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The contrasting roles of aquatic fungi and oomycetes in the degradation and transformation of polymeric organic matter

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Abstract

Studies on the ecological role of fungi and, to a lesser extent, oomycetes, are receiving increasing attention, mainly due to their participation in the cycling of organic matter in aquatic ecosystems. To unravel their importance in humification processes, we isolated several strains of fungi and oomycetes from Anzali lagoon, Iran. We then performed taxonomic characterization by morphological and molecular methods, analyzed the ability to degrade several polymeric substrates, performed metabolic fingerprinting with Ecoplates, and determined the degradation of humic substances (HS) using liquid chromatography-organic carbon detection. Our analyses highlighted the capacity of aquatic fungi to better degrade a plethora of organic molecules, including complex polymers. Specifically, we were able to demonstrate not only the utilization of these complex polymers, but also the role of fungi in the production of HS. In contrast, oomycetes, despite some morphological and physiological similarities with aquatic fungi, exhibited a propensity toward opportunism, quickly benefitting from the availability of small organic molecules, while exhibiting sensitivity toward more complex polymers. Despite their contrasting roles, our study highlights the importance of both oomycetes and fungi in aquatic organic matter transformation and cycling with potential implications for the global carbon cycle.

Freshwater ecosystems are recipients of large amounts of organic matter from their terrestrial surrounding—mainly in the dissolved form (Solomon et al. 2015). Dissolved organic matter (DOM) originates either from these allochthonous (DOM_{allo}) or internally from autochthonous (DOM_{auto}) sources. DOM_{auto} classically results from excretions and leachates of phytoplankton as well as their processing by microbial heterotrophs (McKnight et al. 2001). Thus, DOM_{auto} encompasses low-molecular-weight compounds with high aliphatic content and are considered to be labile organic matter leading to a high degradability of DOM_{auto} by microbial heterotrophs (Hessen and Tranvik 1998). On the other hand, DOM_{allo} mainly stems from degradation products of terrestrial vascular plants (Axmanová and Rulík 2005; Hur et al. 2011). Consequently, DOM_{allo} contains high-molecular-weight (HMW) substances with a higher proportion of aromatic compounds and thus is considered refractory organic matter (Hessen and Tranvik 1998). Due to its recalcitrant properties, DOM_{allo} represents most of the DOM pool (Leenheer and Croué 2003; Catalán et al. 2016) with humic substances (HS) being the most abundant fraction. HS can be defined as a category of naturally occurring, biogenic, heterogeneous organic substances, which generally have a yellow to black coloration, HMW, and refractory nature (McDonald et al. 2004). It has been estimated that HS, mainly derived from lignin, carbohydrates (e.g., cellulose, hemicellulose, etc.), and other macromolecular C-compounds (Stevenson 1982; Varadachari and Ghosh 1984; Inbar et al. 1990, 1992), represent up to 80% of the total DOM pool (Bano et al. 1997; Mattsson et al. 1998; Kisand et al. 2013; Lennon et al. 2013). These precursors are incompletely degraded by the microbial community and, therefore, HS accumulate gradually over time (Fenner and Freeman 2013).

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Unlike bacteria, which exhibit a limited ability to degrade stable macromolecules (Filip and Tesarova 2004), fungal degradation and transformation of HS extracted from soil, coal, compost, groundwater, and freshwater ecosystems is widely accepted (Claus and Filip 1998; Belcarz et al. 2005; Grinhut et al. 2011; Zahmatkesh et al. 2016; Collado et al. 2018). In soil, this capacity is mainly affiliated with white-rot fungi, which represent a physiological group of fungi consisting of a broad and diverse range of species mainly distributed in the family of basidiomycetes and to lesser extent ascomycetes (Eaton and Hale 1993; Grinhut et al. 2011; Collado et al. 2018). A large variety of lignocellulolytic enzymes such as peroxidases, laccases, endoglucanases (endo-1,4-b-glucanases), cellobiohydrolases (exo-1,4-b-glucanases), xylanases, and pectinases of fungi have been confirmed to play a vital role in microbial HS transformation (Kirk and Cullen 1998; Pointing 1999; Pérez et al. 2002; Maciel and Ribeiro 2010).

Yet, in freshwater ecosystems, only a few rare cases of fungal involvement in HS formation have been reported, e.g., formation of laccase and high ligninolytic activities of *Cladosporium cladosporioides* and *Polyporus versicolor* (Claus and Filip 1998). Recently, Rojas-Jimenez et al. (2017) confirmed the role of the fungi *Cladosporium* sp. in the transformation of complex organic compounds such as HS. On the other hand, very little is known about the interactions of oomycetes with organic matter such as HS. This is surprising since they are widely distributed, throughout multiple biomes, and often consist of animal pathogens and predominantly freshwater saprophytes of plant and animal debris (Van west 2006; Phillips et al. 2008). Furthermore, they have some striking similarities with fungi, including a vegetative stage, asexual and sexual reproduction, and their mode of colonization (Latijnhouwers et al. 2003; Shearer et al. 2007; Thines 2014). Consequently, the role of fungi and oomycetes in polymeric organic matter turnover, in particular HS degradation, remains to be explored in detail for most aquatic ecosystems.

Although it has been shown that the accumulation of highly recalcitrant molecules derived from microbial degradation leads to humic polymers (Steger et al. 2011), the formation of HS is one of the least understood aspects of humus chemistry (Tan 2014), especially since its nature is greatly determined by its precursors, environmental conditions, time of humification, and so on (Marbot 1997; Burdon 2001; Peña-Méndez et al. 2005). Interestingly, although fungal involvement in degradation of precursors has been established, it seems they also contribute to HS production, e.g., by generating humic polymers with higher aromaticity (Ryckeboer et al. 2003*a*,*b*). There is a succession of fungi depending on temperature, pH, and availability of nutrients during the process of composting in the soil environment with mesophiles being the most dominant one (Tuomela et al. 2000). In other words, fungi not only act on HS precursors such as plant material to degrade them into aromatic residues, but also actively contribute to the production of more recalcitrant humic molecules (Colberg 1988). In the context of global climate change and rising carbon emissions, inland waters have gained increasing attention regarding their role in the global carbon cycle (Cole et al. 2007; Raymond et al. 2013). Despite covering only a small fraction of the earth surface, the organic carbon sequestration capacity of lakes and reservoirs can exceed the one of all oceans together (Tranvik et al. 2009). Given our recent awareness about freshwater ecosystems as one of the greatest contributors of global carbon cycling (Wurzbacher et al. 2016), investigating the fate of terrestrial organic matter exported to aquatic ecosystems could reveal unseen aspects of the carbon cycle.

In this study, we aimed to investigate the enzymatic capacity of aquatic fungi and oomycetes as well as their involvement in HS mineralization and processing. We hypothesize that fungi and oomycetes have an exceptionally high potential for polymeric DOM mineralization and transformation. Therefore, we isolated and identified 18 fungal and 8 oomycetes strains from Anzali lagoon, Rasht, northern Iran. After morphological and molecular identification, prescreening methods were used to investigate the enzymatic activity of each strain. Then, two strains with the highest enzymatic activity were exposed to HS in order to determine their ability to produce more aromatic structures. Our study highlights the important ecological role of fungi and oomycetes in aquatic carbon cycling and their different affinity toward HS.

Materials and methods

Sampling and culturing

Saprotrophic fungal and oomycetes strains were isolated by collecting wood and leaves along the shoreline of Anzali lagoon (location 1 = 37°25′44.0″N 49°27′29.5″E, location 2 = 37°26′20.1″N 49°27′18.8″E, location 3 = 37°27′55.6″N 49°28′08.4″E) over several months in 2017 (Table 1). Isolation of oomycetes was performed using the baiting method as previously described (Johnson 1956). Briefly, the samples were cut equally and placed in sterile Petri dishes containing boiled hemp seed halves (*Cannabis sativa* L.) floating on the surface of sterile water and then incubated at room temperature. After 3–5 d, sterile needles were used to transfer the mycelia from colonized hemp seeds to another sterile Petri dish containing sterile water and boiled hemp seed halves. This process was repeated three to five times. Finally, bacteria-free cultures were obtained by transferring colonies to corn meal agar (CMA) medium and conducting the hyphal-tip technique three times. For isolation of fungal strains, the same plant materials were moist-incubated in Petri dishes and examined every 3 d under a binocular to detect their fruiting bodies for several weeks (Descals 1997). Pure cultures were obtained by transferring fruiting bodies and mycelia to malt extract agar (MEA) medium and conducting the hyphal-tip technique three times. The last round of hyphal-tipping was conducted in CMA and MEA medium amended with ampicillin (200 μg mL^{-1}) to avoid bacterial contamination.

