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Hypersaline waters in salterns – natural ecological niches for halophilic black yeasts

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Abstract

Hypersaline waters in salterns have so far been considered to be populated only with halophilic algae and bacteria and completely lacking halophilic fungi. In this paper we present population dynamics of polymorphic black yeasts, isolated from hypersaline waters (3–30% NaCl) of a saltern, in relation to different physicochemical parameters. *Hortaea werneckii, Phaeotheca triangularis, Trimmatostroma salinum, Aureobasidium pullulans* and *Cladosporium* spp. were detected with the highest frequency just before the peak of halite (NaCl) concentration. Since *H. werneckii*, *P. triangularis* and *T. salinum* are not known outside saline environments, these results suggest that hypersaline water is their natural ecological niche.

Keywords: Saltern; Hypersaline water; Crystallization pond; Halite; Halophilic fungus; Black yeast

1. Introduction

Only microorganisms are able to populate certain extreme environments. This also holds true for natural or man-made marine salterns. The main characteristics of ponds in the salterns are evaporating water with salinity ranging from 3% to saturated solutions of NaCl (35%), low oxygen concentration, high light intensity, ample nutrient availability and neutral pH [1].

During evaporation, the chemical composition of the sea water is altered by complex processes of dissolution and precipitation. The major compound precipitating during the last stage of salt production at a final concentration above 30% is NaCl (halite) [1]. At that time a bright red phototrophic bacterial biofilm is formed on the water surface. This is an indication of salt crystallization [2].

It is generally assumed that microbial life in concentrated sea water is composed only of algae, protozoa, Archaea and Bacteria and not fungi [1,3,4]. However, xerophilic/halophilic fungi, able to grow on different media with low water activities (a_w), may be expected. The known ones belong to a few genera and mostly cause food spoilage [5]. The term 'halophile' for fungi was introduced in 1975 [6] for those few xerophilic food-borne species that exhibit quite superior growth on media with NaCl as controlling solute. Fungi have subsequently been described in moderately saline environments, such as salt marshes [7], saline soil [8] and sea water [9], but were considered to be unable to grow in highly saline waters.

For our studies we selected the active marine salterns Sečovlje in Slovenia, along the Adriatic coast, protected under the UNESCO Ramsar convention and originating from the 13th century [2].

In the hypersaline waters a surprisingly rich diversity of fungi was discovered [10]. In this study we present data on fungal populations in a crystallization pond during the months of salt production, with salinity in the range of 3–35% NaCl.

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2. Materials and methods

2.1. Description of the saltpans

The salterns Sečovlje, a natural xeric environment, were founded in 1274 by the Venetians. They are situated in the south east part of the Piran bay, at the delta of the Dragonja river, at the border between Slovenia and Croatia. During the winter the climate is humid and there is no salt production. During the summer the climate is arid and salt is being produced. Since evaporation of water is enhanced by strong local winds, temperatures of the shallow water are moderate (18–32°C) even in summer. A gradual concentration of sea water is achieved over 16 consecutive evaporative ponds, separated by canals. When the water reaches a suitable salt concentration, it is led over wooden barriers to the next pond. Therefore it is possible to follow all different stages of evaporation from May to October. The major compound precipitating in the crystallization pond is NaCl, while the water is enriched in $MgCl_2$.

The sampled crystallization pond measures 50×100 m, and contains a 5–10-cm layer of hypersaline water in the central part. It is surrounded by a 30 cm deep canal, to which preconcentrated water is led. The sedimentation surface of the pond is stabilized by a firm microbial mat consisting mainly of the extremely halophilic phototrophic cyanobacterium *Microcoleus chthonoplastes* [2,11].

Hypersaline water of the crystallization pond was sampled every 3 weeks while salt was produced, from the beginning of May to the end of October in 1997.

2.2. Isolation methods

Four different isolation methods were used. The aim was to avoid selection of certain mycobiota favored by any particular method. The population dynamics of fungi was followed using filtration.

1. Filtration of 10–100-ml aliquots of saline water was performed immediately after sampling on Millipore membrane filters (0.45 μm) and placed on two enumeration and six different selective agar media of low water activities, established by either high salt or high sugar concentrations. A drop of the original salty water was applied on the membrane and dispersed with a Drigalski spatula. For every medium four aliquots were filtered in parallel and the average numbers of colony forming units (CFU) were calculated. Plates were incubated from 1–10 weeks at 25°C. CFU on enumeration media were counted every 3, 5, 7, 14, 30 and 60 days of incubation.
2. Agar baits in dialysis tubings and in glass tubes [12] were left for 5 months in the crystallization pond. In autumn all samples showed black or reddish overgrowth. Agar blocks were removed, cut and placed on low water activity media. Natural wood baits were collected as well.
3. An enrichment technique was applied as follows. To 100 ml of saline water 1 g glucose and 0.5 g yeast extract (YE) was added and incubated for 1 week on a rotary shaker (50 rpm) at 25°C. Subsequently 10 ml broth was diluted with 90 ml of sterilized 17% saline with added YE and glucose and incubated again for 1 week. This procedure was repeated five times and growth was recorded weekly.
4. Biofilms were also spread on low water activity selective media.

