ANNUAL REVIEWS

Annual Review of Microbiology

Understanding Fungi in Glacial and Hypersaline Environments

Cene Gostinčar and Nina Gunde-Cimerman

Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia; email: cene.gostincar@bf.uni-lj.si, nina.Gunde-Cimerman@bf.uni-lj.si

Annu. Rev. Microbiol. 2023. 77:89–109

First published as a Review in Advance on March 31, 2023

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-032521-020922

Keywords

fungi, hypersaline water, glacial ice, halophilic, psychrophilic, extremophilic

Abstract

Hypersaline waters and glacial ice are inhospitable environments that have low water activity and high concentrations of osmolytes. They are inhabited by diverse microbial communities, of which extremotolerant and extremophilic fungi are essential components. Some fungi are specialized in only one of these two environments and can thrive in conditions that are lethal to most other life-forms. Others are generalists, highly adaptable species that occur in both environments and tolerate a wide range of extremes. Both groups efficiently balance cellular osmotic pressure and ion concentration, stabilize cell membranes, remodel cell walls, and neutralize intracellular oxidative stress. Some species use unusual reproductive strategies. Further investigation of these adaptations with new methods and carefully designed experiments under ecologically relevant conditions will help predict the role of fungi in hypersaline and glacial environments affected by climate change, decipher their stress resistance mechanisms and exploit their biotechnological potential.

Contents

LIFE IN HYPERSALINE WATER AND GLACIAL ICE

Water is essential for life, and in environments where it is limited, some organisms have evolved ways to survive and even thrive under conditions of extreme water scarcity. In nature, the amount of biologically available water is limited not only by aridity, but also because it exists in unavailable states due to freezing or high concentrations of osmolytes. In fact, high concentrations of extracellular solutes are characteristic of all these conditions: In the case of drought, solutes are concentrated by evaporation of water, and in the case of freezing, by displacement of solutes from expanding ice crystals into the decreasing volume of liquid water between the crystals (55). This review focuses on fungi and their adaptations in two types of environments with low availability of water: glacial ice and hypersaline water (**Figure 1**). These fungal species typically fall into the category of extremophilic fungi (including yeasts), which share some common and novel characteristics.

Glacial and hypersaline environments are attracting growing interest among microbiologists for two main reasons: the rapid changes in these environments due to climate change and the increasing awareness of the novelty of their rich microbial communities. At first, hypersaline environments were thought to be inhabited mainly by archaea and glaciers mainly by bacteria. Only later did it become clear that a considerable diversity of extremophilic fungi was an overlooked but important component of these biotopes.

Despite the well-known salt tolerance of the alga *Dunaliella salina*, eukaryotic fungal cells were thought of as too complex to adapt to extreme concentrations of extracellular salt (54). Eventually, however, it was discovered that extremophilic fungi are an important component of the hypersaline microbiota (13, 56). Thus, certain fungi tolerate high concentrations of Na^+, K^+, Li^+, Ca^{2+}, and, most impressively, Mg^{2+} in natural saline lakes as well as in artificial evaporation ponds used for harvesting salt (136). Fungi populate thalassohaline waters, which are mostly concentrated seawater, as well as athalassohaline waters, the ionic composition of

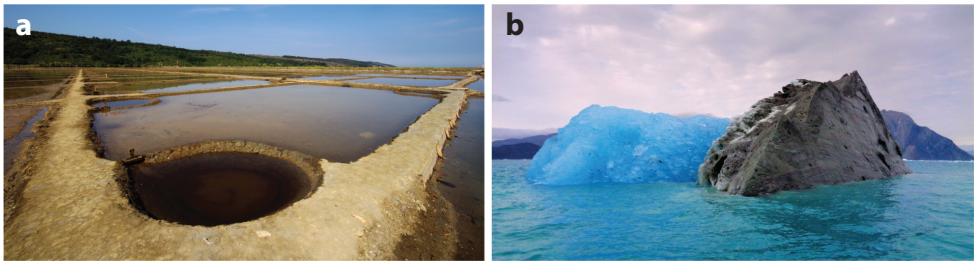

Figure 1

Typical hypersaline and glacial habitats. Shallow ponds for evaporation of sea water in salt production (*a*) and glacial ice with various amounts of inorganic material originating mostly from subglacial bedrock (*b*).

which reflects local geology and environmental conditions. Thus, extremophilic fungi have been isolated worldwide from saltern brines (1, 4, 23, 54, 134, 140); from magnesium-rich bitterns (136); from the athalassohaline hypersaline waters of the Dead Sea, the Great Salt Lake, and the alkaline Wadi El Natrun in Egypt (5, 85, 133); from hypersaline industrial effluents in temperate and tropical climates (39, 66, 72); from cold hypersaline lakes in Antarctica; and from cryopegs in Siberian permafrost (37). As explained below, the survival of these fungi is supported by specialized adaptations at both genomic and phenotypic levels.

Glacial ice was originally thought to be a medium in which microbes could become entrapped (sometimes in stable, chronologically defined layers) and remain viable but dormant for long periods. For example, over the last 30 years viable strains of fungi have been isolated from ice 1 to 2 million years old collected from ice cores at depths of 3.5 km (9, 27, 66, 80). Then, in 2007, fungi were discovered in a much more dynamic glacial habitat: at the base of polythermal glaciers, where local pressure creates a thin film of melted ice that allows unexpectedly abundant, metabolically active fungal communities. Similar observations have been made in the biodiversity sampling of communities in retreating Arctic glaciers on Svalbard and glaciers in Europe, Canada, Alaska, China, Patagonia, and Mexico (9, 14, 15, 18, 19, 24, 75, 124–126, 146). Some of the same fungal species have also been sampled on the surface of melting ice sheets in Greenland and Antarctica (26, 92, 113).

