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Chaophilic or chaotolerant fungi: a new category of extremophiles?

Running title: Chaophilic or chaotolerant fungi?

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Abstract

It is well known that few halophilic bacteria and archaea as well as certain fungi can grow at the highest concentrations of NaCl. However, data about possible life at extremely high concentrations of various others kosmotropic (stabilizing; like NaCl, KCl and $MgSO_4$) and chaotropic (destabilizing) salts (NaBr, $MgCl_2$ and $CaCl_2$) are scarce for prokaryotes and almost absent for the eukaryotic domain including fungi. Fungi from diverse (extreme) environments were tested for their ability to grow at the highest concentrations of kosmotropic and chaotropic salts ever recorded to support life. The majority of fungi showed preference for relatively high concentrations of kosmotropes. However, our study revealed the outstanding tolerance of several fungi to high concentrations of $MgCl_2$ (up to 2.1 M) or $CaCl_2$ (up to 2.0 M) without compensating kosmotropic salts. Few species, for instance *Hortaea werneckii*, *Eurotium amstelodami*, *Eurotium chevalieri* and *Wallemia ichthyophaga*, are able to thrive in media with the highest salinities of all salts (except for $CaCl_2$ in the case of *W. ichthyophaga*). The upper concentration of $MgCl_2$ to support fungal life in the absence of kosmotropes (2.1 M) is much higher than previously determined to be the upper limit for microbial growth (1.26 M). No fungal representatives showed exclusive preference for only chaotropic salts (being obligate chaophiles). Nevertheless, our study expands the knowledge of possible active life by a diverse set of fungi in biologically detrimental chaotropic environments.

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1. Introduction

Water is essential to life, and life can only exist within a narrow range of water availability in a particular environment, expressed as water activity (a_w). Water activity is the effective water content expressed as its mole fraction, therefore pure water has $a_w = 1$, all the other solutions have $a_w < 1$. Types and amounts of solutes present in the environment lower a_w to various values and exert additional effects on the growth of microorganisms – causing osmotic pressure and/or have toxic effects. The lowest a_w known to support life is 0.61, measured for the xerophilic fungus *Xeromyces bisporus* grown on sugar-based media (Pitt and Hocking, 2009), and also for some halophilic Archaea and Bacteria (Stevenson et al., 2014). Many fungi are able to thrive at low a_w, especially the numerous xerophilic filamentous fungi and osmophilic yeasts that grow on drying foods or on foods with high concentrations of sugars (Pitt and Hocking, 1977; 2009).

In the past, fungi were not renowned for growth at high salt concentrations. However, after the first record of fungi as active inhabitants of solar salterns were published (Gunde-Cimerman et al., 2000), the study of halotolerant and halophilic fungi expanded. Since that time numerous fungal species thriving in extremely saline environments around the globe have been described, most of them being halotolerant and extremely halotolerant, and few are obligate halophiles (reviewed in Zajc et al., 2012). The most halophilic fungus known to date is *Wallemia ichthyophaga* as it requires at least 10% NaCl and grows also in solutions saturated with NaCl (Zalar et al., 2005; Zajc et al., 2014).

Fungi have some common characteristics of osmotolerance, for instance they all employ the »compatible solutes« strategy: they balance the osmotic pressure of the surroundings by accumulating small organic molecules (compatible solutes), most commonly glycerol, and maintain low intracellular concentrations of salt (such as toxic Na^+ ions) (reviewed in Gostinčar et al., 2011; Zajc et al., 2012). Sensing and responding to turgor stress (either due to organic osmolytes or due to salt) is under the control of the high osmolarity glycerol (HOG) signalling pathway in all halotolerant and halophilic fungi (Gostinčar et al., 2011). The activation of the HOG pathway results in the production of glycerol, which restores the osmotic balance of the cell (Hohmann, 2009). The cells are equipped with channels allowing for a quick expulsion of glycerol, as well as its active intake when required (Luyten et al., 1995; Ferreira et al., 2005). As the concentration of glycerol is carefully regulated, this strategy allows more flexible adaptations to changing salinity. Besides energetically costly synthesis of high concentrations of organic solutes, the cells also use much energy by using different efflux and influx systems to actively eliminate surplus ions, to preserve membrane potential, regulate intracellular pH, and maintain positive turgor of the cell. Hence, the alkali-metal cation transporters are of high importance of the osmoadaptation to extremely saline environments. In fact, the Na^+- exporting ATPase (EnaA) is the major determinant of salt tolerance in yeasts (reviewed in Ariño et al., 2010). In addition to the above active mechanisms, fungi also employ some strategies for increasing their stress resistance that may be referred as passive – like clustering cells in compact cell clumps (Palkova and Vachova, 2006; Kralj Kunčič et al., 2010), covering the cells with abundant extracellular polysaccharides or increasing the thickness (Kralj Kunčič et al., 2010), and pigmentation (*e. g*. melanin) (Selbmann et al., 2005; Kogej et al., 2006) of the cell wall.

As most hypersaline environments are rich in NaCl, salt tolerance of fungi and other microorganisms, and mechanisms of adaptations were generally tested by using only NaCl as the solute. Therefore, the responses to high concentrations of other chaotropic salts remained unknown. However, other salts such as $MgCl_2$ are also abundantly present in nature and can be important or even life-limiting. Salts in the environment not only lower the biologically available water and cause toxicity due to the penetration of certain cations into the cell, but they also modify structural interactions of cellular macromolecules. The Hofmeister series of ions (K^+> Na^+> Mg^{2+}> Ca^{2+}; SO_4^{2-}> HPO_4^{2-}>Cl^-> NO_3^-> Br^-> ClO_3^-> I^-> ClO_4^-) describes the order of the ability of ions to salt-out or salt-in proteins (Hofmeister, 1888; Kunz et al., 2004). This phenomenon is based on direct interactions between ions and macromolecules and on interactions between ions and water molecules in the first hydration shell of the macromolecule (Zhang and Cremer, 2006). Hofmeister effects of ions on biological structures are either kosmotropic or chaotropic; chaotropes weaken electrostatic interactions and destabilize biological macromolecules, whereas the contrary is true for the kosmotropes (reviewed in Oren, 2013). The difference among the kosmotropic effect of NaCl on one hand and the chaotropic effect of $MgCl_2$ and $CaCl_2$ on the other hand might explain why high concentrations of Mg^{2+} and Ca^{2+} are toxic even to the most halophilic microorganisms (McGenity and Oren, 2012). However, to some extent the chaotropic effects of Mg^{2+} and Ca^{2+} can be counteracted by the presence of kosmotropic ions (Williams and Hallsworth, 2009). In fact, few halophilic Archaea can grow at high concentrations of $MgCl_2$, but only in the presence of significant concentrations of NaCl (Mullakhanbhai and Larsen, 1975; Oren, 1983; Oren et al., 1995). This confirms an early study of interactions among kosmotropic and chaotropic ions on the growth of the halophilic alga *Dunaliella salina* performed by Baas Becking, who discovered that toxicity of Ca^{2+} ions was diminished in the presence of sodium ions (Baas Becking, 1934; Oren, 2011).

Two types of hypersaline brines are distinguished with respect to their origin of formation; thalassohaline and athalassohaline (Oren, 2002). Thalassohaline waters, such as marine ponds, salt marshes and solar salterns, originate by evaporation of sea water and are therefore dominated by sodium and chloride ions. During the progression of evaporation, ionic composition changes due to the consecutive precipitation of calcite ($CaCO_3$), gypsum ($CaSO_4 \cdot 2H_2O$), halite (NaCl), sylvite (KCl) and final carnalite ($KCl \cdot MgCl_2 \cdot 6H_2O$) after their solubilities have been surpassed (Oren, 2002; Oren, 2013). The major change in the ratio of divalent and monovalent cations occurs when the total salt concentration exceeds 300 to 350 g l^{-1} and most of the sodium (as halite) precipitates. In the remaining brine, so-called bittern, the dominate ion becomes Mg^{2+} (Oren, 2013).

While NaCl-rich (thalassohaline) environments are well known to support a rich biodiversity, including of fungi, very little is known about the occurrence of fungi and other microorganisms in athalassohaline, $MgCl_2$- and $CaCl_2$-dominated environments. Several fungi were isolated from the magnesium and calcium-rich water of the Dead Sea (Oren and Gunde-Cimerman, 2012) (~2.0 M and ~0.5 M, respectively; total dissolved salts concentration ~350 g l^{-1} (Oren, 2013); water activity ~0.683 (at 35 °C) (Hallsworth, personal communication)). However, most frequently isolation media were supplemented with different NaCl concentrations (reviewed in Oren and Gunde-Cimerman, 2012) rather than with chaotropic ions such as magnesium and calcium. Recently fungal strains were isolated from the bittern brines of solar salterns (Sonjak et al., 2010), an environment earlier considered sterile due to the high concentrations of magnesium salts (Javor, 1989). These fungal strains showed elevated tolerance to $MgCl_2$, a phenomenon not yet reported for fungi. This raised the issue of the existence of chaophiles among extremophilic fungi. To address the question whether chaotolerant/chaophilic fungi may exist, we have examined a range of them both from bitterns, the Dead Sea and other extreme environments, as well as reference strains from culture collections for their ability to grow at high concentrations of various chaotropic as well as kosmotropic salts.

