Diversity and Characterization of Culturable Fungi from Marine Sediment Collected from St. Helena Bay, South Africa

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Abstract Marine fungi are known to originate from a wide variety of habitats within the marine environment. Marine sediment represents one environmental niche, with most fungi occurring in these sediments being facultative marine fungi with terrestrial origins. It has not been proven whether these fungi merely survive the harsh environmental conditions presented by the ocean sediment, as opposed to playing an active role in this ecological niche. During this study, marine sediment was collected from St. Helena Bay, on the west coast of the Western Cape, South Africa. Using dilution, enrichment, and repetitive culturing techniques, 59 fungal isolates were obtained from marine sediments and identified to at least genus level using morphological and molecular methods. Moreover, a series of tests were performed to characterize the physical and physicochemical attributes of the isolates. Results showed that the isolates not only survived but also had the potential to grow in the natural conditions present in this environment. Extracellular cellulase was produced by the filamentous fungal isolates indicating their probable role in detrital decay processes and therefore the carbon cycle on the ocean bed. Also, denitrification patterns were observed when isolates were grown in liquid media amended with $NaNO_2$, $NaNO_3$, and $(NH_4)SO_4$, implicating that these fungi have the potential to play an active role in denitrification, co-denitrification, and ammonification phases of nitrogen cycles occurring in the marine sediments.

Introduction

Marine fungi are frequently isolated from plant detritus and decomposing wood found in coastal waters [11, 12, 16, 20] but have also been found on sea shells [8, 19], in corals [8], sponges [30], coastal marine sediments [3], deep-sea sediments, deep-sea hydrothermal vents [2], and even anoxic marine sediments [20]. These fungi are the least studied of all marine organisms, forming a group defined by ecology and physiology, rather than taxonomic clusters [12]. Two distinct groups can be distinguished: facultative marine fungi which originate from the terrestrial or fresh water environment and adapt to marine conditions and obligate marine fungi that live and propagate exclusively in the marine aquatic habitat [16, 20]. Most species fall in the facultative group and belong to genera such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Wardomyces*, *Chrysosporium*, and *Chaetomium* [15, 22, 24], as well as *Cephalosporium, Cladosporium*, and *Alternaria* [23, 24]. Less commonly reported species include those that represent the facultative marine genera *Scytalidium, Verticillium*, and *Oidiodendron*, as well as obligate marine fungi from the genus *Dendriphiella* [15].

Spores of facultative marine fungi enter pelagic sediment by being swept from land soils into the ocean or are carried on air currents from land areas, after which these spores gradually descend to the sea bed. Many fungi are known for their ability to withstand harsh environmental conditions and even to grow vegetatively under inhospitable circumstances,

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and the marine environment is no exception. However, it has not been proven whether these species merely survive and endure the prevailing conditions or play an active role in the ecosystem [16, 23, 29]. The marine environment is ultimately a unique habitat presenting marine fungi with a number of physical challenges: (1) salinity and pH of the water—marine fungi are known to grow and produce functional enzymes at pH 7–8; (2) low water potential, which is overcome by accumulating osmolytes such as glycerol, mannitol, polylol, and trehalose; (3) high sodium-ion concentration, which is tolerated by using a very efficient sodium efflux ATPase or storage in vacuoles; (4) low temperatures; and (5) high hydrostatic pressure. These unusual circumstances inevitably lead to organisms with novel genes and unique properties [13, 20].

Marine fungi from sediment had been reported to be involved in denitrification processes [3, 21]. A study by Cathrine and Raghukumar [3] showed that the majority of fungi isolated were common terrestrial fungi that adapted to marine conditions. Fungal denitrification takes place under suboxic conditions (300–900 μM O_2), and excess oxygen (>900 μM O_2) inhibits the process; in contrast, bacterial denitrification requires completely anoxic conditions. Fungal denitrifiers were presumed not to complete the denitrification process to dinitrogen (N_2) but form nitrous oxide (N_2O) instead, thereby contributing to an increase in harmful greenhouse gases. However, some authors found that many denitrifying fungi are capable of producing hybrid N_2 or N_2O molecules, by combining nitrogen (N) atoms from nitrite (NO_2^-) with other N-compounds such as azide or ammonium (NH_4^+), during denitrifying conditions. This process known as co-denitrification would be induced in the presence of NO_2^- or nitrate (NO_3^-) [10, 21, 27]. Furthermore, in anoxic conditions, fungi produce NH_4^+ when reducing NO_3^- through a process known as ammonia fermentations (ammonification). Fungi are therefore capable of performing a "multi-model type of respiration," depending on the O_2 levels in their environment [3, 9, 27].

