to which cross-kingdom amplification occurs largely depends on the choice of specific primers, PCR conditions but also the abundance of specific fungal taxon groups in relation to the co-occurrence of eukaryote nontarget organisms in the environmental samples under study, i.e. when nontarget DNA is much more abundant in an environmental sample than target DNA, it is reasonable to assume that there is a greater than usual chance of nontarget DNA being amplified. Such systematic biases can distort diversity assessments. Furthermore, different fungal-specific primer sets may amplify different subsets of the fungal community (Fig. 4; Anderson et al., 2003). This severely affects the comparability of different fungal 18S rRNA gene data sets obtained from PCR amplification with different primers. A solution may arise from the application of a multiple-primer approach as suggested for general microbial eukaryote diversity surveys (Stoeck et al., 2006).

**Table 3.** Taxonomic assignment of unique nonfungal RFLPs (n = 281) after sequencing and BLAST analysis of one representative sequence from each RFLP pattern (n = 549), obtained after restriction digest of 1440 clones

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Taxonomic assignment of nonfungal RFLPs</th>
<th>Proportion of unique nonfungal RFLPs obtained with each primer set (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fung1</td>
<td>Corallochytrium; Choanoflagellates; Metazoa (Gastrotricha)</td>
<td>48.18</td>
</tr>
<tr>
<td>Fung2</td>
<td>Bacteria (Proteobacteria); Vibrio sp.; Rhizaria; Corallochytrium; Metazoa (Gastrotricha)</td>
<td>35.05</td>
</tr>
<tr>
<td>EukAB</td>
<td>Bacteria (Proteobacteria); Bacillariodermes, Firmicutes; Choanoflagellates; Rhizaria; Euglenozoa; Alveolaria; Metazoa (Anthropods, Nematodes, Annelidia)</td>
<td>74.07</td>
</tr>
</tbody>
</table>

The three primer sets used to generate the individual clone libraries are described in Table 2. Values given in the last column depict the proportion of unique nonfungal RFLP patterns (identified after BLAST analyses of obtained sequences from each unique RFLP relative to the number of total distinct RFLP patterns obtained with the respective primer set) (193 for Fung1, 194 for Fung2, and 162 for EukAB). A table detailing the taxonomic affiliation of each individual nontarget sequence (RFLP pattern) is given as Supporting Information.

**Diversity patterns of fungal communities in the Arabian Sea**

A UPGMA cluster analysis of the \( I_{\text{abundance}} \) index (Fig. 5) reveals that the fungal communities from the different sampling sites (Table 1) are distinctly different from each other. Of all the comparisons, the two coastal sites sampled under anoxic and oxic conditions (25_ANS, 25_OXS) are most similar in their fungal community membership (\( I_{\text{abundance}} = 0.48 \)). The sediment (200_ANS) and water column (200_ANW)-derived fungal communities from the OMZ offshore site cluster together with a \( I_{\text{abundance}} \) of 0.35. The fungal community from the coral reef reference site (03_CRS) showed the highest dissimilarity to all other samples (\( I_{\text{abundance}} = 0.06-0.11 \)).

The Indian Ocean has a characteristic seasonal anoxic condition that develops along the western margin during October to January (Naqvi et al., 2006). In this process, the coastal sites are subjected to seasonal oxygen fluctuations. Our analysis suggests a clear separation of fungal communities adapted to permanently oxic conditions, temporal anoxia and the ones adapted to permanent anoxia. Recent studies have shown that numerous fungi can adapt to alternate modes of respiration depending on oxygen availability (Daiber et al., 2005; Jelbaraj & Raghukumar, 2009). It is then reasonable to assume that the fungal community of the coastal site (25_ANS, 25_OXS), considered as an entity, may be capable of physiological adaptation when the oxygen...
Table 4. Overview of the total number of clones analyzed per library and primer set, total number of distinct RFLP patterns obtained, number of unique RFLP patterns that could be assigned to fungi and nonfungal taxon groups (for each distinct RFLP, we obtained a partial sequence that was analyzed by BLASTN and number of distinct OTUs (called at 99% sequence similarity) obtained after some analyses of full-length fragments for each primer set.