2. Material and methods

2.1 Fungal strains

The fungal strains studied (listed in Table 1) include culture collection strains known for their halotolerance and/or xerotolerance, and reference strains not known to be derived from hypersaline or dry environments. In addition, we tested newly isolated strains from bitterns of the Sečovlje (Slovenia) solar salterns. All fungal strains used are maintained in the Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

2.2 Screening of the fungal growth in media of various salt concentration and composition

Strains were first inoculated on MEA without additional salts, except for the special strains that are obligately xerophilic (*Xeromyces bisporus* FRR525/EXF-9116) or halophilic (*Wallemia ichtyophaga* EXF-1059, -5676, -994, -6068, -8617 and *W. muriae* EXF-753, -2361, -8359, -951). For the latter two species, MEA was supplemented with 2 M NaCl, whereas for *X. bisporus* MEA was supplemented with 30 % (w/v) glucose. After seven to fourteen days of incubation at 24 °C in the dark, spore suspensions were prepared using spore suspension solution (0.05 % (w/v) Tween 80, 0.05 % agar, 0.9 % NaCl). The optical density of the spore suspensions were measured at 600 nm and adjusted to ~0.8. Spore suspension (50 µl) was added to 2 ml of the liquid Malt Extract (ME) medium (pH 7) supplemented with various salts (NaCl, KCl, NaBr, $MgSO_4$, $MgCl_2$, $CaCl_2$) of indicated concentrations (NaCl: 2.0, 2.5, 3.0, 4.0, 5.0 M; NaBr: 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 M; KCl: 2.0, 2.5, 3.0, 4.0, 4.5 M; $MgCl_2$: 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1 M; $MgSO_4$: 2.0, 2.5, 3.0 M; $CaCl_2$: 1.0, 1.2, 1.5, 1.7, 1.9, 2.0 M), and incubated in 12 ml glass test tubes (covered with metal caps and thoroughly wrapped with parafilm) at 24 °C. Inoculated media were examined for visible growth (either in a form of a submerged or surface mycelium or culture turbidity due to growth of yeast cells) after 6 weeks. Negative controls (sterile medium) for each salinity and salt type were included in the experiments. Cultures were examined by light microscopy using Olympus BX51 light microscope equipped with an Olympus DP73 digital camera.

2.3 Data analysis using machine learning

The experiments described above resulted in a dataset with a total of 135 samples. Each of the samples refers to a single fungal strain and is described with environmental conditions (considered as independent or descriptive variables), and the fungal species encountered at each sample (considered as the dependent or the target variable). More specifically, we used the following descriptive variables: habitat (with the possible values of salterns, the Dead Sea, food, freshwater, ice; human, or animal), pigmentation (non-melanized or melanized), cell morphology (filamentous, polymorphic, yeast, or clumps), the lowest a_w salt with observable growth, the type of salt with the lowest a_w still supporting growth, and the highest concentrations of various salts still supporting growth (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$). The target variable is the fungal species, described with its taxonomic rank. Taken together, the samples included information from 94 different species from 31 different genera.

The generic data analysis task that we addressed was a task of predictive modeling, relating the environmental conditions (descriptive variables) and the fungal species (target variable). We have defined seven different scenarios for analysis. The descriptive variable(s) for each were as follows: A) the highest concentrations of salts (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$), B) habitat and salt concentrations, C) pigmentation, morphology and salt concentrations, D) habitat, pigmentation, morphology and salt concentrations, E) habitat, lowest a_w (type of salt), lowest a_w (value) and salt concentrations, F) habitat, lowest a_w (type of salt and lowest a_w value), and G) all descriptive variables.

To analyze the data, we used the machine learning tool CLUS available for download at http://clus.sourceforge.net. More specifically, we used predictive clustering trees (PCTs) for hierarchical classification as models. PCTs are a generalization of decision trees – a machine learning approach commonly used for classification. PCTs are tree-like structures that have internal nodes and leafs. The internal nodes contain tests on the descriptive variables, while leafs represent the predictions about the target variable. PCTs can solve more general task of structured output prediction, including the task of hierarchical classification.

We selected PCTs to model the data because of the specific task at hand. Namely, we used the taxonomic rank of the fungi species to create a hierarchy of classes, where different species can belong to the same genera. This clearly defines the prediction task as a task of hierarchical classification. The predictive clustering trees are able to exploit the information contained in the taxonomic rank of the species during the model construction. Furthermore, the PCTs are easily interpretable predictive models. Detailed information about predictive clustering trees for hierarchical classification has been published before (Vens et al., 2008; Kocev et al., 2013; Levatić et al., 2014).

For each scenario, we have constructed a PCT for hierarchical classification. The PCTs for scenarios A, C, D and F are given in Figure 1. The internal nodes contain tests on individual environmental conditions (e.g., $MgCl_2 > 1.8$) and leaves correspond to a specific combination of environmental conditions. In each leaf, the species encountered under the given conditions are listed.

3. Results

3.1 Screening of the fungal growth at various salts

We have selected 135 fungal strains covering 94 different species and 31 genera. Amongst the genera with the highest number of strains were *Cladosporium* (23), *Aspergillus*, *Wallemia* (both 14) and *Penicillium* (10). The selected strains were previously isolated from different aqueous environments that contain high concentrations of salts (44 strains from salterns, 47 strains from the Dead Sea and also 13 strains from the subglacial ice) and from freshwater (6 strains). Additionally, we have included fungi from various habitats (25 representatives) including food, skin (agents of mycoses), and animals. Among the strains from the Dead Sea almost half (22) belong to the genus *Cladosporium*. We have tested growth of these strains on salts that act as kosmotropes (NaCl, KCl and $MgSO_4$) and chaotropes ($CaCl_2$, $MgCl_2$ and NaBr) that are present in these hypersaline environments. The highest concentrations of salts that allowed growth of individual strains are presented in the Table 1. The microscopic growth characteristics of the selected fungal representatives (cell clumps forming *W. ichthyophaga* EXF-994; a black yeast *Hortaea werneckii* EXF-225; and filamentous *Eurotium repens* EXF-2132 and *Cladosporium cladosporoides* EXF-1824) are represented in Figure 2.

3.2 Predictive clustering trees for fungal growth

The predictive clustering trees obtained with the machine learning analysis are presented in Figure 1. When the highest concentrations of salts at which fungi were able to thrive (scenario A) were used as the only descriptive variables, the decision tree identified chaotropic salts as the most limiting for fungal growth (Figure 1 (A)). The most limiting turned out to be $MgCl_2$ which was at the top of the decision tree, whereas $CaCl_2$ and NaBr occupied internal nodes. In addition, pigmentation (melanized and non-melanized) and cell morphology (yeast, filamentous, polymorphic and clumps) (scenario C, D and F; PCT for scenarios C and D is given in Figure 1 (B), while for scenario G in Figure 1 (C)) turned out to be key features influencing fungal distribution. Finally, when all the descriptive variables were used (including the lowest water activity and the type of salt at the lowest water activity to support growth), pigmentation was again the key variable, whereas morphology divided fungi at internal nodes and finally the lowest water activity and the type of salt in the medium with the lowest a_w led to the leaves (Figure 1 (C)). Among non-melanized filamentous representatives, the ability to grow at KCl > 3.0 M and among non-melanized non-filamentous yeasts NaCl > 2.5 M turned out to be the key variables (Figure 1 (B)). Among melanized filamentous fungi, the genus *Cladosporium* predominated, whereas for so called "black yeasts" the ability to grow at NaCl > 3.5 M was the criterion to differentiate *H. werneckii* from *Aureobasidium* sp., *Exophiala* sp., *Phaeotheca* sp. and *Phaeococcomyces* sp. (Figure 1 (B)). When habitat was added to the other variables, almost no changes occurred in the tree (scenario B, D and F; Supplemental figure 1).

4. Discussion

Few studies have addressed the issue of the tolerance of microorganisms to chaotropic conditions over the years (reviewed in Oren, 2013). Searching for the chaophilic strains from the hypersaline deep-sea Discovery Basin, an environment with the highest salinity ever found in the marine environments – the brine is almost at saturated levels of $MgCl_2$ (5.15 M) (van der Wielen et al., 2005), did not reveal any prokaryotic representatives (Hallsworth et al., 2007). Instead, a fungus *X. bisporus*, with the lowest a_w limit so far reported to support life (Pitt and Hocking, 2009), was the first described species of having the preference to chaotropic conditions/solutes as it was able to grow in highly chaotropic media containing up to 7.6 M glycerol (Williams and Hallsworth, 2009) and was markedly intolerant to NaCl (Pitt and Hocking, 1977). Importantly, its growth on chaotropic solutes like $MgCl_2$ and $CaCl_2$ was not tested.

Indeed, fungi are promising candidates for chaophiles as they can thrive in the environments, such as crystallizer ponds of solar salterns (Gunde-Cimerman et al., 2000; Butinar et al., 2005a; Butinar et al., 2005b), hypersaline water of the Dead Sea (reviewed in Oren and Gunde-Cimerman, 2012) as well as the brine channels of sea ice (Gunde-Cimerman et al., 2003; Sonjak et al., 2006). As these fungi have not previously been examined for their ability to grow in media dominated by chaotropic ions, we have carried out an extensive screening of tolerance to various salts.