St. Helena Bay is a shallow, semi-enclosed bay east of Cape Columbine (Western Cape, South Africa), representing one of the main upwelling centers in the Southern Benguela upwelling system. It is a major controlling factor of the biogeochemical characteristics of the bay, and sediments in the bay come from either atmospheric input or are carried there by the Berg River. Fine-grained offshore sediment from as far north as the Orange River's mouth is also known to be transported to the bay in a subsurface southward current. The Berg River and its tributaries flow through areas dominated by agriculture, wineries, canneries, and textile milling. The upper reaches of the river flow mainly through the Table Mountain Group sandstone, granitic hills, and clay soils consisting of weathered granitic material. Downstream, sandstone had been eroded to expose Malmesbury Group shale that forms the underlying bed rock

for the remaining part of the river channel to the mouth. Many aquatic ecosystems are supported in St. Helena Bay and the area is known to be rich in marine life [18]. The aims of our study were to determine the diversity of culturable fungal strains from marine sediment collected from St. Helena Bay on the west coast of Southern Africa and to typify these isolates using a number of physiological characteristics in an effort to understand their possible role in the ecology of this unique habitat.
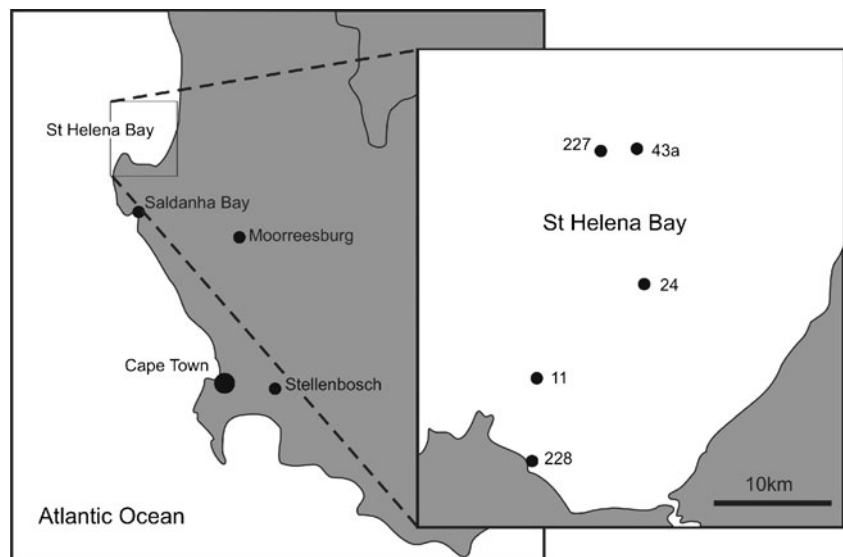
Methods

Sampling

Sediment samples were collected on 14 February 2009 from five sampling stations in St. Helena Bay (and Great Berg River estuary), Western Cape, South Africa. The stations included St 11 (S32 40 21.7, E18 01 25.3, depth 28.0 m), St 24 (S32 35 59.3, E18 07 27.1, depth 32.0 m), St 227 (S32 30 31.6, E18 05 39.3, depth 15.0 m), St 43a (S32 29 32.3, E18 06 57.6, depth 61.0 m), and St 228 (S32 44 38.5, E18 00 45.9, depth 8.0 m) (Fig. 1). Sampling was done by coring (60×300-mm cores), using an Ocean Instruments MC-200 multi-corer operated from the CSIR semirigid vessel, "Wave Rider." Due to strong wind and rough sea conditions, only one coring procedure could be performed at each sampling site. Subsequent to their collection, cores were stored at 4°C.

Isolation

In the laboratory, the cores were removed from the acrylic core barrels by sliding them into a sterile glass bowl. Sterilized spatulas were used to scoop ca. 1 cm³ sediment subsamples from the top 5 cm of each core; each of these subsamples was suspended in 100 ml sterile sea water. Different isolation methods were subsequently employed to ensure optimal culturing conditions and to accommodate possible low spore numbers. For each subsample, a dilution series (10^{-1} to 10^{-6}) was prepared in triplicate, using sterile sea water. MFA medium (2 % D(+) Glucose (Saarchem, Merck Chemicals, South Africa), 1 % soluble starch, 2 % tryptone soy broth (Biolab, Merck, South Africa), 0.3 % meat extract powder (Biolab, Merck, South Africa), 0.5 % yeast extract powder (Biolab, Merck, South Africa), 1.5 % bacteriological agar (Biolab, Merck, South Africa), 0.2 g/l chloramphenicol (Sigma-Aldrich, Germany), and 1 l sea water) was prepared as a culture medium for the dilutions. Other isolation media included malt extract agar (MEA; Biolab, Merck, South Africa) and oatmeal agar (OA; 3 % oat flakes simmered (30 min) in distilled water, clarified by filtration through a stainless steel sieve, mixed with 1.5 % bacteriological agar (Biolab, Merck, South Africa)),

