Limits of life in hostile environments: no barriers to biosphere function?

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Summary

Environments that are hostile to life are characterized by reduced microbial activity which results in poor soil- and plant-health, low biomass and biodiversity, and feeble ecosystem development. Whereas the functional biosphere may primarily be constrained by water activity (a_w) the mechanism(s) by which this occurs have not been fully elucidated. Remarkably we found that, for diverse species of xerophilic fungi at a_w values of ≤ 0.72, water activity per se did not limit cellular function. We provide evidence that chaotropic activity determined their biotic window, and obtained mycelial growth at water activities as low as 0.647 (below that recorded for any microbial species) by addition of compounds that reduced the net chaotropicity. Unexpectedly we found that some fungi grew optimally under chaotropic conditions, providing evidence for a previously uncharacterized class of extremophilic microbes. Further studies to elucidate the way in which solute activities interact to determine the limits of life may lead to enhanced biotechnological processes, and increased productivity of agricultural and natural ecosystems in arid and semiarid regions.

Introduction

Xerophilic fungi are more tolerant to water stress than any other organism; mycelial growth of one species has been previously recorded down to a water activity of 0.656 (Pitt and Christian, 1968). Terrestrial fungi play key roles in the degradation of organic matter and global nutrient cycles, the formation and structure of soils and geological deposits, and via their symbiotic interactions with plants (Ruiz and Azcon, 1995; Gunde-Cimerman et al., 2000; Jeffries et al., 2003; Hoffland et al., 2004; van der Heijden et al., 2008). Furthermore, the substantial fungal biomass of soils in semiarid regions (Smith et al., 1992; Rutz and Kieft, 2004) can act as nutrient and water reservoirs in water-constrained ecosystems (Ruiz and Azcon, 1995; Austin et al., 2004; Kashangura et al., 2006; Collins et al., 2008). Xerophilic microbes have historically been isolated and characterized in the context of food-spoilage studies (Pitt, 1975), but they exist in nature at an indefinite number of biosphere–environment interfaces where life is challenged by physical and chemical barriers. Xerophilic fungi are therefore useful model systems to investigate the feasibility of cellular activity in arid and stressful habitats (Onofri et al., 2004; Beatty and Buxbaum, 2006; Tosca et al., 2008). Recent studies carried out on halophilic prokaryotes and mesophilic bacterial and yeast species from hostile environments found that chaotropicity (or related solute activities) can limit microbial metabolism, replication and survival (Hallsworth et al., 2003a; 2007; Duda et al., 2004; Lo Nostro et al., 2005). For both ionic and non-ionic solutes, neither chaotropic activities nor Hofmeister effects is a colligative property of a solution (see Dixit et al., 2002; Ball, 2008); furthermore the mechanism of chaotropic activity for ions (see Sachs and Wolff, 2003), non-ionic solutes (see Hallsworth et al., 2003a), and hydrophobic substances (McCambick et al., 2009; P. Bhaganna and J.E. Hallsworth, unpublished) may differ. Nevertheless, the Hofmeister series (for ions), chaotropicity and kosmotropicity (activities of diverse chemical species) provide frameworks that can be usefully employed to study the affinities of substances to modify the structural interactions of cellular macromolecules (see Hamaguchi and Geiduschek, 1962; Collins, 1997; Hallsworth et al., 2003a; Ball, 2008). However, such solute activities have not been studied either at ultra-low water activities (≤ 0.8; see Hallsworth et al., 2007), or in xerophilic fungi. Water activity has been highly effective in providing a global measure of the cumulative molecular-level, biochemical and phenotypic effects of decreased solvent availability (see Brown, 1990; Chaplin, 2006). Nevertheless, we recently observed that other solutes activities, most notably chaotropicity that weakens macromolecular interactions and disorders cellular structures, can also limit biosphere function in specific localities (see Hallsworth et al., 2007). We therefore suspected that water activity is not a definitive parameter that dictates the limits of microbial activity in all environmental niches. We carried out this study of xerophiles, obtained from diverse sources, to test the hypothesis that water activity does not...
always act as the barrier to microbial and, by implication, biosphere function in high-solute environments. Here we show that in low water-activity environments that are hostile to life ($\leq 0.72 \text{ a}_w$), water activity per se did not limit microbial activity; and provide evidence that cellular function was determined by the net effect of other environmental parameters (including chaotropic and kosmotropic activities) that impact on macromolecule structure-function. We also identified a new class of extremophilic microbe, *chaophiles*, that may prove to be a source of novel enzymes for biotechnology.

**Results and discussion**

**Xerophilic fungi from high- and low-solute substrates**

We took a two-pronged approach to identifying the ultimate, most xerophilic, microbes. First, we sampled environments in various continents and climatic zones, focusing our search on low-solute substrates: the surfaces of glass, metal, wood, leather, textiles and paper (see Table 1; *Experimental procedures*). Remarkably we found an abundance of xerophilic fungi, and isolated 107 phenotypically distinct cultures from these low-solute environments using glycerol-supplemented (5 M glycerol; $0.845 \text{ a}_w$) or sucrose-supplemented media (2.2 M sucrose; $0.884 \text{ a}_w$), predominantly on samples originating from humid countries such as Japan, Northern Ireland and Thailand (see Table 1). Second, we identified and obtained cultures of the 37 most-xerophilic strains previously reported in the published literature (1900–2008); the majority of these had been isolated from high-solute foods (see Table 2). In addition, we contacted research groups currently working in the field of environmental microbiology and obtained cultures of fungi and yeasts that had been isolated from high-salt or high-sugar environments and/or were suspected to be highly solute-tolerant (i.e. the 14 strains from EXF and UWOPS culture collections; see Table 2). For the purposes of the current study we used multiple criteria to define xerophilicity: that a species must be able to grow below 0.85 \text{ a}_w, under at least two sets of environmental conditions, and must also grow optimally below 0.95 \text{ a}_w (see Pitt, 1975).

For all environmental isolates and the named xerophile species (157 strains in total; see Tables 1 and 2) rates of hyphal extension were determined on low water-activity media containing one of a range of chemically diverse but biologically relevant solutes (see Fig. 1A and B). Generally strains from low-solute substrates grew down to similar water activities, and at comparable growth rates, to those from high-solute environments (data not shown). The solute that facilitated the optimum growth-rate varied depending on the fungal strain, but sucrose was most permissive for the majority of strains (Fig. 1A). By contrast, glycerol facilitated growth down to the lowest water-activity for more than 75% of strains (Fig. 1B) so we used glycerol-supplemented media to test the hypothesis that the stress parameter water activity does not always limit life on low water-activity substrates.