Fungi are essential to the biochemical cycles in polar and alpine regions. However, most studies of fungi focus on their ecological role in soils and their interactions with plants (2, 3), while fungi in glacial ice have been primarily studied by cataloging their biodiversity. Even here, large knowledge gaps remain. For example, little is known about the species of fungi that are deposited on remote supraglacial snow and ice fields by aeolian processes, or about their role in aerosols and in ice nucleation (105). Interestingly, the overlap between the fungal diversity of the ice sheets and that of the overlying fresh snow is very small (92). This supports the hypothesis that glacial microbiota result from migration processes of microbes deposited on the glaciers and ice sheets by rain, snow, and wind, during which these microbes undergo selection and enrichment as they move from the surface to the bottom of the glacier, a process that can take thousands of years.

The selection and enrichment processes of microbes during glacial formation vary according to local conditions. Analyses of glacial communities in geographically distant areas find that ice environments exhibit a great deal of spatial heterogeneity with very little overlap (82). This is true for both subglacial and supraglacial environments. The latter are covered by snow or ice algae (or both), which are responsible for the accumulation of pigmented organic matter. In some cases (e.g., black ice algae on the Greenland Ice Sheet) this leads to a significant reduction in surface albedo and an increase in ice melt and microbial activity. Initial observations suggest that these algae act as environmental filters and structure the supraglacial fungal community. The consequences are complex, ranging from enrichment with pathogens and endophytes of boreal, polar, and alpine plants to saprotrophic fungi contributing to algal biomass degradation (11, 92).

Prokaryotes and fungi of the englacial system (the habitat within glacial ice) are less studied than those in other glacial habitats. Some fungi deposited on the glacier surface gradually migrate to deeper ice layers, possibly reproducing in the veins and the micropockets of brine between ice crystals. These fungal microcommunities may be influenced by mineral dissolution and precipitation processes in the ice, possibly creating local islands of metabolically active fungi (3).

Under temperate and polythermal glaciers, and under large parts of the Greenland and Antarctic Ice Sheets, liquid water can accumulate and create a habitat with high concentrations of weathering products and nutrients. Fungi isolated from such subglacial environments likely contribute to consortia of heterotrophic and chemoautotrophic microbes that accelerate the weathering of rocks. However, their occurrence and role are still unknown (76). Given the

accelerated glacial melt and retreat, fungi released by glacial meltwater from subglacial environments could also be indicators of climate change (3, 9, 24).

Finally, studies of glacial fungi might give a clue as to how and when the first fungi made the transition from water to land: This could have happened—according to the "White hypothesis"—in icy environments of the Cryogenian period also known as Snowball Earth (82). During this period, the diverse, including highly osmotic, glacial microniches on the surface of the melting ice may have favored the transition from zoosporic fungi to hyphal growth and true osmotrophy. Consistent with this, recent studies have shown an unexpected abundance and diversity of (mainly uncultivable) zoosporic (e.g., chytrid fungi) lineages in certain icy environments (3, 8).

DIVERSITY OF FUNGI IN HYPERSALINE WATER AND GLACIAL ICE

Hypersaline and glacial environments differ in several important characteristics. Hypersaline environments, found mainly in temperate and tropical parts of the world, are characterized by high UV radiation, low oxygen levels (at the highest salinities), fluctuating nutrients, and occasional abrupt increases in water activity due to rainfall. Nevertheless, conditions are generally stable (23). Glacial environments, on the other hand, are quite heterogeneous. The supraglacial zone is influenced by the exposure to strong solar irradiation and cycles of freezing and thawing and is enriched in nutrients and airborne microorganisms. The subglacial ice and englacial ice typically receive little to no light, are oligotrophic, and are likely low in oxygen and influenced by local mineral dissolution and precipitation processes (3, 27).

Despite these differences, the challenges encountered by microorganisms in hypersaline and glacial environments overlap substantially. Salinity causes both ionic and osmotic stress through osmotic imbalance. Freezing causes osmotic stress by dehydrating cells due to reduced water absorption and conduction. Under both conditions, osmolytes stabilize the cells by acting as both cryoprotectants and osmoprotectants. This could explain why many fungal species isolated from hypersaline environments are also isolated from cold or glacial ecosystems and why many fungi isolated from glacial ice grow on media supplemented with 10% or even 17% (w/v) NaCl (125).

Unlike prokaryotes found in hypersaline waters, most fungi from these environments can grow without salt but nevertheless tolerate high NaCl concentrations, some up to saturation (32% w/v); i.e., they are extremely halotolerant (54). Few fungal species cannot grow in normal microbiological media but require an increased concentration of salt (78, 83, 107, 147). Fungi found in glacial ice show similar adaptability. Most are psychrotolerant rather than psychrophilic, and some even grow at 37°C (57, 95).

The mycobiota of the two low-water-activity environments, hypersaline water and glacial ice, can be divided into transient taxa that are strongly influenced by environmental conditions and the resident core community, which is found in the majority of similar environments around the world (**Figure 2**). Both categories can be further subdivided into specialists and generalists. Specialists are usually associated with only one of the two environments with low water activity, while many generalists can be isolated from both extreme habitats (4).

