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Stevenson, A., Hamill, P. G., O'Kane, C. J., Kminek, G., Rummel, J. D., Voytek, M. A., Dijksterhuis, J., & Hallsworth, J. E. (2017). *Aspergillus penicillioides* differentiation and cell division at 0.585 water activity. *Environmental Microbiology*, 19(2), 687-697. <https://doi.org/10.1111/1462-2920.13597>

Published in:
Environmental Microbiology

Document Version:
Publisher's PDF, also known as Version of record

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Aspergillus penicillioides differentiation and cell division at 0.585 water activity

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Summary

Water availability acts as the most stringent constraint for life on Earth. Thus, understanding the water relations of microbial extremophiles is imperative to our ability to increase agricultural productivity (e.g., by enhancing the processing and turnover of dead organic matter in soils of arid regions), reduce human exposure to mycotoxins in buildings and our food-supply chain, prevent the spoilage of foods/animal feeds, books, museum specimens and artworks and better control microbiology of industrial fermentations. Only a small number of microbial systems can retain activity at <0.710 water activity (*ISME J* 2015 9: 1333–1351). It has long-been considered that the most resilient of these is *Xeromyces bisporus*, which inhabits sugar-rich substrates (*Appl Environ Microbiol* 1968 16: 1853–1858). The current study focused on germination of *Aspergillus penicillioides*, a xerophile which is also able to grow under low humidity and saline conditions. Investigations of germination differed from those reported earlier: firstly, aerially borne conidia were harvested, and then used for inoculations, in their dry condition; secondly, cultures were incubated at 24°C, i.e. below optimum germination

temperature, to minimize the possibility of water loss from the substrate; thirdly, cultures remained sealed throughout the 73-day study period (microscopic examination was carried out directly 48 through the Petri plate lid); fourthly, the germination parameters determined were: rates and extent of conidial swelling, production of differentiated germination-structures and septate germlings, and subsequent development of mycelium and/or sporulation; fifthly, assessments were carried out over a range of water-activity values and time points to obtain a complete profile of the germination process. Conidia swelled, formed differentiated germination-structures and then produced septate germlings at a water-activity of just 0.585 (≡58.5% relative humidity), outside the currently understood thermodynamic window for life. Furthermore, analyses of these data suggest a theoretical water-activity minimum of 0.565 for germination of *A. penicillioides*. In relation to astrobiology, these findings have an application in understanding the limits to life in extraterrestrial environments. In light of current plans for exploration missions to Mars and other places, and the need to safeguard martian scientific sites and potential resources (including water) for future human habitation, a knowledge-based and effective policy for planetary protection is essential. As it is, Mars-bound spacecraft may frequently be contaminated with aspergilli (including *A. penicillioides*) and other organisms which, when transported to other planetary bodies, pose a contamination risk. In crafting countermeasures to offset this, it is important to know as precisely as possible the capabilities of these potential interplanetary visitors.

Introduction

A number of recent studies indicate that *Aspergillus penicillioides* is active close to the water-activity limit of Earth’s biosphere (Stevenson *et al.*, 2015a; 2015b; Stevenson *et al.*, in press-a; in press-b). This species, sometimes implicated in aspergillosis (Gupta *et al.*, 2015; Paulussen *et al.*, in press), has also been the focus of studies on production of secondary metabolites, ecology of hypersaline environments, food spoilage, damage to cultural artefacts

Received 19 September, 2016; revised 3 November, 2016; accepted 16 November, 2016. *For correspondence. E-mail: j.hallsworth@qub.ac.uk.

and coal-mine bioaerosols (Zhao *et al.*, 2014; Nazareth and Gonsalves, 2014; Okano *et al.*, 2015; Wei *et al.*, 2015; Micheluz *et al.*, 2016). *A. penicillioides* is at the same time xerophilic, osmophilic and halophilic (in relation to low water activity, high sugar- and NaCl concentrations, respectively), grows close to 0°C (and almost certainly at sub-zero temperatures, as well) and can function anaerobically (Chin *et al.*, 2010; Zhang *et al.*, 2013; Nazareth and Gonsalves, 2014; Rummel *et al.*, 2014). When growing in saline conditions, xerophilic aspergilli synthesize and accumulate molar concentrations of glycerol at low water-activity (Nazareth and Gonsalves, 2014; de Lima Alves *et al.*, 2015). Brines which existed in early Mars (and likely also present-day on Mars and other planetary bodies) have a peculiar composition and chemistry, and their exceptional high ionic strength can be the life-limiting parameter in relation to their habitability with terrestrial microbes (Tosca *et al.*, 2011; Fox-Powell *et al.*, 2016). Experiments carried out using analogue martian brines revealed that members of the Eukarya, including fungi, can be prevalent members of the communities which develop following inoculation with microbial consortia obtained from terrestrial environmental samples (Fox-Powell *et al.*, 2016). Glycerol, a biophysically and physiologically versatile stress protectant, can mitigate chaotropicity-mediated stresses caused by MgCl₂, ethanol and benzene (or other chaotropes and hydrophobes), as well as those induced by high turgor, low water-activity and other, mechanistically diverse stresses (Hallsworth *et al.*, 2003; Bhaganna *et al.*, 2010; de Lima Alves *et al.*, 2015; Bhaganna *et al.*, 2016). It is likely that the synthesis and accumulation of glycerol, the polyol of choice for osmotic adjustment in xerophilic fungi at high salt/low water-activity (de Lima Alves *et al.*, 2015), could facilitate fungal colonization of martian brines by mitigating against osmotic stress, low water-activity and ionic strength.