All fungi isolated from selective media were inoculated in parallel on malt extract agar (MEA) and on MEA+17% NaCl. Only those fungal colonies that were able to grow at this concentration of salt were further analyzed. Growth rate and morphological characteristics were determined on both media after 7 and 30 days.

For enumeration of CFU per 100 ml of water, a general purpose medium (DRBC, a_w 1.0) [13] and a medium for the detection of moderate xerophiles (DG-18, a_w 0.96) [14] were used. Selective media with sugar used for isolation of fungi were: 10% glucose–12% NaCl agar (MY10–12, a_w 0.88) [6], 50% glucose malt extract yeast extract agar (MY50G, a_w 0.89) [6] and glucose/fructose malt extract yeast extract agar (MY70GF, a_w 0.76) [6]. For salt-based selective media different salt concentrations were added to MEA [6]: MEA+17% NaCl (a_w 0.861), MEA+24% NaCl (a_w 0.83) and MEA+32% NaCl (a_w 0.78).

To all selective and enumeration media chloramphenicol (100 mg l^{-1}) was added. Water activities (a_w) of the media were determined using the CX-1 system (Campbell Scientific Ltd.).

2.3. Taxonomy

Isolated black yeasts were identified by microscopy, nutritional physiology, restriction fragment length polymorphism of SSU and ITS rDNA, and by sequencing of the 5.8 rDNA gene [15–17]. The isolated strains are maintained in the Culture Collection of the National Institute of Chemistry (MZKI-ex), Ljubljana, Slovenia.

2.4. Determination of environmental parameters

In all water samples the following physicochemical parameters were determined: pH (ISO 10523: 1994E, electrometric method), temperature, turbidity, chemical oxygen demand (COD, ISO 6060), biological oxygen demand (BOD, dilution and seeding method, ISO 5815), dissolved O_2 concentration and O_2 saturation level (iodometric method, ISO 5813), salinity (areometer), chemical analyses of Cl^- and SO_4^{2-} anions (ion chromatography Dionex 4000, ISO 10304-2) and K^+, Na^+, Mg^{2+} cations (ICP, AES Thermo Jarrell Ash). Total phosphorus content was

Table 1
Physico-chemical parameters of the water in saltpans

	Date						
	22-5	10-6	2-7	23-7	13-8	5-9	30-9
Cl^- (g l^{-1})	9.4	56.3	85.5	128	160	184	60.3
K^+ (g l^{-1})	0.46	1.2	1.4	2.6	3.9	4.8	1.1
Na^+ (g l^{-1})	12.1	33.7	36.1	65.3	87.4	95	34.6
Mg^{2+} (g l^{-1})	1.5	4.39	4.96	8.8	13.1	18.8	3.7
SO_4^{2-} (g l^{-1})	0.47	9.55	9.62	14.9	19.4	26.1	7.34
Water activity	0.982	0.924	0.911	0.901	0.818	0.772	0.935
Salinity (%)	4.5	7.5	10	18	22	27	10
N_2 (mg l^{-1})	0.1	2.28	0.1	1.46	13.2	10.6	5.9
P (mg l^{-1})	0.04	0.04	0.04	0.07	0.29	0.06	0.04
O_2 (%)	115	89	134	84	45	65	96
O_2 (mg l^{-1})	10.3	8.5	9	7	3.5	5.4	9.3

determined spectrometrically after mineralization with persulfate (ISO 6878/1: 1986E), organic nitrogen and ammonia content were measured by the macro-Kjeldahl method after mineralization with selenium (ISO 5663: 1984) and water activity (a_w) with the CX-1 system (Campbell Scientific Ltd.).

3. Results

3.1. Environmental parameters

The most extreme environmental values were detected from the middle of August to the beginning of September, during the peak of salt production.

The pH of the crystallization pond varied between 7.4 and 8.1 during the entire season. During the peak of salt production in early September (salt concentration 28%) the water activity decreased to 0.75 due to high Cl^-, SO_4^{2-}, Mg^{2+}, K^+ and Na^+ ion levels. The O_2 concentration decreased as well from 12 mg l^{-1} in the initial sea water concentration at the beginning of July, to 3 mg l^{-1} in August, but its lowest level did not correlate with the highest salinity. Simultaneously the levels of nitrogen and phosphorus increased during the period of salt production (Table 1), reaching a peak in the middle of August, accompanied by high COD and BOD (data not shown).

