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Fungi in hypersaline environments – from brine to microbial mats

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ABSTRACT

Fungi, one of the ecologically most successful lineages, can adapt to natural extreme environmental conditions of water, temperature, pH and salinity. It was only recently that fungi were isolated from natural hypersaline environments. Before systematic studies on halophilic and halotolerant fungi in nature were conducted, it was believed that fungi occur only on food preserved with low water activity (a_w), while their occurrence in natural hypersaline environments was due to a random event caused by airborne inoculums. So far accumulated evidence indicates that diverse polyphyletic halophilic and halotolerant fungal species inhabit salterns and salt lakes world-wide. They grow and reproduce in hypersaline waters, on wood, form biofilms and, as recent evidence suggests, they also grow in microbial mats. Halophilic fungi are adapted to the extreme conditions by a number of different mechanisms, which can be understood by their special morphology, physiological characteristics and by special molecular mechanisms. The study of fungal communities is a challenging process due to the high diversity, complexity, difficulty of direct observations and low cultivability of many species. Molecular methods combined with physiological and biochemical characterizations and traditional, morphology-based techniques are needed to fully understand the composition and function of the fungal community.

BIODIVERSITY OF FUNGI IN THE SALTERNS

Only microorganisms are able to populate certain extreme environments. This holds true for natural haypersaline lakes or man-made salterns. In addition to high salinities (up to 35% NaCl), strong UV irradiation and low oxygen concentrations, represent extreme conditions (Javor, 1989). Until we have started our investigations on the presence of halophilic and halotolerant fungi in naural hypersaline environments (Gunde-Cimerman *et al*., 1997) it was generally assumed that microbial life in concentrated sea water is composed only of Archaea, Bacteria, a few species of algae, and not fungi (Ventosa and Nieto, 1995). The xerophilic fungi known at that time did belong to a few genera that caused food spoilage (Northolt *et al*., 1995), while the term halophile for fungi was introduced only in 1975 (Pitt and Hocking, 1985) for few xerophilic food-borne species that exhibited superior growth on media with NaCl as controlling solute. Fungi have been subsequently described in moderately saline environments, such as salt marshes (Newell, 1996), saline soil (Guiraud *et al*., 1995) and sea water (Kohlmeyer and Volkmann-Kohlmayer, 1991), but were still considered to be unable to grow in highly saline waters. The occasional discoveries of fungi in hypersaline environments were interpreted as random events, caused by deposition of sturdy airborne spores. It was generally accepted that fungi do not have any specific ecological function in natural hypersaline environments.

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Our studies were initiated in the Slovenian Adriatic marine salterns Sečovlje, that originate from the 13th century (Schneider, 1995). By using highly selective media with high concentrations of either NaCl or sugar and chloramphenicol to repress bacterial growth, we discovered a surprisingly rich diversity of fungi in the hypersaline brine (Gunde – Cimerman *et al*., 2000). Later, their presence and diversity were confirmed as well in brine of natural and man-made hypersaline environments in Croatia, France, Spain, Portugal, Israel, Namibia, Dominican Republic, Puerto Rico and Utah (Kis-Papo *et al*., 2003; Gunde-Cimerman *et al*., 2004, Butinar *et al*., 2005a; Díaz-Muñoz and Montalvo-Rodríguez, 2005), with NaCl concentrations up to 32% NaCl.

Meristematic black yeasts, *Cladosporium* sp. and genus *Wallemia* were identified among the most common species, along with certain species of ascomycetous and basidiomycetous nonmelanized yeasts, various species from the genera *Aspergillus*, *Penicillium* and their teleomorphs *(Eurotium)*.