Nine out of the 157 strains grew at $\leq 0.75 \text{ a}_w$, and these had been isolated either from low-solute surfaces during the current study (strains JH06THH; JH06GBM; JH06GBO; JH06JPD from wooden surfaces, see Table 1) or from high-solute substrates by other research groups (strains *Aspergillus penicillioides* FRR 2179; *Eurotium amstelodami* FRR 2792; and three strains of *Xeromyces bisporus*: FRR 0025; FRR 3443; FRR 2347, see Table 2). Mycelial growth rates of these strains were quantified on glycerol-supplemented media over a matrix of temperature and water-activity values (Fig. 1C–K) in order to determine the limits of their biotic windows, and to obtain two-dimensional profiles of their growth phenotypes. We then determined the pH required for optimum growth over a range of water-activity values on glycerol-supplemented media in order to avoid inadvertently causing pH limitation. There were clear phenotypic differences between strains, but growth at low water activity was generally optimal at 30°C (see Fig. 1C–K) and pH 5.75 (data not shown) so these conditions were used throughout the study. Although hyphal growth has previously been recorded at $\leq 0.710 \text{ a}_w$ (see Pitt and Christian, 1968; see later), only two strains grew on glycerol media at water-activity values significantly below 0.714 \text{ a}_w, regardless of temperature or pH (see Fig. 1J and K). The glycerol concentrations used in these media (i.e. $\leq 7.16 \text{ M}$) are consistent with the intra and/or extracellular concentrations to which microbial cells can be exposed in nature (Brown, 1990; Hallsworth and Magan, 1994a; de Jong et al., 1997; Hallsworth, 1998; Zhuge et al., 2001; Bardavid et al., 2008). However, our data as well as earlier studies suggest that glycerol has inhibitory activities at molar concentrations (see Fig. 1J and K; Borowitz and Brown, 1974; Hallsworth et al., 2007), and may act as a chaotropic stressor due to its unusual interactions with water and destabilizing effects on macromolecular structures (Borowitz and Brown, 1974; Hallsworth et al., 2007). We therefore formulated the hypothesis that solute activities other than water activity can determine the limits of microbial-cell function.

**Water activity did not limit life at low water activity**

To test this hypothesis, we designed a range of 14 low water-activity media that were supplemented with either a chaotropic solute (fructose or glycerol) or combinations of glycerol and a number of other solutes: fructose and/or the kosmotropes sucrose, glucose, NaCl and KCl (Collins,
Strains were isolated from diverse substrates during the current study. The third and fourth characters of strain designations indicate the year that sampling and isolation were carried out (i.e. 2005, 2006 or 2007).

| Strain designation | Environmental source (country) | Strain designation | Environmental source (country) |
|--------------------|--------------------------------|--------------------|--------------------------------|
| JH05GB42           | Copper pipe in 12°C constant-temperature room (UK) | JW07JP14           | Dead bamboo (Japan) |
| JH05GB43           | Copper pipe in 12°C constant-temperature room (UK) | JW07JP18           | Surface of firewood in outdoor woodpile (Japan) |
| JH06GBa            | Underside of an antique earthenware-bowl (UK)     | JW07JP20           | External wall of a wooden hut (Japan) |
| JH06GBb            | Dust on the floor of a living room (UK)           | JW07JP21           | Insect pupa (Japan) |
| JH06GbC            | Blue (Stilton) cheese (UK)                        | JW07JP25           | Surface of firewood in outdoor woodpile (Japan) |
| JH06GBB            | Stem of dried protea flower (South Africa)        | JW07JP29           | Aluminium windowsill on the outside of a building (Japan) |
| JH06GBF            | Paint work of a 1922 wooden window-frame (UK)     | JW07JP30a          | Aluminium windowsill inside a building (Japan) |
| JH06GBM            | Underside of an antique sycamore chopping-block (UK) | JW07JP30b          | Aluminium windowsill inside a building (Japan) |
| JH06GBN            | Underside of an antique sycamore chopping-block (UK) | JW07JP30c          | Aluminium windowsill inside a building (Japan) |
| JH06GBO            | Underside of an antique sycamore chopping-block (UK) | JW07JP36           | Glass surface of a window inside a building (Japan) |
| JH06GBW            | Antique felt (UK)                                | JW07JP41a          | Wooden floor (Japan) |
| JH06IL49           | Semi-dried date (Israel)                         | JW07JP41b          | Wooden floor (Japan) |
| JH06IL50           | Semi-dried date (Israel)                         | JW07JP43           | Old glass light-bulb (Japan) |
| JH06IN45           | Semi-dried tamarind pods (India)                 | JW07JP49           | Underside of a stone table – outdoors (Japan) |
| JH06IN46           | Semi-dried tamarind pods (India)                 | JW07JP51           | Surface of wooden bench – outdoors (Japan) |
| JH06IN47           | Antique wooden artefact (India)                  | JW07JP56           | Rotting wood (Japan) |
| JH06IN48           | Antique wooden artefact (India)                  | JW07JP64           | Dead tree-trunk (Japan) |
| JH06JPJ            | Antique wooden artefact (Japan)                  | JW07JP74           | Aluminium windowsill inside a building (Japan) |
| JH06JPD            | Antique wooden rice-scoop (Japan)                | JW07JP75           | Old cotton cushion-cover (Japan) |
| JH06JPE            | Inner surface of an antique bronze bell (Japan)   | JW07JP83           | Tree trunk (Japan) |
| JH06JPF            | Inner surface of an antique bronze bell (Japan)   | JW07JP95           | Surface of wooden bench – outdoors (Japan) |
| JH06JPQ            | Antique wooden rice-pot lid (Japan)              | JW07JP96           | Stone table – outdoors (Japan) |
| JH06JPS            | Antique wooden rice-pot lid (Japan)              | JW07JP96           | Underside of a wooden bench – outdoors (Japan) |
| JH06JPT            | Antique wooden rice-pot lid (Japan)              | JW07JP117a         | Internal surface of dried bamboo (Japan) |
| JH06NAV            | Stem of a wild grape (Namibia)                    | JW07JP117b         | Internal surface of dried bamboo (Japan) |
| JH06THH            | Antique wooden artefact (Thailand)               | JW07JP120a         | Antique wooden artefact (Japan) |
| JH06THI            | Antique wooden artefact (Thailand)               | JW07JP120b         | Antique wooden artefact (Japan) |
| JH06THJ            | Antique wooden artefact (Thailand)               | JW07JP160          | Antique wooden artefact (Japan) |
| JH06THK            | Antique wooden artefact (Thailand)               | JW07JP166          | Rotting bamboo (Japan) |
| JH06ZA44           | Grass basket (South Africa)                       | JW07JP167          | Rotting bamboo (Japan) |
| JH06ZA51           | Tin surface of a food can (South Africa)         | JW07JP168a         | Rotting bamboo (Japan) |
| JH06ZA52           | Tin surface of a food can (South Africa)         | JW07JP168b         | Rotting bamboo (Japan) |
| JH06ZAU            | Glass of a 1940’s picture frame (South Africa)   | JW07JP169          | Rotting bamboo (Japan) |
| JH07JP126 | Antique bronze vase (Japan)                      | JW07JP170a         | Rotting bamboo (Japan) |
| JH07JP127 | Green leaf (Japan)                               | JW07JP170b         | Rotting bamboo (Japan) |
| JH07JP128 | Old earthenware bonsai-container (Japan)         | JW07JP171a         | Rotting bamboo (Japan) |
| JH07JP130 | Green bamboo (Japan)                             | JW07JP171b         | Rotting bamboo (Japan) |
| JH07JP133 | Rotting wood (Japan)                             | JW07JP172          | Rotting bamboo (Japan) |
| JH07JP138 | Old cedarwood-container (Japan)                  | JW07JP173          | Old, dried Reiki mushroom (Japan) |
| JH07JP141 | Bamboo leaf (Japan)                              | JW07JP174          | Old, dried Reiki mushroom (Japan) |
| JH07JP143 | Green bamboo (Japan)                             | JW07JP175a         | Old, dried Reiki mushroom (Japan) |
| JH07JP144 | Leaf surface (Japan)                             | JW07JP175b         | Old, dried Reiki mushroom (Japan) |
| JH07JP146 | Dead bamboo (Japan)                              | JW07JP176          | Old, dried Reiki mushroom (Japan) |
| JH07JP148 | Rotting bamboo (Japan)                           | JW07JP177          | Old, dried Reiki mushroom (Japan) |
| JH07JP149 | Rotting bamboo (Japan)                           | JW07JP179          | Old, dried Reiki mushroom (Japan) |
| JH07JP151 | Rotting leaf (Japan)                             | JW07JP180          | Moulding surface of tree branch (Japan) |
| JH07JP154 | Wooden bathroom wall (Japan)                     | JW07JP181          | Airborne spores (Japan) |
| JH07JP156 | Wooden bathroom wall (Japan)                     | JW07JPc118         | Laboratory contaminant (Portugal) |
| JH07ZA147 | Wooden artefact (South Africa)                   | RS07PT1            | Laboratory contaminant (Portugal) |
| JW07GB158 | Antique mahogany table-top (UK)                  | RS07PT2            | Laboratory contaminant (Portugal) |
| JW07JP12    | Metal surface of an armrest on a 1970’s train (Japan) | RS07PT3            | Laboratory contaminant (Portugal) |
| JW07JP14    | Silicon floor-seal on a 1970’s train (Japan)     | RS07US5            | Soil (North America) |
| JW07JP18    | Silk toy hung on exterior of a building (Japan)   | RS07US10           | Soil (North America) |
| JW07JP13    | Insect faeces on dead bamboo (Japan)              | RS07US10           | Soil (North America) |