There are very few reports of microbial growth or metabolism at <0.710 water activity, due to the extreme thermodynamic constraints imposed by molecular crowding in the cytoplasm (Pitt, 1975; Grant, 2004; Williams and Hallsworth, 2009; Miermont *et al.*, 2013; Nakano *et al.*, 2013; Stevenson *et al.*, 2015a; 2015b; in press-a; in press-b; Lee *et al.*, in press). The most xerophilic microbe thus far identified was the sugar-tolerant xerophile *Xeromyces bisporus* (FRR 0025) (Pitt and Christian, 1968; Stevenson *et al.*, 2015a). Due to the importance and impacts of water activity on all aspects of cellular and ecosystem function, there has been an effort to detect biotic activity of microbes at very low water activity which spans a 50-year period (e.g. Pitt, 1975; Grant, 2004; Lievens *et al.*, 2015; Stevenson *et al.*, 2015b). Nevertheless, the 0.605 water-activity germination of *X. bisporus* has never been surpassed or repeated and, indeed, there have been no reliable reports of either cell division or any other kind of biotic activity

below this value (see Stevenson and Hallsworth, 2014; Stevenson *et al.*, 2015a; 2015b; in press-a). This has given rise to a maxim that this 0.605 water-activity (equivalent to 60.5% relative humidity) acts as an insurmountable thermodynamic barrier for life (Kminek *et al.*, 2010; Rummel *et al.*, 2014). Furthermore, the anomalous performance of *X. bisporus* at low water activity has found little application beyond the spoilage of high-sugar foods due to its intolerance of salts. Microbial cells are extremely sensitive to differences of water activity, even at ± 0.001 (equivalent to 0.1% relative humidity). Recent studies of several fungal xerophiles documented that high intra- and extracellular concentrations of glycerol can enhance spore germination close to the water-activity limit for life (Stevenson *et al.*, in press-a; in press-b). However, there was no empirical evidence of *A. penicillioides* germination below 0.640 water activity, so the current study was carried out to seek evidence for metabolic activity of *A. penicillioides* conidia below this value, or even below the 0.605 limit established by Pitt and Christian (1968). The specific aims were to devise a novel system, which is both permissive for germination and ecologically pertinent, and characterize morphological changes in conidia that are indicative of metabolic activity, at extreme water activities.

Results and discussion

Germination protocol

By utilizing spores which had accumulated glycerol, and supplementing germination media with molar concentrations of glycerol or glycerol plus other stressors, vigorous germination of *A. penicillioides* and other xerophiles has been observed at extremely low water-activity (Stevenson *et al.*, in press-a; in press-b). Nevertheless, germination of *A. penicillioides* did not occur on any media in the water-activity range 0.639–0.575 in these studies, even though 18 media that had been designed to be biologically permissive were assayed in this range (Stevenson *et al.*, in press-a). Here, we developed a germination protocol including the same high-glycerol element, but based a number of key modifications (Fig. S1). First, in earlier studies, spores were harvested in stressor solutions thereby introducing a mid-stage between the dehydrated condition of the spore and the germination-assay medium (Stevenson *et al.*, in press-a; in press-b); in the current study, conidia were both harvested and inoculated in their dry condition (Fig. S1). Second, germination assays in previous studies were carried out at 30°C, which may have accelerated water loss from media thereby reducing water-activity (Stevenson *et al.*, in press-a; in press-b); assays were carried out in the current study at 24°C (Fig. S1). Third, in previous studies, plates were continuously opened throughout the 50-d study period (such that