<table>
<thead>
<tr>
<th>Primer set: Fungi</th>
<th>Clone library</th>
<th>Total number of clones analyzed by RFLP</th>
<th>Total number of distinct RFLP patterns</th>
<th>Number of unique RFLP patterns assigned to fungi after sequencing</th>
<th>Number of unique RFLP patterns assigned to nonfungal taxon groups after sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>25_ANS</td>
<td>96</td>
<td>38</td>
<td>35</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>25_OXS</td>
<td>96</td>
<td>24</td>
<td>23</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>200_ANS</td>
<td>96</td>
<td>36</td>
<td>4</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>200_ANW</td>
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<td>62</td>
<td>18</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
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<td>96</td>
<td>33</td>
<td>20</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
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<td>193</td>
<td>100</td>
<td>25</td>
<td>93</td>
</tr>
<tr>
<td>Primer set: Fungi</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>96</td>
<td>46</td>
<td>29</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>25_OXS</td>
<td>96</td>
<td>30</td>
<td>27</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>200_ANS</td>
<td>96</td>
<td>37</td>
<td>26</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>200_ANW</td>
<td>96</td>
<td>55</td>
<td>20</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td>03_CRS</td>
<td>96</td>
<td>26</td>
<td>24</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
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<td>194</td>
<td>126</td>
<td>54</td>
<td>68</td>
</tr>
<tr>
<td>Primer set: EukAB</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25_ANS</td>
<td>96</td>
<td>42</td>
<td>17</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>25_OXS</td>
<td>96</td>
<td>17</td>
<td>7</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>200_ANS</td>
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<tr>
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<td>96</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>03_CRS</td>
<td>96</td>
<td>39</td>
<td>1</td>
<td>1</td>
<td>38</td>
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<tr>
<td>Total</td>
<td>480</td>
<td>162</td>
<td>42</td>
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<tr>
<td>Overall</td>
<td>1440</td>
<td>549</td>
<td>268</td>
<td>48</td>
<td>281</td>
</tr>
</tbody>
</table>

The total number of OTUs does not equal the sum of OTUs of the individual libraries, but reflects the number of unique OTUs for all five individual libraries (obtained with the same primer set) together. For details, see Materials and methods.

The overall number of OTUs does not equal the sum of OTUs of the three individual primer sets (= 91), but is a result from a separate homology analysis that determined the number of unique OTUs for all three individual primer sets together. For details, see the Materials and methods.

Considering these wide ecological and physiological adaptations of different fungi, it is not unexpected that fungal communities thriving under oxygenated conditions (25_OXS) are distinctively different from fungal communities living under permanently anoxic conditions (25_ANS). The same applies to those from anaerobic sediments (200_ANS) and anaerobic water samples (200_ANW). However, the actual degree of dissimilarity is difficult to assess with our data due to severe undersampling (Fig. 6).

Phylogeny of environmental fungal rDNA sequences

The analyses of the intraspecific variability of the 18S rDNA gene of five strains obtained in this study revealed a maximum divergence of 0.77% (FCAS35, 0.18–0.36%; FCAS36, 0.17–0.5%; FCAS41, 0.25–0.77%; FCAS89, 0–0.14%; and FCAS125, 0%). Thus, we are confident that a 1% divergence between two different OTUs is sufficient to represent distinct genotypes. The environmental OTUs identified in this study are designated as FAS (funga1 OTUs).
Fig. 4. Venn diagram showing the number of OTUs that were recovered simultaneously by two or three of the primer sets used in this study (Fung1, Fung2 and EukAB; see Table 2) and number of OTUs recovered exclusively with a single primer set. All OTUs, which were retrieved by the universal eukaryote primer set (EukAB), were also detected with the two fungal-specific primer sets (Fungi and Fung2).

Fig. 5. Dendrogram resulting from calculated Jaccard index based on incidence (incidence) of unique OTUs, as a measure of community similarity between the samples under study. For information on sampling sites, see Table 1. Similarity values were transformed into a distance matrix and subsequent cluster analysis was performed using the UPGMA algorithm.

from the Arabian Sea). The OTUs from the five sample sites were assigned to the Dikarya (Ascomycota, 27 OTUs; Basidiomycota, 20 OTUs), and one OTU branched within the subphylum Mucoromycotina (phylum Zygomycota).

