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Title: Extremophilic and Extremotolerant Fungi

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The extreme is in the eye of the beholder. Extremophilic organisms are so adapted to their environment that they perceive extreme conditions as optimal for their growth and can sometimes even be stressed by what we perceive as moderate. Stress is therefore not an optimal criterion for defining what is extreme. Instead, extreme conditions can be seen as those in which the majority of species cannot grow or even survive.

Extreme environments have long been considered an almost exclusive domain of prokaryotes. In recent decades, however, fungi have been found to be an integral part of extreme microbial communities. Who are they? Where can we find them? How did they evolve and how do they survive in some of the most extraordinary conditions we find on our planet?

From extremotolerant to extremophilic

Bacteria and archaea that live in extreme environments often cannot survive in temperate conditions. The ecological amplitude of most fungi from these environments is greater - they are extremotolerant and not extremophilic. Among rare exceptions are *Wallemia ichthyophaga*, a phylogenetically distinct basidiomycete that cannot grow without nearly 10% NaCl in the medium, or *Mrakia psychrophila*, a cold-loving basidiomycetous yeast that cannot grow at temperatures above 20 °C and some other similar species.

However, a more typical example of fungi from extreme environments is *Hortaea werneckii*, a black yeast that can grow in almost saturated NaCl solutions, but also thrives in normal mycological media without NaCl. It is easy to imagine how a broad ecological amplitude of extremotolerant species could increase their chances of dispersal and survival. However, while extremotolerant species can grow under temperate conditions in the laboratory, some of them are never found under such conditions in nature. These species can be classified as habitat specialists, along with true, obligate extremophiles. Many are specialised to a single (e.g. high salinity) or a range of similar extremes (e.g. high salinity and aridity). Their distribution in nature is usually limited, but they occur in some of the most extreme conditions on the planet.

More adaptable than extremophilic and extremotolerant specialists are extremotolerant generalists. These can tolerate a number of different extreme conditions, even if they are not the most successful in any of them. They are often ubiquitous and nutritionally versatile, easy to cultivate and difficult to outcompete: the fungal equivalent of weeds. A typical example is the black

yeast *Aureobasidium pullulans*, a species found in an exceptionally wide range of habitats: from house dust to polar glaciers. Based on their adaptability, versatile metabolism and good antagonistic activity, extremotolerant generalists often have a range of biotechnological applications, *A. pullulans* being a prime example.

From glaciers to dishwashers

Fungi have been found in hypersaline lakes and seas around the world, as well as in the brine of man-made salt pans. They share this habitat with another well-known eukaryotic halophile, the unicellular alga *Dunaliella salina*. Their diversity and abundance depend on climatic conditions, the amount of nutrients and, especially, the composition of the brine. Thalassohaline waters are concentrated seawater. Athalassohaline waters are distinctly different from sea water and their composition is not necessarily dominated by Na^+ . These waters often contain high concentrations of other cations, such as Mg^{2+} , Ca^{2+} , Li^+ , depending on local geology. High concentration of Mg^{2+} is particularly challenging, but the tolerance of some fungi to magnesium salts exceeds even that of prokaryotes.

As a consequence of high concentrations of salts (or other solutes), water activity (availability) is reduced. In this regard, salt brines represent similar challenges to microbial life as deserts. Some fungi are able to grow very slowly within rocks using tiny amounts of nutrients and water. Deserts and arid land soils with no plant growth are often occupied by cryptogamic or biological soil crusts. These are aggregations of microbes, including fungi, that form a crust-like structure usually on the first few centimetres of soil. Mature biocrusts are structured by lichen fungi that grow on the soil surface and host a community of microbes with photosynthetic cyanobacteria or sometimes eukaryotic algae. Together, these microbes form a crust-like layer bound by sticky extracellular polymeric substance (EPS), which reduces soil erosion. Fungi, including melanised black yeasts, can thrive in these environments, but their specific ecological functions are still the subject of active research.

Not all deserts are in hot climates. Some of the driest places on earth are in Antarctica, where the lack of water is accompanied by extremely low temperatures, but still supports local fungal life. Fungi are also found in permafrost and glacial ice on both poles as well as in the cold waters of the deep sea. The extent to which these environments support active fungal growth, rather than maintaining cells in a metabolically inactive state, is unclear. At least in some habitats, such as the ice at the bottom of polythermal glaciers, some filamentous fungi and yeasts appear to form active communities with a very different diversity and higher abundance than those deposited as precipitation on glaciers.

In general, fungi are better at handling low than high temperatures. In fact, high temperature is the only extreme where prokaryotes clearly outperform fungi: the current growth limit for archaea is 123 °C and for fungi just above 60 °C. Fungal preference for low temperatures might be linked to their diversification and terrestrialisation during the periods of “Snowball Earth”.