Specialist core species of hypersaline waters are represented by ascomycetous melanized black yeasts *Hortaea werneckii*, *Phaeotheca triangularis*, and *Trimmatostroma salinum* (56) and basidiomycetous filamentous fungi *Wallemia ichthyophaga*, *Wallemia hederae*, and *Wallemia sebi* (1, 61). In comparison, glacial environments show higher variability in core fungal diversity. Specialist basidiomycetous yeast taxa that predominate in the glacial microbiome include members of the *Cryptococcus*, *Glaciozyma*, *Mrakia*, *Naganishia*, *Rhodotorula*, and *Sporobolomyces* genera (16, 25, 121, 132, 143). However, many yeast populations in glaciers are habitat-specific and strongly influenced by the type and temperature of ice, sediment, and salt inclusions, resulting in pockets of local

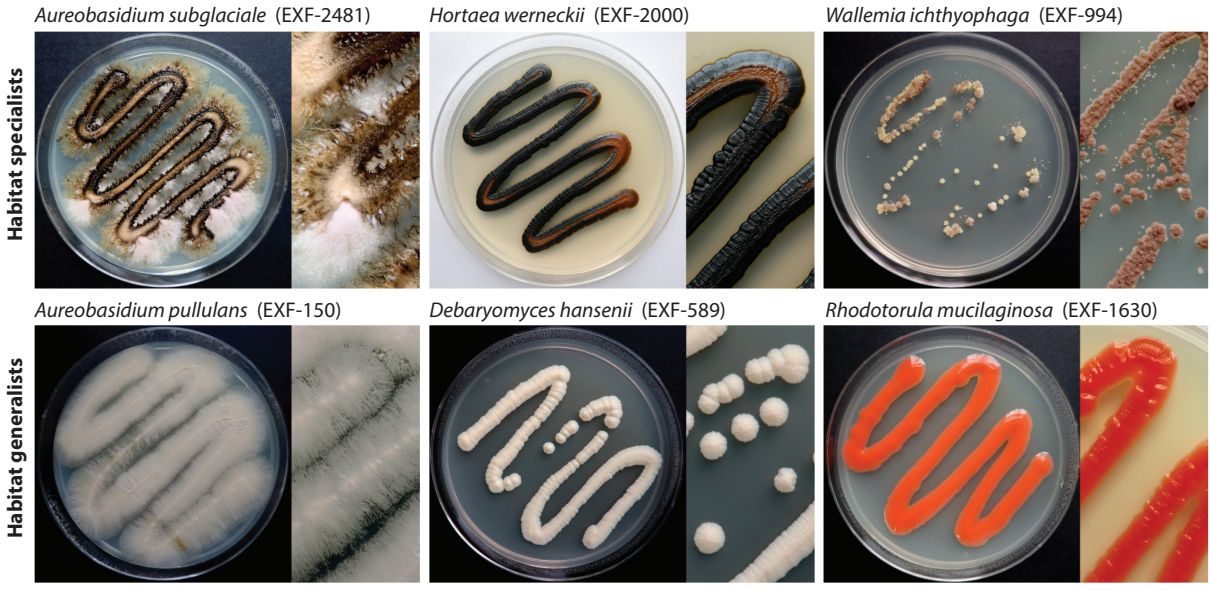

Figure 2

Representative species of extremotolerant and extremophilic fungi. Fungi were grown at 24°C for three weeks on malt extract agar [in the case of *Wallemia ichthyophaga*, with 15% of added NaCl (w/v)].

diversity (91, 93, 148). Although these populations probably play an important role in nutrient cycling and mineralization of organic matter, little is known about these processes.

Specialist species are found in a narrow range of environments and are among the species that survive and grow in some of the most extreme conditions on the planet: the black yeast *H. werneckii* grows in almost the entire range of NaCl concentrations (54). The basidiomycete *W. ichthyophaga* can grow in solutions saturated with NaCl (54). This specialization may explain the limited distribution of the fungus: Only 25 isolates of *W. ichthyophaga* are known worldwide. Even fewer isolates have been found of the black yeast *Aureobasidium subglaciale*, most of which have been isolated from the subglacial ice of Arctic glaciers (135). Interestingly, a handful of *A. subglaciale* strains (and several rare *Penicillium* species) have also been isolated from household refrigerators, suggesting that even highly specialized species can find unexpected refuge in certain artificial habitats (135).

The black yeast *H. werneckii* shows the extremophilic phenotype characteristic of black yeasts: slow, polymorphic growth as yeast cells, hyphae, or meristematic clumps (i.e., thick melanized cell walls covered by extracellular polysaccharides) and the ability to form biofilms (56). Although *H. werneckii* is the predominant fungus in thalassohaline hypersaline waters worldwide, it has also been found in cold environments such as deep-sea water and glacial ice, in association with animals and plants, on salted foods, and as the causative agent of tinea nigra on salty human skin (53). *H. werneckii* thus has a remarkable adaptability to different temperatures and salinities (reflected in its ability to produce enzymes that are active at high salinities) (144).

The ability to grow at low water activity is rare among Basidiomycota. However, all species of the genus *Wallemia* are xerophilic or halophilic (61). The specialist *W. ichthyophaga*, the phylogenetically most distant species from the rest of the genus, is the most halophilic fungus known to date with an obligate requirement for at least 10% (w/v) NaCl. This species also grows in saturated KCl and $MgSO_4$ solutions and at 2.1 M $MgCl_2$ (21, 136). Comparative phylogenomic analyses suggest that *Wallemia* belongs to the Wallemiomycetes, recently elevated to the subphylum

Wallemiomycotina, a 500-million-year-old sister group of Agaricomycotina (147). The unusual extremophilic nature of the taxon is reflected in the production of various toxic metabolites that increases at high NaCl concentrations—a notable exception to the general rule of reduced secondary metabolite synthesis at low water activity (60, 137).

In addition to the core community of habitat specialists, other species occur in both glacial and hypersaline (and often many other) environments. These species usually do not tolerate such extreme values of physicochemical parameters as the specialists, but they are characterized by a high tolerance to many types of stress as well as by their nutritional versatility. Examples of such polyextremotolerant species are *Aureobasidium pullulans*; *Rhodotorula mucilaginosa*; *Debaryomyces hansenii*; and representatives of the genera *Aspergillus*, *Cladosporium*, and *Penicillium* (16, 17, 59, 79, 141, 142). *Penicillium* species from glacial environments are also characterized by the coexistence of a large diversity of species in the same samples and the ability to thrive under extremely nutrient-poor conditions (110, 111).