Our search for the chaophilic characters of fungi based on their isolation from bittern brines (Sonjak et al., 2010), residual water after the precipitation of NaCl, which is highly enriched with magnesium salts, mostly $MgCl_2$. These brines were long considered sterile as high concentrations of Mg^{2+} are often toxic for biological systems. However, it was shown recently that bittern brines of the Sečovlje salterns (Slovenia) are not completely free of living microorganisms. They harbour different filamentous fungi, *Cladosporium* spp., black and other yeasts, albeit their abundance and biodiversity is low when compared to the hypersaline water of the salterns (Sonjak et al., 2010). The lower diversity and abundance might be a consequence of a combination of various factors *in situ*, such as prolonged exposure to solar radiation and magnesium, its life-limiting effect and nutrient availability.

However, the ionic composition of the bittern brine is not completely unfavorable for microbial growth despite extremely low water activity (0.737); the level of toxic ion Mg^{2+} is compensated by a relatively higher concentration of Na^+. An outstanding discovery here was that these fungi isolated either from brine rich in $MgCl_2$ or NaCl were able to grow at high concentrations of $MgCl_2$ (1.5 M) (Sonjak et al., 2010) – higher than previously reported for prokaryotes (1.26 M $MgCl_2$) (Hallsworth et al., 2007). This observation led to the study of the ability of a list of fungi, composed of the isolates from the Dead Sea and the reference strains from our culture collection, to grow in media with low a_w due to high concentrations of not only kosmotropic salts (NaCl, KCl, $MgSO_4$) but also chaotropic salts such as NaBr, $MgCl_2$ and $CaCl_2$.

Among the extremophilic fungi included in our study, 104 (almost 80% of the strains) were able to grow at concentrations of $MgCl_2$ higher than 1.5 M, and among these 16 (12%) were able to grow at the highest concentrations of $MgCl_2$ ($\geq$ 2.0 M). Next, 56 (41.5% of the strains) were capable of growth at concentrations of $CaCl_2$ higher than 1.5 M, with two of these able to grow at the highest concentration (2.0 M).

The decision trees (more specifically PCTs) obtained by machine learning analysis in various scenarios (Figure 1) revealed key types of salts influencing the ability of growth of fungi. The most important salts for the limitation of fungal diversity turned out to be the chaotropic salts $MgCl_2$, $CaCl_2$, and NaBr, whereas KCl and NaCl appeared to be the least limiting and were not present in the nodes of the decision tree. The first decision tree (Figure 1 (A)) revealed that 37 strains, including 11 strains of *H. werneckii* and *W. ichthyophaga* can cope with $MgCl_2$ concentrations higher than 1.8 M. Here, the majority of strains of *W. ichthyophaga* were unique in their ability to tolerate the highest concentrations of $MgCl_2$, but not $CaCl_2$; whereas almost all strains (except for one instance) of *H. werneckii* could grow at the highest concentrations of all tested salts. Another key variable distinguishing the tested fungal strains is pigmentation, which is at the top of the decision tree using all the variables available. However, melanization is known for its role in UV and other stress responses including in osmoadaptation in halotolerant fungi (Jacobson and Ikeda, 2005; Kogej et al., 2007). Melanin impregnates the outer layer of the cell wall, this decreasing the porosity of the cell wall in order to retain more glycerol, which is most often the main compatible solute (Kogej et al., 2007). Next, cell morphology also appeared high in the decision trees. The ability to form dense clumps of meristematic cells, as observed for *W. ichthyophaga* and *Phaeotheca triangularis,* also impacts the ability of fungi to live in stressful conditions (Wollenzien et al., 1995; Palkova, 2004; Palkova and Vachova, 2006).

A simple determination of the type of salt to allow growth of individual strains at the lowest a_w revealed that the largest number of fungi thrived in the media with the lowest a_w when NaCl (52; 38.8 %) or KCl (42; 31.3 %) were used as the main solutes. On the contrary, less than 10% of strains were able to grow in the presence of chaotropic salts, $MgCl_2$ (10; 7.5%) and $CaCl_2$ (2; 1.5%), at their lowest a_w. This again emphasizes the life-limiting effects of chaotropic salts. Whether these fungi have the preference for chaotropic salts is inconclusive, as most of them are able to grow also at the highest concentrations of other – kosmotropic – salts. Nevertheless, the fact that they are not only tolerating but growing at such high concentrations of magnesium and/or calcium salts makes these strains the most chaotolerant organisms described so far.

For comparison, the highest concentration of salts to support growth of *X. bisporus* given our results (Table 1) were 2.5 M NaCl, 3.5 M KCl, 2.5 M $MgSO_4$ and 2.5 M NaBr, albeit growth was poor. In addition it was also able to grow at 1.5 M $MgCl_2$ and 1 M $CaCl_2$. For the inhibition of growth of *X. bisporus* the a_w of the medium was clearly not the determining factor – the lowest a_w of media tested in our study was 0.867 for medium containing 3.5 M KCl, which is far above its lowest a_w enabling growth in a glycerol-based medium (Pitt and Hocking, 1977). Here, it seems that high concentrations of salt, regardless of their chao- or kosmotropicity, limit the growth of *X. bisporus*, which clearly prefers sugar-based media as previously reported. Its chaophilic character on organic solutes such as glycerol (Williams and Hallsworth, 2009) must be reconsidered with caution when addressing ionic chaophilic solutes. Poor growth in the presence of salt might be a consequence of the absence of a gene coding for Na^+-exporting

ATPase (Ena) in the genome of *X. bisporus* (Leong et al., 2014), whereas this pump is present in multiple copies and/or is differentially expressed in the extremely halotolerant *H. werneckii* (Gorjan and Plemenitaš, 2006; Lenassi et al., 2013) and the halophilic *W. ichtyophaga* (Zajc et al., 2013).

Hortaea werneckii is a representative of the polyphyletic group of black (melanized) yeasts that have filamentous and yeast-like growth. It is able to grow across the whole range of NaCl concentrations, from 0 M to saturation, with a broad optimum from 1 M to 2.4 M NaCl (Butinar et al., 2005b), and it is thus considered to be the most extremely halotolerant fungus so far described (reviewed in Gostinčar et al., 2011). Amongst all of the melanized fungi *H. werneckii* is the most abundant in the hypersaline water of salterns (Gunde-Cimerman et al., 2000). Our screening revealed that *H. werneckii* strains are able to grow at the highest tested concentrations of salts; in media saturated with kosmotropes (5.0 M NaCl, 4.5 M KCl, 3.0 M $MgSO_4$) and the highest tested concentrations of chaotropes (2.1 M $MgCl_2$, 1.7 M $CaCl_2$ and 4.0 M NaBr) (Table 2 and Figure 2). This exceptional ability might be linked to the redundancy of plasma membrane Na^+ and K^+ transporters encoded in its duplicated genome (Lenassi et al., 2013).

The genus *Aureobasidium* (de Bary) G. Arnaud is a wide-spread osmotolerant (Kogej et al., 2005) representative of black yeast associated with numerous habitats from hypersaline waters, Arctic glaciers, plant surfaces and household dust (reviewed in Gostinčar et al., 2014). In the genus, recently four new species were introduced *A. pullulans*, *A. melanogenum*, *A. subglaciale* and *A. namibiae* in (Gostinčar et al., 2014). All of them are described as polyextremotolerant (Gostinčar et al., 2010; Gostinčar et al., 2011) capable of surviving also hypersaline conditions (Gunde-Cimerman et al., 2000). The maximum concentrations of NaCl supporting growth of *A. pullulans* was reported to be 2.9 M NaCl (Kogej et al., 2005). Our study confirmed the upper limit of NaCl for *Aureobasidium* sp. and revealed its ability for growth at high concentrations of KCl (4.0 M) and $MgSO_4$ (3.0 M), but not extremely high concentration of $MgCl_2$ (lower than 1.5 M) and $CaCl_2$ (up to 1.2 M). *Aureobasidium* spp. can thus be considered kosmophilic. Recent genome analysis uncovered a large repertoire of plasma-membrane transporters in the four *Aureobasidium* species (Gostinčar et al., 2014). *A. melanogenum*, which is heavily melanized, is able to grow at the highest concentrations of all salts among the four tested species of the genus *Aureobasidium*, and the least melanized *A. subglaciale* on the other hand thrives at the lowest. Here, it seems that melanization is required for the highest salt tolerance. The role of melanin in osmoadaptation was shown previously by modifying the permeability of the cell wall in order to retain the compatible solute glycerol (Jacobson and Ikeda, 2005; Kogej et al., 2006).