Most fungi prefer a slightly acidic pH. However, only a few species are known to colonise the most acidic environments, such as mine drainage waters and volcanic lakes. At the other end of the pH spectrum, some fungi are found in alkaline soda soils.

The remarkable adaptability of fungi to extreme environments also includes resistance to radiation. Thus, some species found at the heavily contaminated sites of the Chernobyl nuclear power plant tolerate levels of ionising radiation that exceed those found in nature by many orders of magnitude. The seemingly counterintuitive evolution of such “unnecessary” resistance can be explained as exaptation – an adaptation to a different selection pressure, but one that is also useful against radiation. As with bacteria, this actual selection pressure is believed to be desiccation, since radiation resistance strongly correlates with desiccation resistance across the tree of life.

Extremotolerant fungal generalists can sometimes cause opportunistic infections – as long as they can grow at the temperature of the human body, an insurmountable obstacle for most fungi. There is little evidence that these opportunists have evolved to overcome our immune defences. Instead, high adaptability and stress tolerance appear to be another exaptation that helps them survive (temporarily) in susceptible patients as in any other extreme environment and cope with oxidative burst of immune cells, antimicrobial compounds and other sources of stress in the body.

If our bodies have evolved to be inhospitable to potential pathogens, then our homes are unfavourable to microorganisms by design, to prevent microbial overgrowth. Limited amounts of nutrients and moisture, challenging chemicals in the form of cleaning products and synthetic materials, new types of household appliances – all of these have created extreme indoor habitats. These conditions prevent the growth of most microorganisms, but select the most resilient and nutritionally versatile. The regular exposure to extremotolerant opportunistic pathogens in household dishwashers and washing machines might represent a largely unrecognized threat to the health of immunocompromised individuals.

Far beyond salt lakes and domestic appliances, the extra-planetary occurrence of microbes has occupied human minds for decades. Some fungi from extreme environments, such as hypersaline and acidic lake Magic in Western Australia, even serve as models for survival of conditions in space. Fungi have been experimentally shown to survive for many months in the near-vacuum and radiation of space. Conditions on the planets and moons of our solar system, even those thought most likely to support life, are extreme compared to Earth, from the cold deserts of Mars to the hot and acidic Venus to the hydrogen peroxide-rich icy Europa and the alkaline, salty subsurface ocean of Enceladus. While extra-terrestrial life is likely very different from ours, Earth extremophiles may provide insight into its potential forms.

Adapted for survival

Extremophilic and extremotolerant fungi have evolved several times and are found in different parts of the fungal tree of life. Their distribution is not uniform. Despite the phylogenetic distance between these groups and the different challenges of the various extreme environments, extremotolerant and extremophilic fungi share some common adaptations. Their cells use efficient sensors and signalling pathways to trigger changes in transcription, as well as in translation, activation and degradation of proteins. Maintenance of the plasma membrane proton gradient and of the physiological concentrations of inorganic cations (especially Na^+ and K^+ , but also Mg^{2+} , Ca^{2+} , Li^+ and others) requires continuous activity of membrane transporters, particularly in hypersaline and acidic environments. These fungi accumulate protective small metabolites, either by synthesis or uptake, and use them to balance osmotic pressure and maintain turgor, prevent intracellular ice crystal formation and scavenge reactive oxygen species (ROS). The length and

saturation of fatty acids and the sterol content of membranes are adjusted to maintain proper membrane fluidity.

The cell wall is remodelled and may be in some cases supplemented with melanin and hydrophobins that alter its structural and functional properties, including permeability. Large phenotypic plasticity is often observed. Thus, species of the genus *Aureobasidium* can form an astonishing number of different morphologies, often in a single petri dish. When *A. subglaciale* is isolated from glacial ice with precipitated salts, it is completely translucent and only turns black after sub-cultivation *in vitro*.

Because large amounts of energy are expended on these processes, growth under extreme conditions is often slow. In addition, accelerated energy metabolism produces more ROS, making the cellular antioxidant machinery an important determinant of extremotolerance.

Many adaptations to extreme environments remain poorly understood. For example, it is unclear why some species code for many functionally redundant ion transporters, while other species thrive in the same environments with only one or two homologues. Experiments on the role of melanin in protecting against stress in different organisms often yield contradictory results. The relationship between the synthesis of small metabolites and their uptake – especially in multi-species communities, including lichens – remains unquantified. Thick cell walls, slow growth, pigmentation and other adaptations make extremotolerant and extremophilic species particularly difficult to study.

Future research

Future research into extremophilic fungi offers many opportunities for exciting discoveries. Some lie in deciphering the ecological functions of fungi in extreme environments. Others relate to their dispersion: some extremophilic fungi are asexual, but not all, and extreme environments are often small and fragmented. How are they colonised? How do extremophilic fungi find each other and recombine? What is the role of “stable parasexuality” – hybridisation into stable diploids in some otherwise clonal and haploid species? Can comparative genomics of extremophilic genomes provide answers to some of the above questions?