Many polyextremotolerant fungi originating from environments with low water activity exhibit a number of exaptations that are considered virulence factors: increased resistance to oxidative stress, growth at human body temperature, oligotrophy, melanization, and flexible morphology (41, 42, 50, 51). Because of these characteristics, they can grow indoors, in close proximity to humans, and in some cases cause opportunistic infections (50). The generalist *R. mucilaginosa* colonizes dishwashers and washing machines and can infect humans (145). Studying these adaptations and the biodiversity of these fungi indoors is important to better understand their evolution and implications for the emergence of novel fungal pathogens (50, 102, 150).

A. pullulans, *R. mucilaginosa*, and *D. hansenii* are ubiquitous, polyextremotolerant species that can be isolated from environments with widely varying water activity and temperature and other extremes (43, 89, 99, 143). This adaptive behavior might be related to their ability to tolerate adverse conditions in a state of anhydrobiosis (i.e., temporarily and reversibly suspended metabolism), their ability to survive freeze-thaw cycles, and (in the case of *A. pullulans* and *R. mucilaginosa*) their highly protective pigmented cell walls (7).

The airborne saprotrophic fungi of the genus *Cladosporium* are associated with dead organic matter. In polar regions, they have been proposed as bioindicators of rapidly melting glaciers and ice sheets and associated with newly exposed land (101). *Aspergillus* and *Penicillium* species are also commonly found in cultured isolates from brines and are known to survive in ice for a long time as "living fossils" (12). Some species that can reproduce in glacial environments appear to evolve there into genetically or phenotypically distinct populations (109, 111). Some are characterized by an even richer profile of secondary metabolites than is seen in their mesophilic relatives despite living in extremely oligotrophic environments (92, 112).

ADAPTATIONS TO EXTREME CONDITIONS

The similarities between hypersaline and glacial environments mean that microorganisms in these environments face some common challenges. The concentrated extracellular solutes in the brine and between the ice crystals may contain ions that are toxic if they enter the cell in large quantities. Low water activity of the medium threatens the cell with water loss. Low temperature and high salinity alter the fluidity of lipid membranes and disrupt membrane-bound processes. Thus, in the plethora of fungal adaptations to life in hypersaline and glacial environments, some clear patterns can be discerned (**Figure 3**).

The first step in responding to extreme conditions is to perceive them. One of the most important signaling pathways that responds to high osmolarity, the high-osmolarity glycerol (HOG) signal transduction pathway, is triggered by sensors in the plasma membrane and leads to the

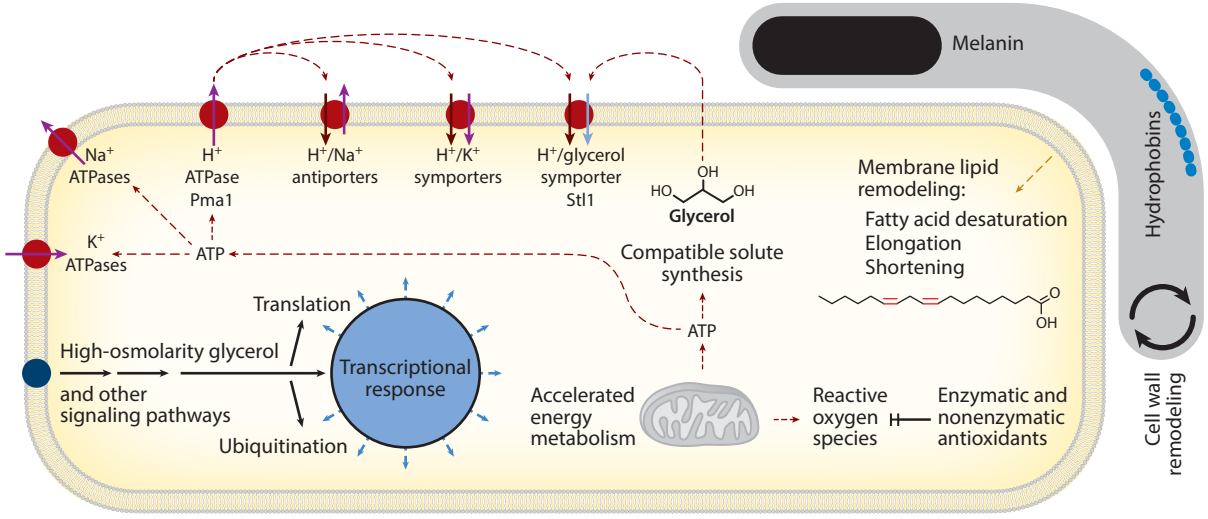

Figure 3

The main stress-mitigating cellular mechanisms of fungi from hypersaline and glacial environments. The cellular structures are not drawn to scale.

MAPK (mitogen-activated protein kinase) Hog1 via two cascades of signaling molecules (58). In addition to hyperosmolarity, this pathway also responds to numerous other stress factors, including low temperatures (88), and leads to a variety of changes in transcription, translation, and ubiquitination. The HOG signaling pathway has been studied in detail in *H. werneckii* and *W. ichthyophaga*, leading to the identification of all major components of the pathway (63, 70, 129). In contrast to what occurs in most other species, Hog1 is constitutively phosphorylated in *W. ichthyophaga* under optimal osmotic conditions and is dephosphorylated under both hypo- and hypersaline conditions. Moreover, only one of the two branches of the signaling pathway leading to Hog1 appears to be conserved in this species (98). In *H. werneckii*, Hog1 is permanently phosphorylated as long as the cells are maintained at high NaCl concentrations, whereas in most fungi (including *H. werneckii* at high KCl or sorbitol concentrations) such phosphorylation is only transient (98). In other extremotolerant fungi, HOG and other signaling pathways have received less attention despite some initial studies, e.g., in *D. hansenii* (100). The same is true for alternative signaling pathways. A promising target for future research is the calcineurin signaling pathway, which regulates ion homeostasis (62). The signaling pathway is involved in tolerance to NaCl in *Aspergillus oryzae* (62) and to freezing in *Saccharomyces cerevisiae* (87).