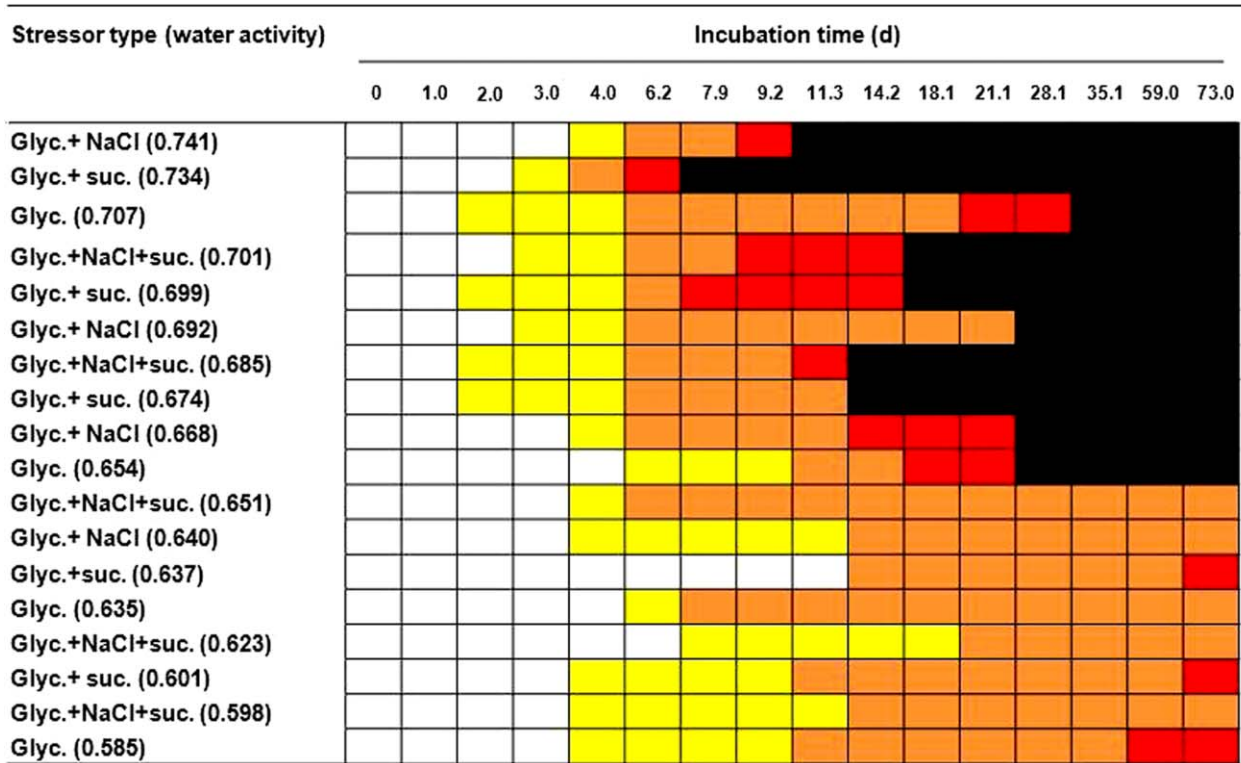


Fig. 1. Key stages of conidial germination of, and subsequent development of mycelium and/or spore production from, *Aspergillus penicillioides* (JH06THJ) conidia which had been inoculated onto malt extract yeast extract phosphate agar supplemented with either glycerol + NaCl; glycerol + sucrose; glycerol only; or glycerol + NaCl + sucrose (Table S1): (yellow) swelling of $\geq 40\%$ mean by volume; (orange) differentiation to form polarized, tapered germination structures; (red) production of fully-formed, septate germlings and (black) development of mycelium and/or sporulation of the newly established mycelia colony. Conidia used in germination assays had been harvested from cultures grown on malt extract yeast extract phosphate agar supplemented with 5.5 M glycerol (0.821 water activity), at 24°C; intracellular glycerol content was 70.1 mg g dry weight⁻¹ (Stevenson *et al.*, in press-a). White regions indicate times at which swelling to $\geq 40\%$ of initial volume was not observed in the sample of 50 conidia inspected. Methodological approach is detailed in 'Experimental procedures' section and outlined Fig. S1.

small but significant quantities of water were lost from the medium during the assay period (Stevenson *et al.*, in press-a); in the current study, plates remained sealed throughout the (extended, 73 days) period of the germination assay. This was achieved by carrying out microscopic examination directly through the Petri-plate lid (Fig. S1). Fourth, in the previous study, germination was determined according to whether germ-tube length exceeded the diameter of the spore, germ-tube length was also quantified (Stevenson *et al.*, in press-a; in press-b); the current study quantified (i) rates and extent of conidial swelling during the uptake of water; production of (ii) differentiated germination structures and (iii) septate germlings and (iv) development of mycelium and/or sporulation: elaboration of branched, adventitious germ-tubes followed by production of aerial hyphae and/or production of conidiophores and conidia (Fig. S1). Some earlier studies, which have produced seminal findings, reported only single data-points (e.g., Pitt and Christian, 1968); here, we carried out assessments over a range of water-activity values and

time points to obtain a complete profile of the germination process (Figs 1 and S1).

Conidial swelling during uptake of water

During conidial swelling, water rehydrates the cytosol and the spore's macromolecular systems, facilitates cell turgor and enables metabolic activity. The process can be part-mechanical, and part-ATP-driven, and represents the first stage of germination (Liu *et al.*, 2016; Turgeman *et al.*, 2016). Differentiation of conidia into tapered germination structures (see below) did not take place until after conidial volume had increased by a threshold value of $\geq 40\%$, so this criterion was used as an indicator for the pre-germination phase (Fig. 1). Conidia had swollen to $\geq 40\%$ (volume) at all water-activity values by days 2–7.9, depending on the medium (Figs 1 and S2–S4). Significant differences ($P \leq 0.05$) were observed between mean volume-increases observed at time zero (time of inoculation) and day 4, regardless of the medium (Fig. 2).

