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# Survival of Filamentous Fungi in Hypersaline Dead Sea Water

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## ABSTRACT

A variety of filamentous fungi have recently been isolated from the Dead Sea (340 g/L total dissolved salts). To assess the extent to which such fungi can survive for prolonged periods in Dead Sea water, we examined the survival of both spores and mycelia in undiluted Dead Sea water and in Dead Sea water diluted to different degrees with distilled water. Mycelia of *Aspergillus versicolor* and *Chaetomium globosum* strains isolated from the Dead Sea remained viable for up to 8 weeks in undiluted Dead Sea water. Four Dead Sea isolates (*A. versicolor*, *Eurotium herbariorum*, *Gymnascella marismortui*, and *C. globosum*) retained their viability in Dead Sea water diluted to 80% during the 12 weeks of the experiment. Mycelia of all species survived for the full term of the experiment in Dead Sea water diluted to 50% and 10% of its original salinity. Comparison of the survival of Dead Sea species and closely related isolates obtained from other locations showed prolonged viability of the strains obtained from the Dead Sea. Spores of isolates obtained from the terrestrial shore of the Dead Sea generally proved less tolerant to suspension in undiluted Dead Sea water than spores of species isolated from the water column. Spores of the species isolated from the control sites had lost their viability in undiluted Dead Sea water within 12 weeks. However, with the exception of *Emericella* spores, which showed poor survival, a substantial fraction of the spores of Dead Sea fungal isolates remained viable for that period. The difference in survival rate between spores and mycelia of isolates of the same species points to the existence of adapted halotolerant and/or halophilic fungi in the Dead Sea.

## Introduction

The Dead Sea is one of the most extreme habitats for microorganisms on Earth. Not only is the salt concentration of its waters extremely high (around 340 g/L total dissolved salts), but the unique ionic composition makes the lake a uniquely hostile environment even for halophilic and halotolerant microorganisms adapted to life at high concentrations of NaCl. The increased drying of the Dead Sea in recent years has caused a massive precipitation of

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halite. Divalent cations (presently about 1.89 M $Mg^{2+}$ and 0.44 M $Ca^{2+}$) now dominate over monovalent cations (about 1.6 M $Na^+$ and 0.2 M $K^+$). $Cl^-$ makes up 99% of the anion sum. The water activity ($a_w$ = vapor pressure of the solution divided by vapor pressure of pure water) of the Dead Sea brine is presently below 0.669 [11], and the pH is relatively low (5.5–6.0).

The biota of the Dead Sea are dominated by the unicellular green alga *Dunaliella parva* Lerche, being the main or sole primary producer, and by different species of halophilic Archaea of the family Halobacteriaceae (*Halobaculum gomorrense*, *Halorubrum sodomense*, *Haloarcula marismortui*, *Haloferax volcanii*, and possibly others as well) as main consumers. Since the full overturn of the water column in the winter of 1979, blooms of these microorganisms occur only when extensive rain floods in exceptionally rainy years cause a significant dilution of the upper water layers of the water column [15–18].

Recently, a remarkable diversity of filamentous fungi has been discovered in the Dead Sea, including from the surface water layers, down to a depth of 300 m [2–5, 9, 10]. Little information is available on the possible function of fungi in the Dead Sea. Fungi could be expected to be well adapted to life in this environment, as many xerophilic species grow well under very low water activities [8] and generally prefer low pH values as well. Some of the species recovered proved to be true halophiles. The newly described *Gymnascella marismortui* Buchalo et al. [2] grows optimally at NaCl concentrations between 0.5 and 2 M. Its growth rate is not affected by ionic composition, and it is able to grow in Dead Sea water (DSW) diluted to 50% of its original salinity and possibly at even higher salt concentrations [13]. It was thus suggested that *G. marismortui* is endemic to the Dead Sea [3].

Many of the other fungal isolates recovered from Dead Sea water samples belong to non-halophilic, terrestrial species, known for their cosmopolitan distribution. Examples are *Aspergillus niger* Tiegh., *Cladosporium cladosporoides* (Fres.) de Vries, and many others [4, 5, 9]. Most of these species were also isolated from sand and mud on the surrounding shore [Grishkan, personal communication] or from inflowing fresh water of floods and springs [9, 10]. It is probable that the constant inflow of species such as *C. cladosporoides* and *C. sphaerospermum* Penz from the Jordan River and the surrounding springs can explain the high recovery rate of these species [10].