A universal response of fungi to hyperosmotic stress is intracellular accumulation of small organic molecules such as glycerol. These compatible solutes balance the osmotic pressure, preventing cytosol dehydration and loss of turgor (96). Glycerol and some other compatible solutes also have a cryoprotective function (52).

The synthesis of large quantities of compatible solutes contributes substantially to the high energy requirements of the extremophilic lifestyle (84). Because of its small size, glycerol is cheaper to produce than larger compatible solutes, but it is also susceptible to leakage from the cell (84). In contrast to the deliberate release of glycerol during hypoosmotic shock by plasma membrane aquaglyceroporins (119), such leakage is undesirable and is countered by active transport of glycerol back into the cell by transporters of the major facilitator superfamily (MFS) (33). These proteins, Stl1 and its homologs, are one of the most commonly identified mechanisms of fungal

adaptation to hypersaline conditions. In *H. werneckii*, *Aspergillus sydowii*, *Aspergillus salisburgensis*, *Aspergillus sclerotialis*, *D. hansenii*, and several *Hyphopichia* species, transcription of genes encoding homologs of Stl1 is induced in response to high NaCl concentrations in the environment (40, 63, 73, 90, 117). The multiplication of genes encoding Stl1 has also been observed in several halotolerant and halophilic fungi, including *A. pullulans* and *A. subglaciale* (127), *W. ichthyophaga* (139), and *Hyphopichia* spp. (73). The costs required to synthesize compatible solutes could also be reduced by efficient uptake of glycerol produced by other organisms, or even by active predation—a possibility that has been largely neglected in the existing literature focused on the study of pure cultures.

The synthesis of glycerol is controlled by the rate-limiting enzyme glycerol-3-phosphate dehydrogenase 1 (Gpd1). Its expression is strongly upregulated during hyperosmotic shocks, as shown in *H. werneckii* (63), *A. pullulans* and *A. subglaciale* (127), *A. sydowii* (90), *Eurotium rubrum* (65), and *Hyphopichia* spp. (73). However, under long-term hypersaline conditions, expression often returns to the level of nonsaline conditions, while Stl1 expression remains elevated, suggesting that cells in stable hypersaline environments prioritize the energetically more efficient import of glycerol over its synthesis (90).

In *S. cerevisiae*, expression of Gpd1 and Stl1 is also upregulated by freezing and improves yeast freezing tolerance (32). Since regulation occurs via the HOG pathway and the cryoprotective role of glycerol is well known (77), the same may be expected in other fungi. For example, *Mrakia psychrophila* accumulates glycerol as a compatible solute at 4°C due to upregulation of Gpd1 expression (115).

While glycerol is the main compatible solute of most fungi, many other osmolytes warrant further investigation. It has been suggested that *Rhodotorula frigidialcoholis* lowers its freezing point with ethanol (123), and a variety of organisms produce antifreeze proteins—a nonosmolyte alternative to prevent freezing damage (64). In addition to glycerol, *H. werneckii* accumulates arabitol, mannitol, erythritol (especially in the stationary growth phase) (69), and even mycosporines and mycosporine-like amino acids (67). *W. ichthyophaga* accumulates glycerol and arabitol (138). Trehalose is known to protect against extreme temperatures, but its role in halotolerance is questionable (90). The genomes of *A. sydowii*, *Aspergillus versicolor*, and *Hyphopichia* spp. are enriched in genes for amino acid uptake (73, 90), while *A. salisburgensis*, *A. sclerotialis*, and *M. psychrophila* are enriched in the number of genes encoding transporters of the MFS (115, 117).

Not all of the above osmolytes are necessarily directly associated with halotolerance or psychrotolerance. The accumulation of small organic molecules in the cytosol may play other roles in stress tolerance that remain to be explored, including the mitigation of oxidative stress (50). The production of reactive oxygen species (ROS) in the cell typically increases under stressful conditions, including high salinity and low temperatures (77, 120). In *H. werneckii*, the ability to cope with ROS is thought to determine the upper limit of salt tolerance (97). Increased expression of antioxidant response genes has been observed in *A. sclerotialis* and *Aspergillus salisburgensis* at high salinity (117) and in the psychrotolerant *Penicillium olsonii* following cold shock (38).

The classic route of ROS detoxification is via antioxidant enzymes, the three most important being superoxide dismutases, catalases, and peroxiredoxins. There is some evidence that the copy number of genes encoding these enzymes correlates with the maximum tolerated salinity of selected halotolerant fungi (42). An alternative, nonenzymatic way of ROS detoxification has emerged in recent years, particularly in the context of resistance to ionizing radiation: scavenging ROS with low-molecular-weight complexes of manganese, short peptides, and other small metabolites. The model was first discovered in bacteria and later extended to other organisms, including fungi (106), and to ROS that are formed during aging and desiccation (35). *Geomyces pannorum*, a weak producer of enzymatic antioxidants, uses phenolics to increase ROS scavenging

activity after cold shock (77), but otherwise the potential role of small osmolytes in mitigating ROS under hypersaline and cold conditions remains largely unexplored.