a. Strains were isolated on glycerol-supplemented and sucrose-supplemented MYPiA medium; see Experimental procedures. Strains RS07PT1, RS07PT2, RS07PT3, RS07US5 and RS07US10 were isolated by Ricardo dos Santos, Laboratório de Análises of the Instituto Superior Técnico, Portugal. Entries in bold correspond to strains selected for more detailed study (see Figs 1C–K, 2 and 3A).

b. The third and fourth characters of strain designations indicate the year that sampling and isolation were carried out (i.e. 2005, 2006 or 2007).
| Species                        | Strain designation | Environmental source (country) | Relevant reference(s) |
|-------------------------------|--------------------|-------------------------------|-----------------------|
| Aspergillus glaucus           | IMI 053242         | Microscope objective (Sri Lanka) | Fennell and Raper (1955) |
| Aspergillus nidulans var. echinulatus | CBS 120.55; IMI 061454 | Not stated (Argentina) | Fennell and Raper (1955) |
| Aspergillus penicillioides     | ATTC 14567; FRR 3722 | Binocular lens (Australia) | Gock et al. (2003) |
| Aspergillus penicillioides     | ATTC 16910; FRR 3722 | Human lobomycosis (Australia) | Gock et al. (2003) |
| Aspergillus penicillioides     | FRR 2179           | Dried chillies (Australia)    |                      |
| Aspergillus penicillioides     | FRR 3795           | Audio tape (Australia)        |                      |
| Aspergillus wentii            | CBS 104.07; IMI 017295 | Soybeans (Indonesia) |                      |
| Basipetospora chlamdospora    | IMI 332258         | Soil (Chile)                  |                      |
| Brettanomyces bruxellensis    | UWOPS 94-239.3     | Tequila fermentation (Mexico) |                      |
| Candida apicola               | UWOPS 01-663       | Merremia tuberosa flower (Costa Rica) |                      |
| Candida berthetii             | ATCC 18808; CBS 5452 | Arabic gum (Cameroon) | Boidin et al. (1963) |
| Candida etchellsii           | UWOPS 01-168.3     | Bee hive (Costa Rica)        |                      |
| Candida hawaiiana            | UWOPS 04-206.8     | Proceros c. bifer (Malaysia)  |                      |
| Chrysosporium fastidium      | ATTC 18053; FRR 0077 | Improperly sundried prunes (Australia) | Hocking and Pitt (1980); Pitt and Hocking (1977) |
| Chrysosporium xerophilium    | ATTC 18052; FRR 0530 | High-moisture prunes (Australia) | Kinderlerer (1995) |
| Cladosporium sphaerospermum  | EXF 738            | Bathroom (Slovenia)           | Zalar et al. (2007) |
| Debaryomyces hansenii        | DSMZ 7090          | Spoilt sake (Chile)           |                      |
| Debaryomyces melissophilus   | UWOPS 01-677       | Conotelus nitidulid beetle from Merremia tuberosa flower (Costa Rica) |                      |
| Eurotium amstelodami         | ATTC 16465; FRR 0153 | Dates (Australia)             | Tamura et al. (1999) |
| Eurotium chevalieri          | ATCC 28248; FRR 0471 | Dates (Australia)             | Tamura et al. (1999) |
| Eurotium echinulatum         | ATCC 62930; FRR 2471 | Dates (Australia)             | Tamura et al. (1999) |
| Eurotium halophilicum        | ATCC 16465; FRR 2792 | Dates (Australia)             | Tamura et al. (1999) |
| Eurotium herbariorum         | ATCC 16465; FRR 5004 | Dates (Australia)             | Tamura et al. (1999) |
| Hortaea werneckii            | EXF 225            | Hypersaline saltern (Slovenia) |                      |
| Kodamaea ohmeri               | UWOPS 05-228.2     | Beetle, Bertam Palm (Malaysia) |                      |
| Pichia sydowiorum            | UWOPS 03-414.2     | Nectar, Bertam Palm (Malaysia) |                      |
| Polypaecilum pisce           | FRR 2732; IMI 288726 | Dried fish (Indonesia)       |                      |
| Saccharomyces cerevisae      | CCY 21-4-13        | Spoilt sake (Chile)           |                      |
| Saccharomyces ludwigii       | UWOPS 92-218.4     | Tequila fermentation (Mexico) |                      |
| Starmerella bombicola        | UWOPS 01-123.1     | Bee from Ipomoea trifida (Costa Rica) |                      |
| Wallemia ichthyophaga        | CBS 818.96         | Sunflower seed (Sweden)       | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Wallemia muriae              | MZKI B-952         | Hypersaline saltern (Slovenia) | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Wallemia sebi                | EXF 994            | Dead Sea (Israel)             | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Wallemia sebi                | EF 994             | Dead Sea (Israel)             | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Wallemia sebi                | FRR 3443           | Raisins (Australia)           | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Wallemia sebi                | IMI 317902         | Chinese dates (Australia)     | Vaupotic and Plemenitas (2007); Zalar et al. (2005) |
| Xeromyces bisporus           | ATCC 9656; FRR 1522 | Spoilt liquorice (Australia)  | Hocking and Pitt (1980); Pitt and Hocking (1977) |
| X. bisporus                  | ATCC 9656; FRR 3699 | Table wine (Australia)        | Hocking and Pitt (1980); Pitt and Hocking (1977) |
| X. bisporus                  | ATCC 9594; FRB 3699 | Table wine (Australia)        | Hocking and Pitt (1980); Pitt and Hocking (1977) |
| Z. rouxii                    | FRB 9594           | Maple syrup (Australia)       | Andrews and Pitt (1980) |
| Z. rouxii                    | FRB 9594           | Table wine (Australia)        | Andrews and Pitt (1980) |
| Z. rouxii                    | FRB 9594           | Table wine (Australia)        | Andrews and Pitt (1980) |