To obtain information on the possible role of fungi in the Dead Sea ecosystem, the question should be asked to what extent mycelia and spores of Dead Sea and other fungi may survive in its hypersaline brines for prolonged periods. Furthermore, the possibility that Dead Sea fungal isolates have adapted to life in its highly salty waters should be investigated by comparison of their properties with those of related isolates obtained from other less stressful environments.

In this study we examined the survival of fungal mycelia and spores of species obtained from the Dead Sea, from its terrestrial shore, and from Mediterranean forest soil ("Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee) [16], in an attempt to evaluate the degree of adaptation of fungi isolated from the Dead Sea.

## Methods

### *Fungal Isolates*

The eight species used in the experiment are presented in Table 1. The fungi were grown at 25°C on Petri dishes with glucose–yeast extract agar (GYA) (yeast extract 1 g/L, glucose 10 g/L, agar 15 g/L, prepared in distilled water); in the case of the halophile *G. marismortui* and *Eurotium* spp. the medium was prepared in 30% (vol/vol) of Dead Sea water (DSW). *C. globosum* isolates were grown for at least 1-month on the following cellulose-containing medium (CA) for production of ascospores (g/L): carboxymethyl cellulose 10; $K_2HPO_4$ 1; KC1 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; $Fe_2SO_4 \cdot 7H_2O$ 0.01; yeast extract 0.5; agar 15.

### *Experimental Design*

*Mycelium Survival Experiments.* Mycelium experiments were conducted for 12 weeks in DSW diluted with sterile distilled water to a percentage of its original salinity. Four dilutions were tested in the experiment: 10%, 50%, 80%, and undiluted DSW. Fungal spores were harvested from 14-day-old cultures on agar plates (1 month for *C. globosum* on CA plates) as described previously [1]. One-mL portions containing about 200 spores/mL were added to 100-mL glucose-yeast extract (GY) liquid medium made with autoclaved DSW (sampled from 200 m depth in the center of the lake) diluted with sterile distilled water to 10% of its original salinity (10% DSW) in 250-mL Erlenmeyer flasks. The spores were left to germinate for 4 days on a shaker (150 rpm) to prevent sporulation. When the mycelium clusters reached a diameter of about 1–2 mm, they were transferred to 250-mL Erlenmeyer flasks containing a series of dilutions of DSW (10%, 30%, 50%, and 80% DSW) to prevent osmotic shock. Microscopic examination did not reveal the presence of spores after this treatment. The stepwise incubation was continued in each dilu-

**Table 1.** Fungal strains used in the experiments[a]

| Species | Sampling location | | |
|---|---|---|---|
| | Dead Sea water (DS) | Dead Sea shore (DSS) | Evolution Canyon II (ECII) |
| *Aspergillus niger* | — | HAI 3087 | HAI 3088 |
| *Aspergillus versicolor* | HAI 2504 | HAI 3097 | HAI 3098 |
| *Chaetomium globosum* | HAI 2777 | HAI 3037 | HAI 3038 |
| *Emericella nidulans* | HAI 2760 | — | HAI 3045 |
| *Eurotium herbariorum* | HAI 2033 | HAI 3033 | — |
| *Eurotium* sp. | — | — | HAI 3051 |
| *Gymnascella marismortui* | HAI 2002 | — | — |
| *Mycelia sterilia* | — | HAI 3385 | — |

[a] The isolates were obtained from the Dead Sea water column [2, 9], from the terrestrial shore of the Dead Sea [Griskan, unpublished results], and from soil of the south-facing slope of "Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee [Griskan, unpublished results]. Isolates were identified by morphological and cultural characters. The accession numbers in the culture collection of the Institute of Evolution, the University of Haifa (HAI), are indicated.

tion for 1 day and then the clusters were transferred to the final dilution tested. To prevent sporulation, incubation of 50% and 10% DSW was performed in 125-mL Erlenmeyer flasks on a rotating shaker (100 rpm). For 80% and undiluted DSW, portions of 10-ml suspensions, containing about 20 mycelium clusters, were filtered on sterile gauze and resuspended in glass test tubes containing 20 mL of sterile DSW or 80% DSW. The tubes were aerated once a week by introduction of sterile air, and the medium was replaced every 2 weeks. Five replicate tubes were prepared for each combination of mycelium and DSW concentration. All media were supplemented with 1 g/L glucose and 1 g/L yeast extract. At the start of the experiment and after 1, 2, 4, 6, 8, 10, and 12 weeks of incubation at 25°C, samples were withdrawn from all dilutions. Mycelium clusters (2–5) were inoculated on Petri dishes with GYA medium or GYA prepared in 30% DSW. *C. globosum* strains were also inoculated on CA media. The plates were incubated at 25°C for 1 week (up to 1 month for CA media), and growth and sporulation were recorded.