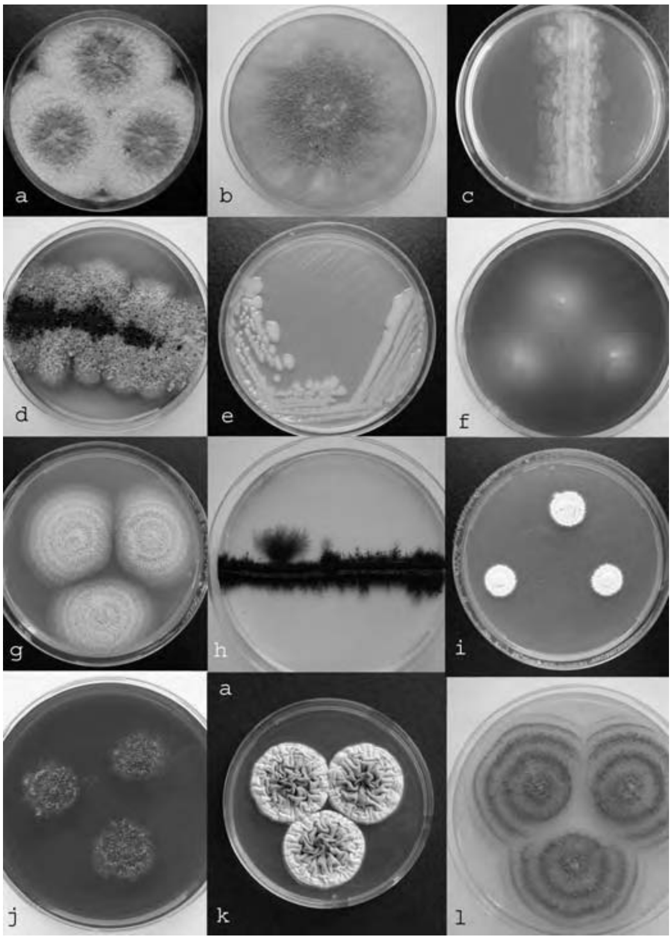

Fig. 1. Colorful fungal colonies and dynamic patterns of their life cycles. Colonies are richly pigmented and display a variety of colors, from black to white, with shades of orange, red, green.
-a- *Eurotium amstelodami*; -b, d, g- *Epicoccum nigrum*; -c- *Mucor* sp.; -e- *Rhodotorula mucilaginosa*; -f- *Penicillium chrysogenum*; -h- *Hortaea werneckii*; -i- *Aspergillus candidus;* -j- *Emericella variecolor*; -k- *Penicillium corylophilum*; -l- *Aspergillus flavus*;

Black yeasts of the order Dothideales have surprising abilities to grow in many different extreme environments and also in a wide range of salt concentrations (from 0 to 32% NaCl). Due to their constant presence in saltern waters, their polymorphism as a possible adaptation to a wide range of salinities, we described black yeasts as the natural inhabitants of hypersaline waters (Gunde – Cimerman *et al*., 2000). These melanized polymorphic fungi, as the group of black yeasts was also named, are able to grow in metabolically different forms. They represent an extremophilic

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ecotype, characterized by slow, often meristematic growth, reproduction by endoconidiation and thick, melanized cell walls (Zalar *et al*., 1999). These organisms only rarely appear outside saline environments, which is probably a consequence of adaptive evolutionary processes. They can tolerate different concentrations of salt, but cannot grow at the same osmotic values of substrate, if water activity is lowered with sugar. The main representatives are halophilic *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and halotolerant *Aureobasidum pullulans* (Gunde – Cimerman *et al*., 2000; 2004).