Figure 1 Map showing the location of the sediment core samples collected in 2009 from St. Helena Bay in the Great Berg River estuary along the Western Cape of South Africa

supplemented with distilled water to 1 l and autoclaved (121°C for 15 min). In addition to this method, a second method involved the filtering of a spatula-scooped subsample suspended in 100 ml sterile sea water, through a sterile Sartorius Filter system, fitted with a cellulose acetate filter (0.2 μm; Sartorius Stedim Biotech, Germany); filters were subsequently placed in Petri dishes containing MFA, MEA, and OA. A third isolation method was also employed, wherein 1 ml of the sediment suspensions of all subsamples was enriched in 100 ml MF broth (MFA without agarose) in an Erlenmeyer flask incubated on a shaker at 28°C for 24 h in the dark. Isolations of the enriched microorganisms were done by transferring 100 μl liquid culture from each Erlenmeyer flask to a culture plate using a pipette (done in triplicate). A sterilized glass rod was used to spread the suspension across the entire surface of each culture plate. All plates were incubated at 25°C in the dark. The plates were examined daily for developing fungal colonies which appeared after 5 to 7 days. Colonies were counted on day 7 and transferred to fresh plates (repetitive culturing). Stock cultures of all fungal isolates were kept on MEA at 8°C, and all subsequent tests were performed using these stock cultures.

Identification

Fungal isolates were examined and identified to genus level using light microscopy. Classical morphological features of the fungal isolates were used for these preliminary identifications of the fungal isolates [6]. Genomic DNA was then extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research Corp., USA) according to the manufacturer's instructions. The internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA operon (including the 5.8S gene) of the filamentous fungi were subsequently amplified using the primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [32]. For the yeast spp., the D1/D2 (600–650 bp) region of the large subunit (26S) rDNA was amplified by the polymerase chain reaction (PCR) employing the universal forward primer F63 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and reverse primer LR3 (5′-GGTCCGTGTTTCAAGACGG-3′). The PCR used for the D1/D2 region was performed in a 50-μl reaction mixture, containing 10 ng DNA, 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 0.1 μM of each primer, and 0.5 U Fermentas *Taq*. Amplification was in a GeneAmp PCR system (Applied Biosystems Inc, USA) with the following cycling parameters for 30 cycles: initial denaturation for 3 min at 94°C, denaturation for 1 min at 94°C, followed by annealing for 1 min at 61°C, elongation for 2 min at 72°C, followed by a final elongation of 7 min at 72°C. The amplified product was separated on an agarose gel (0.8 %, *w/v*) and visualized by ethidium bromide staining. The cycling parameters for the PCR employed to amplify the ITS-regions of the filamentous fungi [32] were the same as the PCR for the D1/D2 region, except for the annealing temperature which was set at 56°C. PCR products were purified by means of a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals (Pty) Ltd., S.A.) and sequenced using a Hitachi 3730xl DNA Analyzer (Applied Biosystems, USA). The fungal sequences were compared to reported sequences using a BLAST search on the NCBI database (http://www.ncbi.nlm.nih.gov/blast/), as well as the database of the Centraalbureau for Schimmelcultures (http://www.cbs.knaw.nl). The results obtained for each isolate were compared to morphological data to determine the most likely identity. In addition, only sequence matches above 98.5 % were regarded as meaningful for identification to species level. In cases where sequence matches were above 99 % for two or more species in a genus that were different in

morphology and colony characteristics, only the genus name with an allocated strain number were used in subsequent tests.

Physiological Tests

Physiological growth tests were conducted on filamentous fungal isolates by studying radial growth (measured on an *x*- and *y*-axis, across the bottom surface of each plate) on differential or growth media in Petri dishes for each selected environmental parameter. For all these tests, growth of the fungal colonies was measured by taking the average of two colony diameter measurements for each plate. Three Petri dishes were used at each test criterion (e.g., temperature) and

the experiments were conducted in the dark and repeated once. The average colony diameters from the two experiments were then calculated. For the yeast species, similar physiological tests were performed but in liquid media and growth was measured spectrophotometrically at 600 nm, using a SmartSpec™Plus spectrophotometer (Biorad Laboratories Ltd, South Africa).

Physical Physiology

Isolates were cultured on MFA medium from stock cultures, and disks of fungal growth, 5 mm in diameter, were cut from culture plates with the back end of a sterilized Pasteur

Table 1 List of fungal isolates from sediment collected in St. Helena Bay in February 2009 and assessment of their physiological attributes