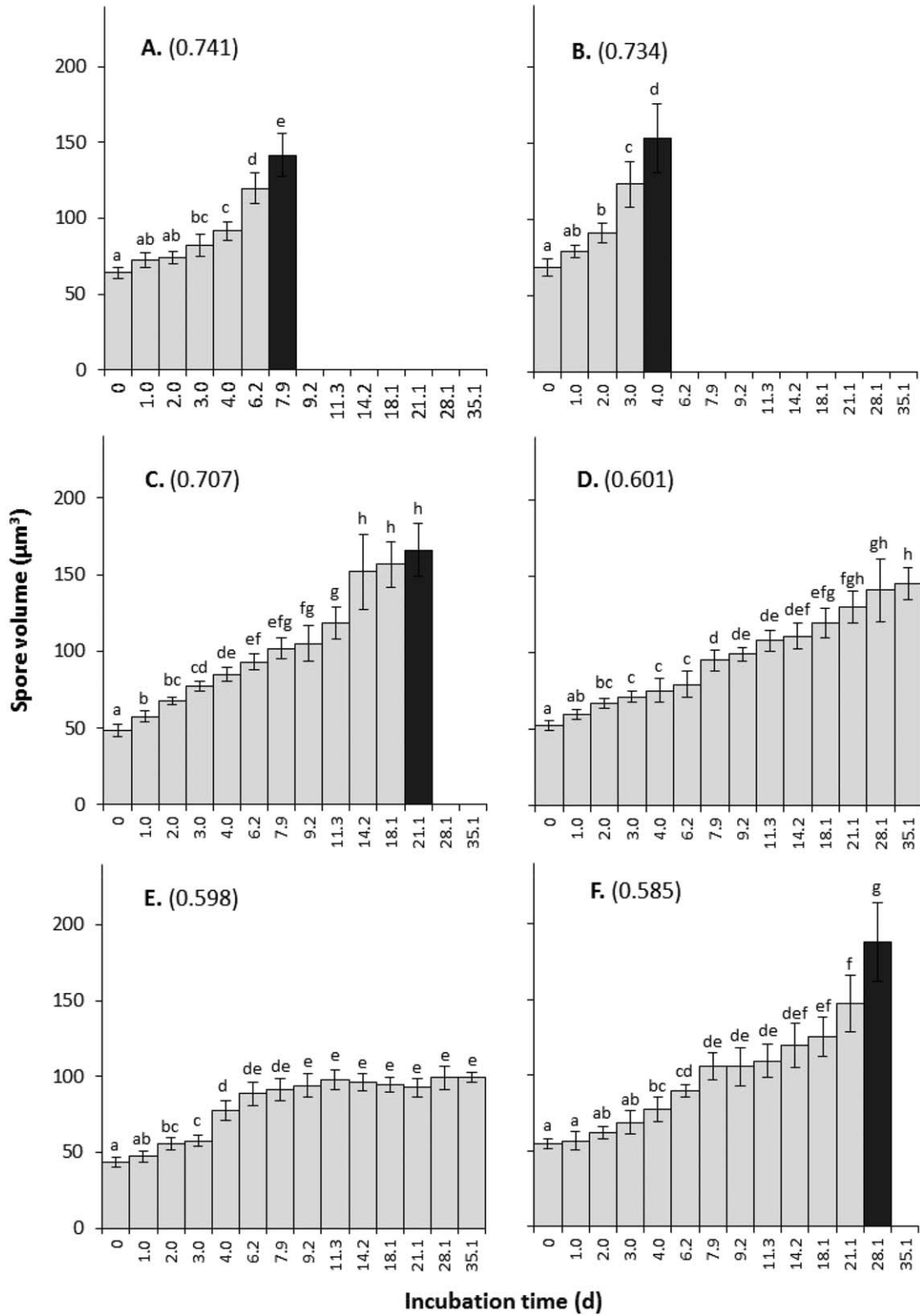


Fig. 2. Volumes of *Aspergillus penicillioides* (JH06THJ) conidia, which had been inoculated onto the three highest water-activities, at (A) 0.741 water activity on media supplemented with glycerol + NaCl; (B) 0.734 water activity on media supplemented with glycerol + sucrose and (C) 0.707 water activity on media supplemented with glycerol, and the three lowest water-activities, at (D) 0.601 water activity on media supplemented with glycerol + sucrose; (E) 0.598 water activity on media supplemented with glycerol + NaCl + sucrose and (F) 0.585 water activity on media supplemented with glycerol (Table S1). Volumes (μm^3) were calculated from the empirically determined cross-sectional areas (Fig. S2), as explained in the 'Experimental procedures' section. Grey columns indicate volumes of conidia during the swelling process; black columns indicate the maximum spore size attained (which was generally followed by formation of sporulating mycelium; see Fig. 1). Lower-case letters indicate significant differences ($P \leq 0.05$) between groups (i.e., time of measurement), as determined by ANOVA followed by Tukey's multiple comparison test (see 'Experimental procedures' section). Further details of each germination-assay medium are given in Table S1; the methodological approach is detailed in *Experimental procedures* and outlined Fig. S1.

Maximum conidial volume was attained on the highest water-activity media by days 7.9, 4.0 and 21.1 at 0.741, 0.734 and 0.707 water activity, respectively (Fig. 2A–C) and by day 57 and 28.1 at low water-activities, i.e., 0.601 (data not shown) and 0.585 (Fig. 2F). On some media, e.g., at 0.598 swelling took place but did not pass the 40% threshold-value, and there was no evidence of further development (Figs 1 and 2E). Likewise, there was no further development of differentiated germination structures at 0.623 water activity (Fig. 1), even though there was a volume increase of 49.6% by day 35.1 (Fig. S3O). Given the evidence that water uptake by conidia is, in part at least, an active process, these data are indicative of both (i) cellular metabolism at water activities as low as 0.601 and 0.585, which is unprecedented for any type of microbial system, and (ii) a capacity to differentiate and germinate down to 0.585 water activity.