Representatives of the cosmopolitan genus *Cladosporium* are frequently found in habitats characterized by low a_w, like foods preserved with sugar or salt (Samson et al., 2002), salt marshes of Egypt, in the rhizosphere of halophytic plants, and the phylloplane of Mediterranean plants (Abdel-Hafez et al., 1978). They are therefore considered xerotolerant with 0.82 being the minimal a_w for growth of *Cladosporium sphaerospermum* (Hocking et al., 1994). *Cladosporium* spp. are among the most abundant melanized fungi throughout the year in the solar salterns in Sečovlje (Gunde-Cimerman et al., 2000; Butinar et al., 2005b) and Cabo Rojo in Puerto Rico (Cantrell et al., 2006). Five species of the genus *Cladosporium* were isolated from the Dead Sea (reviewed in Oren and Gunde-Cimerman, 2012). The highest concentration of NaCl for *in vitro* growth of various representatives of the genus *Cladosporium* was reported to be 2.9 M to 3.5 M (Zalar et al., 2007). Strains of the genus *Cladosporium* exhibited variable tolerance to different types of salts, ranging from the lowest concentrations used in the study to the highest (Table 2). The highest growth concentrations of kosmotropic NaCl, KCl and $MgSO_4$

among *Cladosporium* spp. are respectively 2.5 – 4.0 M, 2.5 – 4.5 M and 2.0 – 3.0 M. Two strains, *C. tenuissimum* EXF-1943 and *C. cladosporoides* EXF-1824, were able to grow at 2.0 or 2.1 M $MgCl_2$ and 1.7 M $CaCl_2$ (Figure 2).

Species of the basidiomycetous genus *Wallemia* Johan-Olsen can be found in a wide variety of environments characterized by low a_w (Samson et al., 2002; Zalar et al., 2005), such as dried, salty and sweet foods like chocolate, indoor and outdoor air in urban and agricultural environments, hypersaline water of the salterns on different continents and salt crystals (Zalar et al., 2005). Two species of the genus *Wallemia*, *W. muriae* and *W. ichthyophaga*, are obligate xerophiles with the a_w growth ranges 0.984 - 0.805 and 0.959 - 0.771, respectively (Zalar et al., 2005), whereas *W. sebi* is xerotolerant with the ability to grow in media without additional solutes (a_w growth range: 0.997 - 0.690) (Pitt and Hocking, 1977). However, in media supplemented with NaCl as the major solute, the lowest a_w for the growth of *W. sebi* was reported to be 0.80 (Zalar et al., 2005) corresponding to 4.5 M NaCl. *W. muriae* can grow up to 4.3 M NaCl, while *W. ichthyophaga* can thrive only in media with NaCl above 1.7 M, has an optimum at 2.6 to 3.5 M NaCl and can grow up to saturating levels of NaCl (5.2 M) (Zalar et al., 2005; Zajc et al., 2014). Here, we determined that strains of *W. ichthyophaga* grew well at highest concentrations of NaCl (above 4.0 M), NaBr (4 M) and saturated KCl and $MgSO_4$, but show quite a variability when cultivated at different concentrations of chaotropes like $MgCl_2$ and $CaCl_2$ (Table 2 and Figure 2). A type strain from the hypersaline waters of salterns (*W. ichthyophaga* EXF-994) grew also at the 2.1 M $MgCl_2$, whereas it was not able to tolerate high concentrations of calcium (not even 1 M $CaCl_2$). *W. ichthyophaga* is indeed the most halophilic fungus ever described. Interestingly, its genome analysis showed that the life in extremely saline environments is possible even with low number of cation-transporter genes, and seems independent of their low transcription and non-responsiveness to variable salinity. In this case, the role of passive barriers against high salinity conditions seems crucial. The cell wall is unusually thick, the cells are joined into thick multicellular clumps and the cell-wall proteins, hydrophobins, are among the highly expressed genes in saline environments (Zajc et al., 2013).

The filamentous fungi of the order *Eurotiales*, comprised of teleomorphic genera *Eurotium* and *Emericella*, and the anamorphic *Aspergillus* and *Penicillium*, are commonly found in different salterns around the World (Cantrell et al., 2006; Butinar et al., 2011) as well as in the Dead Sea (reviewed in Oren and Gunde-Cimerman, 2012). Tolerance for high salt concentrations has been known for many food-borne species (Tresner and Hayes, 1971). The representatives of *Aspergillus* and *Penicillium* are most abundant at salinities below 1.7 M NaCl in the solar salterns (Butinar et al., 2011); however, the *in vitro* determined salinity growth ranges of the *Eurotium* spp. are broad, ranging from 0 up to 4.7 M (Butinar et al., 2005c). Given our results the highest concentrations of salts in which species from the order Eurotiales are able to thrive are highly diverse, ranging from the lowest to highest concentrations tested depending on individual strain (see Figure 2 for *Eurotium repens* EXF-2132). However, all strains were capable to grow in concentrations higher than 3.0 M NaCl, 3.5 M KCl, 2.0 M $MgSO_4$, 2.5 M NaBr and even over 1.5 M $MgCl_2$ and 1.2 M $CaCl_2$ (except in one incident in the case of $MgCl_2$ and $CaCl_2$). Few halophilic Archaea can grow at high concentrations of $MgCl_2$, but only in the presence of significant concentrations of NaCl (Mullakhanbhai and Larsen, 1975; Oren, 1983; Oren et al., 1995). For instance, *Haloferax volcanii* is tolerant to high magnesium as growth is still possible at 1.4 M Mg^{2+} in the presence of 2 M Na^+ (Mullakhanbhai and Larsen, 1975). Also, *Halobaculum gomorrense* is moderately tolerant to Mg^{2+} with optimal growth at 0.6 to 1.0 M Mg^{2+} in the presence of 2.1 M NaCl (Oren et al., 1995). Another archaeon isolated from the Dead Sea, *Halobacterium sodomense*, has an extremely high magnesium requirement. It grows optimally even at 1.2 M $MgCl_2$ and 2.0 M NaCl and still grows, albeit poorly, at 1.8 M $MgC1_2$ and 1.7 M NaCl and at 2.5 M $MgC1_2$ and 0.5 M NaCl (Oren, 1983). The upper concentration of solely $MgCl_2$ still supporting life was suggested to be 2.3 M and it based on the presence of specific mRNA indicators of active life, (Hallsworth et al., 2007). However, the highest concentration of $MgCl_2$ (without compensating kosmotropes) showing microbial growth (after 18 months of cultivation) of deep-sea Discovery brine samples was 1.26 M (Hallsworth et al., 2007). Given the fact that it was not uncommon for fungi to thrive at concentrations of $MgCl_2$ higher than 1.5 M without compensating NaCl, it is clear that fungi are truly tolerant to magnesium. Some of these were able to grow at 2.1 M $MgCl_2$, a concentration that is close to the chaotropicity limit of possible life (2.3 M) (Hallsworth et al., 2007).

Fungi from diverse environments (salterns, Dead Sea, ice, freshwater and other) can not only tolerate but also thrive at high concentrations of salts, which are either kosmotropic like NaCl, KCl and $MgSO_4$ or– to biological systems more toxic – chaotropic like NaBr, $MgCl_2$ and $CaCl_2$. A few representatives of various species, such as *H. werneckii*, *E. amstelodami*, *E. chevalieri* and *W. ichthyophaga* were able to thrive in media with the highest tested salinities of all salts (except in $CaCl_2$ in case of *W. ichthyophaga*). In addition, several fungi (*Aureobasidium* spp., *Exophiala* spp.) exert a tendency towards kosmotropes, as they are able to grow at relatively high concentrations of NaCl, KCl and $MgSO_4$, but not at high concentrations of chaotropes, like $MgCl_2$ and $CaCl_2$. However, no fungal representatives showed the preference for the highest concentrations of only chaotropic salts but not for the kosmotropic, *i.e.* being obligately chaophilic. Nevertheless, our study revealed many representatives of the novel group of chaophiles among fungi, which thrive well above the highest previously determined concentration of $MgCl_2$. The ability to grow in the presence of high concentrations of another potent chaotrope - $CaCl_2$ was addressed for the first time. This expands our knowledge of possible life performance under diverse and most extreme environmental parameters.

Conflict of interest

The authors declare that they have no conflict of interests.

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Table 1. The highest concentrations of various salt for a list of fungi.