Species isolated	Isolate numbers	Sampling station	Temperature (°C) range + (optimum)	pH range + (optimum)	Salinity (%) range + (optimum)	Anaerobic growth	Hemolytic ability	Cellulase activity	Ammonia production
Acremonium alternatum	58	24	4–37 (26)	5–10 (8)	0–6 (3)	+	+	+	+
A. potronii	1, 2, 3, 4, 11	43a	15–37 (26)	5–10 (7–8)	0–6 (3)	+	+	+	+
Acremonium sp. 1	10	43a	4–37 (26)	5–10 (7–8)	0–6 (3)	+	+	+	−
Aspergillus candidus	35, 36	228	15–37 (26)	5–10 (5)	0–6 (3)	+	+	+	+
A. terreus	12, 13, 14	43a	15–37 (37)	5–10 (5–6)	0–6 (1.5)	+	−	+	+
Aspergillus sp. 1	45, 49, 56	227	15–37 (26)	5–10 (5)	0–6 (1.5)	+	+	+	+
Aspergillus sp. 2	46, 48	227	15–37 (26)	5–10 (10)	0–6 (3)	+	+	+	+
Aspergillus sp. 3	53, 55	227	15–37 (26)	5–10 (5)	0–6 (1.5)	−	+	+	+
Aspergillus sp. 4	52	227	15–37 (26)	5–10 (6)	0–6 (3)	+	−	+	+
Aspergillus sp. 5	51	227	4–26 (26)	5–10 (7)	0–6 (1.5)	+	−	+	+
Aspergillus sp. 6	54	227	15–37 (26)	5–10 (6)	0–6 (6)	+	+	+	+
Aspergillus sp. 7	57	227	15–37 (26)	5–10 (7)	0–6 (3)	+	+	+	−
Eurotium amstelodami	5, 6	43a	15–37 (26)	5–10 (8)	0–6 (6)	+	+	+	+
Paraconiothyrium cyclothyrioides	40a, 40b	228	15–26 (26)	5–10 (8)	0–6 (3)	+	−	+	+
Penicillium canescens	20, 21	43a	4–37 (26)	5–10 (5–8)	0–6 (1.5)	+	−	+	+
P. chrysogenum	47, 50	227	4–37 (26)	5–10 (5–7)	0–6 (3)	+	−	+	+
P. crustosum	25, 26	43a	4–37 (26)	5–10 (7)	0–6 (1.5)	−	−	+	−
Penicillium sp. 1	8, 9	43a	4–37 (26)	5–10 (6)	3–4.5	+	+	+	+
Penicillium sp. 2	15, 16	43a	15–37 (37)	5–10 (5)	0–6 (3)	−	−	+	+
Penicillium sp. 3	18	43a	4–37 (26)	5–10 (6)	0–6 (1.5)	+	−	+	+
Penicillium sp. 4	19	43a	4–37 (26)	5–10 (6)	0–6 (6)	+	+	+	+
Penicillium sp. 5	7, 17	43a	15–37 (37)	5–10 (5)	0–6 (1.5)	+	+	+	−
Trichoderma sp.	38, 39, 41, 42, 43	228	15–37 (26)	5–10 (6)	0–6 (0)	+	−	+	+
Rhodotorula mucilaginosa	59	227	4–37 (26)	5–10 (6)	0–6 (0)	−	−	−	
Yarrowia lipolytica	29, 30, 31, 32, 33, 34, 44	228	15–37 (26)	5–10 (9)	0–6 (3)	−	+	−	+
* West coast seawater values			10–14[*]	7.5-8.4[*]	3.5[*]				

pipette and used to inoculate plates. Yeast strains were grown in test tubes containing 10 ml YPD broth (2 % D (+) glucose (Saarchem, Merck Chemicals, South Africa), 2 % peptone (Biolab, Merck, South Africa), and 1 % yeast extract (Biolab, Merck, South Africa) in distilled H_2O). The effect of temperature was determined at 4, 15, 26, 37, and 45°C. Subsequent experiments were incubated at the respective optimal growth temperatures determined in this experiment. Growth at different concentrations of salinity was determined by modifying concentrations of sea salts (Instant ocean synthetic sea salt, Aquarium systems, France) in the MFA medium to 0, 1.5, 3.0, 4.5, and 6.0 %. The effect of pH was determined by altering the pH of MFA medium to 5, 6, 7, 8, 9, and 10 respectively, with 10 M NaOH or concentrated HCl. The ability of isolates to grow under anaerobic conditions was studied by incubating, inoculated MFA plates and test tubes containing YPD broth, in anaerobic chambers containing an Anaerocult A (Merck, Germany) set and Anaerotest (Merck, Germany) strip.