Differentiation and germ-tube formation

The production of differentiated germination structures was the first visually identifiable stage of germ-tube

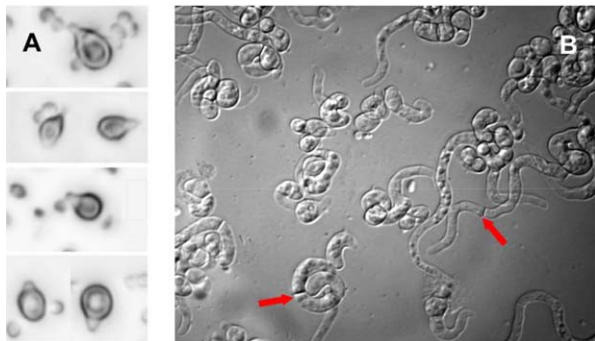


Fig. 3. Germinating conidia of *Aspergillus penicillioides* (JH06THJ), which had been inoculated onto malt extract yeast extract phosphate agar supplemented with stressors: (A) differentiated germination structures which have a polarised, tapered morphology at 0.598 water activity (18.1 days) on media supplemented with glycerol + NaCl + sucrose and (B) septate germ-lings at 0.585 water activity (57 days) on media supplemented with glycerol (see Table S1). Red arrows show examples of septa within germ-lings. Micrographs were taken at 400 \times using a Nikon MULTIZOOM AZ100 microscope; the methodological approach is detailed in the 'Experimental procedures' section and outlined Fig. S1.

formation (Fig. 3A). During this process, the cytoskeleton, and other components of the cell's ultrastructural machinery, drive the localized deposition of cell-wall material and germ-tube emergence (Harris and Momany, 2004). These tapered structures were produced throughout the water-activity range by days 4–18.1, depending on the medium, with the exception of the 0.640 (glycerol + NaCl) medium (Figs 1 and S4). Upon completion of the swelling process (Figs (1 and 2), S2 and S3) and formation of differentiated, tapered germination structures, there was a substantial increase in conidial size; equivalent to a two-fold increase in spore diameter and approximately four-fold increase in spore area (Fig. 3A). This is consistent with previous studies of conidial germination, whereby the germination process is characterized by two distinct stages: the initial, isotropic swelling of the conidium, followed by polarized development of germ-tubes (Hayer *et al.*, 2013). On 13 out of the 18 germination media used in the current study, these differentiated structures went on to produce germ-lings which had clearly visible septa within 6–73 days (Figs 1 and S4). Nevertheless, septate germ-lings were clearly visible by days 57 and 73 at 0.585 and 0.601 water activity, respectively (Fig. 2a and 3B), indicating that microbial metabolism, cellular differentiation and cell division take place beyond the currently understood 0.605 water-activity limit for life. This finding is consistent with the theoretical 0.600–0.570 water-activity minima for germination of *A. penicillioides* that were reported previously (Stevenson *et al.*, in press-a). The window from 0.690 to 0.605, considered to be the biophysical fringe of Earth's biosphere (Stevenson *et al.*, 2015b), actually, therefore, spans the range from 0.690 to 0.585 water activity; in other words, the findings reported here represent a 24% increase in this window, and a 5% increase in the 1–0.605 water-activity window-for-life. The equivalent temperature window for microbial metabolism on Earth, approximately -40 to $+121^\circ\text{C}$, spans a range of 161°C (see reviews in Stevenson *et al.*, 2015b; Rummel *et al.*, 2014). A 5% increase in the water-activity window for life is, therefore, equivalent to an 8°C expansion of the temperature limit for the functional biosphere.

Generally, the period during which differentiated germination structures are visible, but before septate germ-lings have formed, lasts for >7 days (Figs 1 and S4). By

contrast, the period between the appearance of septate germlings and production of mycelium and/or sporulation of colonies was typically shorter; <7 days (Figs 1 and S4). At one level, this is counterintuitive because the former process involves one to three cell-divisions (see Fig. 3), whereas the latter involves development of mycelium, conidiophores and conidiogenesis, which requires a greater number of cell divisions as well as further differentiation. However, the delay between formation of differentiated germination structures and septate germlings is consistent with fundamental changes known to take place early on during germination of conidia. These include the initiation of respiration, or fermentation followed by respiration; synthesis of proteins, including aquaporins and those involved in substrate uptake; synthesis of DNA and RNA; critical changes in compatible-solute metabolism (assimilation and/or synthesis of trehalose and specific polyols); generation of cell-available energy; and additional changes including upregulation of other genes involved in sensing the environment, substrate uptake, stress responses and developmental processes (Osharov and May, 2001; van Leeuwen *et al.*, 2013; Hagiwara *et al.*, 2016; Novodvorskaa *et al.*, 2016; Turgeman *et al.*, 2016).

The inability to form germlings on the 0.651, 0.640, 0.635, 0.623 and 0.598 water-activity media by day 73 may relate to unfavorable combinations of chaotropes, kosmotropes, water activity, temperature, nutrients and/or turgor (Williams and Hallsworth, 2009; Chin *et al.*, 2010; Stevenson *et al.*, 2015a; de Lima Alves *et al.*, 2015; Wyatt *et al.*, 2015a). Such windows-of-inactivity have been observed previously in studies of mycelial growth of xerophiles at extreme water activities (Williams and Hallsworth, 2009; Stevenson *et al.*, 2015a). The lack of germlings on glycerol-supplemented media at 0.635 water activity, even by day 73, appears anomalous given that germlings were produced by day 57 on glycerol-only media at 0.585 water activity (Fig. 1). At extreme glycerol concentrations, however, biophysical aspects of molecular motion, cytosolic viscosity and flexibility-stability of biomacromolecules are both complex and dynamic. It may be that viscosity of the cytosol is prohibitively high at 7.4 M glycerol in the 0.635 water-activity medium (Wyatt *et al.*, 2015a), whereas the extreme chaotropicity observed at 7.7 M glycerol in the 0.585 water-activity medium (Hallsworth *et al.*, 2007; Williams and Hallsworth, 2009; Cray *et al.*, 2013a; de Lima Alves *et al.*, 2015) can offset this biophysical constraint (Ball and Hallsworth, 2015; Wyatt *et al.*, 2015a). The lack of germling formation on the media listed above is consistent with the slow progress and relatively low level of water intake by the conidia (Figs 3, S2 and S3). Hostile combinations of stress parameters, i.e., 'black holes' in relation to functionality of biological systems, are the rule rather than the exception under very extreme conditions (Williams and Hallsworth, 2009; Yakimov *et al.*, 2015; Cray *et al.*, 2016).