Strain accession no.	*Genus*	*species*	Habitat	The highest concentration of salt with observable growth (M)						Type of salt with the lowest a_w supporting growth	The lowest a_w supporting growth (value)
				NaCl	KCl	$MgCl_2$	$CaCl_2$	NaBr	$MgSO_4$		
EXF-2277	*Acremonium*	*strictum*	salterns	2	3	0.75	0.5	2	3	KCl	0.885
EXF-174	*Alternaria*	*alternata*	salterns	3	3	0.75	1.2	2.5	1.5	NaCl	0.884
EXF-2340	*Alternaria*	*arborescens*	salterns	2	3	1.5	1	2	3	KCl	0.885
EXF-2332	*Alternaria*	*infectoria*	salterns	2	2.5	1.5	1.2	2.5	3	$MgSO_4$	0.886
EXF-1730	*Alternaria*	sp.	Dead Sea	2.5	3	0.75	1.2	2	2	KCl	0.885
EXF-2318	*Alternaria*	*tenuissima*	salterns	3	3	1.5	1.2	2	3	NaCl	0.884
EXF-5007	*Aspergillus*	*caespitosus*	salterns	4	4.5	1.9	1.5	3	3	NaCl	0.825
EXF-6616	*Aspergillus*	*candidus*	salterns	4	4.5	1.5	1.2	3.5	2.5	NaCl	0.825
EXF-6615	*Aspergillus*	*flavipes*	salterns	3.5	4.5	1.5	2	3	2.5	$CaCl_2$	0.830
EXF-1751	*Aspergillus*	*flavus*	Dead Sea	3	4	1.6	1.5	2.5	3	KCl	0.849
EXF-1760	*Aspergillus*	*niger*	Dead Sea	3.5	4	1.5	1.5	3	3	KCl	0.849
EXF-5077	*Aspergillus*	*ochraceus*	salterns	4	4.5	1.7	1.7	3.5	3	NaCl	0.825
EXF-138 (226)	*Aspergillus*	*penicillioides*	salterns	3.5	3.5	1.5	1.5	2.5	2	NaCl	0.854
EXF-1946	*Aspergillus*	*proliferans*	Dead Sea	4	4.5	2.1	1.9	3	3	$MgCl_2$	0.808
EXF-1752	*Aspergillus*	*sclerotiorum*	Dead Sea	4	4.5	1.9	1.7	3.5	3	NaCl	0.825
EXF-1847	*Aspergillus*	*sydowii*	Dead Sea	4	3.5	1.9	2	4	3	NaBr	0.803
EXF-5006	*Aspergillus*	*tubingiensis*	salterns	4	4	1.9	1.7	2.5	3	NaCl	0.825
EXF-189	*Aspergillus*	*ustus*	salterns	4	4	2	1.2	3	3	$MgCl_2$	0.822
EXF-4284	*Aspergillus*	*versicolor*	salterns	4	4.5	1.5	1.7	3.5	2.5	NaCl	0.825
EXF-4303	*Aspergillus*	*wentii*	various	3.5	4	0.75	1.5	3	2	KCl	0.849
EXF-3400	*Aureobasidium*	*melanogenum*	freshwater	3	3	1.5	1.2	3	2	NaBr	0.861
EXF-8429	*Aureobasidium*	*melanogenum*	freshwater	2.5	3.5	1.5	1	2	2	KCl	0.867
EXF-3382	*Aureobasidium*	*melanogenum*	salterns	3	3.5	1.7	1.5	3	3	NaBr	0.861
EXF-3405	*Aureobasidium*	*melanogenum*	various	2.5	4	0.75	0.5	2	2	KCl	0.849
EXF-3398	*Aureobasidium*	*namibiae*	various	2.5	3	0.75	1.2	2	2	KCl	0.885

EXF-150	*Aureobasidium*	*pullulans*	salterns	3	4	0.75	1.2	2	3	KCl	0.849
EXF-2481	*Aureobasidium*	*subglaciale*	ice	2	2	0.75	1.2	1.5	2	KCl	0.922
EXF-1830	*Bjerkandera*	sp.	Dead Sea	1.5	3	0.75	0.5	0.75	1.5	KCl	0.885
EXF-6603	*Candida*	*glabrata*	various	2	2.5	0.75	0.5	1.5	2	KCl	0.903
EXF-1987	*Candida*	*parapsilosis*	Dead Sea	3.5	4	1.6	1.7	3	2.5	KCl	0.849
EXF-517	*Candida*	*parapsilosis*	salterns	3	3.5	1.7	1.2	3	3	NaBr	0.861
EXF-1574	*Candida*	*parapsilosis*	ice	3	4	1.7	1.2	3	3	KCl	0.849
EXF-253	*Chaetomium*	*globosum*	salterns	2	2.5	0.75	1	0.75	1.5	KCl	0.903
EXF-1060	*Cladosporium*	*aff. herbarum*	Dead Sea	4	4.5	1.5	1.5	3	3	NaCl	0.825
EXF-2036	*Cladosporium*	*aff. herbarum*	Dead Sea	3	4	1.7	0.5	2.5	2.5	KCl	0.849
EXF-1930	*Cladosporium*	*aff. inversicolor*	Dead Sea	2.5	2.5	0.75	1.2	4	1.5	NaBr	0.803
EXF-2034	*Cladosporium*	*aff. sphaerospermum*	Dead Sea	4	4.5	1.5	1.7	3	3	NaCl	0.825
EXF-1728	*Cladosporium*	*cladosporioides*	Dead Sea	4	3.5	0.75	1	2.5	3	NaCl	0.825
EXF-1071	*Cladosporium*	*cladosporoides*	Dead Sea	3.5	4	1.9	1.7	3.5	3	NaBr	0.832
EXF-1824	*Cladosporium*	*cladosporoides*	Dead Sea	4	4.5	2	1.7	3	3	$MgCl_2$	0.822
EXF-1081	*Cladosporium*	*halotolerans*	Dead Sea	4	4.5	1.5	1.7	3	3	NaCl	0.825
EXF-2513	*Cladosporium*	*halotolerans*	ice	3.5	4.5	1.8	1.5	3.5	3	KCl	0.831
EXF-572	*Cladosporium*	*halotolerans*	salterns	3	4	1.5	1.2	2.5	3	KCl	0.849
EXF-1066	*Cladosporium*	*herbarum*	Dead Sea	4	4.5	1.5	1.7	3	2.5	NaCl	0.825
EXF-1000	*Cladosporium*	*langeronii*	various	2.5	3.5	0.75	1	3	3	NaBr	0.861
EXF-2287	*Cladosporium*	*macrocarpum*	salterns	2.5	3.5	0.75	1.2	4	2.5	NaBr	0.803
EXF-1736	*Cladosporium*	*ramotenellum*	Dead Sea	3.5	4.5	0.75	1.7	3	2	KCl	0.831
EXF-335	*Cladosporium*	*salinae*	salterns	3	4.5	1.8	1	2.5	3	KCl	0.831
EXF-1079	*Cladosporium*	sp.	Dead Sea	4	4.5	1.5	1.7	3	2.5	NaCl	0.825
EXF-1741	*Cladosporium*	sp.	Dead Sea	2.5	4	1.5	1.5	3	2	KCl	0.849
EXF-2012	*Cladosporium*	sp.	Dead Sea	3.5	4	1.5	1.5	3	3	KCl	0.849
EXF-2015	*Cladosporium*	sp.	Dead Sea	3.5	4.5	1.5	1.7	3	2	KCl	0.831
EXF-2016	*Cladosporium*	sp.	Dead Sea	3.5	4	1.5	1.5	3	2.5	KCl	0.849
EXF-2038	*Cladosporium*	sp.	Dead Sea	4	4	1.5	1.2	3	2.5	KCl	0.825

EXF-2040	*Cladosporium*	sp.	Dead Sea	2.5	3	0.75	0.5	2	2	KCl	0.885
EXF-1986	*Cladosporium*	sp.	Dead Sea	2.5	3	0.75	1	1.5	2	KCl	0.885
EXF-1997	*Cladosporium*	sp.	Dead Sea	3.5	4.5	1.7	1.7	3	2.5	KCl	0.831
EXF-7632	*Cladosporium*	sp.	salterns	4	4	1.8	1.7	3	3	NaCl	0.825
EXF-7634	*Cladosporium*	*sp.*	salterns	4	4	1.8	1.5	3	3	NaCl	0.825
EXF-7635	*Cladosporium*	*sp.*	salterns	4	4	1.8	1.5	3	3	NaCl	0.825
EXF-2037	*Cladosporium*	*sphaerospermum*	Dead Sea	4	4.5	1.5	1.5	3	2.5	NaCl	0.825
EXF-1735	*Cladosporium*	*tenellum*	Dead Sea	3.5	3.5	0.75	0.5	2.5	2.5	NaCl	0.854
EXF-1943	*Cladosporium*	*ttenuissimum*	Dead Sea	3.5	4.5	2.1	1.7	2.5	3	$MgCl_2$	0.808
EXF-6893	*Cryptococcus*	*albidus*	various	3.5	3.5	1.5	1	1.5	3	NaCl	0.854
EXF-2008	*Cryptococcus*	*albidus var. kuetzingii*	Dead Sea	3.5	3.5	1.5	1.2	2.5	2.5	NaCl	0.854
EXF-8012	*Cryptococcus*	*diffluens*	freshwater	2.5	2.5	1.5	1	1.5	3	$MgSO_4$	0.886
EXF-3360	*Cryptococcus*	*magnus*	ice	1.5	2.5	1.5	1.2	1.5	3	$MgSO_4$	0.886
EXF-3792	*Cryptococcus*	*victoriae*	salterns	2.5	2.5	1.5	1	2	3	$MgSO_4$	0.886
EXF-1928	*Emericella*	*purpurea*	Dead Sea	4	4.5	2.1	1.9	3.5	3	$MgSO_4$	0.808
EXF-1929	*Emericella*	*purpurea*	Dead Sea	3	4	1.5	1	3	2.5	KCl	0.849
EXF-1840	*Eurotium*	*amstelodami*	Dead Sea	4	4.5	1.9	1.5	2.5	3	NaCl	0.825
EXF-5620	*Eurotium*	*chevalieri*	various	4	4.5	2.1	1.9	4	3	NaBr	0.803
EXF-1453	*Eurotium*	*herbariorum*	salterns	4	4.5	1.9	1.7	3.5	3	NaCl	0.825
EXF-2132	*Eurotium*	*repens*	Dead Sea	4	4	2.1	1.5	2.5	2.5	$MgCl_2$	0.808
EXF-441	*Eurotium*	*rubrum*	salterns	4	4	1.9	1.7	3.5	2.5	NaCl	0.825
EXF-5573	*Exophiala*	*dermatitidis*	freshwater	2.5	2.5	0.75	0.5	0.75	3	$MgSO_4$	0.886
EXF-2060	*Exophiala*	*oligosperma*	ice	3	3	1.5	1.2	2.5	3	NaCl	0.884
EXF-5575	*Exophiala*	*phaeomuriformis*	freshwater	2.5	2	0.75	1	1.5	3	$MgSO_4$	0.886
EXF-4024	*Exophiala*	*xenobiotica*	ice	1.5	1.5	1.8	1.7	1.5	3	$MgSO_4$	0.886
EXF-2275	*Fusarium*	*aff. equiseti*	salterns	2	2.5	1.5	1.5	2	3	$MgSO_4$	0.886
EXF-2254	*Fusarium*	*graminearum*	salterns	2	2.5	1.5	1.2	1.5	3	$MgSO_4$	0.886
EXF-132	*Hortaea*	*werneckii*	various	5	4.5	2.1	1.2	4	3	NaCl	0.766