Physiochemistry

Three replicates for each isolate were used for all experiments described in this section. Ability to grow or secrete a specific enzyme or substance was documented as positive (+), while inability to grow or absence of the latter as negative (−). Protease activity of fungal isolates was determined by growing them on a solid medium in Petri dishes containing skimmed milk and a pH indicator (0.1 % yeast extract, 1 % skim milk, 2 % bacteriological agar, and 0.01 % bromocresol purple indicator (pH 5.2, Riedel De Haën AG Zeelze, Germany)). Subsequent to the incubation (24 h), plates were examined for the formation of clearing zones. Purple zones were assumed to indicate the secretion of ammonia, which raises the ambient pH and allows extracellular protease production and activity [25]. Cellulase production by the isolates was tested on carboxymethylcellulose medium (0.67 % yeast nitrogen base (Becton, Dickinson and Company Sparks, USA), 0.5 % carboxymethylcellulose (Sigma-Aldrich, Germany), and 2 % bacteriological agar) plates. After 7 days, the plates were stained using 0.1 % Congo red (B & M Scientific, South Africa) for 10 min, followed by de-staining with 1 M NaCl (KIMIX, South Africa) for 5 min. Clearing zones surrounding the fungal colony indicated cellulase activity. Hemolytic activity was determined by inoculating plates of Columbia agar base containing 5 % whole sheep blood. Plates were incubated at 26°C for 7 days and small yellow zones around the colonies indicated hemolysis.

Anaerobic Nitrogen Utilization

To determine nitrogen utilization under anaerobic conditions, Czapek Dox agar (CDA) medium was modified to

contain the following in 1 l distilled water: 5.2 g KH_2PO_4, 6.4 g K_2HPO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g KCl, 0.02 g $FeSO_4$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.008 g $CuSO_4 \cdot 5H_2O$, 16.7 g glucose, 1.32 g $(NH_4)_2SO_4$, 0.85 g $NaNO_3$, and 0.28 g $NaNO_2$. Test tubes, each containing 10 ml of the medium, were inoculated with a small amount of spores or hyphae and transferred with an inoculation loop from fungal colonies grown on the CDA medium. Negative controls, without any fungal growth, were included, along with positive controls inoculated with *Botrytis cinerea* spores. Regarding the positive controls, Shoun and coworkers [21] showed that *B. cinerea* is capable of evolving N_2O, nitric oxide (NO), and/or N_2 when incubated anaerobically with NO_2^- as N source. The test tubes were incubated in anaerobic jars, as described above, for 3 weeks at 26°C. The NH_4 and NO_3^- concentrations were determined colorimetrically with the method described by Kartal et al. [14], and the NO_2^- concentration was also verified colorimetrically with the method described by Egli et al. [7] using a BioRad SmartSpec Plus spectrophotometer. Each isolate was prepared in triplicate, and the average absorbance of the isolate was used to determine

Table 2 List of fungal isolates from sediment collected in St. Helena Bay in February 2009 with Genbank Accession numbers

Species isolated	Isolate numbers	Genbank accession numbers
Acremonium alternatum	58	JN021556
A. potronii	1, 2, 3, 4, 11	JN021531
Acremonium sp. 1	10	JN021536
Aspergillus candidus	35, 36	JN021544, JN021545
A. terreus	12, 13, 14	JN021537
Aspergillus sp. 1	45, 49, 56	JN021548
Aspergillus sp. 2	46, 48	JN021550
Aspergillus sp. 3	53, 55	JN021554
Aspergillus sp. 4	52	JN021553
Aspergillus sp. 5	51	JN021552
Aspergillus sp. 7	57	JN021555
Eurotium amstelodami	5, 6	JN021532
Paraconiothyrium cyclothyrioides	40a, 40b	JN021546, JN021547
Penicillium canescens	20, 21	JN021541
P. chrysogenum	47, 50	JN021549, JN021551
P. crustosum	25, 26	JN021542, JN021543
Penicillium sp. 1	8, 9	JN021534, JN021535
Penicillium sp. 2	15, 16	JN021538
Penicillium sp. 3	18	JN021540
Penicillium sp. 5	7, 17	JN021533, JN021539
Rhodotorula mucilaginosa	59	JN021557
Yarrowia lipolytica	29, 30, 31, 32, 33, 34, 44	JN021559, JN021560, JN021558

the ratio of absorbance to the average absorbance of the negative controls. The experiment was performed three times and the average ratio values of these experiments were calculated.

Results

Identities of Isolates

Only 10^{-1} and 10^{-2} dilutions of the diluted marine sediment yielded colonies. The 59 isolates included 25 different species of *Acremonium, Aspergillus, Penicillium, Trichoderma, Paraconiothyrium, Eurotium, Rhodotorula,* and *Yarrowia* (Tables 1 and 2). Using morphological as well as ITS data, many isolates could be identified to species level, and only sequence matches above 98.5 % were regarded as meaningful. Species of *Aspergillus, Acremonium, Penicillium,* and *Trichoderma* were also present, although in some cases, the methods and characteristics used were not specific enough to distinguish closely related species. In these cases, the sequence matching analysis was above 99 % for a number of different species within the genus. Sampling station 43a yielded 11 species, St 227 yielded 9 species, St 228 yielded 4 species, St 24 yielded only 1 species, and St 11, no isolates (Table 1 and Fig. 2).