Germination of diverse xerophile species was observed in the water-activity range 0.738–0.605 by between 19 and 120 days by Pitt and Christian (1968). By contrast, the production of *A. penicillioides* germination structures reported here was observed after unprecedented periods of only 4 days at 0.734 water activity, 7.9 days at 0.635 water activity and 11.3 days at 0.585 water activity (Figs 1 and S4).

Production of mycelium and sporulation: the culmination of development

Upon germination, both the development of new mycelium and subsequent sporulation are key events in the natural ecology of fungi. These processes facilitate survival, dispersal, and ultimately enable location of new resources, colonization of new habitats, etc. In the context of space-exploration missions, bacterial and fungal spores may act as an effective vehicle for the transfer of life from Earth to extraterrestrial locations in a dormant yet viable state (Rummel *et al.*, 2014; Gibney, 2016; Nagler *et al.*, 2016). Spores of aspergilli and closely related fungi are highly stress resistant (van Leeuwen *et al.*, 2013; Rangel *et al.*, 2015; Wyatt *et al.*, 2015b) and can remain viable after exposure to space at least 22 months, as demonstrated by the EXPOSE-R experiment performed on the outer surface of the International Space Station (Novikova *et al.*, 2015), though may potentially do so for decades. In the current study, production of aerial hyphae and/or conidiophores and lawns of (pigmented) spores were recorded on 10 out of the 18 germination media (Figs 1 and S4). The lowest water-activity at which this level of development occurred during the experimental period was 0.654 (Fig. 1). In previous studies, germination of xerophilic aspergilli occurred in a comparable time period only at the relatively high water activity of 0.771; production of spores was only recorded 64–116 days in the water-activity range 0.708–0.663; and no sporulation occurred below 0.663 (Pitt and Christian, 1968). In the current study, mycelium developed and/or sporulation had occurred at 0.734, 0.674 and 0.654 water activity by 7.9, 14.2 and 28.1 days, respectively (Fig. 2). Extrapolations of the time zone in which each dataset lies indicate that mycelial development and/or sporulation may take up to 131 days on 0.585 water-activity media (Fig. S4C). Further studies are needed to determine whether conidiogenesis occurs at, and whether germination takes place below, 0.585 water activity.

Concluding remarks

The current study reveals unprecedented rates of metabolism, active cellular differentiation, and cell division at extremely, low water-activity. Furthermore, it demonstrated microbial cell differentiation and cell division at a water activity outside the currently understood window-for-life.

Understanding the constraints on microbial systems is essential to understanding habitability in relation to hostile conditions on Earth, within industrial systems, and in extraterrestrial locations (Kminek *et al.*, 2010; Rummel *et al.*, 2014; Hu *et al.*, 2016). A number of policies, protocols and processes are based on the water-activity limit for life; in light of the data presented here, which revealed fungal germination at 0.585 water activity, and the circumstantial evidence of potential microbial germination and growth at even lower water-activity values, these may now require revision. They include: water-activity and relative-humidity thresholds considered safe for preservation of foods and feeds (Brown, 1990; Shehbal, 2012); relative humidities considered safe to prevent growth of mycotoxin-producing fungi within habitations (van Laarhoven *et al.*, 2016); conditions needed to preserve books, artworks and museum specimens; and the international policy for planetary protection in relation to exploratory space missions which has been formulated based (albeit with some safety margin, to allow for potential new findings) on previous data that no microbe could multiply below 0.605 water activity (Rummel *et al.*, 2014; Stevenson *et al.*, 2015b; Kminek *et al.*, 2016). Intriguingly, abiotic glycerol (and structurally similar molecules) has been identified in both terrestrial and extraterrestrial environments (Cooper *et al.*, 2001; Kaiser *et al.*, 2015; McCollom *et al.*, 2015), and fungi – like other microbes – efficiently take up extracellular glycerol when present at nanomolar concentrations, and possibly even below this range (Hallsworth and Magan, 1995; Lages *et al.*, 1999; Holst *et al.*, 2000). Application of our findings may also help to protect health by reducing exposure to mycotoxins in airborne spores for inhabitants of buildings or astronauts within spacecraft, or via the food-supply chain.