Molecular and morphological characterization

DNA extraction followed the protocol proposed of Montero-Pau et al. (2008) with minor modifications. Briefly, 100 μL of alkaline lysis buffer (NaOH 25 mmol L^{-1}, disodium ETDA 0.2 mmol L^{-1}, pH 8.0) was aliquoted into 1.5 mL tubes. A clot of mycelia of

Table 1. Fungal and oomycetes strains obtained from Anzali lagoon (Rasht County, Iran).

Strain	Taxonomy	Time of isolation	Sampling site	Accession number	
				LSU	ITS
RT1	*Sarocladium* sp.	Jan 2017	1	MH367070	MH367052
RT2	*Sclerotium* sp.	Feb 2017	1	MH367071	MH367053
RT3	*Fusarium* sp.	Mar 2017	1	MH367072	MH367054
RT4	*Volutella* sp.	Mar 2017	1	MH367073	MH367055
RT5	*Plectosphaerella* sp.	Apr 2017	1	MH367074	MH367056
RT6	*Sarocladium* sp.	Apr 2017	1	MH367075	MH367057
RT7	*Arthrobotrys* sp.	May 2017	2	MH367076	MH367058
RT8	*Pleosporales* sp.	May 2017	2	MH367077	MH367059
RT9	*Myrmecridium* sp.	May 2017	2	MH367078	MH367060
RT10	*Paecilomyces* sp.	Aug 2017	2	MH367079	MH367061
RT11	*Fusarium* sp.	Sep 2017	2	MH367080	MH367062
RT12	*Arthrobotrys* sp.	Sep 2017	2	MH367081	MH367063
RT14	*Arthrobotrys* sp.	Sep 2017	2	MH367082	MH367064
RT15	*Fusarium* sp.	Sep 2017	3	MH367083	MH367065
RT16	*Aspergillus* sp.	Sep 2017	3	MH367084	MH367066
RT17	*Scedosporium* sp.	Sep 2017	3	MH367085	MH367067
RT18	*Fusarium* sp.	Oct 2017	3	MH367086	MH367068
RT19	Fungal sp.	Oct 2017	3	MH367087	MH367069
O961-3	*Dictyuchus* sp.	May 2017	1	—	MH367091
F962-15	*Achlya* sp.	Apr 2017	2	—	MH367058
O962-13	*Achlya* sp.	May 2017	2	—	MH367063
O962-14	*Dictyuchus* sp.	May 2017	2	—	MH367064
O963-5	*Dictyuchus* sp.	May 2017	3	—	MH367090
O963-13	*Achlya* sp.	May 2017	3	—	MH253591
T963-33B	*Dictyuchus* sp.	Jul 2017	3	—	MH253593
M963-8A	*Dictyuchus* sp.	Aug 2017	3	—	MH253582

1 = 37°25′44.0″N 49°27′29.5″E, 2 = 37°26′20.1″N 49°27′18.8″E, 3 = 37°27′55.6″N 49°28′08.4″E.

fungi and oomycetes zoospores growing in the malt extract broth were added to the alkaline lysis buffer and centrifuged for 30 min at 9000 rpm. Samples were incubated at 95°C for 30 min and then cooled on ice for 5 min. Finally, 100 μL of neutralizing solution (Tris-HCl 40 mmol L^{-1}, pH 5.0) was added to the tubes. The samples were vortexed and stored at −20°C. The large subunit (LSU) and ribosomal internally transcribed spacer (ITS) were amplified in a Flexible polymerase chain reaction (PCR) Thermocycler (Analytikjena, Germany) using primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGGGAAAC TTCG-3′) (Stielow et al. 2015), ITS1 (5′-TCCGTAGTGAA CCTGCGG-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990(, respectively. All reactions were performed with a program of 94°C for 2 min initial denaturation followed by 32 cycles of 94°C for 15 s, 53°C for 15 s, 72°C for 30 s, and a final extension at 72°C for 5 min. Samples were kept at −20°C and sent to a sequencing platform (Macrogen, The Netherlands) for Sanger sequencing. The resulting sequences were edited using the BioEdit software (Hall 1999) and submitted to GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) (Table 1). Molecular classification was performed by comparing strain sequences with those stored in GenBank and UNITE sequence databases (https://unite.ut.ee/index.php). Morphological identification of strains was based on available features such as shape and color of colony, conidia, and some asexual and sexual structures (Seymour and Padgett 2002; Dugan 2006).

Screening for lignolytic, cellulolytic, pectolytic, and chitinolytic activities

Lignolytic activity

Mycelia from the edge of 7- to 15-d-old cultures were transferred to six-well culture plates containing the cultivation medium proposed by Rojas-Jimenez et al. (2017) (i.e., 0.94 g KH_2PO_4, 1.9 g K_2HPO_4, 1.6 g KCl, 1.43 g NaCl, 0.15 g NH_4Cl, 0.037 g $MgSO_4$, 0.1 g yeast extract, 10 g malt extract, 15 g agar per liter, pH 7.0) and mPmTG agar medium amended with one of the following substrates: (1) 0.1% wt/vol 2,20-Azino-bis 3-ethylbenzo thiazoline-6-sulfonic acid diammonium salt (ABTS), (2) 0.02% and 0.005% wt/vol bromocresol green (BG), (3) 0.02% and 0.005% wt/vol Congo red (CR), (4) 0.02% and 0.005% wt/vol malachite green (MG), (5) 0.02% and 0.005% wt/vol phenol red (PhR), (6) 0.02% and 0.005% wt/vol PolyR-478 (PR) (in pH 5

and 7), (7) 0.02% and 0.005% wt/vol remazol brilliant blue R (RBBR), and (8) 0.02% and 0.005% wt/vol toluidine blue (TB) (Pointing 1999; Swamy and Ramsay 1999; Moreira et al. 2000; Novotny et al. 2001; Gill et al. 2002; Rojas-Jimenez et al. 2017). We have used different concentrations for different dyes in the different experiments. Therefore, two different concentrations were used in order to ensure that concentration does not affect enzymatic activity of the tested strains. The capacity of each strain to produce lignolytic activity was determined by decolorization of the mentioned substrates in the area around the mycelia or as a color change of the media during 3 weeks. A decolorization of the medium by 0–33%, 33–66%, and 66–100% in all screening treatments were considered as weak, medium, and strong, respectively. This takes into account the different growth rates of the tested strains.

Cellulolytic and pectinase activity

The same media used in the evaluation of cellulolytic and pectinase activity were amended with the following enzymatic carbon source: (9) 7.5 g carboxymethilcellulose (CMC), (10) 7.5 g Avicel (AVL), (11) 5 g D-cellobiose (DCB), and (12) 5 g polygalacturonic acid (PGA) (Wood and Bhat 1988; Pointing 1999; Yoon et al. 2007; Jo et al. 2010; Florencio et al. 2012). After 3 weeks of incubation, Congo red (1 mg mL^{-1}) was amended to the medium and incubated at room temperature for 15 min. Subsequently, the medium was rinsed with distilled water and 30 mL of 1 mol L^{-1} NaCl added. Degradation of CMC, AVL, DCB, and PGA was confirmed by a transparent appearance of the medium (and mycelia) (Teather and Wood 1982; Pointing 1999).