Finally, can the application of new methods such as CRISPR/Cas9 release the full biotechnological potential of extremophilic fungi, as well as help us understand the limits and limitations of life as we know it and as it might have evolved beyond our planet?

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Box Text: Record-breakers.

The thresholds that separate mesophiles from extremotolerant and extremophilic fungi are not always clearly defined. However, some of the commonly applied values and some of the record-setting fungi that far surpass them are listed below.

Psychrotolerant/psychrotrophic and psychrophilic fungi grow at or near 0 °C. Psychrophiles grow optimally below 15 °C and are unable to grow above 20 °C. *Rhodotorula frigidialcoholis* grows at -10 °C and *Pseudogymnoascus pannorum* as low as -20°C.

Thermotolerant and thermophilic fungi grow above 45-50 °C. Thermophiles are unable to grow below 20 °C. *Chaetomium thermophilum* grows up to 60 °C.

Xerotolerant and xerophilic fungi grow below a water activity of 0.85. Xerophiles cannot grow in normal mycological media. *Xeromyces bisporus* grows at water activity of 0.61 and grows optimally at 0.85, with absolute requirement for lowered water availability.

Halotolerant and halophilic fungi grow above 17% NaCl (w/v). Halophiles cannot grow in normal mycological media. Black yeast *Hortaea werneckii* and filamentous *Aspergillus sydowii* grow almost across the whole salinity range. *Wallemia ichthyophaga*, *W. muriae*, *Phialosimplex salinarum*, *Aspergillus baarnensis*, *A. salisburgensis* and *A. atacamensis* are obligate halophilic fungi that strictly require from 5 to 10% NaCl (w/v).

Acidotolerant and acidophilic fungi grow in acidic conditions (below pH 4 in some studies). Acidophiles have a growth optimum below pH 3 and are capable of growth at pH 1. Extremely acidotolerant *Neohortaea acidophila* and *Acidomyces acidophilus* grow at pH 0.6. No obligate acidophilic fungus has been described to date.

Alkalitolerant and alkaliphilic fungi grow above pH 8. Alkaliphiles have a growth optimum above pH 8-9. Studies differ substantially in the growth conditions used (particularly the use of buffer systems), with reports of *Aspergillus glaucus*, *Emericellopsis alkalina*, *Exophiala dermatitidis*, and some *Sodiomyces* spp. growing above pH 11.

Radiation resistant bacteria have 10% survival above 1 kGy of ionizing radiation, while there is no generally accepted threshold for fungi. Surprisingly, a strain of *Saccharomyces cerevisiae* was shown to have a 10% survival at 3.2 kGy of gamma radiation, with survival increasing substantially if desiccated or frozen, since both of these treatments reduce the reactive oxygen species created by radiolysis of water. Some black yeasts are also highly radiation resistant, with desiccated *Cryomyces minteri* 10% survival measured at 23.5 kGy.

Box Text: Extremes in our kitchens.

Extreme environments are often seen as exotic and distant, but for microorganisms, extreme conditions are found in much more ordinary places. Extreme environments indoors are no accident – we deliberately maintain conditions that are unfriendly to microorganisms to prevent them from overgrowing our homes. This works well for most species, but the most resilient find such conditions quite inviting. We encounter these fungi every day. They live in our dishwashers, washing machines and refrigerators, in kitchen sinks and between bathroom tiles. Most of them are harmless, but some are known to be rare opportunistic pathogens of susceptible individuals.

Box Text: **Sprints or marathons?**

Many studies of extremophiles focus on cellular responses to environmental shocks, for example by transferring cells from a normal medium to one with high salinity. Such sudden changes often do not reflect conditions in nature. Desiccation, evaporation of sea water, sharp temperature changes and most other conditions do not usually occur within a few seconds, but take hours or days, sometimes even weeks. Such gradual changes allow for slow adaptation with anticipatory stress responses that prepare the cells for further deterioration of conditions. A sudden increase in water activity due to precipitation and an increase in temperature and radiation due to sunlight are among the very few rapid changes that microorganisms experience often. Therefore, in most cases studies of adaptation to long-term extreme conditions (and not sudden shocks) should be a priority, even if they are methodologically less convenient. The difference is not only academic but may have a crucial impact on our understanding of extremophilic lifestyles. The available data show that cellular responses to shocks can be fundamentally different from the responses required for constant growth under extreme conditions.

Figures

Figure 1: Examples of extreme environments (left to right): hypersaline water rich in microbial pigments, glacial ice breaking into the sea, desert soil crust.

Figure 2: Pure cultures of selected extremophilic fungi on malt extract agar, incubated at 24 °C for ten days (top and middle rows) and *Aureobasidium subglaciale* for 25 days (bottom row, illustrating the great phenotypic plasticity of the species).

Figure 3: Schematic summary of the most common adaptations to extreme environments (cellular components are not drawn to scale).