*Spore Survival Experiments.* Spores were harvested from 14-day-old cultures on agar plates (1 month for *C. globosum* on CA plates). The harvesting was performed as described previously [1]. The spores were introduced into glass test tubes containing 10 mL sterile DSW, 80% DSW, and 10% DSW, and the tubes were shaken thoroughly. The initial spore density was standardized to approximately 200 spores/mL. Five replicate tubes were prepared for each combination of fungal isolate and DSW concentration. At the start of the experiment and after 1, 2, 4, 8, and 12 weeks of incubation at room temperature in diffuse daylight the tubes were shaken, and samples were withdrawn.

Portions of samples (0.5 mL) were poured into sterile Petri dishes and mixed by rotation with 17 mL molten GYA medium or GYA prepared in 30% DSW. After solidification of the agar, the plates were incubated at 25°C for up to 2 weeks, and colonies were counted.

*Data Analyses.* Survival in mycelium experiments was defined as positive if any of the mycelium clusters retained the ability to visibly grow and sporulate in solid medium after suspension in the dilution of DSW.

In spore experiments mean colony numbers and standard deviations were calculated, and *t*-tests were performed to determine the degree of significance between the number of colonies in GYA and GYA with diluted DSW. Fraction of viable spores was determined as the relative number of viable spores after suspension compared to the initial number of spores at the onset of the experiment. The sign test [20] was applied to the state of survival at the end of the incubation for the undiluted and 80% DSW to test whether the Dead Sea isolates show a greater fraction of survival.

## Results

### *Viability of Mycelia in Dead Sea Water*

Figure 1 shows the results of the viability tests of vegetative mycelia of different fungal species suspended in different concentrations of Dead Sea water. After 10 weeks of incubation in undiluted DSW, all mycelia had lost their viability. Mycelia of Dead Sea (DS) isolates of *Aspergillus versicolor* (Fig. 1B) and *Chaetomium globosum* (Fig. 1C) remained viable for up to 8 weeks in undiluted Dead Sea water. Twelve isolates were viable for 4 weeks or less in undiluted Dead Sea water. Four Dead Sea isolates (*Eurotium herbariorum*, *A. versicolor*, *C. globosum*, and *Gymnascella marismortui*) retained their viability in Dead Sea water diluted to 80% during the 12 weeks of the experiment. In 80% DSW the Dead Sea Shore (DSS) isolate of *A. versicolor* and the dark sterile mycelium remained viable for 10 weeks and *E. herbariorum* DSS (Fig. 1A) and *C. globosum* from "Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee (ECII), for 8 weeks. Comparison of the survival of Dead Sea isolates and isolates obtained