The balancing of osmotic pressure is complemented by careful management of intracellular concentrations of inorganic ions. This usually means retaining sufficient amounts of intracellular K^+ and expelling excess Na^+ and other toxic ions (e.g., Mg^{2+}, Li^+, or others, depending on the environment). *H. werneckii* and *A. pullulans* appear to do this very efficiently (68), but in *D. hansenii* and *W. ichthyophaga* the concentration of intracellular Na^+ is relatively high (99, 138).

Active membrane transport of inorganic ions provides another important energy sink under hypersaline conditions. The energy is provided by ATP or by the proton motive force, which is mainly generated by the H^+-ATPase Pma1. Black yeasts, including *H. werneckii* and *Aureobasidium* spp., contain a large collection of transporter genes consisting of both paralogs and unrelated but functionally redundant transporters (e.g., three types of K^+ importers in *A. pullulans*) (44). In these and other halotolerant fungi, expression of transporter genes responds to environmental salinity (65, 73, 90, 131). Heterologously expressed transporters from *D. hansenii* increase halotolerance of *S. cerevisiae* (100).

The role of alkali metal cation transporter genes in fungal halotolerance is well established, but an abundance of these genes is neither sufficient nor necessary for halotolerance. The diversity and multiplicity of transporter genes is high in many black fungi, not all of which are halotolerant (44). In contrast, the halophilic *W. ichthyophaga* has only a modest diversity and an abundance of transporter genes, the transcription of which is mostly unresponsive to salt (139).

Membrane transporters, other membrane-bound proteins, and cellular integrity depend on adequate cell membrane fluidity. At low temperatures, psychrophilic fungi decrease the saturation and average chain length of fatty acids and lower the sterol:phospholipid ratio (6, 77, 103, 104). Additional compounds such as carotenoids may help to balance the higher proportion of unsaturated fatty acids (123). In halotolerant and halophilic fungi, fluctuation of plasma membrane fluidity is kept low even at extreme salt concentrations (128), and at least some of these changes are achieved by altered expression of fatty acid desaturases, elongases, and enzymes involved in ergosterol synthesis (48, 90). Since these changes can affect membrane permeability, this could reduce the need for active transport counteracting the leaking of various substances through the membrane. For example, in *D. hansenii* osmotic stress induces membrane depolarization and reduces permeability to cationic substances (22).

The barrier of the plasma membrane is supplemented by the cell wall. In *H. werneckii*, the melanization of the cell wall is thought to reduce the leakage of glycerol at high salinity (69). Some of the (very few) changes observed in *H. werneckii* after seven years of experimental evolution at high salinity were associated with the cell wall (45). In *W. ichthyophaga*, the cell wall thickens more than threefold at high salinity and accounts for more than half of the cell dry mass. The otherwise contracted genome of the species is significantly enriched with genes encoding hydrophobins, small hydrophobic proteins that could further impermeabilize and strengthen the cell wall (71, 138, 139).

The elasticity of the cell wall is crucial for the survival of fungi after osmotic shock. Architectural changes in the cell wall can occur within seconds of shock (30). In several halotolerant and halophilic fungi, the changes in the cell wall are accompanied by altered expression of enzymes involved in the synthesis, restructuring, or degradation of chitin, β-glucans, and hydrophobins (65, 71, 90, 117) and also of mannoproteins on the cell wall surface (40). A phosphomannomutase from the polyextremotolerant yeast *Rhodotorula mucilaginosa*, an enzyme catalyzing an early step of O- and N-linked mannosylation, increased halotolerance of *S. cerevisiae* when heterologously expressed (43). Mannose is also a component of mannan, an extracellular polysaccharide of *Rhodotorula* spp. that protects against desiccation and freeze-thaw damage (123).

Although the above adaptations keep the intracellular environment as undisturbed as possible even under extreme conditions, this balance is not absolute. Membrane transporters and the synthesis of compatible solutes are energetically costly. Antifreeze compounds prevent damage from intracellular ice crystals, but low temperatures still slow down enzymatic reactions. As a result, growth of extremotolerant and extremophilic organisms is often slow (51, 103). In response, *H. werneckii* upregulates genes of the glycolytic pathway, tricarboxylic acid cycle, pentose phosphate pathway, and mitochondrial biogenesis at high salinity (131). *D. hansenii* upregulates genes encoding proteins of mitochondrial functions (40). At low temperatures *M. psychrophila* upregulates genes involved in ribosome and energy metabolism (115). In contrast, *R. frigidialcoholis* downregulates electron transport chain and tricarboxylic acid cycle genes, but overexpresses fermentation and pentose phosphate pathway genes (123). Psychrophilic enzymes with higher activity at low temperatures have also been intensively studied, not least because of their potential biotechnological applications (28).

Other global adaptations to high salinity include a proteome with an increased proportion of acidic amino acid residues, a feature also observed in halophilic prokaryotes (65, 117). At low temperatures, codon usage bias and frequent alternative splicing were observed in *M. psychrophila* (115) and an increased role of small RNAs in *R. frigidialcoholis* (123).

In some cases, single genes have been reported to have significant effects on fungal extremotolerance. 3′-Phosphoadenosine-5′-phosphatases from *H. werneckii* and *A. pullulans*, or even a 21–amino acid region of these enzymes when inserted into the homologous protein of the recipient, increased halotolerance of *S. cerevisiae* and *Arabidopsis thaliana* (36, 130). A phosphoglucomutase from *R. mucilaginosa* proved to be more lithium resistant than its very lithium-sensitive *S. cerevisiae* homolog and increased the halotolerance of the latter yeast (43). The changes in the carboxy-terminal domain of RNA polymerase II in fungi from polar and hypersaline environments affect the ability of this enzyme to undergo phase separation in vitro and localize in vivo, potentially allowing it to overcome energetic barriers to metabolic activity (86).