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Fig. 1. Stress tolerance of xerophilic fungi to (A and B) single stressors and (C–I) temperature : water-activity regimes. Proportion of the 157 fungal strains tested (see Tables 1 and 2) that (A) grew optimally and (B) grew to their water-activity minimum on media containing either no added solute (control) or those supplemented with ethanol, NaCl, ethylene glycol, glycerol, MgCl₂, fructose, sucrose or PEG 400. For each medium type, fungi were grown over a range of concentrations from zero (control media) to the concentration limit that prevented growth (data not shown). For three fungal strains growth-rate data obtained from single-stressor screens were plotted according to the chaotropic or kosmotropic activity of media (see later). For C–K: growth profiles for the nine most xerophilic fungi incubated at 15, 20, 25, 30 and 37°C on glycerol-supplemented media [water-activity values ranged from 0.810 to 0.653; isopleth contours indicate growth rates (mm day⁻¹)] and were plotted using Sigmaplot, Version 8.0. The fungal strains were (C) JH06THH, (D) JH06GBM, (E) JH06GBO, (F) JH06JPD, (G) Aspergillus penicilloides FRR 2179, (H) Eurotium amstelodami FRR 2792, (I) Xeromyces bisporus FRR 0025, (J) X. bisporus FRR 3443 and (K) X. bisporus FRR 2347 (see Tables 1 and 2).

A number of recent studies provide evidence that some ions can penetrate the hydrophobic domains of macromolecular systems by shedding their hydration water and that, via their physical bulk, they disorder the tertiary/quaternary structure (see Sachs and Woolf, 2003), i.e. that – by our earlier definition – they act chaotropically. However, NaCl and KCl that were used to depress water activity in Media 7, 9 and 12 are kosmotropic (i.e. solutions of their ions have a net kosmotropic activity; see Table 3), and this is consistent with their stabilizing effects on membranes, proteins and other cellular structures (see Brown, 1990). The range of water-activity values tested (0.760–0.644 aw) lies at the extreme edge of the water-activity window for all nine xerophile strains under study, and growth optima at 30°C lay between 0.95 and 0.85, as shown for one X. bisporus strain in Fig. 2J. Remarkably there was no correlation between rates of radial extension for these nine fungi (the most xerophilic microbes thus far identified) on glycerol-containing media at ≤ 0.72 aw, and the water activity of their culture media (Fig. 2A–I). Generally, on glycerol-supplemented media at ≤ 0.85 aw, the growth rates of all strains decreased in proportion to medium water activity (e.g. see Fig. 2J). On the glycerol-supplemented medium at 0.644 aw and the fructose-supplemented medium at 0.760 aw (despite the relatively high water activity of the latter; see Table 3) there was no hyphal growth of any xerophile strain (therefore data are not shown for Media 13 or 14 in Fig. 2). Paradoxically, for Medium 1 (at 0.714 aw) eight out of the nine fungal strains either failed to grow (Fig. 2A–D) or grew 65–90% more slowly than predicted (see Fig. 2E–G and I), whereas at lower water-activities (0.670–0.647) growth rates were up to 580% greater than predicted (i.e. the mixed-solute Media 6–9 and 12; see Fig. 2A–I).

The lowest water activity previously reported for sustained growth of fungi was 0.656: for X. bisporus after a 90 day incubation period (Pitt and Christian, 1968). By comparison several fungal strains grew in the current study at 0.656 aw, and hyphal growth was observed at this water activity for one strain after only 11 days (Fig. 2F; Table 4). Remarkably we observed growth at water-activity values as low as 0.647, and did so in as little as 5–8 weeks, on mixed-solute media (see Fig. 2A–I; Table 4). Furthermore, four out of the five strains that were able to grow at 0.647 aw had been isolated from low-solute surfaces (in the current study; see Table 1) and were therefore more xerophilic than all but one of the strains