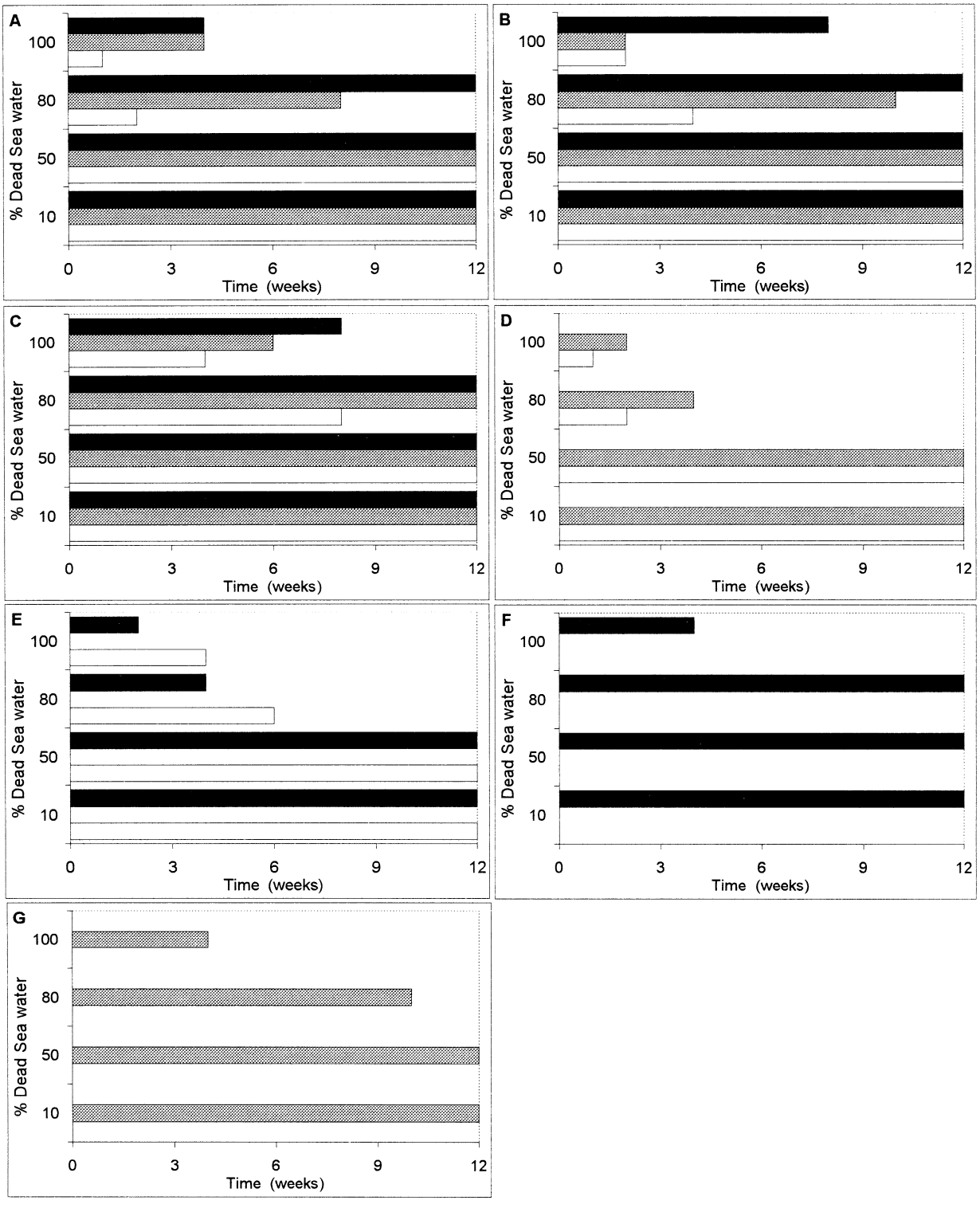


Fig. 1. Survival of vegetative mycelia of filamentous fungi isolated from the Dead Sea in different concentrations of Dead Sea water, as compared to the survival of isolates of the same species obtained from the terrestrial shore of the Dead Sea and from Mediterranean forest soil, Nahal Keziv ("Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee). The survival of the Dead Sea isolates-(black bars), Dead Sea shore isolates (gray bars), and Nahal Keziv isolates (white bars) (where available) of *Eurotium* spp. (**A**), *Aspergillus versicolor* (**B**), *Chaetomium globosum* (**C**), *A. niger* (**D**), *Emericella nidulans* (**E**), *Gymnascella marismortui* (**F**), and a dark sterile mycelia strain isolated from the Dead Sea shore (**G**) was measured in undiluted Dead Sea water, and in 80%, 50% , and 10% dilutions in distilled water. The graphs present the time in weeks during which the mycelia (one isolate for each bar) remained viable.

from other locations generally showed prolonged viability of the strains obtained from the Dead Sea.

Mycelia of all species survived in Dead Sea water diluted to 50% and 10% of its original salinity. In 10% Dead Sea water all species continued to grow during the experiment and maintained their typical morphological features, reaching a final cluster size of 10–15 mm. In 50% DSW there was no evident growth in the test medium after 2 weeks. The *C. globosum* ECII produced abundant mature ascomata within only a few days after transfer to CA agar following suspension of 1 week in undiluted and 80% DSW. After 2 weeks suspension the 50% DSW culture produced mature ascomata as well. When no salt stress was applied, this isolate produced only a few mature ascomata even after 6 weeks in CA medium. No ascomata were produced in 10% DSW. *A. niger* ECII (Fig. 1D) showed profuse sporulation after 8 weeks in 50% DSW; in the same dilution *E. nidulans* (Fig. 1E) ECII had a slower growth rate and produced fewer ascomata.