Chitinolytic activity

The method proposed by Agrawal and Kotasthane (2012) was used in order to evaluate the chitinolytic properties of the isolated strains. Crab shell flakes were ground in a mortar and sieved through the top piece (130 mm) of a polypropylene Buchner filter. Twenty grams of the sieved crab shell flakes were then treated with 150 mL of ~ 12 mol L^{-1} concentrated HCl. HCl was added gently and continuously stirred for 45 min under a chemical fume hood. The final mixture was passed through eight layers of cheesecloth to remove large chitin chunks. The product was treated with two liters of ice cold distilled water and incubated overnight under static conditions at 4°C. Sufficient amount of tap water was then passed through the product until the pH of the product reached a value of 7.0. The final product was squeezed between coffee paper and then sterilized by autoclaving at standard temperature and pressure (15 psi, 20 min, 121°C) (Murthy and Bleakley 2012). The chitinase detection medium consisted of a basal medium comprising (per liter) 0.3 g of $MgSO_4$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 μL of Tween-80, 4.5 g of colloidal chitin (CC), and 0.15 g of bromocresol purple; the pH was adjusted to 4.7 and the neutralized medium autoclaved.

For each strain, the experimental procedures mentioned above were repeated on two occasions with three replicates in each instance. When a positive result was observed, this was confirmed on a third experimental instance in tissue culture plates (TPP®) with the aforementioned medium. We never observed a conflict in the response of the strains to each substrate between any of the experimental instances. Strains used in Rojas-Jimenez et al. (2017) were considered as positive controls.

Substrate utilization assays

Seven fungal and eight oomycetes strains with different lignocellulolytic activities were chosen for physiological profiling, using Ecoplate™, which contain 31 carbon sources in triplicate (Biolog, U.S.A.). For fungal strains, 20 mL of distilled sterile water were poured in the plate to favor detachment of spores. The spore suspension was transferred to a 50 mL Falcon tube, using sterile pipette. In order to measure the concentration of the suspension, spores were counted using a hemocytometer and an approximately equal concentration for all strains was reached. For oomycetes strains, the mycelia from colonized hemp seeds were first rinsed and then transferred to another distilled Petri dish containing sterile water and boiled hemp seed halves with the help of sterile needles. Petri dishes were observed every day and once the intended concentration of zoospores was reached, the spore suspension was transferred to a 50 mL Falcon tube. A 100 μL suspension of each strain was added to each well of the Ecoplates. Ecoplates were incubated at 24°C for 9 d. The activity in the plates was measured using a Synergy™ 2 Multi-Mode Microplate Readers (BioTek, U.S.A.) set for absorbance at 590 nm.

Growth inhibition test

During the degradation assay of polymeric substrates, significant inhibition was observed in several strains. Petri dishes containing mPmTG medium and one of the five abovementioned polymeric substrates with 0.02% wt/vol concentration (BG, CR, PR, RBBR, and TB) were inoculated with mycelia from the edge of 7–15 d-old cultures of fungi and oomycetes and then incubated at 20°C for 7 d under dark conditions. Growth inhibition was determined based on the radial growth at the seventh day compared to the control lacking any polymeric substrate. Ward clustering (Murtagh and Legendre 2014) was applied to draw the dendrogram.

In addition, the effect of two different HS extracted from two bog lakes, i.e., acidic Lake Grosse Fuchskuhle (FUKU) and more neutral Schwazersee (SZ) on vegetative growth of the strains was measured by inoculation of fungal and oomycetes strains to Petri dishes containing mPmTG and one of the HS (200 ppm). The whole experiment was repeated twice, each time with three replicates. Both HS were extracted by Elke Zwirnmann from IGB's chemical lab via reverse osmosis. Details are given by Hutalle-Schmelzer et al. (2010).

HS production capacity

The strains *Aspergillus* sp. RT16 and *Achlya* sp. O962-13 with the highest enzymatic activity were selected for further

detailed analyses as representative of fungi and oomycetes, respectively.

We aimed to reproduce the previous study of Rojas-Jimenez et al. (2017) to evaluate the capacity of fungal and oomycetes strains to transform HS. Thus, we incubated five equal plugs of each strain in the following medium: (1) T1: Ma, containing Test medium (1 g maltose as sole carbon source, 0.94 g KH_2PO_4, 1.9 g K_2HPO_4, 1.6 g KCl, 1.43 g NaCl, 0.15 g NH_4Cl, 0.037 g $MgSO_4$, pH 7.0), (2) T2: Ma + HA, containing Test medium and 50 mg Suwannee River humic acid (2S101H, International Humic Substances Society, U.S.A.), and (3) T3: Ma + HA + Fe, containing Test medium, humic acid (as above), and 10 mg of $FeSO_4$. This treatment was applied since the notion of fungi employing reactive oxygen species (ROS) to degrade HS and other large polymers was discovered in the middle of the last century. Grossart and Rojas-Jimenez (2016) proposed a model to explain the nonspecific HS degradation in aquatic environments by the production and extracellular action of ROS, such as singlet oxygen and peroxide, as well as hydroxyl radical generation via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH^+ + OH^-$). The latter includes the degradation of the subsequent products mainly by laccases, manganese peroxidases, lignin peroxidases, among other nonspecific catalytic enzymatic activities.

Samples were inoculated in tissue culture flasks 25 (TPP®) containing respective media and kept for 20 d at room temperature with constant shaking (100 rpm). Each treatment consisted of five replicates with respective controls.

At the end of the incubation period, the mycelia were separated by filtration on 5 μm polycarbonate filters (Millipore, U.S.A.). Fungal biomass was determined by drying the filters at 55°C for at least 48 h. The dissolved organic carbon content of 1:100 dilutions was measured with a total organic carbon analyzer (TOC-VCPH, Shimadzu). Major compound classes of natural organic matter (NOM) were separated and quantified by coupled size-exclusion chromatography with UV and organic carbon detection (liquid chromatography-organic carbon-organic nitrogen detection [LC-OCD-OND], Huber et al. 2011; Rojas-Jimenez et al. 2017). Briefly, this technique differentiates between HMW substances (e.g., polysaccharides), humic and humic-like substances (HS), and low-molecular-weight substances (LMWS) (e.g., organic acids). Specific UV absorption at 254 nm wavelength (SUVA254) served as a proxy for the degree of aromaticity and/or abundance of unsaturated NOM bonds.

Statistical analyses

Statistical differences between the treatments were estimated for each carbon fraction according to the Kruskal-Wallis test, implemented in R (R Development Core Team 2006).

In regard to our LC-OCD-OND test, data were first visually inspected using scatter plots to evaluate the presence of obvious outliers—but no sample was removed from the analysis. Then, data were z-transformed prior to performing a principal component analysis (PCA, prcomp function, vegan package, Oksanen et al. 2018). We retrieved the scores on the first two PCA axes and used an *F* test for testing if the axes were able to separate the different strains used in this study. We used Spearman rank coefficient analysis and an *F* test to evaluate the correlations between DOM variables and the two PCA axes. More specifically, we correlated the scores of all samples on one axis with the concentration of each variable used to design the PCA. Our motivations for performing these analyses were to evaluate if gradients in DOM composition, e.g., a gradient in aromaticity, were correlated with the nature of the strains, i.e., "fungi" or "oomycetes," or a specific experimental treatment. To evaluate differences in HS transformation, we computed Euclidean distances between HS amended treatments and control conditions. Euclidean distances were computed using only two dimensions, i.e., the two PCA axes, for each fungus and for the control condition. We tested differences in Euclidean distances between "fungi and oomycetes" using a Wilcoxon test. Ultimately, we compared differences in the aromaticity of HS (SUVA_HS) produced in presence or absence of HS by the two fungi using a Wilcoxon test.

In regard to Biolog assays, a threshold optical density (OD) was established at OD590 > 0.25 according to the manufacturer's instructions. For each substrate replicate, we subtracted the average measurement for the blank and then estimated substrate utilization as calculated by integrating the area under the curve (AUC), where the OD_{590} exceeded 0.25 (Miki et al. 2018). For each substrate, we averaged the AUC across the three replicates. The average metabolic response (AMR) and the strain metabolic diversity (SMD) of each strain were calculated by averaging the AUC for all positive substrates and summing the number of positive responses ($OD_{590} > 0.25$) (purple-colored wells) observed following incubation, respectively. We tested for significant differences between strains and, more generally, between fungi and oomycetes across all substrates using a permutational multivariate analysis of variance (PERMANOVA). Substrate-specific differences, between strains were assessed using a two-way ANOVA, with subsequent pairwise assessments, accounting for multiple comparisons, assessed using Tukey's test (*see* Supporting Information Materials 1 and 2).