Table 3. Chaotropic-activity and water-activity values for solutes and solute combinations used to supplement growth media.*

| Medium designation* | Added solute(s); concentration [M] | Water activity² | Chaotropic activity (kJ kg⁻¹)² |
|---------------------|------------------------------------|----------------|-------------------------------|
| 1                   | Glycerol 0.84 | NaCl 0 | KCl 0 | Fructose 0 | Glucose 0 | Sucrose 0 | Highly chaotropic (15.27) | 0.714 |
| 2                   | 7.06 | 0 | 0 | 0 | 0 | 0 | Highly chaotropic (16.84) | 0.702 |
| 3                   | 5.34 | 0 | 0 | 0 | 0 | 0.73 | Relatively neutral (12.48)² | 0.699 |
| 4                   | 7.48 | 0 | 0 | 0 | 0 | 0 | Highly chaotropic (18.05) | 0.686 |
| 5                   | 7.48 | 0 | 0 | 0 | 0 | 0 | Highly chaotropic (18.05) | 0.685 |
| 6                   | 5.97 | 0 | 0 | 0 | 0 | 0.73 | Relatively neutral (11.11)² | 0.670 |
| 7                   | 3.91 | 1.20 | 0.13 | 0 | 0 | 0.73 | Relatively neutral (11.11)² | 0.670 |
| 8                   | 4.34 | 0 | 0 | 1.11 | 1.11 | 0 | Relatively neutral (9.73)² | 0.665 |
| 9                   | 4.67 | 1.20 | 0.13 | 0 | 0 | 0.73 | Relatively neutral (12.75)² | 0.656 |
| 10                  | 7.60 | 0 | 0 | 0 | 0 | 0.73 | Highly chaotropic (28.80) | 0.655 |
| 11                  | 7.60 | 0 | 0 | 0 | 0 | 0 | Highly chaotropic (20.80) | 0.653 |
| 12                  | 6.19 | 1.20 | 0.13 | 0 | 0 | 0 | Relatively neutral (2.79)² | 0.647 |
| 13                  | 7.65 | 0 | 0 | 0 | 0 | 0 | Highly chaotropic (20.88) | 0.644 |
| 14                  | 0 | 0 | 0 | 4.80 | 0 | 0 | Highly chaotropic (20.80) | 0.760 |

a. See Fig. 2A–I. The pH of all media was 5.75, except for Medium 4 (pH 4).
b. See Hallsworth and colleagues (2003a).
c. Measured at 30°C.
d. Extrapolated from agar gel-point curve.
e. Media were slightly kosmotropic so the activity value is negative.

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isolated from high-solute environments during the past 100 years (see Fig. 2A–I; Table 4). One fungal strain, isolated in 2006 from the wooden (sycamore) surface of a 19th Century, kitchen chopping-block (JH06NIM; see Table 1), was observed to be growing at 0.647 aw after only 34 days incubation at 30°C (Fig. 2B; Table 4).

For a given fungal species, the lower water-activity limit for the germination of propagules is typically lower than that for hyphal growth (Pitt, 1975). However, numerous studies of spore germination of xerophiles at low water-activities (see Table S1) have found that growth ceases upon germ-tube production (Pitt and Christian, 1968). Whereas a number of reviews cite germ-tube formation by *X. bisporus* spores at 0.605 aw as evidence of cellular function at ultra-low water activity (Pitt, 1975; Grant, 2004; see also Table S1), further hyphal growth and mycelium development were not recorded (Pitt and Christian, 1968). In the current study hyphal growth of *A. penicillioides* and *E. amstelodami* occurred at considerably lower water-activity values (0.647 and 0.656 aw on mixed-solute media; see Fig. 2E and F; Table 4) than those previously reported for germination (i.e. 0.680 and 0.703 respectively; see Table S1). For each xerophile strain at water-activity values below their growth optimum, growth rates were proportionally reduced (for an example, see Fig. 2J). However, at extremely low water activity (= 0.72 aw), growth rates were no longer proportional to water activity so we concluded that other stress parameters limited cellular activity. Furthermore, we asked the scientific questions whether the chaotropicity of glycerol-supplemented media (6.84–7.65 M glycerol) limited hyphal growth at low water activity, and whether this inhibition was reversed by the kosmotropic activity of other substances present in the mixed-solute media (3.91–6.19 M glycerol).

**Chaotropic compounds limited cell function, but their effects were reversible**

The fructose-supplemented medium and the seven glycerol-supplemented media to have chaotropic-activity values of 15–21 kJ kg solution\(^{-1}\) (Table 3); values that are consistent with the chaotropicity limits for other microbial species (see Hallsworth et al., 2007). However, the activity values of the six mixed-solute media that contained kosmotropes were relatively neutral (12.48 to –2.75 kJ kg solution\(^{-1}\); see Table 3). Generally there were either low rates of radial extension on chaotropic media, or no growth at all (for glycerol-only media see Fig. 2A–I, orange columns; for fructose-only media data not shown). By contrast, remarkably high growth-rate values were obtained on the other media that were neutral or mildly chaotropic, and these were several hundred per cent higher than those predicted from water-activity values (see Fig. 2A–I, black columns). There was a strong inverse correlation between chaotropic activity and fungal growth (see Figs 2A–I and 3A): three strains that were able to grow down to 0.647 aw did not grow on any glycerol-supplemented media even at the relatively less-stressful water activity of 0.714 (Fig. 2A–C).

Glycerol, which is neutral or only weakly chaotropic below concentrations of 3–4 M (F.D.L. Alves and J.E. Hallsworth, unpublished), is widely known for its activities as a stress protectant that can both protect the structure and function of cellular macromolecules, and act as an intracellular osmolyte to control cell turgor (Brown, 1990; Dashnau et al., 2006). At higher concentrations (≥ 6 M), however, we have demonstrated the extreme chaotropicity of glycerol (Table 3). On high-solute substrates xerophile cells can fail due to the prohibitive energy expenditure required to retain the intracellular glycerol that is needed as an osmolyte (Hocking, 1993). We propose that glycerol itself can disorder and permeabilize the plasma membrane, via its chaotropic activity, thereby resulting in the leakage of this protectant from the cell.

Whereas the biochemical mechanisms by which chaotropic solutes disorder cellular macromolecules are not yet fully understood (see above), we have illustrated the structural consequences for macromolecular systems in a cell stressed by a chaotropic solute, and the way in which kosmotropic solutes counter cha trope-induced stress (Fig. 3B–E). For a cell growing under optimal conditions...
There are several classes of solute-tolerant microbe:

- NaCl (3.59 M, 0.812 aw), (III) sucrose (2.34 M, 0.831 aw), (IV) PEG 400 (1.25 M, 0.855 aw), (V) glycerol (4.90 M, 0.828 aw), (VI) fructose

- (VII) fructose (3.94 M, 0.804 aw), (VIII) fructose (4.36 M, 0.791 aw), (IX) glycerol (6.66 M, 0.747 aw), (X) ammonium nitrate

- (XI) ammonium nitrate (5.15 M, 0.817 aw). Values are means of three replicates and bars represent standard errors.

The data approximate to a Normal distribution (see dotted line), although it may be that the osmotic stress or other stress parameters associated with kosmotropic stressors ultimately limit hyphal growth. Diagrammatic illustrations (B–E) of the way in which chaotropic and kosmotropic activities impact on macromolecule and membrane structure in relation to an unstressed cell (B); in a chaotrope (e.g. urea)-stressed cell (C), a kosmotrope (e.g. sucrose)-stressed cell (D), and a cell exposed to both chaotropes and kosmotropes (E).