GENOMICS AND TRANSCRIPTOMICS

It was expected that genomic data would accelerate genetic research of typically recalcitrant extremotolerant fungi. However, explaining such complex phenotypes as psychrotolerance or halotolerance with genome analysis only leads to very general and often inconclusive observations (49, 114). Sequencing the transcriptomes of fungi grown at high salinity or low temperatures provided more informative insights (90, 117), but even transcriptional responses were often difficult to interpret (139).

Nevertheless, the availability of a genome sequence is a valuable first step in deciphering the halotolerance and psychrotolerance of fungi. On the one hand, it can support and direct targeted in vitro genetic studies. On the other hand, a sequenced genome provides a reference point for further sequencing, followed by comparative and population genomics. Such studies have, for example, revealed an unexpected diversity of reproductive strategies of fungi from extreme environments (**Table 1**). Evidence of recombination has been discovered in three species previously considered asexual: *A. pullulans* (49), *Wallemia mellicola* (116), and *W. ichthyophaga* (47). The degree of recombination observed was higher in *A. pullulans* than in most other fungi (49). This species was confirmed to be a true generalist, without cryptic specialization, although it is capable of inhabiting an unusually wide variety of environments, both temperate and extreme (49). While the observed recombination of the *A. pullulans* and *W. mellicola* is not difficult to explain, due to their ubiquitous distribution, it is surprising in *W. ichthyophaga*, an extremely rare species with a narrow range of habitats and therefore very limited opportunities for recombination (61).

Table 1 Representative extremophilic and extremotolerant fungi and their traits discovered by population genomics

Species	Extreme phenotype	Distribution	Recombination	Ploidy
Aureobasidium pullulans	Polyextremotolerant	Generalist	Yes	Haploid
Aureobasidium melanogenum	Polyextremotolerant	Generalist/specialist	No	Haploid or diploid (hybrids)
Aureobasidium subglaciale	Psychrotolerant	Specialist	No	Haploid
Hortaea werneckii	Extremely halotolerant	Specialist	No	Haploid or diploid (hybrids)
Wallemia ichthyophaga	Halophilic	Specialist	Yes	Haploid
Wallemia mellicola	Extremely osmotolerant	Generalist	Yes	Haploid

In contrast to the generalists *A. pullulans* and *W. mellicola*, the specialists *A. subglaciale* (135) and *H. werneckii* (46) are strictly clonal. Despite known pitfalls, including Muller's ratchet, clonality also eliminates the recombination load that would disrupt efficient genome configurations of evolving extremophiles, and it also saves the energy required for sexual reproduction (46). Nevertheless, clonality in *Aureobasidium melanogenum* and *H. werneckii* is amended by unusual intraspecific hybridization, described as "stable parasexuality" (46, p. 12). This results in highly heterozygous diploids that make up about two-thirds of the species' wild isolates and are stable enough to spread over large geographical distances—without haploidization, which is generally typical of parasexuality (46). In addition to altered ploidy, which alone can affect phenotype (122), paralogous genes are not necessarily functionally identical—as shown in the case of glycerol-3-phosphate dehydrogenases of *H. werneckii* (74). Hybrid genomes have also been found in some other, nonextremotolerant fungi (34), but the prevalence of this phenomenon in fungi, the mechanism of hybridization, and its ecological implications in extreme environments are as yet unknown.

FUTURE RESEARCH

The number of studies on the microbiology of glacial ice and hypersaline waters is increasing, but fungi still receive far less attention than prokaryotes. The diversity and geographical distribution of fungi in these environments are generally well known, with some exceptions. For example, our knowledge of the fungi growing in magnesium-rich bitterns is rudimentary. In addition, published studies on biodiversity in glacial and hypersaline environments are mainly based on culturing techniques.

A few studies using culture-independent methods reported fungal DNA in glacial and hypersaline environments (4, 29, 93, 134). These studies showed a considerable diversity of uncultivable fungi (e.g., Chytridiomycota) in glacial environments (8), but none so far in hypersaline environments. However, molecular methods for analyzing fungal diversity are also not without problems. In such studies, particular care should be taken not to amplify environmental DNA derived from organisms that cannot survive in extreme environments. In addition, some fungal species are particularly recalcitrant to DNA extraction, due to their thick, often melanized cell walls (44). Standard methods may not succeed in extracting DNA from these cells, thus distorting inferences about the composition of the extremotolerant fungal community.

The ecological functions of fungal diversity in glacial and hypersaline environments are largely unexplored. Proposed functions range from actively shaping their environment to interactions with plants and animals. Some of these fungi are opportunistic human pathogens, and many hold untapped biotechnological potential, e.g., the production of cold-active enzymes (28) and biocontrol of cold-stored produce (135). More research is also needed in the face of climate changes, which pose particular threats to the cryosphere. Glacial fungi are directly affected by these processes, but they can also help shape them. For example, certain fungi may form a symbiotic,

lichen-like relationship with pigmented, albedo-reducing algae, and in this way contribute to accelerating ice melt. This phenomenon needs to be studied further, especially with regard to its impact on global warming (94).

The role of fungal populations in salterns in modulating halite precipitation and the organoleptic quality of harvested salt is largely unknown. The role of fungi as ecological drivers in hypersaline habitats has not yet been investigated. Open questions include the following: (*a*) the influence of available nutrients, such as phosphorus and nitrogen, on fungal abundance; (*b*) possible correlations and interactions of fungi with the alga *D. salina* (81); and (*c*) the role of fungi in the microbial mats that form the floor of the precipitation basins of salterns (which in some ancient salterns may have been continuously cultivated for hundreds of years) (20). Other important research topics are fungal degradation of wood immersed in hypersaline water (144) and the role of hypersaline water as a reservoir of species pathogenic to corals and other marine animals (e.g., *A. sydowii*), a role possibly exacerbated by global warming (108). Finally, some of these halophilic fungi could have important biotechnological applications in reversing the trend of global loss of agricultural land due to salinization; that is, if fungal genes conferring halotolerance and/or stress tolerance could be successfully transferred to plants of agricultural importance (36).