### *Viability of Fungal Spores in Dead Sea Water*

Following suspension of spores in undiluted Dead Sea water, viable spores were recovered for all Dead Sea isolates after 12 weeks, with the exception of *E. nidulans*. Spores of the shore and Mediterranean isolates had all lost their viability in undiluted Dead Sea water after 12 weeks, with the exception of *C. globosum* DS (Fig. 2). At the end of the experiment (12 weeks incubation), 18% of the *A. niger* DSS spores suspended in 80% DSW had retained their viability. No viable spores of the shore isolate were recovered in undiluted DSW, and no viable spores of the ECII isolate remained in both undiluted and 80% DSW. The *A. versicolor* DS showed low spore survival in all dilutions, but spores of the control isolates (DSS and ECII) perished even faster upon exposure to Dead Sea water. All *C. globosum* strains showed 42–45% survival in 80% DSW after 3 months. However, in undiluted Dead Sea water survival of the DSS isolate was only a third of the sea isolate's survival. Both *E. nidulans* DS and ECII showed less than 5% survival after 1 week, whereas in 10% Dead Sea water the fraction of viable spores was not affected. A similar pattern was seen in *Eurotium* sp. ECII. Spores from the ECII isolate rapidly lost their viability in 80% DSW, and no viable spores remained after incubation in undiluted Dead Sea water after only 1 week. No viable spores were detected after 2 weeks in 80% DSW. Two isolates of another species of this genus, *E. herbariorum* DS and DSS, still had viable spores in 80% DSW after 3 months. Finally, G. *marismortui*, a truly halophilic species that may be endemic to the Dead Sea, had the highest survival rate, with 70% viable spores in undiluted Dead Sea water and nearly 100% in 80% DSW at the termination of the experiment (Fig. 2).

The results of the sign test indicated that the DS isolates showed better survival than both shore and ECII isolates. There was a significant difference between germination on GYA and GYA made with 30% DSW for *E. herbariorum*, *G. marismortui*, and the *A. versicolor* DS. The germination was better in the GYA 30% DSW. There were no statistically significant differences in the presence of germination for *A. niger*, *C. globosum*, and *E. nidulans*; however, the lag period before germination was longer on GYA 30% DSW.

## Discussion

This study was initiated to assess the survival of fungi, either as vegetative mycelia or as spores, in the Dead Sea, in order to obtain information on the possible autochthonous nature of the fungal species recovered from the lake. To test for the presence of special adaptations of the Dead Sea isolates to their hypersaline environment, we compared their behavior with that of isolates of the same species or of closely related ones, from soil on its terrestrial shore and from a low-salt control Mediterranean environment (Nahal Keziv, "Evolution Canyon" II [14]) The species were chosen for their distribution, because of either their frequent recovery from the Dead Sea or their cosmopolitan occurrence.

Vegetative mycelia of all species tested had died after 10 weeks of suspension in undiluted DSW. The longest survival (up to 2 months) was observed in *A. versicolor* DS and *C. globosum* DS. A considerable percentage of the spores of the Dead Sea isolates of *A. versicolor*, *E. herbariorum*, *C. globosum*, *G. marismortui* survived exposure to undiluted DSW through the period of the experiment (12 weeks). Spores and mycelia of all Dead Sea strains remained viable after a 3-month suspension in 80% DSW.

*C. globosum* DSS demonstrated the same survival pattern as the corresponding DS strain. This species was chosen for its cosmopolitan distribution and is abundant in many regions of Israel. The shore isolate produces ascospores that are somewhat larger than typical for the species [21], which normally are up to $12 \times 9$ μm in size [19]. *C. globosum* DS exhibited the same trait. Notwith-