**Fig. 3.** Growth rates of three representative xerophilic fungi (A): *Xeromyces bisporus* FRR 3443, *Eurotium amstelodami* FRR 2792, and isolate JH06THAJ (see Tables 1 and 2) in relation to chaotropic and kosmotropic activities of culture media: (I) NaCl (4.28 M, 0.775 aw), (II) NaCl (3.59 M, 0.812 aw), (III) sucrose (2.34 M, 0.831 aw), (IV) PEG 400 (1.25 M, 0.855 aw), (V) glycerol (4.90 M, 0.828 aw), (VI) fructose (3.51 M, 0.829 aw), (VII) fructose (3.94 M, 0.804 aw), (VIII) fructose (4.36 M, 0.791 aw), (IX) glycerol (6.66 M, 0.747 aw), (X) ammonium nitrate (4.30 M, 0.855 aw) and (XI) ammonium nitrate (5.15 M, 0.817 aw). Values are means of three replicates and bars represent standard errors.

In summary, the data presented here (Figs 1, 2 and 3A; Table 3) support the hypothesis that the chaotropic activity of glycerol, not the stress parameter water activity, limits cell metabolism for these xerophilic fungi at ≤ 0.72 aw. This is consistent with evidence from other reports that chaotropicity limits microbial function (Hallsworth, 1998; 2003a; Duda et al., 2004; Lo Nostro et al., 2005), with a study showing that compatible solutes can reduce ethanol stress in conidia of *Aspergillus nidulans* (Hallsworth et al., 2003b), and with our recent studies of halophilic prokaryotes which demonstrated that the macromolecule-structuring activities of kosmotropic salts can reduce or reverse the inhibitory effects of chaotropic salts (Hallsworth et al., 2007).

**Implications and conclusions**

We already have an understanding of environmentally relevant solute stresses [osmotic stress (Dutrochet, 1826), matric stress (Griffin, 1977) and chaotrope-induced water stress (Hallsworth et al., 2003a)]; how chaotropic agents determine the limits of macromolecule function (see Hallsworth et al., 2003a; 2007; Duda et al., 2004); indications of the cellular components that fail...
Table 4. Fungal strains capable of hyphal growth ≤ 0.71 water activity. a

| Species and/or strain designation | Nature of substrate of origin | Lowest recorded water activity for hyphal growth (1) | Earliest observation of hyphal growth (day) | Rate of hyphal extension (mm day−1) | Method used to reduce water activity (reference) | Chaotropic or kosmotropic activity of culture medium (kJ kg−1) |
|----------------------------------|-------------------------------|-----------------------------------------------|------------------------------------------|-----------------------------------|-----------------------------------------------|--------------------------------------------------|
| JH06GBM                         | L-S                           | 0.647                                         | 34                                       | 0.05                             | Glycerol (6.19 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (2.79)                           |
| Aspergillus penicillioides FRR 2179 | H-S                           | 0.647                                         | 46                                       | 0.13                             | Glycerol (6.19 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (2.79)                           |
| JH06THH                         | L-S                           | 0.647                                         | 46                                       | 0.06                             | Glycerol (6.19 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (2.79)                           |
| JH06GBO                         | L-S                           | 0.647                                         | 60                                       | 0.06                             | Glycerol (6.19 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (2.79)                           |
| JH06THJ                         | L-S                           | 0.647                                         | 60                                       | 0.03                             | Glycerol (6.19 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (2.79)                           |
| Xeromyces bisporus FRR 3443     | H-S                           | 0.653                                         | 41                                       | 0.03                             | Glycerol (7.60 M) | Highly chaotropic (20.80)                                 |
| X. bisporus FRR 2347            | H-S                           | 0.653                                         | 41                                       | 0.01                             | Glycerol (7.60 M) | Highly chaotropic (20.80)                                 |
| X. bisporus FRR 1522            | H-S                           | 0.653                                         | 41                                       | 0.01                             | Glycerol (7.60 M) | Highly chaotropic (20.80)                                 |
| Eurotium amstelodami FRR 2792   | H-S                           | 0.656                                         | 11                                       | 0.12                             | Glycerol (4.67 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| X. bisporus FRR 0025            | H-S                           | 0.656                                         | 22                                       | 0.39                             | Glycerol (4.67 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| A. penicillioides FRR 3722      | H-S                           | 0.656                                         | 29                                       | 0.13                             | Glycerol (4.67 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| E. amstelodami FRR 0475         | H-S                           | 0.656                                         | 29                                       | 0.12                             | Glycerol (4.67 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| JH06THI                         | L-S                           | 0.656                                         | 60                                       | 0.03                             | Glycerol (4.67 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| X. bisporus                     | H-S                           | 0.656                                         | 90                                       | Not quantified                   | Thin layer of medium on a glass surface in a humidity-controlled chamber (Pitt and Christian, 1968) | Not quantified                                   |
| X. bisporus                     | H-S                           | 0.663                                         | 120                                      | Not quantified                   | Thin layer of medium on a glass surface in a humidity-controlled chamber (Pitt and Christian, 1968) | Not quantified                                   |
| JH06JPD                        | L-S                           | 0.667                                         | 11                                       | 0.10                             | Glycerol (3.91 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| JH07JP128                       | L-S                           | 0.667                                         | 94                                       | 0.02                             | Glycerol (3.91 M), sucrose (0.73 M), NaCl (1.20 M), KCl (0.13 M) | Relatively neutral (−2.75)                        |
| Eurotium halophilicum FRR 2471  | H-S                           | 0.675                                         | 38                                       | Not quantified                   | Equal weights of glucose and fructose added to growth media (Andrews and Pitt, 1987) | Not quantified                                   |
| Chrysosporium xerophilum FRR 0530 | H-S                           | 0.686                                         | 118                                      | 0.01                             | Glycerol (7.48 M) | Highly chaotropic (18.05)                                 |
| Chrysosporium fastidium         | H-S                           | 0.697                                         | 64                                       | Not quantified                   | Thin layer of medium on a glass surface in a humidity-controlled chamber (Pitt and Christian, 1968) | Not quantified                                   |
| C. xerophilum                   | H-S                           | 0.708                                         | 80                                       | Not quantified                   | Thin layer of medium on a glass surface in a humidity-controlled chamber (Pitt and Christian, 1968) | Not quantified                                   |
| Eurotium chevalieri PIL 119     | H-S                           | 0.710                                         | 16                                       | 0.1                              | A thin layer of medium enclosed in a humidity-controlled, bung-sealed glass test tube (Ayerst, 1969) | Not quantified                                   |
| E. amstelodami PIL 120          | H-S                           | 0.710                                         | 32                                       | 0.1                              | A thin layer of medium enclosed in a humidity-controlled, bung-sealed glass test tube (Ayerst, 1969) | Not quantified                                   |

a. Data for the yellow-shaded entries were obtained from the current study.