Fungal Physiology

Physical Physiology

The optimum temperature for most of the fungi was 26°C. Seven isolates (numbers 7, 12-14, 15, 16, and 17) had an optimum temperature of 37°C (Table 1). All isolates were also able to grow at 15°C and some as low as 4°C. Most isolates preferred a 1.5–3 % salt concentration, although a number of isolates were more halotolerant, showing enhanced growth at 6 % salt, and only one isolate preferred 0 % salt (Table 1). The pH range for growth of the isolates was between pH 5 and 10 (Table 1). Most isolates were able to grow anaerobically, with only numbers 15, 16, 25, 26, and the yeast spp. as exceptions (Table 1).

Physiochemistry

All of the mycelial fungi were able to produce cellulase under aerobic conditions, although the two yeast species were unable to utilize cellulose as carbon source (Table 1). Aerobic ammonia production varied among isolates (Table 1). Hemolytic activity was observed in 13 of the species, as opposed to the 12 displaying no hemolytic ability (Table 1).
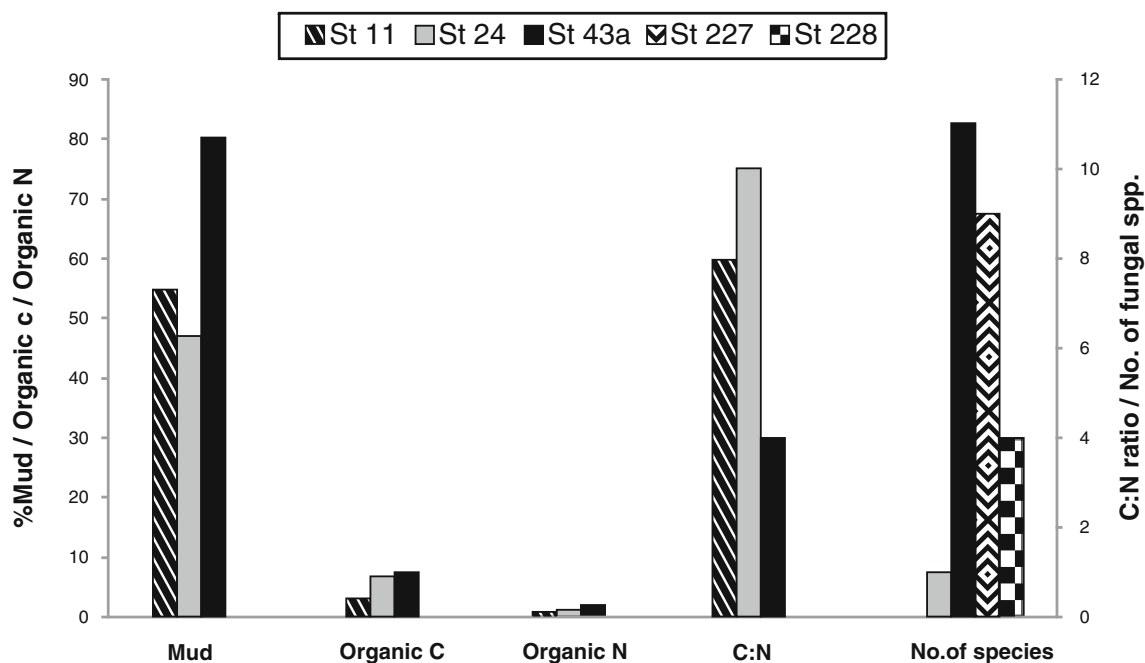
Figure 2 Graph showing percentage mud, organic carbon and nitrogen, as well as carbon–nitrogen ratio and the number of species isolated from available sampling stations (St 11 = sampling station 11, St 24 = sampling station 24, St 43a = sampling station 43a, St 227 = sampling station 227, St 228 = sampling station 228)

Table 3 Sedimentological data from the February 1999 sampling of the sediments of Saldanha Bay

Sampling station	% Mud	% Organic C	% Organic N	C/N	AVS (mmol/g)	Fe (g/kg)	Cu (mg/kg)	Zn (mg/kg)	As (mg/kg)	Cd (mg/kg)	Pb (mg/kg)
St 11	53.6	3.39	0.32	10.7	433	11	9	45	16	4	5.5
St 24	50.0	6.40	0.65	9.8	11,337	24	24	68	21	4	10
St 43a	79.1	6.56	1.40	4.7	24,874	32	32	85	20	4	14

Anaerobic Nitrogen Utilization

Of the 33 isolates tested, 15 showed a nitrogen utilization pattern with a simultaneous decrease in NO_2^- and a slight decrease in NH_4^+, with elevated NO_3^- concentrations. A different denitrification pattern was observed in the other isolates, where NO_3^- was utilized and NO_2^- produced or both NO_2^- and NO_3^- utilized (Table 5). The positive control showed a significant decrease in NO_2^- and an increase in NH_4^+, consistent with literature [21].