EXF-2682	*Hortaea*	*werneckii*	various	5	4.5	1.9	1.5	4	3	NaCl	0.766
EXF-6651	*Hortaea*	*werneckii*	various	5	4.5	2.1	1.7	4	3	NaCl	0.766
EXF-225 (2000)	*Hortaea*	*werneckii*	salterns	4	4	2	1.7	4	3	NaBr	0.803
EXF-6602	*Meyerozyma*	*guilermondii*	various	4	4	1.5	0.5	2.5	2	NaCl	0.825
EXF-519	*Meyerozyma*	*guilliermondii*	salterns	3	4	1.5	1.7	2.5	2.5	KCl	0.849
EXF-2006	*Meyerozyma*	*guilliermondii*	Dead Sea	3.5	3.5	1.5	1.2	2.5	2.5	NaCl	0.854
EXF-518	*Meyerozyma*	*guilliermondii*	salterns	3	3.5	1.7	1.2	2.5	3	KCl	0.867
EXF-224	*Paecilomyces*	*farinosus*	various	2	3.5	0.75	0.5	1.5	1.5	KCl	0.867
EXF-4108	*Penicillium*	*antarcticum*	ice	3.5	4.5	1.5	1.5	3.5	2.5	KCl	0.831
EXF-6614	*Penicillium*	*brevicompactum*	salterns	4	4.5	1.5	1.5	3	2	NaCl	0.825
EXF-1774	*Penicillium*	*chrysogenum*	Dead Sea	4	4.5	1.9	1.7	3.5	3	NaCl	0.825
EXF-3655	*Penicillium*	*comunae*	ice	4	4	2.1	1.2	2.5	3	$MgCl_2$	0.808
EXF-1778	*Penicillium*	*corylophylum*	Dead Sea	3.5	4	1.8	1.5	3	3	KCl	0.849
EXF-1788	*Penicillium*	*crustosum*	Dead Sea	4	4.5	1.5	1.2	3	2.5	NaCl	0.825
EXF-1781	*Penicillium*	*glabrum*	Dead Sea	4	4	1.7	1.9	3	2.5	NaCl	0.825
EXF-6613	*Penicillium*	*nordicum*	salterns	4	4	1.5	1.2	3	2.5	NaCl	0.825
EXF-3675	*Penicillum*	*palitans*	ice	4	3.5	1.6	1	2.5	2.5	NaCl	0.825
EXF-1822	*Penicillium*	*stecki*	Dead Sea	4	4.5	1.9	1.9	3.5	3	NaCl	0.825
EXF-3663	*Phaeococcomyces*	sp.	ice	2	3	0.75	1.2	0.75	1.5	KCl	0.885
EXF-6160	*Phaeococcomyces*	sp.	various	2	1.5	0.75	1	2	1.5	NaBr	0.919
EXF-206	*Phaeotheca*	*triangularis*	salterns	3.5	4	1.9	1.9	4	2.5	NaBr	0.803
EXF-657	*Phoma*	*leveillei*	various	4	4.5	1.5	1.2	2.5	2	NaCl	0.825
EXF-513	*Rhodosporidium*	*babjevae*	salterns	3.5	2.5	1.5	1.2	2.5	3	NaCl	0.854
EXF-3361	*Rhodosporidium*	*iobovatum*	ice	3	3	1.5	1.5	2	3	NaCl	0.884
EXF-6425	*Rhodotorula*	*glutinis*	various	2	2	1.5	1	1.5	3	$MgSO_4$	0.886
EXF-1450	*Rhodotorula*	*laryngis*	Dead Sea	1.5	1.5	0.75	1.5	3.5	1.5	NaBr	0.832
EXF-3871	*Rhodotorula*	*mucilaginosa*	ice	2.5	2.5	0.75	1.2	2	3	$MgSO_4$	0.886
EXF-5543	*Rhodotorula*	*mucilaginosa*	freshwater	2.5	2.5	0.75	1.2	2	3	$MgSO_4$	0.886
EXF-6896	*Rhodotorula*	*mucilaginosa*	various	1.5	4	1.5	1.2	0.75	3	KCl	0.849

EXF-1630	*Rhodotorula*	*mucilaginosa*	ice	2.5	3	0.75	1.2	2	3	KCl	0.885
EXF-3527	*Stachybotrys*	*atra*	various	2	3	0.75	1	1.5	1.5	KCl	0.885
EXF-1811	*Stereum*	*gausapatum*	Dead Sea	1.5	1.5	0.75	0.5	0.75	1.5	nd	nd
EXF-1806	*Trametes*	*versicolor*	Dead Sea	1.5	1.5	0.75	1	0.75	1.5	$CaCl_2$	0.952
EXF-1742	*Trichoderma*	*aff. atroviride*	Dead Sea	1.5	3.5	1.5	1.2	0.75	1.5	KCl	0.867
EXF-1444	*Trichosporon*	*mucoides*	salterns	3	2.5	1.5	1	2	1.5	NaCl	0.884
EXF-1447	*Trichosporon*	*mucoides*	Dead Sea	4	3.5	2	1.5	3.5	3	$MgCl_2$	0.822
EXF-295	*Trimmatostroma*	*salinum*	salterns	4	4	1.7	1	3.5	3	NaCl	0.825
EXF-1835	*Ulocladium*	*tuberculatum*	Dead Sea	3.5	4	2	1.2	3	3	$MgCl_2$	0.822
EXF-5753	*Wallemia*	*hederae*	various	5	4.5	1.8	1	4	2.5	NaCl	0.766
EXF-1059	*Wallemia*	*ichthyophaga*	various	5	4.5	1.9	1	4	3	NaCl	0.766
EXF-5676	*Wallemia*	*ichthyophaga*	various	5	4.5	2	1.2	4	3	NaCl	0.766
EXF-6069	*Wallemia*	*ichthyophaga*	salterns	3.5	4	1.9	0.5	2	2	$MgCl_2$	0.836
EXF-6070	*Wallemia*	*ichthyophaga*	salterns	4	4.5	1.9	0.5	3	3	NaCl	0.825
EXF-8617	*Wallemia*	*ichthyophaga*	various	4	4.5	2	1	4	3	NaBr	0.803
EXF-6068	*Wallemia*	*ichthyophaga*	salterns	4	4.5	1.9	0.5	2	3	NaCl	0.825
EXF-994	*Wallemia*	*ichthyophaga*	salterns	5	4.5	2.1	0.5	4	3	NaCl	0.766
EXF-753	*Wallemia*	*muriae*	various	4	4.5	1.9	1.2	1.5	2.5	NaCl	0.825
EXF-2361	*Wallemia*	*muriae*	various	4	4.5	1.9	1.2	1.5	3	NaCl	0.825
EXF-8359	*Wallemia*	*muriae*	salterns	3.5	4.5	1.9	1.2	2.5	2.5	KCl	0.831
EXF-951	*Wallemia*	*muriae*	salterns	4	4.5	1.9	0.5	4	3	NaBr	0.803
EXF-956	*Wallemia*	*sebi*	various	4	4.5	1.9	1.5	1.5	2.5	NaCl	0.825
EXF-2298	*Wallemia*	*sebi*	salterns	4	4.5	1.5	1.2	2.5	2.5	NaCl	0.825
EXF-9116	*Xeromyces*	*bisporus (T)*	various	2.5	3.5	1.6	1	2.5	2.5	KCl	0.867

Figure legends

Figure 1. Visualization of the decision tree of fungal species obtained by machine learning tool CLUS when using (A) the highest concentrations of various salt (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$); (B) pigmentation (melanized, non-melanized), morphology (yeast, filamentous, polymorphic, clumps) and the highest concentrations of various salt (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$); and (C) when using all the descriptive variables as follows the highest concentrations of various salt (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$), habitat (salterns, Dead Sea, freshwater, various: ice, human associated, animal associated, food), pigmentation (melanized, non-melanized), morphology (yeast, filamentous, polymorphic, clumps) and the lowest a_w (type of salt and value) with observable growth. The target variable was the fungal species (leaves of the decision tree). The number in the brackets defines the number of strains for model (A), (B) or (C).Figure 2. Micromorphological characteristics of liquid culture of four strains, namely *Wallemia ichthyophaga* (EXF-994), *Hortaea werneckii* (EXF-225), *Eurotium repens* (EXF-2132) and *Cladosporium cladosporoides* (EXF-1824) grown in malt extract medium without salt (control condition) and at their highest concentrations of salt that allow growth at 24 °C. Concentrations of various salts are indicated. The scalebar represent 20 µm.