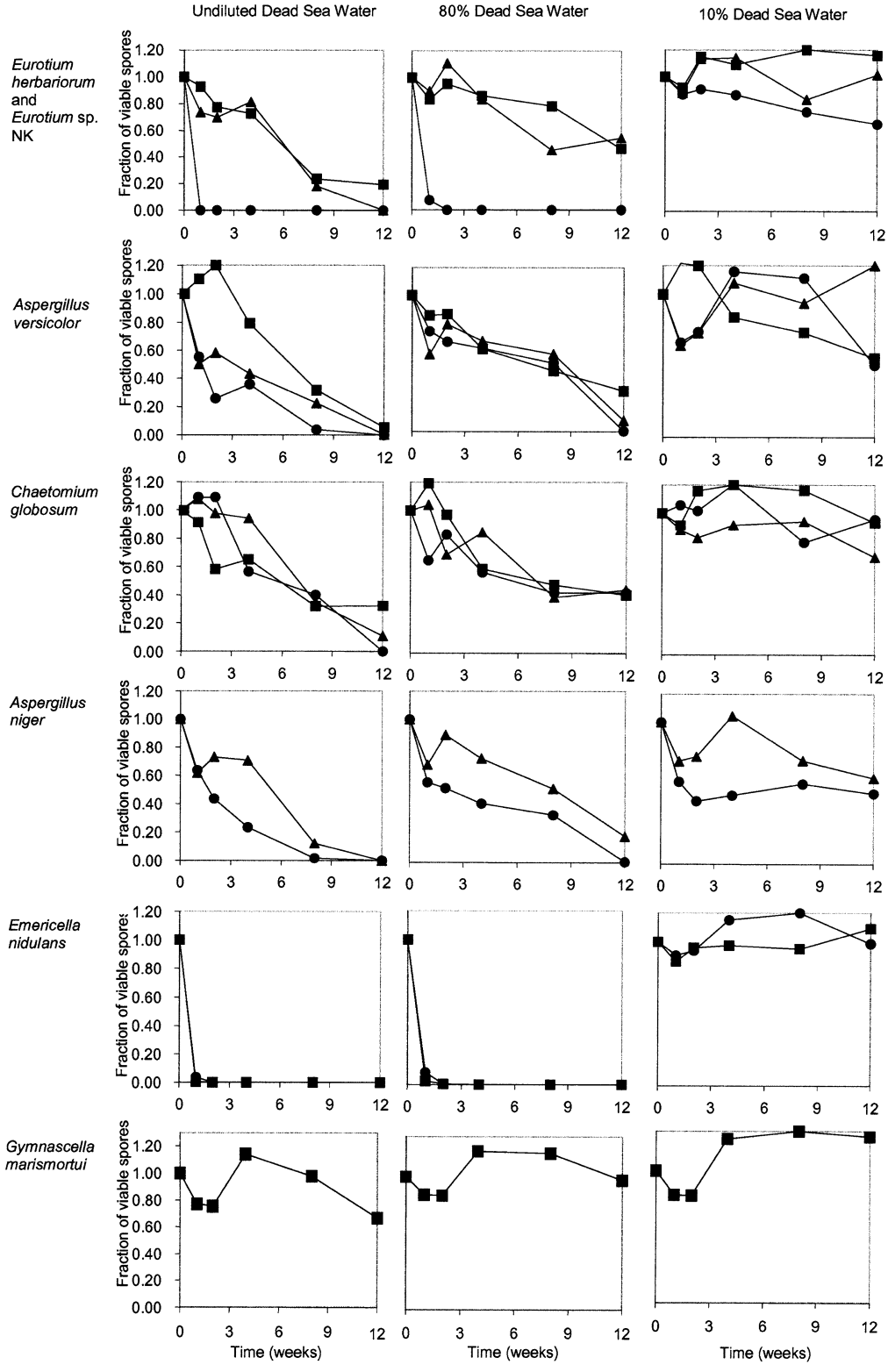


Fig. 2. Survival of spores of different species of filamentous fungi isolated from the Dead Sea in different concentrations of Dead Sea water, as compared to the survival of spores of similar isolates obtained from the terrestrial shore of the Dead Sea and from Mediterranean forest soil, Nahal Keziv ("Evolution Canyon" II, Lower Nahal Keziv, western Upper Galilee). Spores of Dead Sea isolates (squares), Dead Sea shore isolates (triangles), spp., *Aspergillus versicolor*, *Chaetomium globosum*, *A. niger*, *Emericella nidulans*, and *Gymnascella marismortui* were suspended (between 148 ± 22 and 324 ± 52 spores/mL) in undiluted Dead Sea water (left panels), and in 80% (middle panels) and 10% dilutions of Dead Sea water in distilled water (right panels). The fraction of viable spores was assessed as the number of viable spores remaining after different periods of incubation at

standing the fact that this species had only been isolated once from the Dead Sea water, the high percentage of survival of its spores and mycelia and the morphological deviation of both shore and water isolates may define a salinity-adapted variant of this species.