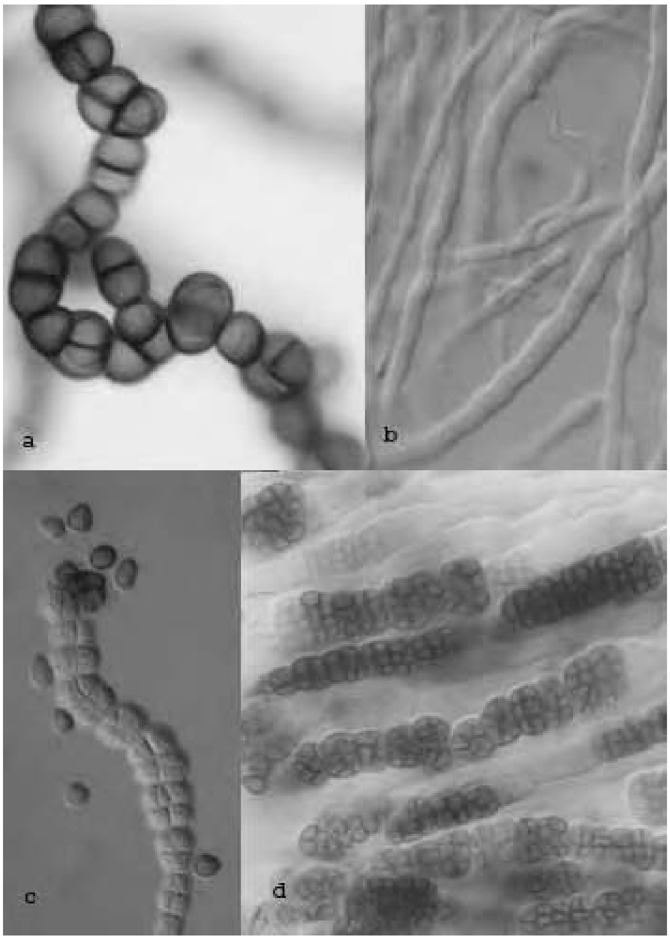

Fig. 2. Adaptations and diversity of forms of fungal mycelium.
-a- *Trimmatostroma salinum*; -b- *Aspergillus candidus*; -c, d- *Phaeotheca triangularis*.

The ubquitous, saprobic genus *Cladosporium* represents another, very adaptable group of fungi inhabiting the salterns. Morphologically *C. sphaerospermum*-like and *C. herbarum*-like isolates form a complex of several species, of which most showed halotolerance as a recurrent feature. Mainly on the basis of phylogenetic analyses but also of cryptic morphological and physiological characters, nine additional species were newly described (Schubert *et al*., 2007; Zalar *et al*., 2007).

Certain species of the ubiquitous genera Aspergillus and Penicillium with their associated teleomorphs constitute a stable fungal community in the hypersaline waters. The species geographically most widely distributed, numerous and consistently present in hypersaline waters worldwide were *A. niger*, *E. amstelodami* and *P. chrysogenum*. In the course of the taxonomic study of this fungal group five new species were retrieved: one from the genus *Eurotium* (Butinar *et al*., 2005b) and four from *Penicillium*.

The presence of non melanized yeasts was investigated as well. Although twelve species were isolated, *Pichia guilermondii*, *Candida parapsilosis* and *Trichosporon mucoides* had the highest

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frequency of occurrence. Three novel yeast species were recognized; one from the genus *Pichia* and two from the genus *Candida* (Butinar *et al.*, 2005c). It is noteworthy that most of the isolated yeasts are recognized for their opportunistic pathogenic nature, as well as that they occurred primarily in oligotrophic waters, characterized by both a high NaCl content, and a high $MgCl_2$ content.

From numerous hypersaline environments we also isolated osmophilic fungi, known as important contaminants of sweet and salty food. The representative of this group was the genus *Wallemia*, which appeared in environments with low water activity, not necessarily associated with the presence of salt (Samson *et al.*, 2002). Our results have shown that *Wallemia* presents one of the most xerophilic fungal taxa. To acknowledge its unique morphology, evolution and xerotolerance, a new basidiomycetous class Wallemiomycetes covering an order Wallemiales was proposed. The genus *Wallemia* now contains three species, *W. sebi, W. muriae* and *W. ichthyophaga*, the latter being the most halophilic eukaryote known up to date (Zalar *et al.*, 2005a).

Marine fungi are classified as halotolerant microorganisms, which are only rarely occurring outside this specific habitat. From hypersaline waters we isolated the only marine representative fungi belonging to the genus *Emericella*, otherwise known from tropical and subtropical regions. Two species were recorded in salterns, *E. filifera* and *E. stella-maris*, the latter described as a new species (Zalar *et al.*, in press).