Supplementary figure S1. Visualization of the decision tree of fungal species obtained by machine learning tool CLUS when using (A) habitat (salterns, the Dead Sea, food, freshwater, ice, human, or animal) and the highest concentrations of various salts (NaCl, KCl, $MgCl_2$, $CaCl_2$ NaBr and $MgSO_4$); (B) habitat, the lowest a_w (type of salt), the lowest a_w (value) and the highest concentrations of various salts; (C) habitat, the lowest a_w (type of salt) and the lowest a_w (value). The target variable was the fungal species (leaves of the decision tree). The number in the brackets defines the number of strains for model (A), (B) or (C).

Figure 1.TIF

A

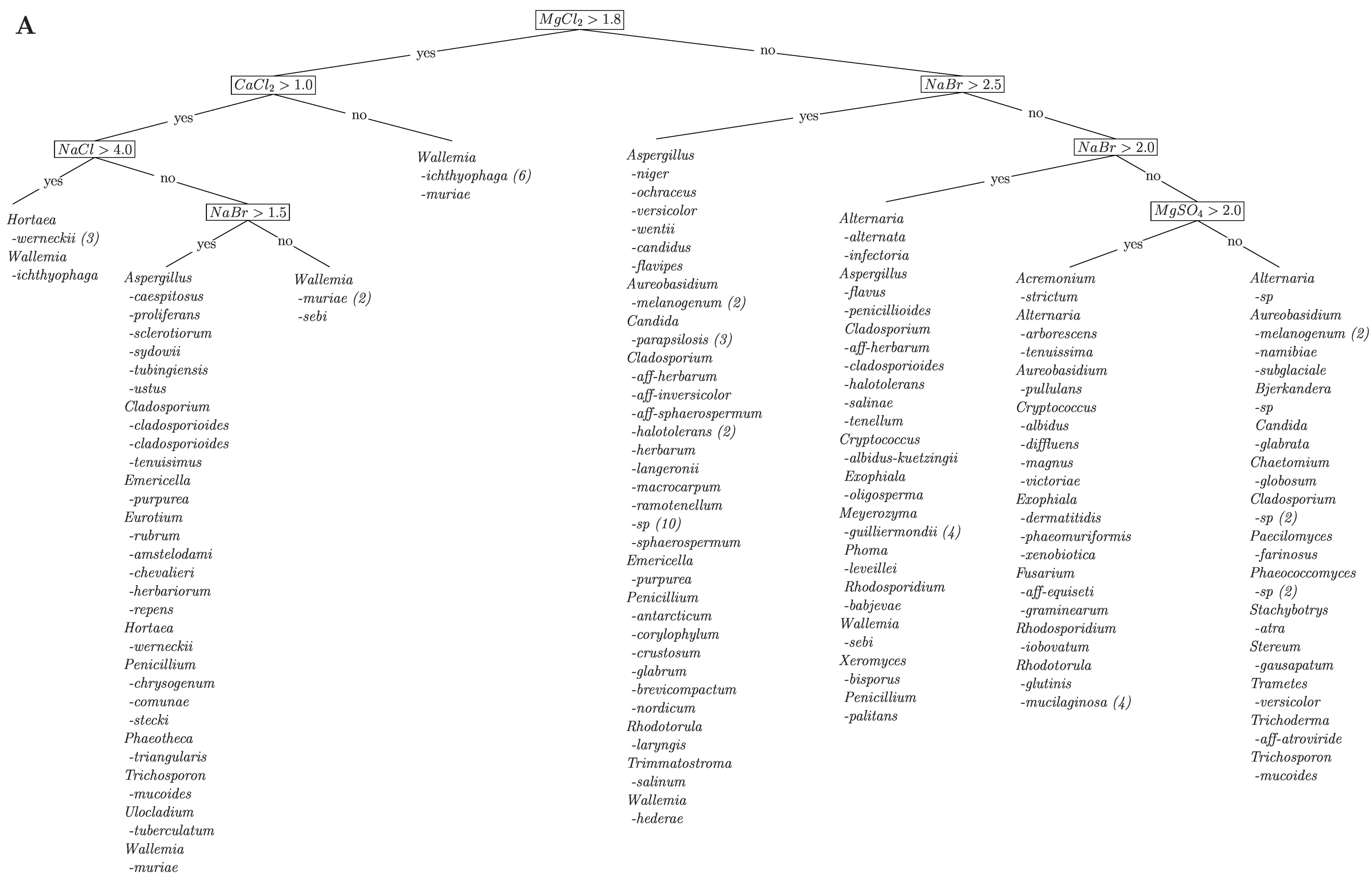


Figure 2.TIF


B
Pigmentation = non − melanized
yes
no
Morphology = clumps
Morphology = filamentous
yes
no
Wallemia
-ichthyophaga (6)
Morphology = filamentous
yes
no
KCl > 3.0
NaCl > 2.5
yes
no
Aspergillus
-caespitosus
-flavus
-flavipes
-niger
-candidus
-ochraceus
-penicillioides
-proliferans
-sclerotiorum
-sydowii
-tubingiensis
-ustus
-versicolor
-wentii
Emericella
-purpurea (2)
Eurotium
-rubrum
-amstelodami
-chevalieri
-herbariorum
-repens
Paecilomyces
-farinosus
Penicillium
-antarcticum
-chrysogenum
-comunae
-corylophylum
-crustosum
-glabrum
-stecki
-brevicompactum
-nordicum
-palitans
Trichoderma
-aff-atroviride
Ulocladium
-tuberculatum
Wallemia
-hederae
-muriae (4)
-sebi (2)
-ichthyophaga
Xeromyces
-bisporus
Acremonium
-strictum
Alternaria
-alternata
-arborescens
-infectoria
-sp
-tenuissima
Stereum
-gausapatum
Candida
-parapsilosis (3)
Cryptococcus
-albidus
-albidus-kuetzingii
Meyerozyma
-guilliermondii (4)
Rhodosporidium
-babjevae
-iobovatum
Trichosporon
-mucoides (2)
Candida
-glabrata
Cryptococcus
-diffluens
-magnus
-victoriae
Rhodotorula
-glutinis
-laryngis
-mucilaginosa (4)
yes
no
Cladosporium
-aff-herbarum (2)
-aff-inversicolor
-aff-sphaerospermum
-cladosporioides (3)
-halotolerans (3)
-herbarum
-langeronii
-macrocarpum
-ramotenellum
-salinae
-sp (12)
-sphaerospermum
-tenellum
-tenuisimus
Trimmatostroma
-salinum
NaCl > 3.5
yes
no
Hortaea
-werneckii (4)
Aureobasidium
-melanogenum (4)
-namibiae
-pullulans
-subglaciale
Exophiala
-dermatitidis
-oligosperma
-phaeomuriformis
-xenobiotica
Phaeococcomyces
-sp (2)
Phaeotheca
-triangularis