b. H-S = isolated from a high-solute substrate; L-S = isolated from a low-solute surface.

c. Compiled using data from the current study and from published xerophile studies; refs. Pitt and Christian (1968); Ayerst (1969); Andrews and Pitt (1987).

d. Data were obtained from the current study unless otherwise stated.

e. The culture medium was MYPA (pH 5.75, 30°C); see Experimental procedures.

f. N.B. Media were slightly kosmotropic so the activity value is negative.

g. The culture medium was Czapek Invert Malic Agar (pH 3.8, 25°C).

h. The culture medium was Yeast Nitrogen Base + 2% glucose w/v + 2% agar w/v.

i. The culture medium was Malt Extract Agar; MEA (30–40°C).

j. The culture medium was MEA (24–30°C).
under extreme forms of stress (see current study; Hocking, 1993; Ferrer et al., 2003; and other factors that determine the limits of microbial function in hostile environments (see Fig. 4; Pitt, 1975; Golyshina et al., 2006; Hallsworth et al., 2007; Marris, 2008). The current study illustrates how hitherto unidentified stress parameters can limit microbial cell function under certain environmental conditions, and may thereby constrain the biosphere in specific locations. Further work is needed to identify and characterize the stress mechanisms that act as failure points for ecosystems in hostile environments (Fig. 4). Many informative studies of the geochemical composition of extreme environments have already been carried out, including those of other planets (Mustard et al., 2008). Although chaotropicity has been shown to limit the functional biosphere in specific locations on Earth (Hallsworth et al., 2007), this stress parameter has not yet been factored into the mathematical models used to predict the feasibility of life in as-yet-unexplored environments on Earth or other planetary bodies (Beaty and Buxbaum, 2006; Marion and Kargel, 2008; Mustard et al., 2008; Tosca et al., 2008). We believe that chaotropicity should be accounted for in future models that aim to predict what types of environment can potentially support cellular activity (Fig. 4).

Global climate change, and changes in land-use, have accelerated the expansion of biologically hostile arid and semiarid regions over the past 30 years (Thomas et al., 2005; Seager et al., 2007). Polluted environments also represent a challenge to microbes that are exposed to the chaotropic activities of xenobiotics (Hallsworth et al., 2003a). Microbes in other natural habitats, as well as substrates used in industrial processes, may also be subjected to low water-activity conditions and/or high concen-

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tations of chaotropic stressors such as formamide, ethanol, urea, ethylene glycol, butanol, NH$_4$NO$_3$, glycerol, phenol, MgCl$_2$, CaCl$_2$ and sodium benzoate (Brown, 1990; Hallsworth, 1998; 2003a; 2007; Bardavid et al., 2008). For both low water-activity and highly chaotropic substrates, analysis of the ways in which these (and other) stress parameters interact to limit biological activity can shed light on how to optimize or eliminate microbial activity, as required. Chaotropes and high temperatures disorder cellular structures, whereas kosmotropes and low temperatures have a stabilizing/ordering effect (Hamaguchi and Geiduschek, 1962; Collins, 1997; Hallsworth et al., 2003a) and we utilized the counteracting solute activities of glycerol and kosmotropic substances in order to extend the biotic window for xerophile growth at low water activity (Fig. 2A–I; Table 4). It may be that microbial cells in laboratory culture could, and that cells in nature do, function below the water-activity limit of 0.647 established in the present study (Fig. 2), and that we have not yet understood how to manipulate solute activities sufficiently well to maintain macromolecular function under high-solute conditions. Furthermore, the vast majority of microbes cannot be cultivated in vitro (Whitman et al., 1998; Rutz and Kieft, 2004; Ward and Fraser, 2005) and some of these may remain elusive as long as the physicochemical parameters that determine their biotic windows for growth are poorly understood. We propose that manipulation of solute activities will facilitate the cultivation and study of numerous microbial species that can currently only be detected in situ (using metagenomic techniques).

Knowledge-based approaches to manipulating environmental conditions could lead to strategies for the regeneration of desertified regions (see Kashangura et al., 2006), and for providing both food and biofuels to support human population whilst maintaining a sustainable biosphere. Diverse approaches based on manipulation of environmental conditions or stress parameters have already given rise to quantum improvements in the growth windows of mesophilic species (see Hallsworth and Magan, 1994b; 1995; Thomas et al., 1994; Hallsworth et al., 2007); there is evidence that kosmotropic substances reduce ethanol stress in yeast (see Hallsworth, 1998), and that kosmotropic ions can increase Halobacterium activity under chaotropic conditions (see Oren, 1983; Hallsworth et al., 2007). The bioremediation of chaotrope-polluted soils (Hallsworth et al., 2003a) may be most efficient at temperatures low enough to minimize the chaotrope-induced disordering of cellular structures. The effectiveness and efficiency of products and processes such as biocides, food preservatives (e.g. sodium benzoate), food and drinks fermentations, bioalcohol production from microbes (Hallsworth, 1998), and industrial biocatalysis in solvent systems, which utilize or generate chaotropic solutes could be enhanced via manipulation of solute activities. Further studies are needed so that more effective interventions can be made based on exploitation of phenotypic plasticity, employing recombinant technologies and/or systems biology approaches to obtaining stress-resistant cells (see Fig. 4).

Xerophilic fungi have most commonly been isolated from kosmotrope-containing substrates (Pitt, 1975), so chaotolerant species and/or their enzymes may have potential for diverse applications. One focus of our ongoing studies is to identify novel stress parameters that prevent life processes: it may be possible to further enhance microbial function, and ecosystem development, in hostile environments once the stress biology of microbes has been more completely elucidated.

Experimental procedures

Sampling strategies and environmental isolates

Fungi were isolated from diverse environments (see Table 1) using sterile cotton-tip swabs and inoculated onto slants of Malt-Extract, Yeast-Extract Phosphate Agar [MYPiA; 1% Malt-Extract w/v (Oxoid, UK), 1% Yeast-Extract w/v (Oxoid, UK), 1.5% Agar w/v (Acros, USA), 0.1% K$_2$HPO$_4$ w/v] supplemented with 5 M glycerol (0.845 a$_w$) in 1.8 ml, internal-thread cryovials and transferred, once back in the laboratory, to Petri plates containing MYPiA supplemented with glycerol (5 M; 0.845 a$_w$) or sucrose (2.2 M; 0.884 a$_w$). Petri plates were incubated at temperatures between 20°C and 30°C in sealed bags (see below) and checked periodically for up to 6 months. Upon visual inspection, all isolates that had grown were subcultured onto MYPiA supplemented with 6.52 M glycerol, and incubated at 30°C.