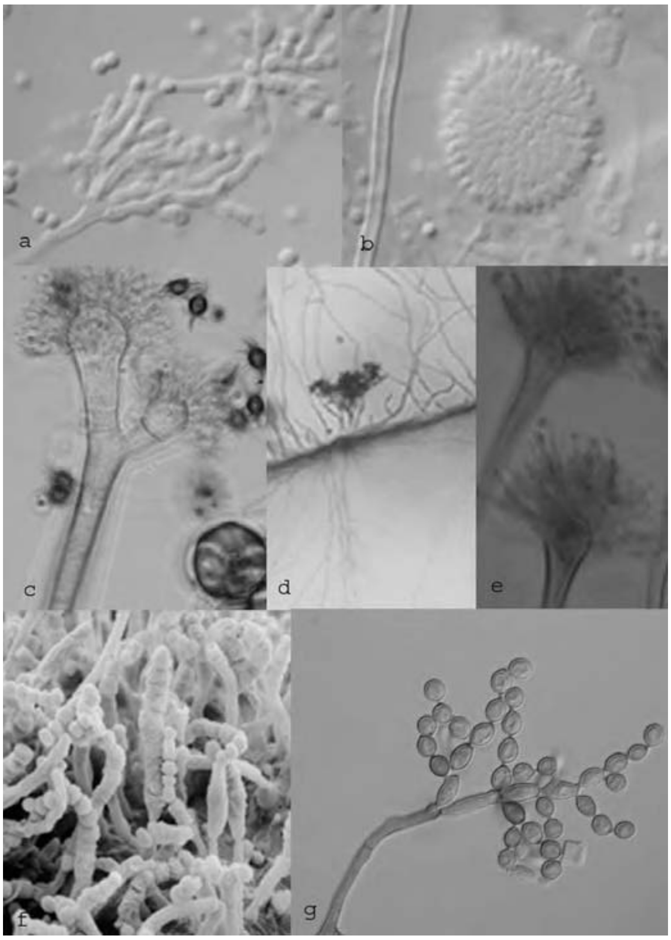

Fig. 3. Fungal conidiophores.
-a, b- *Aspergillus candidus*; -c- *Emericella stellamaris*; -d- *Cladosporium sphaerospermum*; -e- *Emericella appendiculata*; -f- *Wallemia ichthyophaga*; -g- *Cladosporium halotolerans*.

In conclusion, halophilic and halotolerant mycobiota have been found in two of four fungal phyla: in Ascomycota and in Basidiomycota. Comparison of phylogenetically divergent fungi has shown that species actively growing in hypersaline environments have evolved several times. Halophily

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and halotolerance are features most frequently encountered in Dothideales and Eurotiales. It seems that different groups of fungi are in different stages of adaptation to hypersaline environments, varying from a recent radiation (e.g. genus *Cladosporium*) with eurytolerance opening a new window of opportunity, to extremely flexible halotolerant fungi (e.g. black yeasts of genera *Hortaea*, *Phaeotheca*, *Trimmatostroma*) and stenotolerant phylogenetic end-stages of evolution, represented by the genus *Wallemia* (de Hoog *et al*., 2005).

Fungal counts of about 10 cells per litre are typical for ocean water while in polluted waters they can increase up to several thousands (Slavikova *et al*., 1992),. In the hypersaline waters of the salterns, temporal fluctuations in the density of fungi ranged between 10 and 40 000 CFU per litre. Their dynamic pattern across salinity gradient appeared generally in two pronounced peaks: the first at approx. 15% salinity (up to 5000 CFU per litre) and the second at approx. 20% salinity (up to 40 000 CFU per litre) (Gunde-Cimerman *et al*., 2000; 2004). Both peaks correlated with increased phosphorpus and nitrogen levels. Statistical canonical correspondence analysis showed the importance of temporal factor, a_w as well as nutrients on the distribution of species that form a stable core of fungal community in hypersaline waters.

OCCURRENCE OF FUNGI IN SALTERN’S MICROBIAL MATS

Microbial mats are a laminated consortial system, functionally integrated and self-sustained,that can be found in tropical and temperate hypersaline waters (Van Gemerden, 1993; Paerl *et al*., 2000). Fluctuating diel and seasonal physicochemical gradients (predominantly the light regime) characterize these organosedimentary ecosystems and result in both strata and microenvironments that harbor specific microbial communities (Dupraz and Visscher, 2005; Visscher and Stolz, 2005). In the surface green layer, which is typically the most active metabolically, these consortia are dominated by phototrophs (cyanobacteria), heterotrophs and chemoautotrophs. In deeper layers, the role of phototrophs diminishes. During the formation of the microbial mat, two layers are typically formed: a surface oxic (green) and a lower, anoxic (black). A third pink stratum, which may form between these two layers, represents a redox transition zone. In a well-developed mat, the surface oxic layer is dominated by diverse cyanobacteria that are responsible for the primary production, fueling heterotrophic activity in the entire mat. The anoxic layer is dominated by colorless sulfur, anoxygenic phototrophic and sulphate reducing bacteria. Some eukaryotic organisms can be found there as well, including flagellates, ciliates, and algae (particularly diatoms) (Casillas-Martínez *et al*., 2005).