OTUs belonging to the Ascomycota (Fig. 1) grouped within the subphylum Saccharomycotina and Pezizomycotina. FAS_2 is the most frequently represented OTU with a close similarity to the yeast Kodamaea sp. (99.67%) belonging to Saccharomyces. Saccharomyces yeasts are able to cope with anoxia while producing ethanol (Prior et al., 1989). A large number of OTUs (FAS_6, FAS_7, FAS_8, FAS_9, FAS_10, FAS_11 and FAS_12) belong to Sordariomycetes. Their closest known relatives belong to the genus Fusarium, which serves as a model to study fungal denitrification under low-oxygen conditions (Takaya et al., 1999; Daiber et al., 2005). Six OTUs from the Arabian Sea (Fig. 1) branch within the Eurotiomycetes. These sequences are affiliated with a number of environmental sequences obtained from a hypersaline anoxic Mediterranean deep-sea basin (Alexander et al., 2009), anoxic meromictic lake sediments (Takishita et al., 2007a), anoxic coastal sediments (Dawson & Pace, 2002), acidic mine drainages (Baker et al., 2004), hydrothermal vent habitats (López-García et al., 2007) and boiling springs' lake sediment (Wilson et al., 2008). This strongly indicates that the respective phyloclade includes extremophile fungi that are capable of thriving in the most extreme aquatic environments known to date. Described representatives in this clade are Penicillium and Aspergillus, versatile ubiquitously distributed species that are capable of anaerobic denitrification (Takasaki et al., 2004). This agrees with our observation that under laboratory conditions, isolate FCA-S3 (99.45% similarity to Penicillium namyslowskii) grows anaerobically while reducing nitrate (Jebaraj & Raghu-kumar, 2009). The widespread distribution of ascomycete fungi in oxygen-depleted and extreme habitats is also indicated by the close affiliation of a number of further OTUs from the Arabian Sea to environmental clones obtained from other anoxic habitats and extreme environments. For example, FAS_5 is 99.88% similar to P1_3m2 obtained from anoxic aquifer sediment (Brad et al., 2008), and the dothideomycete OTU FAS_24 is 98.92% similar to an environmental clone from the Mid-Atlantic hydrothermal vent sediment (López-García et al., 2007).
Within the phylum Basidiomycota (Fig. 2), we identified sequences branching in the subphylum Pucciniomycotina, Agaricomycotina and Ustilagomycotina. Only a few are closely (> 97% sequence similarity) related to previously deposited sequences of described basidiomycetes. For example, FAS_47 is 99.46% similar to the wood-decaying saprophyte Globulicium hiemale, and FAS_28 and FAS_29 are similar to the nitrogenous compound scavenger Rhodotorula casticola (99.83% and 98.60% sequence similarity, respectively). Most OTUs are more divergent from sequences of described taxa such as the Ustilagomycetes FAS_38 and FAS_39, exhibiting only 93% sequence similarity to the plant pathogen Thecaphora spilanthis. Many basidiomycete OTUs from the Arabian Sea branch in clades that include other environmental fungal sequences from a variety of oxygen-depleted habitats. Examples are three exobasidiomycete OTUs (FAS_30, FAS_31 and FAS_32), which are related to sequences from an anoxic Norwegian Fjord (A. Behnke, K. Barger, J. Bunge & T. Stoeck, unpublished data), an anoxic Mediterranean deep-sea basin (Alexander et al., 2009) and an anoxic basin in the central Baltic Sea (Stock et al., 2009). The closest described relative of this sequence clade is the plant pathogen Exobasidium gracile, with at least 37.3% sequence divergence. The OTUs FAS_40 and FAS_41 branch together with sequences from hydrothermal vent ecosystems (López-García et al., 2007; T. Le Calvez, S. Mahe & P. Vandenkoomhuyse, unpublished data), anoxic intertidal sediment (Dawson & Pace, 2002), an anoxic Mediterranean deep-sea basin (Alexander et al., 2009) and other deep-sea sites (Bass et al., 2007). This cluster, belonging to Ustilagomycotina, is within a clade that has been recognized as a ‘hydrothermal and/or anaerobic fungal group’ (López-García et al., 2007). The closest described species branching in this clade is the rare skin pathogen Malassezia restricta, with 0.39% and 2.36% sequence divergence to the OMZ OTUs FAS_40 and FAS_41. Finding a sequence related to a human skin pathogen in anoxic marine environments seems unusual at first glance. However, sequences closely related to M. restricta were also reported from hydrate-rich deep-sea sediments of South China Sea (Lai et al., 2007), indicating a wide ecological diversity of taxa falling in this sequence clade. Interestingly, OTUs obtained exclusively fromoxic regions of our study grouped together within the agaricomycetes, along with culture sequences and environmental clones obtained from anaerobic aquifers, hydrothermal sediment and agricultural soil (Euringer & Luenders, 2008; Le Calvez et al., submitted; Lesaulnier et al., 2008).

One OTU (FAS_49) obtained from our study (Fig. 3) originates from a fungus other than Dikarya. This clone, together with clone CCW35 from anoxic salt marsh water (Stoeck & Epstein, 2003, sequence similarity 90.45%), branches among zygomycetes. The closest named species was Mortierella wolfii, subphylum Mucoromycotina, with only 90.02% sequence similarity. There are contrasting reports on the capability of zygomycetes to grow anaerobically (Kurakov et al., 2008; Schmidt et al., 2008). Even though we have a poor representation of zygomycetes in our study, they are reported to be a major component of fungal communities in oxygen-depleted environments (Slapeta et al., 2005; Takishita et al., 2007b; Brad et al., 2008). Similarly, we did not discover Chytridiomycetes in our clone libraries. This was unexpected, because the latter taxon group includes fermentative anaerobes (Orpin, 1977) and was found in previous 18S rRNA gene diversity surveys in anoxic habitats (Stoeck et al., 2007; Takishita et al., 2007a, b). This poor representation of fungal groups other than ascomycetes and basidiomycetes may be due to undersampling (Fig. 6), combined with a higher efficiency of the primers to amplify genes from Ascomycota and Basidiomycota (Anderson et al., 2003; Malosso et al., 2006). Indeed, previous studies also failed to obtain chytridiomycete sequences using Fungi (Gomes et al., 2003) and Fung2 primers (Malosso et al., 2006).