Discussion

Most previous studies on marine fungi focused mainly on fungal diversity [2, 20, 22, 23] and the presence of these fungi cannot testify to their participation in physiological processes, ecological role, or adaptations. This study was a pioneering effort to explore the physiology and possible ecological role of marine fungi in biogeochemical processes along the west coast of South Africa. These biogeochemical processes along the west coast, including St. Helena Bay, are primarily regulated by a wind-driven ocean upwelling occurring in the region [18]. This upwelling of cold nutrient-rich water drives the biological pump and hydrodynamics along the coast, determining the fate of sediment, organic matter, and trace elements, ultimately affecting the ecosystem of the region. During previous studies in this region, the physicochemical composition of sediment samples taken from the seabed at different sampling stations was used to construct plots of the sediment composition of St. Helena Bay [4, 5]. The sediments at the sampling stations were found to differ regarding percentage mud and the C/N ratio [5]. Sampling station 43a was characterized by a high

percentage of mud (80 %) with a low C/N ratio (4.0), as opposed to samples from St 24, which had a much lower mud percentage (47 %) with a higher C/N ratio (10.0) (Tables 3 and 4). Samples from St 11 had a mud percentage of 55 % with a C/N ratio of 7.93 and were therefore more similar to samples from St 24 than to samples from St 43a, in percentage mud and C/N ratio. Stations 227 and 228 were not sampled during the 1999 [4] and 2007 [5] surveys. However, according to the constructed plots portraying sediment composition in the bay [4, 5], the physicochemical composition of the sediment at St 227 is similar to that of St 43a.

Our study showed a greater fungal diversity at St 43a (11 species) versus stations 24 (one species) and 11 (zero isolates); we attribute this phenomenon to differences in the physicochemical composition of the sediment at the different sampling stations (Tables 3 and 4). The west coast of Southern Africa is characterized by water temperatures between 10 and 14 °C, with even lower temperatures (2–3 °C) in the deep-sea [18]. Water salinity along the coast would be 3.5 % and pH between 7.5 and 8.4. Our findings showed that a number of fungal isolates were able to grow at low temperatures (as low as 4 °C), in the pH range of sea water and even at salinity levels higher than that of the bay (Table 1). Interestingly, most isolates were able to grow anaerobically, suggesting that they have adapted to anaerobic conditions presented by the oxygen-deficient environment within the sediment. These results indicated that the majority of fungal isolates not only survive but have the potential to grow at conditions presented by the ocean on the west coast of South Africa.

Sampling station 43a yielded the highest number of isolates, although this station represented the deepest sampling station in this study. We hypothesize that the high mud

Table 4 Sedimentological data from the 2007 sampling of the sediments of Saldanha Bay

Sampling station	% Mud	% Organic C	% Organic N	C:N	AVS (mmol/g)	Fe (g/kg)	Cu (mg/kg)	Zn (mg/kg)	As (mg/kg)	Cd (mg/kg)	Pb (mg/kg)
St 11	55	3.04	0.38	7.93	2,500	10	10	32	16	4.5	9
St 24	47	7.0	0.70	10	4,000	23	24	68	20	4	11
St 43a	80	7.0	1.75	4	4,250	30	33	87	21	4	13.5

fraction, high levels of organic matter, and low C/N ratio of St 43a may provide an environment supporting growth of a larger number of microbes. It must be noted, however, that the potential nutrients in the sediment might not necessarily be bioavailable but fixed in microbial biomass. Moreover, all of the taxa isolated from St 43a (*Acremonium, Aspergillus, Penicillium,* and *Eurotium*) are well-known terrestrial fungi, supporting contentions that spores of these fungi had been transported from land areas into the deep sea [22, 29]. The sampling station closest to St 43a, i.e., St 227 also yielded a variety of other known terrestrial fungi (*Aspergillus* and *Penicillium* spp.), as well as the basidiomycetous yeast, *Rhodotorula mucilaginosa* (Tables 1 and 2). The latter species has a worldwide distribution in terrestrial, aquatic, and marine environments and has even been isolated from the Mariana Trench (11,500 m deep) in the Pacific Ocean [26]. Sampling station 228, situated in the shallow water of the harbor area (Fig. 1) yielded only four species, although it is of value to note that at least one of these species is known to be associated with higher anthropogenic activity. *Yarrowia lipolytica* had been shown to occur in hydrocarbons, kerosene, aviation fuel, contaminated gas or oil fuel, *n*-paraffin [28], and even human cornea, skin, and finger nails [1]. This species is well known for its unique physiological properties of industrial value [17, 20], including the utilization of *n*-paraffin, and therefore, most likely plays a role in the degradation of crude oil on the harbor bed [20]. Interestingly, it has previously been found that areas with intense anthropogenic loads were dominated by hemolytically active fungal strains. Genera containing the latter included *Chaetomium, Penicillium, Aspergillus,* and *Trichoderma* [15]. Our study also revealed the presence of *Trichoderma* and hemolytically active *Aspergillus candidus* isolates in sediment from the harbor area (Tables 1 and 2). However,

hemolytically active fungi were not exclusively found in the harbor sediment but also in sediment from all our sampling stations. The ecological role of fungi with this characteristic is largely unknown, although it is tempting to speculate that it may play a role in the decay of dead animals; a role of which relatively little is known [11].