Results

Identification of strains

In total, 26 strains were isolated from Anzali Lagoon, consisting of 18 fungal and eight oomycetes strains (Table 1). We confirmed the presence of *Arthrobotrys* spp. (three strains), *Aspergillus* sp., *Byssochlamys* sp., *Fusarium* spp. (four strains), *Pleosporales* sp., *Myrmecridium* sp., *Phoma* sp., *Plectosphaerella* sp., *Sarocladium* spp. (two strains), *Scedosporium* sp., *Sclerotium*, *Volutella* sp., *Achlya* (three strains), and *Dictyuchus* sp. (five strains) in Anzali Lagoon. Due to inconsistent results pertaining to the LSU and ITS regions of the rRNA operons of RT8, and the lack of any clear morphological features, we considered this strain as unclassified within the order *Pleosporales*. For all remaining taxa, molecular classification of genera was confirmed by morphological approaches (Fig. 1).

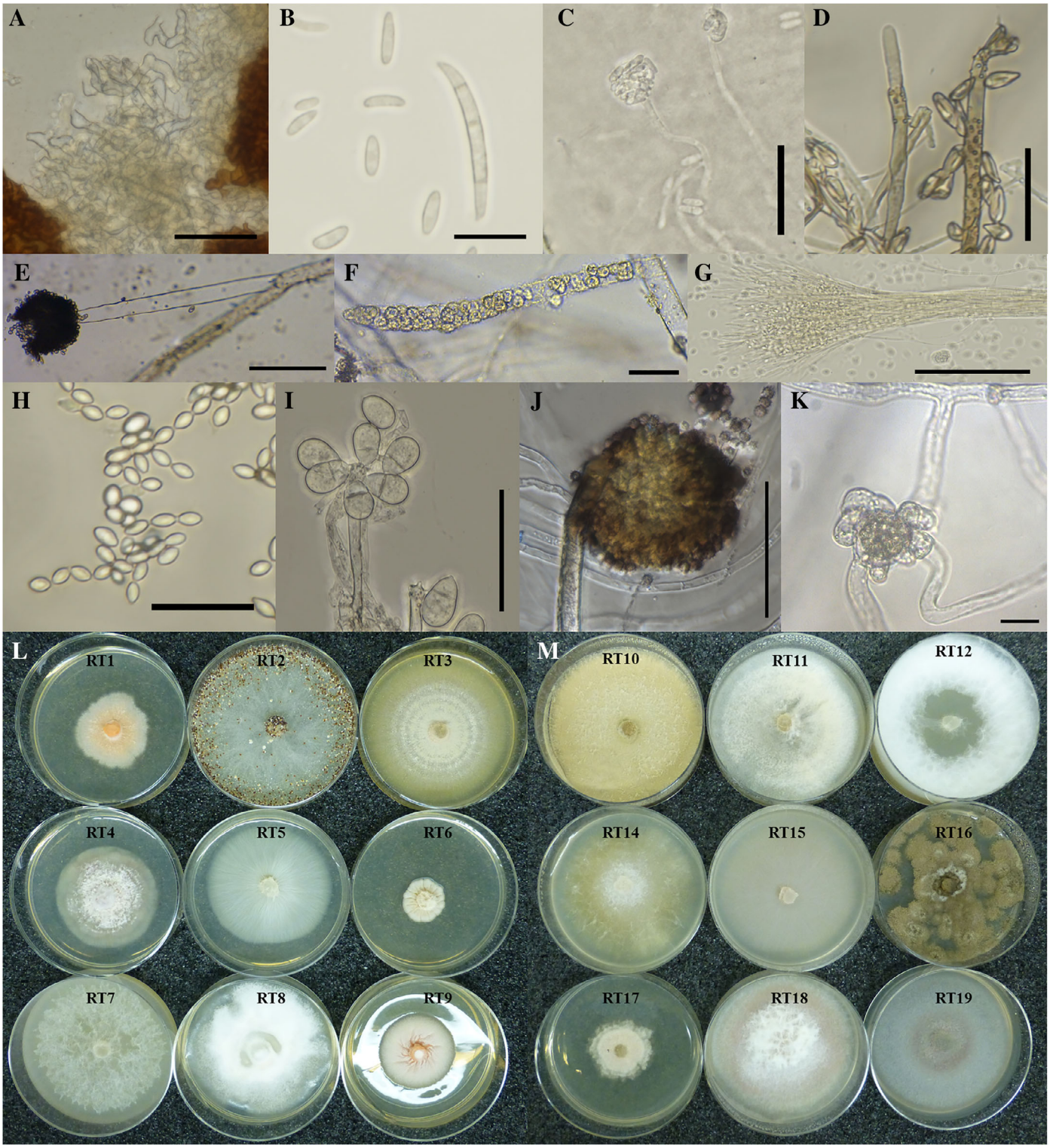

Fig. 1. General morphological characteristics of some strains used in this study. (**A**) Fragments of hyphae inside sclerotia of *Sclerotium* sp. RT2. (**B**) Micro- and macroconidia of *Fusarium* sp. RT3. (**C**) Solitary conidiophore and conidia of *Plectosphaerella* sp. RT5. (**D**) Conidium-bearing rachis with pimple-shaped denticles of *Myrmecridium* sp. RT9. (**E**) Empty sporangium with encysted primary zoospores at the top of it in *Achlya* sp. F962-15. (**F**) Mature sporangium of *Dictyuchus* sp. O961-3. (**G**) Gregarious sporodochia of *Volutella* sp. RT4. (**H**) Conidia in chains of *Paecilomyces* sp. RT10. (**I**) Conidia and conidiophores of *Arthrobotrys* sp. RT14. (**J**) Conidiophore, vesicle, and conidia around it in *Aspergillus* sp. RT16. (**K**) Mature oogonium and antheridium of *Dictyuchus* sp. of O961-3. (**L**, **M**) Colony pattern of hyphomycetes strains after 12 d on MEA cultures. Scale bar: A, G, H, J = 50 μm; B, E, F = 20 μm, C = 100 μm, D = 25 μm, and I = 200 μm.

Table 2. Results of experiment in which we have screened for lignolytic, cellulolytic, pectinolytic, and chitinolytic activities as well as tested for growth inhibition by two different HS (given as percentage of growth without HS) of fungal and oomycetes strains isolated from Anzali lagoon, Rasht, Iran.

		BG*												Inhibition (%)	
Strain	ABTS*	0.02%	0.005%	CR*	PhR*	PR*	RBBR*	TB*	CMC†	AVL†	DCB†	PGA‡	CC§	FUKU HS‖	SZ HS‖
RT1	0	0	0	AD	1	0	0	0	0	0	0	0	1	−111.11	−14.28
RT2	3	1	1	AD	2	0	0	0	3	3	3	2	1	0	3.70
RT3	1	1	1	AD	1	0	1	0	2	1	1	0	0	−9.09	−30.52
RT4	0	0	1	AD	1	0	0	0	0	0	0	0	3	−21.15	−34.78
RT5	0	1	2	AD	1	0	0	0	0	1	2	0	1	0	−10.29
RT6	0	1	1	AD	1	0	1	0	0	1	0	0	1	−5	2.94
RT7	0	0	1	AD	0	0	0	0	1	1	0	0	0	0	−41.93
RT8	1	0	1	AD	1	0	0	0	2	1	2	2	1	12.50	−10.93
RT9	0	0	0	AD	1	0	0	0	0	0	0	0	1	6.780	22
RT10	1	2	2	2	0	0	1	0	2	1	1	2	0	−67.74	16
RT11	0	1	1	AD	1	0	1	0	2	0	0	2	2	−2.20	−16.53
RT12	0	0	2	AD¶	0	0	0	0	0	0	0	0	2	10.34	−8.88
RT14	0	1	1	AD	2	0	0	0	0	0	0	2	2	−5.71	−17.39
RT15	0	1	2	AD	1	0	0	0	0	0	0	0	3	−14.61	−26.54
RT16	2	3	3	2	0	0	0	0	3	3	3	3	2	−15.38	−11.53
RT17	1	0	0	AD	1	0	2	0	2	0	1	0	1	−3.12	−22.22
RT18	1	1	1	AD	1	0	0	0	2	1	1	2	1	0.89	−36.08
RT19	3	1	1	AD	1	0	1	0	1	1	1	1	0	4.22	13.55
O961-3	0	0	1	AD	0	0	0	0	1	0	0	0	0	12.14	100
F962-15	0	1	0	AD	0	0	0	0	0	1	0	0	0	100	100
O962-13	0	2	2	AD	0	0	0	0	1	1	1	0	2	33.11	100
O962-14	0	0	1	AD	0	0	0	0	0	1	0	0	1	52.12	100
O963-5	0	0	0	AD	0	0	0	0	0	1	0	0	2	100	100
O963-13	0	2	2	AD	1	0	0	0	0	1	1	0	1	100	100
T963-33B	0	0	0	AD	1	0	0	0	0	1	0	0	0	9.52	100
M963-8A	0	0	1	AD	0	0	0	0	0	0	0	0	0	100	100