The finding that *A. penicillioides* cells can function at 0.585 water activity in experimental high-glycerol media (current study) – rather than 0.680 in the absence of extracellular glycerol (Pitt and Hocking, 2009) – represents a profound change in its window, equivalent to 9.55% relative humidity or, in terms of typical temperature windows for growth, ~6°C (Stevenson *et al.*, 2015b). A recent study revealed cell division of Bacteria and Archaea close down to 0.635 water activity (Stevenson *et al.*, 2015a), and in the current study, the rate of *A. penicillioides* germination at 0.585 water activity was more rapid than we had anticipated, and lines of best fit in which each dataset lies suggested that differentiated germination structures could plausibly be produced by 20 days, and septate germ-lings by 82 days, at 0.565 water activity (Fig. S4B). Collectively, these findings hint that microbial metabolism and cell division may function in nature at <0.585, or even <0.565, water activity. Furthermore, they provide proof-of-principle that the currently understood water-activity limit for life not an insurmountable thermodynamic barrier, but

can be circumvented by microbial cells (and biotechnologists and microbiologists). Recent studies suggest that both water activity and glycerol are key drivers for the evolutionary biology of microbial systems (e.g. Cray *et al.*, 2013b; Stevenson *et al.*, 2015b; Mattenberger *et al.*, in press; Oren, in press). Studies based on the use of transcriptomics have provided insights into the biophysical limit of the functional biosphere in chaotrophic habitats (Hallsworth *et al.*, 2007; Yakimov *et al.*, 2015); it will be intriguing to discover whether such approaches can also be employed to determine *in situ* activity(ies) of microbes within the stressed ecosystems of terrestrial, water-constrained environments, and to answer the question whether (or how much) microbial metabolism occurs below the water-activity threshold for cell division? As water activity is a key determinant for many biological, biochemical and biotechnological systems/processes, a reassessment of the biophysical constraints which determine their functionality, may now be warranted.

Experimental procedures

Aspergillus penicillioides strain

Aspergillus penicillioides strain JH06THJ was maintained on malt-extract yeast-extract phosphate agar (MYPiA; 1% malt extract, 1% yeast extract, 0.1% anhydrous K₂HPO₄ and 1.5% w/v agar) supplemented with 5.5 M glycerol (0.821 water activity) and incubated at 30°C. This strain was originally isolated in 2006 from an antique wooden artefact (country of origin, Thailand) by Williams and Hallsworth (2009) and is available from the corresponding author. The water-, pH- and temperature-relations for spore germination and mycelial growth of *A. penicillioides* (JH06THJ) have been characterized on glycerol-supplemented medium (Williams and Hallsworth, 2009; Stevenson *et al.*, in press-b); both germination and hyphal growth have been observed down to 0.640 water activity (Williams and Hallsworth, 2009; Stevenson *et al.*, in press-a).

Production and dry-harvesting of high-glycerol conidia

Cultures were incubated on MYPiA supplemented with 5.5 M glycerol 30°C for 10–14 days; until sporulation was sufficiently advanced to be clearly visible by eye (Stevenson *et al.*, in press-a). High-glycerol conidia (up to 7% dry wt; see Stevenson *et al.*, in press-a) were harvested from these cultures by gently stroking the surface of colonies with a sterile glass-rod; in the same motion, this glass rod was immediately used to inoculate germination assays (see below).

Inoculation and incubation of germination assays

The MYPiA-based media used for germination assays were supplemented with either glycerol + NaCl; glycerol + sucrose; glycerol only; or glycerol + NaCl + sucrose (Table S1); and were chosen to represent the high-solute environments (both natural and anthropogenic) in which *A. penicillioides* and other

microbes occur (Hallsworth and Magan, 1995; Wang *et al.*, 2001; Sformo *et al.*, 2010; Santos *et al.*, 2015; Stevenson *et al.*, 2015a; Cray *et al.*, 2015; 2016). The water activities of these media ranged from 0.741 to 0.585 (Table S1); the same batch of media was used for the study Stevenson and colleagues (in press-a). Water activity was determined using a Novasina Humidat-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; Stevenson *et al.*, 2015a). Conidia were inoculated onto germination-assay media by stroking with the glass rod that had been used to harvest spores (see above). Petri plates were then immediately sealed with Parafilm® and incubated in polythene bags at 24°C.

Inspection of spores, germinating and mycelia through sealed Petri plates

Once inoculated, Petri plates remained sealed with Parafilm® throughout the 73-days duration of the study. Conidia and other structures were examined through the lids of Petri plates, and images were captured at regular intervals using a MULTI-ZOOM AZ100 microscope (Nikon Corporation Instruments) at 400× magnification.

Criteria for assessment of the germination process and subsequent sporulation

Photomicrographs of conidia were taken at regular time intervals (at 400× magnification) and analyzed using Adobe Bridge CS5.1 (Adobe Systems Incorporated, CA, USA) to determine the cross-sectional areas of 50 individual spores which were spatially separate from any others. The Magnetic Lasso Tool within Adobe Bridge CS5.1 was used to delineate the edge of each spore, and the number of pixels within this outline was determined using the Adobe Bridge CS5.1 pixel counter. Cross-sectional areas of conidia were, therefore, initially determined according to the number of pixels. Mean cross-sectional area was determined for conidia at each water activity and at each time point (Fig. S2).