Besides this type of mat found in most salterns around the world, Slovenian Sečovlje salterns harbour a firm man-made mat called “petola”, consisting mainly of the extremely halophilic phototrophic cyanobacterium *Microcoleus chthonoplastes* (Schneider, 1995). Petola was introduced to Sečovlje salterns in the 14th century, from the Adriatic island of Pag, famous for centuries throughout the Mediterranean for its production of very high quality salt. After its introduction six centuries ago, it has been continuously cultivated and maintained, because it is believed that the halophilic microorganisms in petola contribute to the quality of the salt by removing certain metals and other impurities. Due to its origin and longevity, it is protected as part of the Sečovlje National Park.

Until recently there were no reports of fungi in microbial mats. Their presence was for the first time investigated in tropical Puerto Rico salterns (Cantrell *et al*., 2006), and later on in a preliminary, not yet published study also in the temperate microbial mats of Slovenian Sečovlje salterns. A total of 30 species were isolated from both salterns: black yeasts, *Aspergillus niger*, *A. flavipes, Penicillium flavigenum, Cladosporium* spp. and *Rhodotorula* spp. were the most frequently encountered isolates. The number of isolates decreased from the first (green) to the third (black) layer.

When the upper layer was stained with Calcofluor, specific for fungal cell walls, fungal mycelium were observed. Phospholipid fatty acid analysis (PLFA) profiles generated from the different layers of the mat indicated that the uppermost layers contained fungal biomarker, 18:2w6. This fatty acid decreased with depth, and disappeared in the black bottom anoxic layer. DNA was extracted from the different layers of the mats and the fungal ITS (Internal Transcribe Spacer) rDNA region was

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amplified for terminal restriction fragment length polymorphism (TRFLP) analysis. The TRFLP profiles of the different layers indicated that diversity decreased from the green layer to the black layer and revealed the presence of 24 phylotypes. The preliminary results demonstrated that the diversity and abundance of fungi were higher during the wet season, as well as in the oxic (green) layer (Cantrell *et al*., 2006).