0 = no activity, 1 = weak, 2 = medium, and 3 = strong activity.

*Dyes as indicators of lignolytic activity, (1) ABTS (as specific indicator of laccase activity), (2) BG, (3) CR, (4) PhR, (5) PR, (6) RBBR, and (7) TB.

†Three carbon sources as indicators of cellulolytic activity, (8) 7.5 g CMC, (9) 7.5 g AVL, (10) 5 g DCB, as indicator of Endo-1,4-β-glucanase, cellobiohydrolase, and β-glucosidase.

‡(12) PGA as indicator of pectinolytic activity.

§CC as indicator of chitinolytic activity.

‖Inhibition percentage in the presence of humic substances extracted from Grosse Fuchskuhle (FUKU) and Schwazersee lake (SZ).

¶Adsorption.

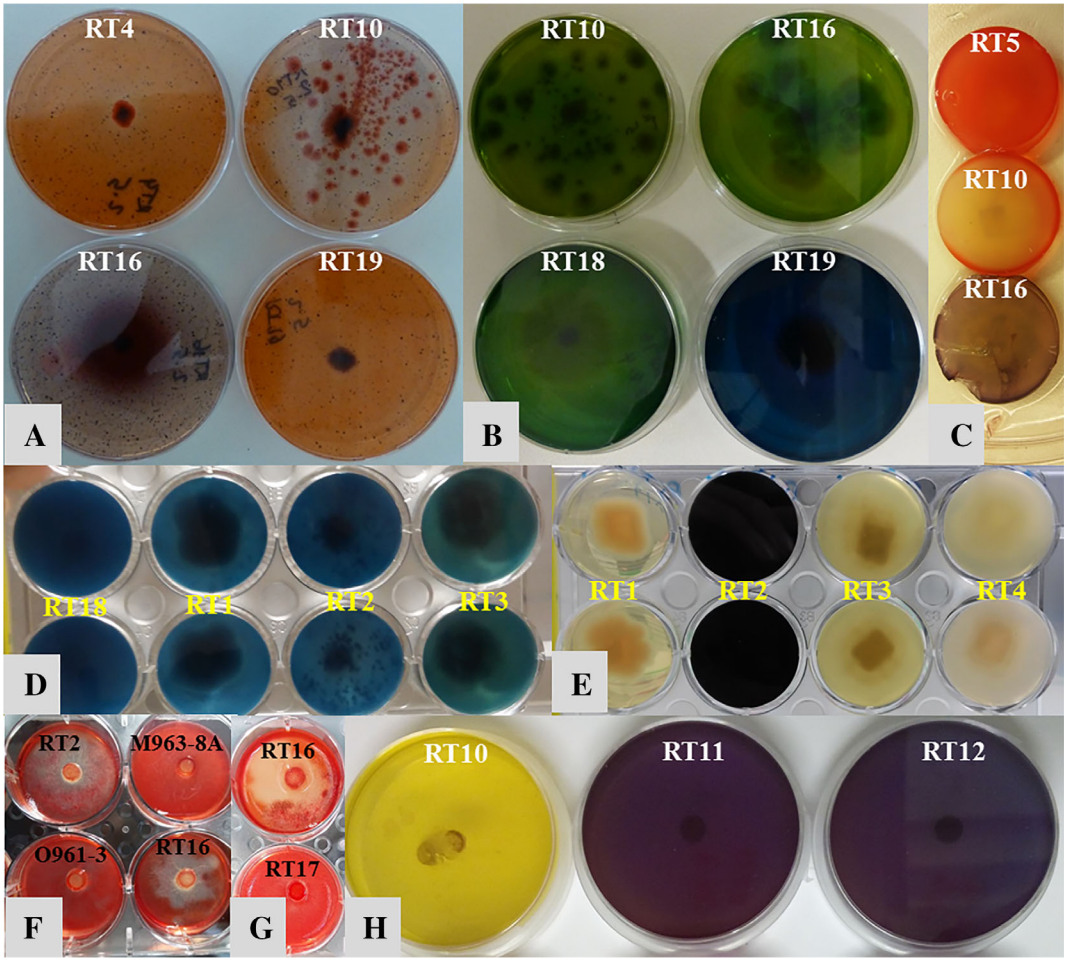

Fig. 2. Decolorization of (**A**) CR (RT10 and RT16), (**B**) BG (RT16 and RT18), (**D**) RBBR (RT3), and (**E**) ABTS (RT2) after during 21 d of inoculation by different strains. Creating transparent area in media containing (**C**) CMC (RT10 and RT16), (**F**) DCB (RT2 and RT16), and (**G**) AVL inoculated by positive strains. (**H**) Change in color of media containing colloidal chitin from dark yellow to purple by positive strains.

Degradation assay of polymeric substrates

All fungal strains produced a positive result on at least two substrates (Table 2). The strongest activities were observed for *Aspergillus* sp. RT16 (five strong and three medium activities), *Sclerotium* sp. RT2 (four strong, two medium and two weak activities), and *Paecilomyces* sp. RT10 (four medium and four weak activities). The most diverse activity was observed in *Sclerotium* sp. RT2, *Fusarium* sp. RT18, *Aspergillus* sp. RT16, and *Phoma* sp. RT19 with nine positive activities. None of the fungal strains could degrade PR and TB. No positive results were obtained for oomycetes growing on media containing ABTS, PR, RBBR, TB, and PGA. The highest activity was observed in *Achlya* sp. O962-13 on one medium and four weak activities (Fig. 2). When two different concentrations of BG were used, we observed slight differences in lignolytic activity of 10 tested strains. MG was eliminated from the assays, due to complete inhibition of vegetative growth in both groups. With the exception of RT10 and RT16, no decolorization was observed in the Petri dishes amended with Congo Red. Thus, the change in colony color must have been a result of adsorption and not degradation. There was no difference in using different concentrations of PR at two pH values (5 and 7).