The fungal strains recovered from the highly saline sand of the DSS (*A. niger*, *A. versicolor*, *E. herbariorum*, and *C. globosum*) showed an intermediate pattern between that of the isolates from the DSW and the control in both spore and mycelium testing. They were less tolerant to incubation in undiluted Dead Sea water than the isolates obtained from the water column (less then 10% survival after 3 months in undiluted DSW), but in 80% DSW spores remained viable to the same extent as the DS isolate. The dark sterile mycelia frequently isolated from the DSS survived for a relatively long time (10 weeks) in 80% DSW. A similar isolate was recovered from the near-shore area of the DSW in 1999.

Spores of *E. nidulans* DS and ECII rapidly perished upon suspension in undiluted and 80% DSW. Similarly, spores of an *E. nidulans* isolate from the Mediterranean non-saline soil, "Evolution Canyon" I, Lower Nahal Oren, Mt. Carmel [7], did not survive exposure to Dead Sea water (data not shown). Thus, the viability of spores and mycelia of this species are not influenced by the source of isolation or show little adaptation to the Dead Sea. Interestingly, the mycelium of this species survived for longer periods in Dead Sea water than its spores.

The genus *Eurotium* is regarded as xerophilic, and its representatives have been reported to live in concentrated salt or sugar solutions at water activities as low as 0.70 [12]. Although *E. herbariorum* DS survived well, both as spores and as mycelium, the species obtained from the shore was found to be sensitive, and no viable spores or mycelium of *Eurotium* sp. ECII were found after 2 weeks in undiluted DSW or 4 weeks of suspension in 80% DSW. *Eurotium* sp. ECII exhibited the same xerophilic growth patterns as *E. herbariorum* and *E. amstelodami* when tested (data not shown). Therefore, the xerophily of the genus in general, and of the ECII species in particular, supports the conclusion that the nature of the environment from which it was isolated influences the survival of spores and mycelium at high salt concentrations.

Filamentous fungi are highly adaptable organisms. Some species of *Aspergillus* and *Penicillium* grow at above 25% NaCl [22]. However, Dead Sea brine, with its high concentration of divalent cations, is more stressful than NaCl solutions. Xerophilic species such as *E. herbariorum* and *E. rubrum* grow poorly on agar plates prepared with 50% DSW.

New fungi may be expected to enter the Dead Sea continuously as fungal hyphae or spores by wind, rain, and runoff as well as by the Jordan River and the nearby springs; migration of fungi between the shore and the water column is also probable. Undiluted Dead Sea water does not support growth of any known fungal species. However, all isolates tested maintained viable mycelium in Dead Sea water diluted to 50% and 10% of its original salinity, and in the diluted water the spores germinate and develop mycelia and mature conidiophores. Areas near freshwater springs or the Jordan River estuary may be diluted havens. Dormant spores present in the surrounding area may, therefore, obtain a chance of developing in the water column during those rare episodes in which the salinity of the upper water layers becomes reduced as a result of massive fresh water inflow in exceptionally rainy years. While the salinity in the lower water layers remains relatively constant at a density of 1.236 g/mL, the upper meters of the Dead Sea water column may become diluted to a significant extent. Thus, in the winters of 1980–1981 and of 1991–1992, at the end of the rainy season, the salinity of the upper 4–5 m of the sea was decreased by about 15% and about 28%, respectively [15–18]. Our experiments performed in 80% DSW can therefore be considered a simulation of the conditions following the formation of a diluted upper layer.

The results presented in this study suggest that fungal species not adapted to life at high salinity cannot survive for prolonged times in the Dead Sea brines. The potential for the introduction of foreign fungal species into the Dead Sea is therefore very low. We have shown here that at least certain species may maintain viable mycelium in 80% DSW and viable spores in undiluted DSW. This survival may be important as an adaptation of fungi to life in the hypersaline waters of the Dead Sea, where development of mycelium may be possible only during rare episodes in which the salinity of the upper water layers becomes reduced as a result of massive inflow of fresh water. This study implies that filamentous fungi develop a complete life cycle in the Dead Sea of the lake remains an open question.

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