Cross-sectional areas in pixels (A) were converted to spore volumes in pixels² (V_{pix}) using the equation:

$$V_{pix} = \frac{4}{3}\pi \times \left(\sqrt{\frac{A}{\pi}} \right)^3$$

based on the spherical form of the conidia. All cross-sectional areas (and corresponding volumes) were recorded only for conidia which had not yet differentiated. The mean diameter of conidia at time zero (upon inoculation) was 4.75 μm, as determined from 1800 measurements made using a gratulated eyepiece and calibration slide (data not shown). This value was used to calculate the initial cross-sectional area of conidia, which was 17.72 μm². Accordingly, the mean volume of conidia upon inoculation was 56.12 μm³; this value was used to convert all volumes expressed in pixels² to volumes expressed in μm³ (V_{mic}) (Fig. S3). For each water activity and at each time point, V_{mic} was determined via the equation:

$$V_{mic} = \frac{\left(\frac{V_{pix}}{MV_{pix}} \times 100 \right) \times 56.12}{100}$$

where MV_{pix} is the mean volume of all spores at time zero in pixels². For the three highest and three lowest water-activity media, log-transformed values for V_{mic} were tested for significant differences ($P \leq 0.05$) in relation to incubation time via one-way ANOVA followed by Tukey's multiple comparison test (Fig. 2).

For each water activity and time point, the first appearance of differentiated conidia (Figs 3A and S1) was recorded upon inspection of at least 200 individual conidia. For each water activity and time point, the appearance of the first germlings was recorded once germ tubes were longer than the diameter of the conidium and septa were visible (Figs 1 and S1). Following germination, production of mycelium and production of pigmented spores were visible by eye. Sporulation (production of aerial hyphae, conidiophores and conidia) was verified by light microscope and then recorded (Figs 1 and S1).

Acknowledgements

This article is based on work funded by the Department of Agriculture, Environment and Rural Affairs (DAERA, Northern Ireland) who supported A. Stevenson and P. G. Hamill and Biotechnology and Biological Sciences Research Council (BBSRC, United Kingdom) project BBF003471. We would like to thank D. Blain (Queen's University Belfast) for the technical assistance provided.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Flow diagram showing the experimental approaches used (A) in the current study that reports germination of *A. penicillioides* at 0.585 water activity, (B) the study that demonstrated that glycerol can enhance xerophile germination at low water-activity, and reported germination of *A. penicillioides* at 0.640 water activity but used extrapolations to predict water-activity minima for germination of <0.600 (6) and (C) the study that reported germination of *Xeromyces bisporus* aleuriospores at 0.605 water

activity (Pitt and Christian, 1968). For full methodological details, see 'Experimental procedures' section.

Fig. S2. Cross-sectional areas of *Aspergillus penicillioides* (JH06THJ) conidia, which had been inoculated onto malt extract yeast extract phosphate agar supplemented with either glycerol + NaCl; glycerol + sucrose; glycerol only; or glycerol + NaCl + sucrose (Table S1) to give water activity values of (A) 0.741, (B) 0.734, (C) 0.707, (D) 0.701, (E) 0.699, (F) 0.692, (G) 0.685, (H) 0.674, (I) 0.668, (J) 0.654, (K) 0.651, (L) 0.640, (M) 0.637, (N) 0.635, (O) 0.623, (P) 0.601, (Q) 0.598 and (R) 0.585. Grey columns indicate cross-sectional areas of conidia (arbitrary units) during the swelling process; black columns indicate the maximum spore size attained (which was generally followed by formation of sporulating mycelium; see Fig. 1). Further details of each germination-assay medium are given in Table S1; the methodological approach is detailed in the 'Experimental procedures' section and outlined in Fig. S1.

Fig. S3. Volumes of *Aspergillus penicillioides* (JH06THJ) conidia, which had been inoculated onto malt extract yeast extract phosphate agar supplemented with diverse stressors (Table S1) to give water-activity values of: (A) 0.741, (B) 0.734, (C) 0.707, (D) 0.701, (E) 0.699, (F) 0.692, (G) 0.685, (H) 0.674, (I) 0.668, (J) 0.654, (K) 0.651, (L) 0.640, (M) 0.637, (N) 0.635, (O) 0.623, (P) 0.601, (Q) 0.598 and (R) 0.585. Volumes (μm^3) were calculated from the empirically determined cross-sectional areas (Fig. S2), as explained in the 'Experimental procedures' section. Grey columns indicate volumes of conidia during the swelling process; black columns indicate the maximum spore size attained (which was generally followed by formation of

sporulating mycelium; see Fig. 1). Further details of each germination-assay medium are given in Table S1; the methodological approach is detailed in the 'Experimental procedures' section and outlined in Fig. S1.

Fig. S4. Key stages of conidial germination of, and subsequent spore production from, *Aspergillus penicillioides* (JH06THJ) conidia which had been inoculated onto malt extract yeast extract phosphate agar supplemented with diverse stressors (Table S1) showing: (A) the scatter of data, (B) lines of best fit and (C) the time zone in which each dataset lies; for (yellow) swelling of $\geq 40\%$ by volume; (orange) differentiation to form polarised, tapered germination structures; (red) production of fully-formed, septate germlings and (black) sporulation of the newly established mycelia colony. Conidia used in germination assays had been harvested from cultures grown on malt extract yeast extract phosphate agar supplemented with 5.5 M glycerol (0.821 water activity), at 24°C; intracellular glycerol content was 70.1 mg g dry weight⁻¹ (41). The shaded regions indicate extrapolated domains where: (yellow) swelling of $\geq 40\%$ by volume; (orange) differentiation to form polarized, tapered germination structures; (red) production of fully-formed, septate germlings; and (black) sporulation of the newly established mycelia colony may occur beyond the time frame of the current study. Methodological approach is detailed in the 'Experimental procedures' section and outlined in Fig. S1.

Table S1. Media used for germination assays of *Aspergillus penicillioides* JH06GBM.