Physiological profiling

We observed a significant difference in the abilities of fungal and oomycetes strains to utilize 31 different carbon sources (Pseudo-F = 15.557, p[perm] < 0.001). Furthermore, we identified for both the fungi and oomycetes, large differences among individual strains (Pseudo-F = 18.19, p[perm] < 0.001). *Aspergillus* sp. RT16 and fungal sp. RT18 exhibited the highest AMR and SMD (28/31), respectively. *Volutella* sp. RT4 and *Sarocladium* sp. RT1 exhibited the lowest AMR and SMD (21/31), respectively (Fig. 3, upper panel; Supporting Information Table S1). Among the oomycetes strains, *Achlya* sp. O963-13 and *Dictyuchus* sp. O962-14 exhibited the highest and lowest AMR, respectively (Fig. 3, bottom panel; Supporting Information Table S1). *Dictyuchus* sp. T963-33B and *Achlya* sp. O962-13 exhibited the highest (28/31) and lowest SMD (22/31) (Fig. 3, bottom panel; Supporting Information Table S1). Neither fungal nor oomycetes strains could utilize γ-hydroxy butyric acid. There was no significant difference in the SMD of

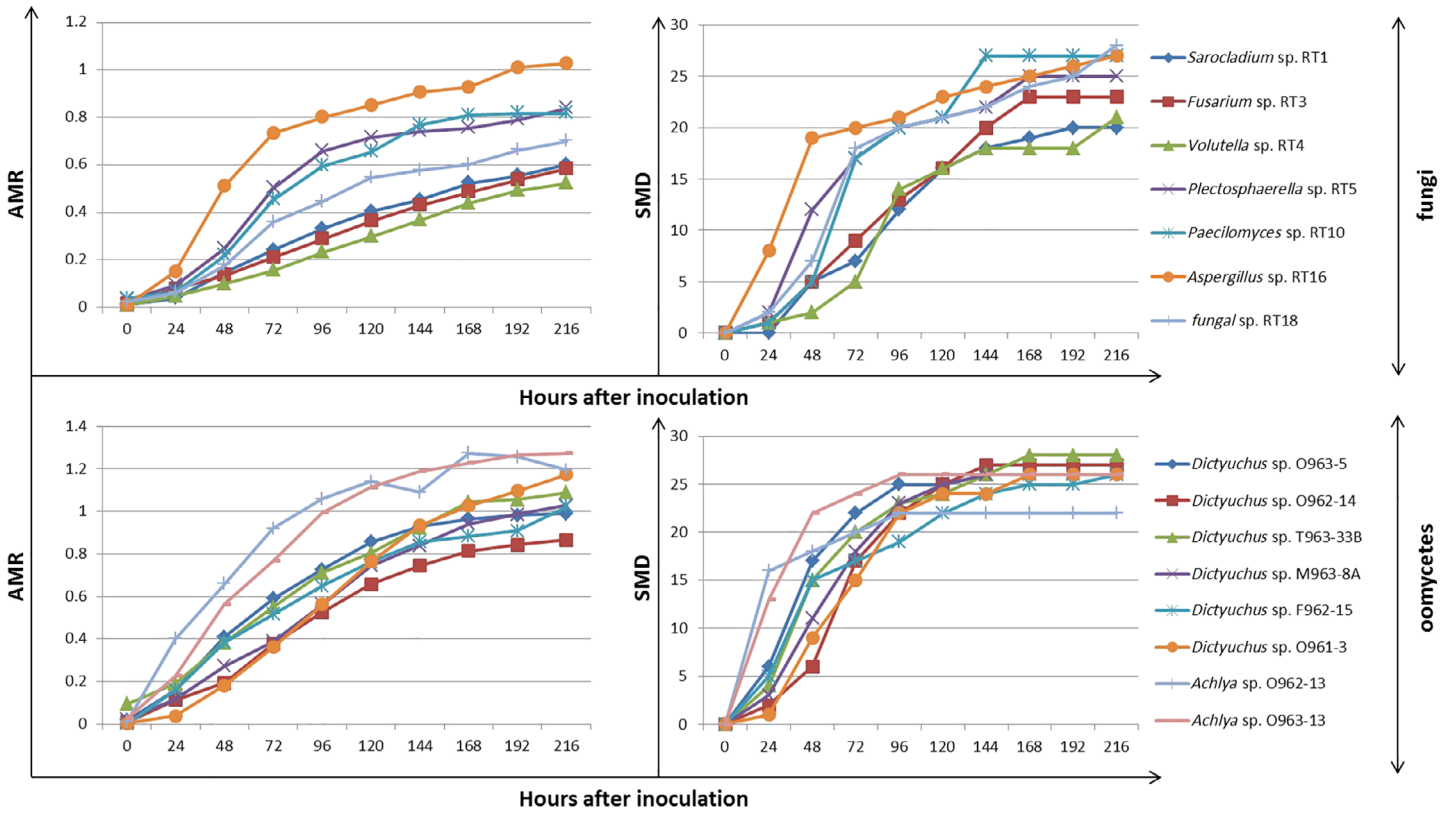

Fig. 3. Physiological profiles of seven fungal (upper panels) and eight oomycetes strains (bottom panels), comparing the AMR and SMD after nine consecutive days based on the utilization of 31 different carbon sources with three replicates each.

oomycetes and fungal strains (p = 0.282); however, oomycetes strains exhibited a significantly higher AMR ($p < 0.001$) (Fig. 4).

Growth inhibition

Differences in the inhibition of vegetative growth among fungal and oomycetes strains were evident following growth in the presence of any of the five polymeric substrates. All fungal strains (except for *Sclerotium* sp. RT2, *Myrmecridium* sp. RT9, *Arthrobotrys* sp., and RT19) and oomycetes strains clustered separately based on the inhibition percentage of five polymeric substrates (Fig. 5). We could readily differentiate between fungal and oomycetes strains based on their inhibitory response to five polymeric substrates. The impact on growth in the presence of HS derived from two lakes differed between fungal and oomycetes strains. Not only did we observe any significant inhibition of fungal strains, in several cases, we observed an increase in vegetative growth up to 20% (Table 2). In contrast, the addition of FUKU and/or SZ HS completely inhibited the growth of eight and four oomycetes strains, respectively (Table 2).

Processing capacity of HS

The potential of *Aspergillus* sp. RT16 and *Achlya* sp. O962-13 to transform different carbon sources was tested using a PCA. The two strains and the controls (without any biomass) were separated in three distinct clusters based on their score on the first PCA axis (PC1, Fig. 6; Supporting Information Table S2). PC1 was positively correlated with the concentration of HS and negatively correlated with the concentration of LMWS. PC1 also negatively correlated with the aromaticity index (SUVA). This gradient highlighted the potential of the two tested fungi to produce HS. Moreover, in all tested conditions, we noted *Aspergillus* sp. RT16 produced clearly more HS than *Achlya* sp. O962-13 (Fig. 6).

The differences induced by the initial carbon sources were visible on the second PCA axis (PC2, Fig. 6). However, the ordination was not able to separate the two treatments amended with HS (T2 and T3 treatments). PC2 was negatively correlated with the aromaticity of the organic matter ($SUVA_{254}$) and HS aromaticity (SUVA_HS). As expected, the addition of HS increased the $SUVA_{254}$ in all conditions. However, in the presence of either strain, the increase in $SUVA_{254}$ was higher than in the control condition. Moreover, the addition of HS to the initial medium induced a production of more aromatic HS than the ones originating solely from the transformation of maltose.

To highlight this process, we computed the Euclidean distance between maltose and HS-amended conditions (T2 and T3 treatments). Thus, we were able to highlight if the production of HS and their aromaticity were higher when the medium was amended with HS. It revealed that solely *Aspergillus* sp. RT16 was able to produce HS with a higher aromaticity than the initial HS inoculum. Interestingly, HS produced by