Named xerophile species

Named xerophile species were obtained from Culture Collection of Yeasts (CCY, Bratislava, Slovakia), German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), Extremophilic Fungi Culture Collection (EXF, Ljubljana, Slovakia), Food Research Ryde (FRR, North Ryde, Australia), International Mycological Institute (IMI, Egham, UK), and University of Western Ontario, UWOPS Collection (Ontario, Canada); see Table 2. Cultures were maintained on MYPiA supplemented with either 2.78 M glucose (0.911 a$_w$) or 2.2 M sucrose (0.884 a$_w$) in sealed bags (see below) and incubated at 30°C.
Culture conditions and growth-rate determinations
Through this study, all media were sterilized in Schott bottles in a water bath (100°C, 60 min), cooled to within 7.5°C of the medium gel-point, and then poured into 9 cm vented Petri plates; inoculations were carried out using 4 mm diameter plugs from the periphery of actively growing cultures growing on MYPiA media supplemented with 5.43 M glycerol; and plates containing identical media were sealed in polyethylene bags to maintain a constant water activity (see Hallsworth et al., 2003b). Growth was assessed at periodic intervals by taking two measurements of colony diameter (in perpendicular directions), that were used to calculate rates of radial extension as described previously (Pitt and Hocking, 1977; Hallsworth and Magan, 1994a). Two-dimensional stress-tolerance profiles were plotted as described previously (Hallsworth and Magan, 1999) and mean values were plotted, and variation indicated in each display. Growth of yeast was carried out on the same media and quantified using spot tests as detailed by Albertyn and colleagues (1994).

Modification of media to investigate stress tolerance

Single-stressor solute-tolerance screen. All environmental isolates and named fungal strains were screened for stress tolerance on MYPiA media supplemented with different solutes over a range of concentrations; either ethanol (0.4–1.3 M), NaCl (2.1–3.4 M), ethylene glycol (2.0–3.4 M), glycerol (2.5–5.0 M), MgCl₂ (0.7–1.5 M), fructose (3.0–4.8 M), sucrose (1.1–2.1 M), or polyeethylene glycol (PEG) 400 (0.8–1.3 M), without addition of pH buffer, and incubated at 30°C for a period of up to 90 days. All inoculations were carried out in triplicate.

pH-tolerance study. All environmental isolates and named fungal strains were grown on MYPiA media supplemented with glycerol (at 3.8 M; 0.92 aw and 4.4 M; 0.88 aw respectively) and buffered to pH values of: 3.75, 4.5, 5.75 (citric acid; Na₃HPO₄), 6, 6.75 (MES; NaOH) and 7.5 (Hepes; NaOH; see Hallsworth and Magan, 1996). The pH of each medium was adjusted prior to autoclaving using appropriate buffers then measured postautoclave using a Mettler Toledo Seven Easy, pH-probe (Switzerland).

Temperature: water-activity growth-response study. The nine most xerophilic strains were inoculated onto MYPiA media supplemented with four different concentrations of glycerol (5.43, 6.19, 6.84 and 7.44 M) with water activity values ranging from 0.81 and 0.65 (see Fig. 1C–K) and incubated at 15, 22.5, 30 and 37.5°C. Quantification of water activity is described below.

Mixed-solute media for limits-of-cell function at low water-activity study. The nine xerophilic strains were inoculated onto 14 ultra-low water-activity media (0.714–0.644 aw), consisting of MYPiA media supplemented with combinations of glycerol, fructose, or glycerol plus kosmotropic solutes (see Fig. 2, Table 3, Fig. S1). The pH of all media was adjusted to 5.75, unless stated otherwise, using citric acid: Na₃HPO₄ buffer; following inoculation Petri plates were incubated at 30°C. For water-activity and chaotropic-activity values of Media 1–14 see Table 3; the methodologies used to obtain these values are described below.

Quantification of water activity
The water-activity values of media were measured at 30°C or, if different, at the temperature of incubation using a Novasina IC-II water-activity machine fitted with an alcohol-resistant humidity sensor and eVALC alcohol filter (Novasina, Pfäffikon, Switzerland), as described previously (Hallsworth and Nomura, 1999). This equipment was calibrated using saturated salt solutions of known water activity (Winston and Bates, 1960). Values were determined three times using replicate solutions made up on separate occasions. The variation of replicate values was within ±0.002 aw.

Determination of chaotropic activity
The chaotropic activity of solute(s) used to supplement growth media (see Table 3) was measured as a function of their ability to destabilize the polysaccharide macromolecule agar (Extra-Pure Reagent-grade agar, gel strength 600–700 g cm⁻², from Nacalai Tesque, Kyoto, Japan), and thereby lower gel-point (Hallsworth et al., 2003a). Agar was melted in distilled water, cooled to 55°C, and added to a solution of the solute or solute-mixture to be tested, also at 55°C, to give a final concentration of agar of 1.5% w/v and concentration(s) of solute(s) as used for the growth study. The agar–compound solutions were allowed to cool gradually and the gel-point temperature (± 0.3°C) was recorded using a temperature probe (Jenway, UK). The gel points determined were used to calculate the chaotropic activity of each compound in kJ kg⁻¹ (mole added compound)⁻¹, based on the fact that the heat capacity for a 1.5% agar w/v gel is 4.15 kJ kg⁻¹ °C⁻¹ (see Hallsworth et al., 2003a).

Acknowledgements
We are grateful for scientific discussions with A.N. Bell, P. Bhaganna, A.G. Maule, J.P. Quinn, D.J. Timson (Queen’s University Belfast, Northern Ireland), K.D. Collins (University of Maryland, USA), M.J. Danson (University of Bath, UK), J.L. Finney (University College London, UK), F. Franks (BioUpdate Foundation, UK), E.A. Galinski (University of Bonn, Germany), A.D. Hocking (CSIRO Division of Food Science and Technology, Australia), T.J McGenity and P. Nicholls (University of Essex, UK), A.Y. Mswaka (University of Harare, Zimbabwe), R.P. Rand (Brock University, Canada), R.J.P.R. dos Santos (Laboratório de Análises of the Instituto Superior Técnico, Portugal), and K.N. Timmis (Helmholtz Centre for Infection Research, Braunschweig, Germany); and also the Reviewers of the manuscript who offered new insights into the data. We wish to thank N. Gunde-Cimerman (University of Ljubljana, Slovenia), M.-A. Lachance, University of Western Ontario, Canada) and R.J.P.R. dos Santos (Portugal) for providing strains of yeast and fungi. Funding was received from the Department of Education and Learning (Northern Ireland), the Great Britain Sasakawa Foundation (London, UK), the Natural Environment Research Council.
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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Flow-chart to illustrate the progression of research activities during the current study.

**Table S1.** Fungal species capable of germination $\leq 0.71$ water activity.

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