It is widely accepted that marine fungi are able to degrade plant material [11]. It was therefore not surprising to find that with the exception of the two yeast species, all the fungal isolates were able to produce extracellular cellulase, indicating their potential involvement in detrital processes and the crucial reminerilization processes occurring in detritus formation, as well as concomitant changes in the C/N ratio [31]. Aside from their role in the microbial regulation of the C/N ratio, marine fungi have also been documented to aid in the release of secondary metabolites from dead plant material. These metabolites include compounds such as omega-3 fatty acids, which are essential for the growth and development of crustaceans [20].

In this study, the highest number of isolates originated from the sampling site with the highest percentage of organic nitrogen. Consequently, the question arose as to whether these fungi are able to participate in the nitrogen cycle within the sediment. We therefore designed an experiment in which liquid cultures of the fungal isolates were challenged with inorganic nitrogen under anaerobic conditions. We found that in these liquid cultures amended with $NaNO_2$, $NaNO_3$, and $(NH_4)SO_4$, approximately 50 % of isolates showed nitrogen utilization patterns characterized by a decrease in NO_2^- levels, a slight but simultaneous decrease in NH_4^+ levels, as well as elevated NO_3^- concentrations (Table 5). These results suggest the occurrence of co-denitrification [21]. Another denitrification pattern was

Table 5 Fungal isolates that showed possible co-denitrification ability

These isolates were able to utilize NO_2 and NH_4 with a simultaneous production of NO_3. Each value represents the ratio of the absorbance obtained after colorometric determination of the N compound in the culture liquid, compared to that of the negative control. Values are the means (± SD) of three repetitions. Values higher than1.0 would indicate production and values below 1.0, consumption of the specific nitrogen source

Isolate no.	Species identity	NO3	NO2	NH4
3	*Acremonium potronii*	1.350±0.983	0.378±0.202	0.996±0.041
12	*Aspergillus terreus*	1.292±0.492	0.393±0.33	0.841±0.193
17	*Penicillium* sp. Nov	1.188±0.774	0.314±0.178	0.744±0.29
18	*Penicillium* sp. 3	1.098±0.772	0.564±0.032	0.836±0.337
20	*P. canescens*	1.464±0.246	0.272±0.01	0.918±0.138
25	*P. crustosum*	1.228±0.819	0.444±0.062	0.765±0.299
29	*Yarrowia lipolytica*	1.436±0.705	0.72±0.284	0.87±0.079
40	*Paraconiothyrium cyclothyrioides*	1.176±0.906	0.172±0.108	0.723±0.33
41	*Trichoderma* sp.	1.283±0.862	0.262±0.187	0.739±0.484
47	*P. chrysogenum*	1.126±0.647	0.64±0.419	0.971±0.201
48	*Aspergillus* sp. 2	1.420±0.827	0.52±0.397	0.941±0.117
50	*P. chrysogenum*	1.228±0.587	0.497±0.54	0.966±0.056
54	*Aspergillus* sp. 6	1.562±0.339	0.509±0.467	0.949±0.131
58	*Acremonium alternatum*	1.386±0.457	0.678±0.346	0.956±0.147
Control (+)	*Botrytis cinerea*	1.376±0.613	0.205±0.125	1.02±0.024

observed in cultures of most other isolates utilizing NO_3^- and producing NO_2^- or utilizing both NO_2^- and NO_3^-; this pattern is known to occur in fungi [10]. These data therefore suggest that these fungal species indeed have the potential to be involved in different parts of the very complex nitrogen cycle, even in this challenging environmental niche, and confirm the observations of Shoun et al. [21] concerning denitrifying abilities that occur in a variety of fungal taxa. The positive control included in the experiment (Table 5) showed a significant decrease in NO_2^- and an increase in NH_4^+, consistent with literature [21], suggesting the occurrence of ammonification. This study therefore supports the sentiment of Raghukumar [20] that fungi may potentially play a role in the nitrogen cycle of the marine environment where hypoxic and anoxic conditions prevail.

This study showed that culturable fungi, originating from sea sediment in St. Helena Bay, have the potential to survive and grow in the physicochemical conditions presented by this marine environment and have the potential to contribute to carbon and nitrogen cycles. We therefore believe that this study contributes to knowledge of this unique environmental niche, since this is the first study on fungi and their role in the sediments of the nutrient-rich Southern Benguela system.

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