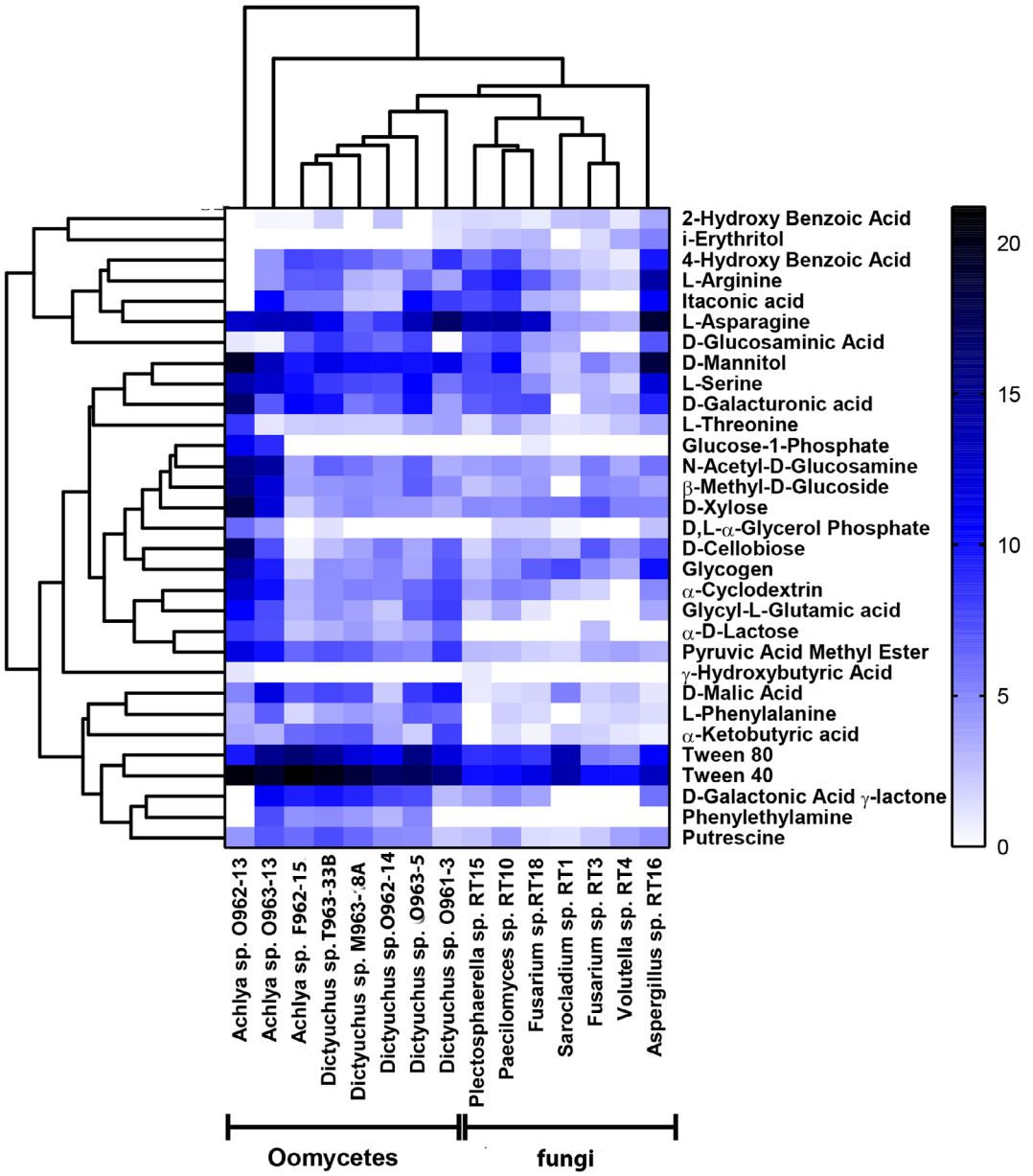

Fig. 4. Comparative heat map of representative fungal (RT1, RT3, RT4, RT10, RT15, RT16, and RT18) and oomycetes strains (O962-13, O963-13, F962-15, T963-33B, M963-8A, O962-14, O963-5, and O961-3) strains grown on Ecoplates after nine consecutive days. White to black gradient indicates poor to high growth on specific substrate (bar = the specific AUC value).

Achlya sp. O962-13 in the presence of other HS was not different from that produced in the presence of only maltose. It was obvious that addition of Fe to the medium (T3 treatment) reveals no difference when compared to the T2 treatment.

Discussion

Although the role of fungi and/or their extracellular enzymes in the decomposition of HS have been established (Kües 2015), their involvement in aquatic DOM degradation and cycling has been rarely investigated. In our study, many fungal strains (e.g., *Aspergillus* sp. RT16) exhibited a broad range of enzymatic activities. Our results indicate that the more enzymatically active a fungus is, the more it is involved in HS transformation.

Methodological obstacles

Dye decolorization assays are cost-efficient and simple methods to test for enzymatic capabilities of isolated fungi with high enzymatic activity. However, there are several factors,

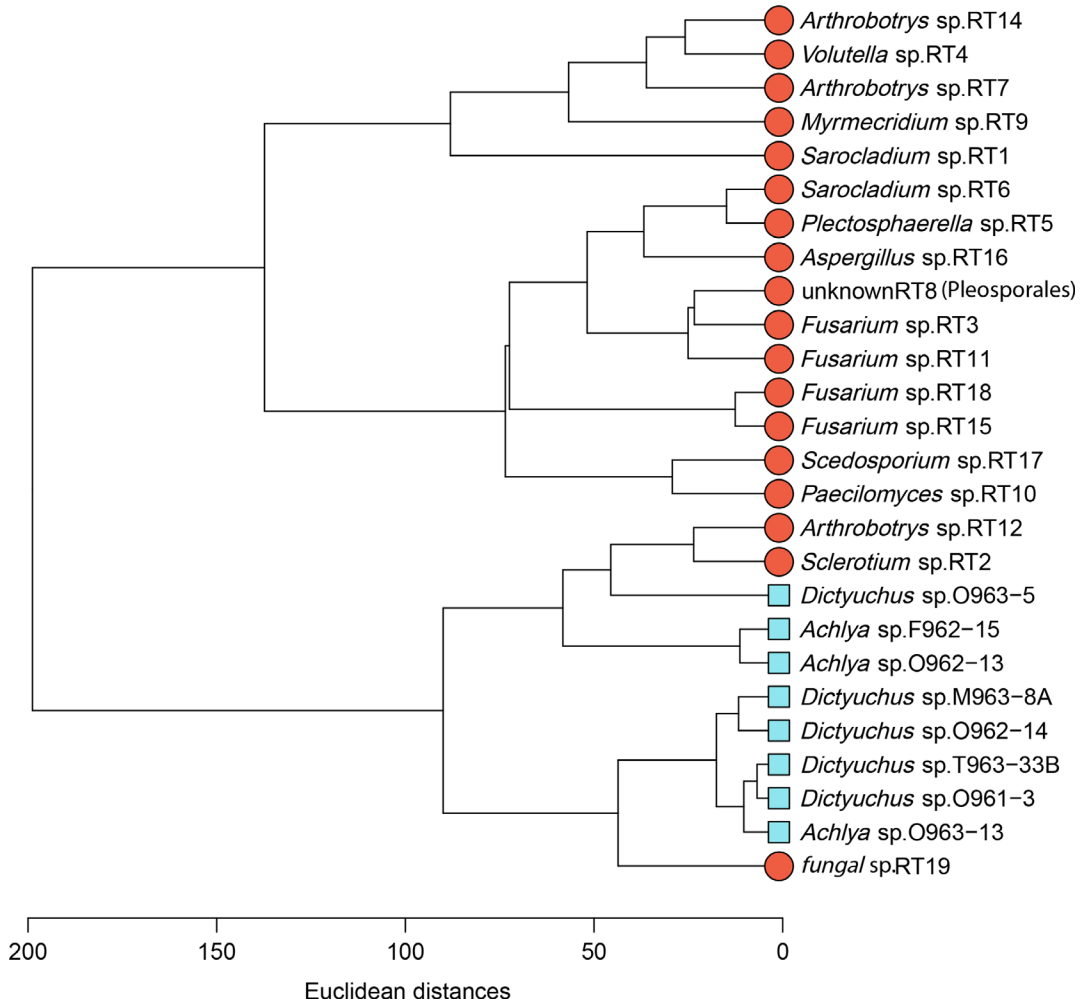

Fig. 5. WARD clustering of fungal and oomycetes strains based on their response to five different polymeric substrates after 7 d.

especially media composition, pH value, initial dye concentration, and its core structure affecting the assay's results, that need to be carefully considered before performing further experiments (Knapp et al. 2001; Singh 2006). No significant differences were observed when using different media, concentration, or pH with our strains. However, percentage of vegetative growth inhibition of fungal and oomycetes strains reveals a clearly different impact of any of the five polymeric substrates used (Fig. 5). This indicates that the sensitivity of the decolorization assay to a certain extent may have been biased by these methodological constraints.