Figure 3.TIF

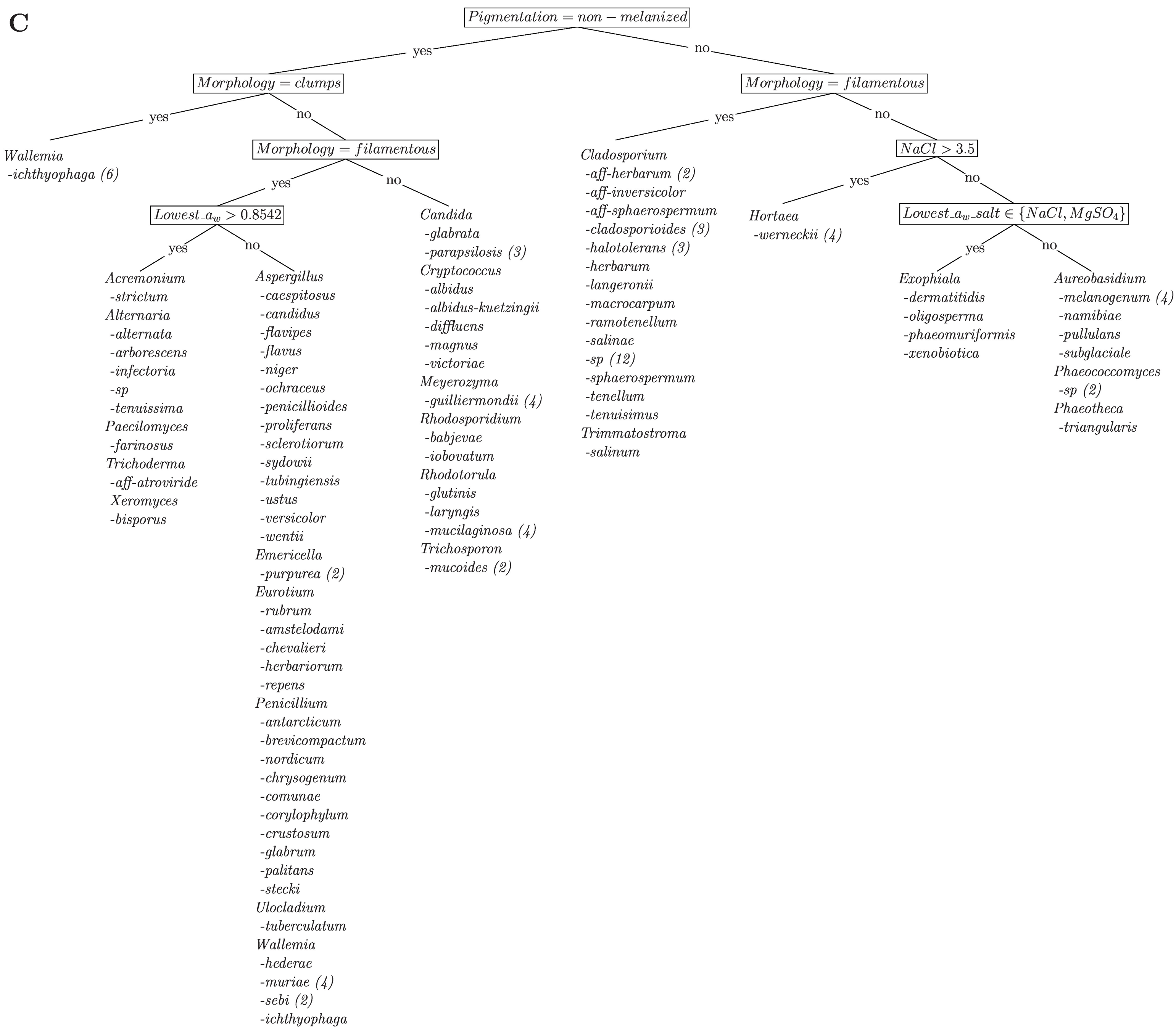
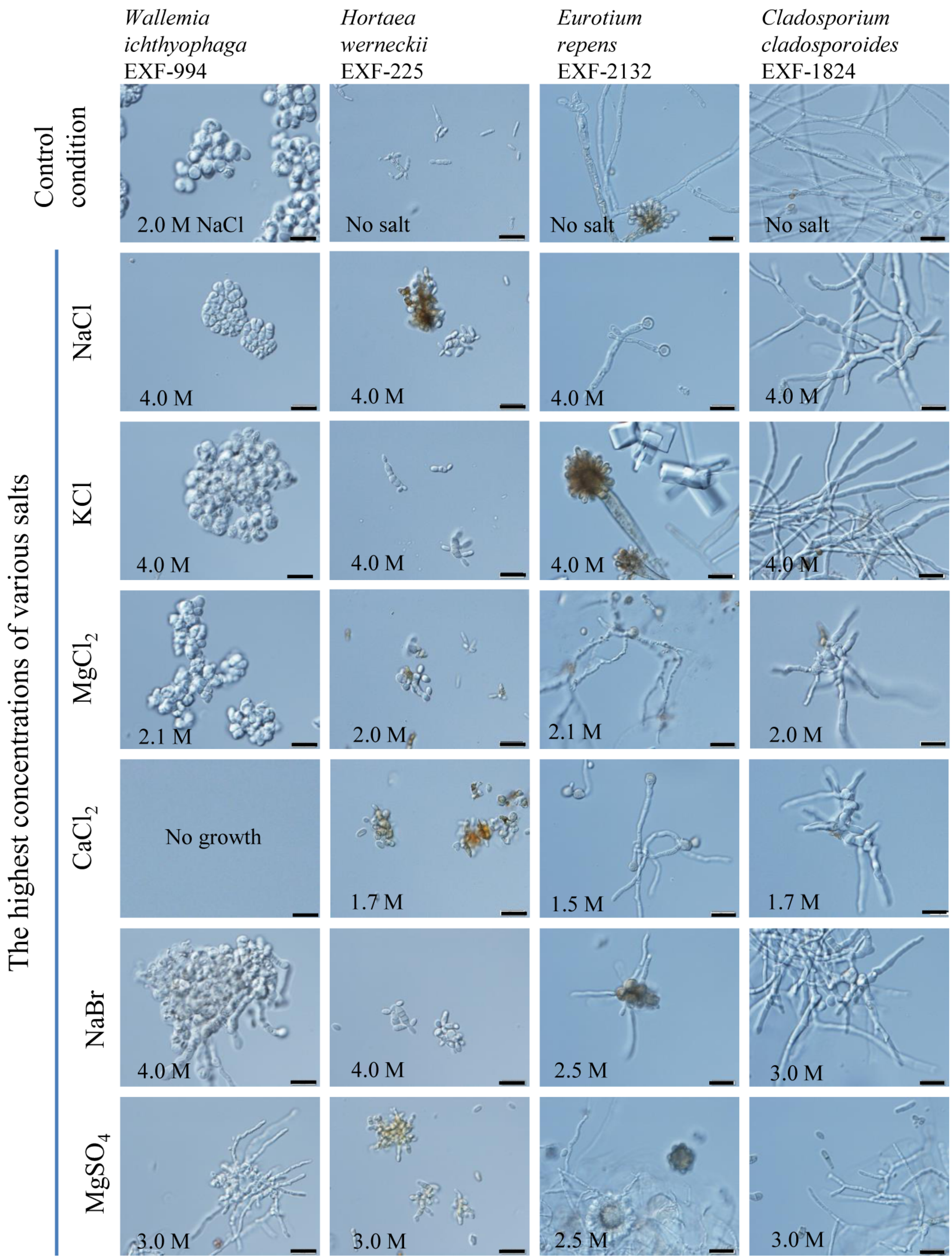

C
Pigmentation = non − melanized
yes
no
Morphology = clumps
Morphology = filamentous
yes
no
Wallemia
-ichthyophaga (6)
Morphology = filamentous
yes
no
Lowest_aw > 0.8542
yes
no
Acremonium
-strictum
Alternaria
-alternata
-arborescens
-infectoria
-sp
-tenuissima
Paecilomyces
-farinosus
Trichoderma
-aff-atroviride
Xeromyces
-bisporus
Aspergillus
-caespitosus
-candidus
-flavipes
-flavus
-niger
-ochraceus
-penicillioides
-proliferans
-sclerotiorum
-sydowii
-tubingiensis
-ustus
-versicolor
-wentii
Emericella
-purpurea (2)
Eurotium
-rubrum
-amstelodami
-chevalieri
-herbariorum
-repens
Penicillium
-antarcticum
-brevicompactum
-nordicum
-chrysogenum
-comunae
-corylophylum
-crustosum
-glabrum
-palitans
-stecki
Ulocladium
-tuberculatum
Wallemia
-hederae
-muriae (4)
-sebi (2)
-ichthyophaga
Candida
-glabrata
-parapsilosis (3)
Cryptococcus
-albidus
-albidus-kuetzingii
-diffluens
-magnus
-victoriae
Meyerozyma
-guilliermondii (4)
Rhodosporidium
-babjevae
-iobovatum
Rhodotorula
-glutinis
-laryngis
-mucilaginosa (4)
Trichosporon
-mucoides (2)
yes
no
Cladosporium
-aff-herbarum (2)
-aff-inversicolor
-aff-sphaerospermum
-cladosporioides (3)
-halotolerans (3)
-herbarum
-langeronii
-macrocarpum
-ramotenellum
-salinae
-sp (12)
-sphaerospermum
-tenellum
-tenuisimus
Trimmatostroma
-salinum
NaCl > 3.5
yes
no
Hortaea
-werneckii (4)
Lowest_aw_salt ∈ {NaCl, MgSO4}
yes
no
Exophiala
-dermatitidis
-oligosperma
-phaeomuriformis
-xenobiotica
Aureobasidium
-melanogenum (4)
-namibiae
-pullulans
-subglaciale
Phaeococcomyces
-sp (2)
Phaeotheca
-triangularis

Figure 4.TIF


Wallemia ichthyophaga EXF-994
Hortaea werneckii EXF-225
Eurotium repens EXF-2132
Cladosporium cladosporoides EXF-1824
Control condition
The highest concentrations of various salts
NaCl
KCl
$MgCl_2$
$CaCl_2$
NaBr
$MgSO_4$
2.0 M NaCl
No salt
No salt
No salt
4.0 M
4.0 M
4.0 M
4.0 M
4.0 M
4.0 M
4.0 M
4.0 M
2.1 M
2.0 M
2.1 M
2.0 M
No growth
1.7 M
1.5 M
1.7 M
4.0 M
4.0 M
2.5 M
3.0 M
3.0 M
3.0 M
2.5 M
3.0 M