Even though we are not able to distinguish native from transitory fungi (both spores and mycelium contain rDNA; Osherov et al., 2002), it is reasonable to assume that the majority of fungi detected in this study are indigenous. The reasoning for this assumption is their close relation to a number of fungal sequences detected (some of them exclusively) in oxygen-deficient environments. Furthermore, we conducted a laboratory experiment to largely exclude the possibility of PCR amplification of DNA from nonindigenous fungi. Wind or water currents are vectors to disperse fungal spores. Such transitory material could theoretically be sources of genomic DNA, although the corresponding organisms are not active members of the fungal communities under study. Therefore, we harvested spores from two cultured isolates originating from the sampling sites (the basidiomycete isolate FCAS11 and the ascomycete isolate FCAS21). The protocol that we used to extract nucleic acids from the environmental samples under study failed to extract DNA from these spores. This largely (even though certainly not entirely) excludes the possibility that the clone libraries constructed from the Arabian Sea samples include significant proportions of nonindigenous transitory fungi (spores) (results not shown, but available from the authors upon request).

Cultured fungi

Traditionally, fungal diversity studies in environmental samples are based on cultivation approaches. Limitations of this strategy—such as the inability to separate biomass from particulate material and lack of growth media and cultivation conditions suitable for all members of the community—are
analyses of clone libraries constructed from environmental diversity (Anderson et al., 2003). Molecular phylogenetic analyses of clone libraries constructed from environmental samples have become the gold standard in fungal diversity research (Pang & Mitchell, 2005). However, this strategy is no panacea, as it has biases such as PCR-primer (un)specificities as discussed above. Combining cultivation-based and cultivation-independent methods may allow for a more complete picture of fungal diversity as each of the methodological strategies may compensate for the biases of the other.

We obtained 26 cultures from all the sampling locations. The taxonomic breadth of the isolated strains is restricted: five isolates (FCAS11, FCAS87, FCAS88, FCAS89 and FCAS90) could be assigned to Basidiomycota, predominantly Pucciniomycotina (Fig. 2), and 21 of them belonged to Pezizomycotina of Ascomycota (Fig. 1). Both divisions of fungi have a high representation of cultured taxa, indicating the accessibility of these groups to cultivation. With the exception of two ascomycetes (FCAS31 and FCAS129) and three basidiomycetes (FCAS87, FCAS90 and FCAS11), all cultures have > 99% sequence similarities to previously cultured and well-described fungi. This indicates that cultivation using standard techniques generally produces more isolates of already known taxa. We note that cultivation under anoxic conditions may have produced a different set of fungi being more divergent to known taxa. Current efforts are in order to stimulate growth under anoxic conditions using a variety of different media. Thus far, we were able to grow one of the isolates (isolate FCAS11) discussed in this study under anoxic cultivation conditions. This supports the hypotheses that at least some fungi from the OMZ region, subjected to changing oxygen conditions, can readily adapt to anoxia.

We also obtained a few isolates that show only a low similarity to previously described and sequenced fungi. These are, for example the basidiomycete isolates FCAS90 exhibiting 97.10% sequence similarity to Graphiola cyindrical, FCAS87 with only 62.5% similarity to Sporisorium relumianum and FCAS11 with 92.1% sequence similarity to Rhodotorula aurantia. These examples reveal another decisive advantage of cultivation over clone library analysis. Having access to (putatively) novel cultures in a living condition paves the way for an in-depth analysis of their phylogeny, morphology and ecology. A current multigene analysis of isolate FCAS11, along with ultrastructural analyses, points to the discovery of a novel fungal taxon on class level with the capability of anaerobic denitrification (C.S. Jebaraj, T. Boekhout, W. Muller, F. Kauff & T. Stoeck, unpublished data). Detailed analysis of the enzymes involved in the nitrate-reducing pathways of cultured fungi and their expression profiles in situ can help us to understand their role in the OMZ of the Arabian Sea and other anoxic aquatic systems.

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References


Fungal diversity in the Arabian Sea


Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Closest BLASTN match of nonfungal sequences.

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