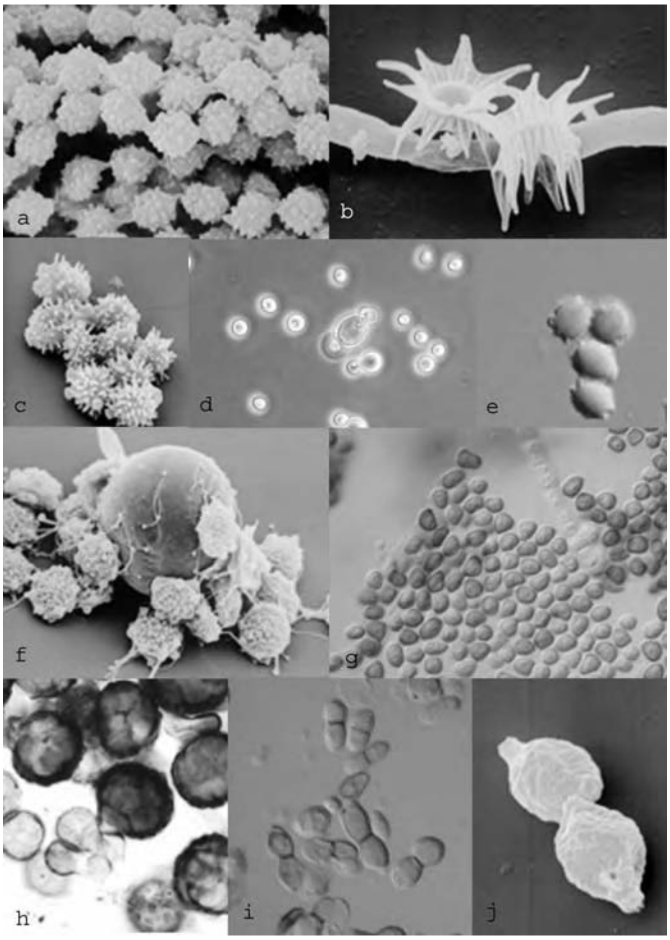

Fig. 4. Most of the filamentous fungi found in the salterns reproduce asexually, by conidia or in the case of yeasts, by budding. Some fungi undergo sexual reproduction, forming ascopores or basidiospores. Some are very sturdy, while others compensate for the weaker nature of their spores by producing spores in extremely large numbers, ensuring survival in this way. Many spores become richly ornamented or have long appendages, helping them to float in the water or glide through the air.
-a- *Aspergillus versicolor,* conidia; -b- *Emericella stellamaris,* ascospores; -c- *Cladosporium echinulatum.,* conidia; -d- *Candida albicans,* yeast cells; -e- *Eurotium amstelodami,* ascospores; -f- *Emericella filifera,* ascospores and Hülle cell; -g- *Phaeotheca triangularis,* conidia; -h- *Epicoccum nigrum,* conidia; -i- *Hortaea werneckii,* yeast cells; -j- *Cladosporim sphaerospermum,* conidia.

CONCLUSIONS

The study of the diversity of microbial communities in hypersaline environments is a challenging process due to the high complexity created by some of the mineral substrates and high salt content, the difficulty of directly observing microorganisms in this complex matrix and the low cultivability of many species. Although in recent years the study of microbial communities has benefited from the development of new culture-independent techniques such as TRFLP, qPCR, and large scale sequencing, most of the molecular microbial diversity studies have focused on bacteria, and only recently have a few studies been published on fungi. For fungi, most of the published molecular-based studies have used TRFLP or DGGE (Edel-Hermann *et al*., 2004; Lord *et al*., 2002; Viaud *et al*., 2000; van Elsas *et al*., 2000), and these only yield general indications of gross shifts in

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microbial communities (e.g., changes in fungal to bacterial ratios). A few of these studies, however, have applied qPCR and DNA sequencing from soil samples to assess fungal communities at a finer scale (Fierer *et al*., 2005; O'Brien *et al*., 2005). Not only can these techniques provide a better understanding of the fungal diversity in environmental samples, but they also can detect novel and unknown fungal groups (Jumpponen and Johnson, 2005).

The identification of halophilic and halotolerant fungi in the hypersaline waters, as well as in microbial mats, have been performed so far mainly using traditional cultivating techniques. To fully understand the composition and function of fungal communities inhabiting salterns and salt lakes, data obtained with physiological and biochemical characterizations and traditional, morphological-based techniques should be supplemented by molecular approaches. Future efforts should be focused on the extraction of DNA directly from the brine and microbial mats in temperate and tropical salterns, with the aim to construct comparable genomic libraries and to study the structure of the fungal community using such methods as TRFLP and TGGE. The experiments should include the use of other fungal primers and restriction enzymes as well as more extensive cloning efforts for the identification of fungal species *in situ* and subsequent comparisons with sequences obtained from pure cultures.

The use of molecular techniques will reveal unknown phylotypes and species. It is certain but likely, based on work carried out in other environments, that besides, more importantly, these techniques will shed more light on the understanding of the potential role of fungi in these extreme environments.

In terrestrial ecosystems fungi are key players in the decomposition process of detritus material. The different chemical compounds in detritus can be divided in recalcitrant that includes very complex carbohydrates such as lignin and labile that includes more simple carbohydrates such as sugars, hemicellulose and cellulose. Recalcitrant compounds are generally broken down by fungi, while labile ones by fast growing organisms such as bacteria. Thus during the decomposition process a succession of microorganisms occurs, mediated by the interaction of different organisms, resource composition and abiotic factors.

For example – prokaryotes, predominantly cyanobacteria in mats – produce large quantities of EPS (extracellular polymeric secretions) which are very complex carbohydrates (Decho *et al*., 2005; Dupraz and Visscher, 2005). These are consumed in the mats and it is highly likely that fungi in concert with the bacterial community contribute to the degradation of EPS and the subsequent mineralization of carbon. Evidence of an underestimated saprotrophic role of fungi in the salterns comes from the repeated isolation of black yeasts and other halophilic fungi from slimy EPS layers on the surface of the brine in the crystallizers and from wood immersed in hypersaline water. Isolated black yeasts *H. werneckii* and *T. salinum*, which colonized the wood, showed xylanolytic and lignolytic activity under hypersaline and non-saline conditions, while *T. salinum* displayed cellulolytic activity as well (Zalar *et al*., 2005b). These results suggest an active saprobic role of halophilic and halotolerant fungi in the environment.