The growing knowledge of fungal adaptations to extreme environments has been accompanied by increasing interest in the field, leading to the development of methods accessible beyond the short list of mesophilic model organisms in the laboratory. The ability to sequence tens or hundreds of fungal genomes can be used in genome-wide association studies, although the small differences in stress tolerance and large intraspecific genotypic variability may hinder this approach (49). Alternatively, successful genetic manipulation and the ability to mate strains in a laboratory setting would support the identification of quantitative trait loci. Valuable insights can be gained by subjecting extremotolerant fungi to experimental evolution under extreme conditions followed by whole-genome sequencing of the evolved strains (45). Although the possibilities are not yet fully exploited and are more difficult than for *S. cerevisiae*, genetic manipulation of extremophilic and extremotolerant fungi with CRISPR-Cas9 is finally within reach (31, 149). This should renew interest in hypothesis-driven research, which has been overshadowed for a while by the data-driven research of the genome and transcriptome era.

For best results, the study of extremotolerance should abandon the implicit and erroneous assumption that the cellular response to an environmental shock is a good proxy of the response required for constant growth under extreme conditions (54). A rapid environmental perturbation can cause a large shift in the cell's transcriptome and proteome, but when cells recover, many of the affected genes return to their preshock transcription levels despite the continued presence of the stressor (48, 115). The overlap between genes responding to shock and those responding to sustained stress is generally small—in *H. werneckii* at high salinity (131) and also in *S. cerevisiae* at low temperature (118), to name just two examples. Moreover, the study of shocks is less relevant to survival in nature than is often assumed. While rain can lead to hypoosmotic shock, cells in nature are not normally moved from freshwater into brine or suddenly shifted from room temperature to a freezing state. Instead, a slow deterioration of conditions likely allows organisms to activate the stress response toolbox in a less dramatic way, possibly through anticipatory stress responses (10).

Finally, approaches such as cocultivation, single-cell sequencing, and metatranscriptomics might enable us to study fungal stress tolerance under less-artificial conditions than pure, nutrient-rich, and dense cell cultures of extremotolerant and extremophilic fungi.

Fungi are an essential component of microbial communities in hypersaline and glacial habitats. Their diversity and adaptation to stress in selected model organisms have been well studied. Further progress can be made by describing the ecological role of fungi in situ and their

interactions with extremophilic prokaryotic microbial communities. Careful design of experiments and adaptation of new methods to work with generalist, nonmodel, extremotolerant fungi will allow us to better understand their role in a rapidly changing environment and explain how they survive some of the most extreme conditions on the planet.

SUMMARY POINTS

1. Extremotolerant and extremophilic fungi are an essential component of microbial communities in hypersaline and glacial environments.
2. Hypersaline water and glacial ice are both characterized by a low availability of water and a high concentration of inorganic ions.
3. Some specialized fungi are rarely found outside their preferred environment—either hypersaline (*Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, some *Wallemia* species) or glacial (some species of the genera *Aureobasidium*, *Cryptococcus*, *Glaciozyma*, *Mrakia*, *Naganishia*, *Rhodotorula*, and *Sporobolomyces*).
4. Some fungi are generalists, occurring in hypersaline, glacial, and often many other environments, such as stressful artificial environments, e.g., *Aureobasidium pullulans*; *Rhodotorula mucilaginosa*; *Debaryomyces hansenii*; and some species of the genera *Aspergillus*, *Cladosporium*, and *Penicillium*.
5. Common mechanisms of extremotolerance in fungi from hypersaline and glacial environments include optimized signaling pathways, balancing of cellular osmotic pressure and ion concentration, maintenance of optimal cell membrane fluidity, cell wall strengthening, optimized energy metabolism, and efficient neutralization of intracellular oxidative stress.
6. Fungal species from hypersaline and glacial environments use different reproductive strategies, from intensive recombination to strict clonality interspersed with occasional hybridization ("stable parasexuality") that produces heterozygous stable diploids.
7. Some fungi from hypersaline and glacial environments have been shown to have considerable biotechnological potential.

FUTURE ISSUES

1. What is the size of the uncultivable fungal microbiota in different hypersaline and glacial environments?
2. What is the role of fungi in nutrient cycling in hypersaline waters and glacial ice?
3. How do fungi interact with each other and with other microorganisms in hypersaline waters and glacial ice; e.g., how do they influence algal blooms that reduce albedo and accelerate the melting of the Greenland Ice Sheet?
4. Can the effects of climate change on extreme environments, particularly the increasing amount of glacial meltwater, lead to the release of fungi that are potentially harmful to animals or plants?

5. Can CRISPR-Cas9, single-cell sequencing, quantitative trait locus analysis, and other methods be adapted for use with recalcitrant cells of most extremotolerant and extremophilic fungi?
6. Can the study of fungal adaptations to hypersaline and glacial conditions be carried out under more ecologically relevant conditions—under constant stress rather than shocks, in cocultures rather than pure cultures, with a combination of multiple stressors rather than just one?
7. How can the biotechnological potential of fungi from hypersaline and glacial environments be exploited in practice?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Børge Diderichsen for critical reading and for commenting on the manuscript. This study was supported by funding from the Slovenian Research Agency to Infrastructural Centre Mycosmo (MRIC UL, I0-0022), programmes P4-0432 and P1-0198, and project J4-2549.

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