Origin of strains

Identification of fungal strains showed that most strains (e.g., *Sclerotium* sp. RT2; *Fusarium* spp. RT3, RT4, RT15, and RT18; and *Sarocladium* spp. RT1 and RT6) are closely affiliated with potential components of the rice sclerotial and sheath rot disease complex (Lanoiselet et al. 2002; Yang et al. 2007; Hu et al. 2008; Xu et al. 2010; Bigirimana et al. 2015). They are, however, not among the most common Hyphomycetes species reported from freshwater ecosystems, which might be explained by our isolation method. Although determining the distribution of these uncommon fungal strains in freshwater ecosystems requires applying different sampling methods and advanced environmental DNA surveys, it might indicate that the aquatic fungal communities can be influenced by its drainage basin. In our case, pumping water from lagoon for fields' irrigation and lack of sustainable drainage systems may facilitate the distribution of these uncommon fungal strains. In our study, it appears that these fungi can exploit both terrestrial and freshwater environments. It is not clear to us whether they are entering to the lagoon from rice fields or vice versa. This possible duality in fungi life style has been suggested previously by several other authors (Selosse et al. 2008; Chauvet et al. 2015) and highlights the difficulty in the delimitation of this ecologically distinct group (Shearer 1993; Dhanasekaran et al. 2006).

Park (1972) tried to present an outline scheme for the ecological classification since the 1970s by dividing aquatic fungi into indwellers (constant activity), migrants (periodic or

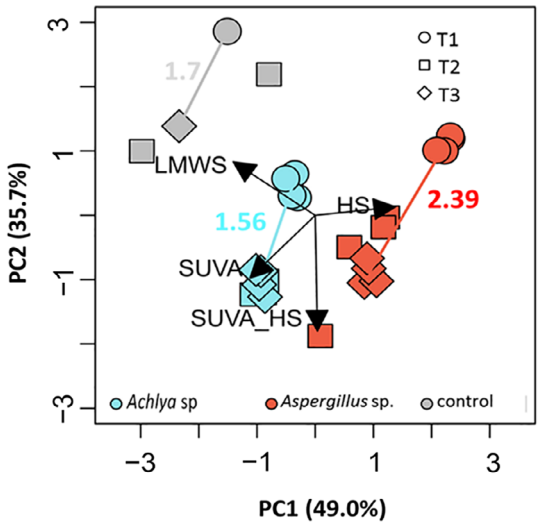

Fig. 6. Principal component analysis of three treatments (T1, T2, and T3) in media after 15 d of incubation with *Aspergillus* sp. RT16 (red) and *Achlya* sp. O962-13 (blue). T1 (circular shape), containing 1% maltose as sole carbon source; T2 (square shape), containing maltose and 50 mg L^{-1} of HS; T3 (diamond shape), containing maltose, HS, and 10 mg L^{-1} of FeSO4.

sporadic activity), versatiles (sporadic activity), and transients (those exhibiting no activity). They have also been called amphibious hyphomycetes (Webster 1977; Michaelides and Kendrick 1978) suggesting they occur in both aquatic and terrestrial habitats (Descals and Moralejo 2001). However, the diversity of aquatic fungi is not limited to a certain phylogenetic clade (Wurzbacher et al. 2016; Nawaz et al. 2018) and could be influenced by the topography of the waterbody's surrounding. Thus, addressing the question whether they are ecologically active, when entering waterbodies, is a legitimate quest.

Identification of oomycetes strains, however, was unsurprising as *Achlya* sp. and *Dictyuchus* sp. along with *Saprolegnia* sp., *Aphanomyces* sp., and so on are common freshwater saprotrophs (Phillips et al. 2008; Svoboda et al. 2014). However, their role in the energy transfer from allochthonous and autochthonous particles to other components of the food chain have received less attention due to their importance as aquatic animal pathogenic oomycetes. For the first time, we showed that oomycetes and fungi may have complementary roles as demonstrated by their distinct enzymatic activities (Table 2) and carbon utilization capacity (Figs. 3–4).

Enzymatic activity of strains

A big fraction of aquatic DOM_{allo} originates from vascular plant cell walls (mainly lignin, cellulose, hemicellulose, and pectin), soil organic matter, and less recalcitrant molecules such as chitin (derived from fungal cell walls and arthropods exoskeleton) (Burdon 2001; Toming et al. 2013). Here, we confirmed that fungi, via their capacity to produce a broad range of extracellular enzymes, are involved in the transformation of these molecules. The enzymatic repertoire of aquatic fungi renders them one of the most important players of the microbial loop in various aquatic ecosystems (Meyer 1994; Sime-Ngando et al. 2011). Together with other heterotroph microorganism, they are placed at the cross junction of production, transformation, and mineralization of DOM and POM (particulate organic matter).

Oomycetes, however, have been mainly considered as animal and plant pathogens (Van West 2006) and, for the first time, we have evaluated the role of oomycetes in aquatic carbon cycling. None of our oomycetes strains exhibited any laccase or pectinase activity (Table 2). In contrast, all 18 laccase genes and expansion of effector protein families including pectinases are present in plant pathogenic oomycetes such as *Phytophthora* sp. (Feng and Li 2012, 2014; Feng et al. 2015; McGowan and Fitzpatrick 2017). This may hint to a sharp dichotomy between mostly terrestrial plant pathogens and freshwater saprophytic oomycetes such as *Saprolegnia* sp., *Achlya* sp., and *Leptolegnia* sp. (Diéguez-Uribeondo et al. 2009; Jiang et al. 2013). To better generalize these findings, future investigations are needed to test whether lack of laccase and pectinase activity is a general trend in solely saprophytic oomycetes.

Physiological profiling

Although our oomycetes strains showed less enzymatic activities to degrade large polymers, their rate of utilization of small carbon sources was generally higher than fungal strains'. Most oomycetes and fungal strains were separated according to their utilization capability (Fig. 4). This separation is in agreement with the recent realization of evolutionary separation between oomycetes and fungi (Beakes et al. 2012). However, more genera from both groups need to be investigated to test this separation.

HS processing capabilities

Our LC-OCD experiment shows that *Aspergillus* sp. RT16 is not only involved in the degradation of HS precursors, but also transforms HS by further increasing their aromatic nature (Fig. 6). The simultaneous occurrence of HS precursor degradation and transformation has been previously shown for *Cladosporium* sp. and *Paecilomyces* sp. from freshwater and terrestrial environments, respectively (Rojas-Jimenez et al. 2017). This is an important finding since HS are one of the most abundant reservoirs of carbon in freshwater ecosystems (Lennon et al. 2013). They are also involved in the transport, fate, and redox conversion of complex compounds affecting distribution and transport of pollutants (Kisand et al. 2013). Our data confirm that HS are not necessarily inert, but are taken up and degraded by enzymatically active fungi (Nardi et al. 2002). In addition, we showed the ability of fungi to degrade large polymers by using ligninolytic enzymes, e.g., laccases. We also show the ability of these species to produce HS from different types of carbon sources available with similar degradation byproducts for each of the tested substances. HS recycling could be mediated by coupled oxidative processes via

laccases, of which very little is known (Mikolasch and Schauer 2009; Rojas-Jimenez et al. 2017). Interestingly, several oomycetes could be frequently isolated from plant debris in freshwater ecosystems—similar to fungi but no HS production was observed for strain *Achlya* sp. O962-13. The relatively low enzymatic activity and/or the high sensitivity of our oomycetes strains to HS (Table 2) are possible causes. Inhibition of different HS on growth of *Saprolegnia* sp. has been previously mentioned by several authors (Gamygin et al. 1991, 1992; Meinelt et al. 2007).

In conclusion, this study uncovers fungi as one of the major players in organic carbon cycling of aquatic ecosystems, and highlights the opportunistic nature of oomycetes. Therefore, the ultimate question regarding the fate of allochthonous carbon in aquatic and global C cycles needs to take into account their interactions with aquatic fungi. Our fungal strains demonstrate a high enzymatic potential to initiate humification by degrading its precursors and mediating the formation, mobilization, transformation, and storage of NOM. In particular, they may also act on highly aromatic structures including HS and transform them to even more aromatic compounds. Aside from their ecological relevance, applicability of fungi as potential biological agents in removal of industrial and agricultural effluents is getting more attention. Currently, their ability to decolorize various synthetic dyes in different reactor systems of laboratory size has been tested (Tavčar et al. 2006), revealing their high potential to decolorize synthetic dyes. Thus, fungi are well suited to tackle increasing problems of organic waste pollution of freshwater systems worldwide.

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Conflict of Interest

None declared.

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