3.2. Population dynamics

The CFU numbers obtained on general enumeration media were higher on DG-18 than on DRBC, reaching the highest numbers in August (3.9×10^4 l^{-1} on DG-18; 3.75×10^4 l^{-1} on DRBC; data not shown). The initial incubation time of 9 days on DRBC and 7 days on DG-18 could be shortened in August to 5–7 days and 5 days respectively, due to the faster appearance of colonies. The fungi detected on both enumeration media were filamentous and non-melanized. No melanized fungi were detected.

On selective media containing high sugar concentrations, the highest CFU values were found in August, all belonging to non-melanized filamentous fungi. A significant number of CFU of melanized fungi (3.9×10^3 l^{-1})

Table 2
Fungal CFU obtained after filtration of 100 ml of water on different selective media

Date		MY10–12	MY50G	MY70GF	MEA+17% NaCl	MEA+24% NaCl	MEA+32% NaCl
22-5	a[a]	383	394	0	405	–	261
	b	36	11	0	8	–	0
10-6	a	14	15	0	2	–	0
	b	26	6	0	5	–	3
2-7	a	4	0	0	4	7	1
	b	47	76	1	8	40	0
23-7	a	7	4.5	0	0	0	1
	b	3	2.5	1	3	0	0
13-8	a	1118	0	0	270	705	387
	b	355	1000	20	81	46	13
5-9	a	28	0	4	27	8	16
	b	142	100	2	67	0	0
30-9	a	17	11	0	18	155	9
	b	141	131	2	965	25	1

[a]a: Melanized fungi; b: non-melanized fungi.

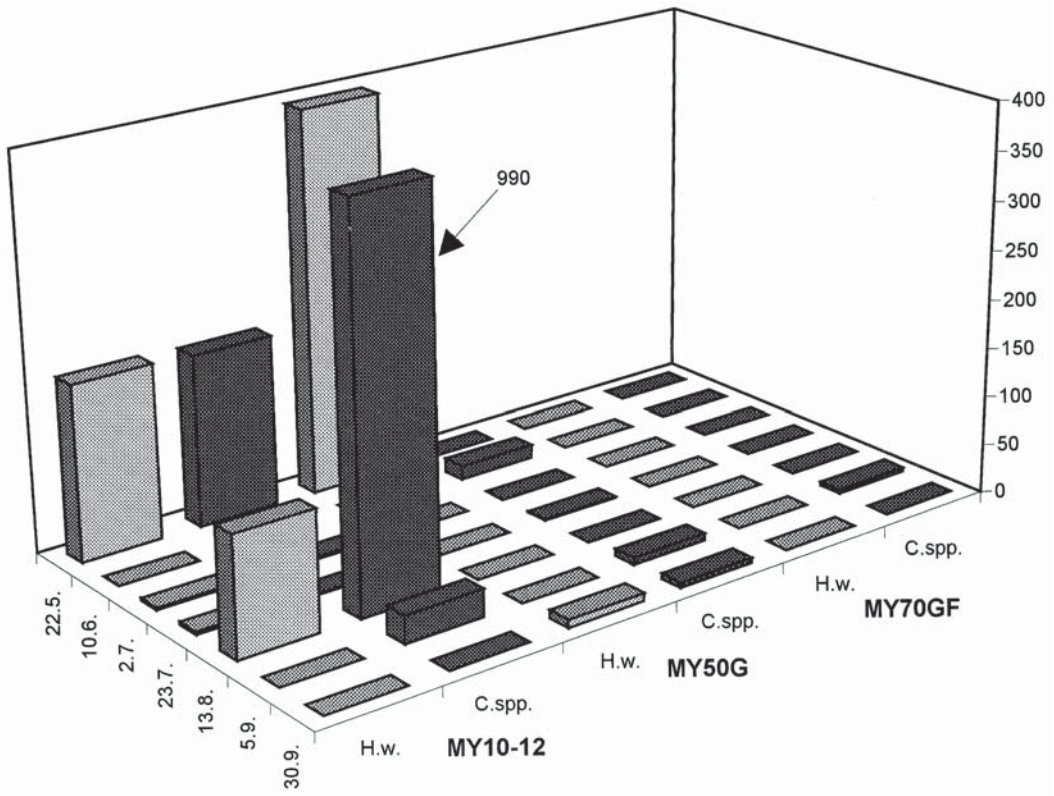

Fig. 1. CFU of prevailing species of melanized fungi in 100 ml of water, detected on selective sugar-based media. Abbreviations: H.w. = *Hortaea werneckii*, C.spp. = *Cladosporium* spp.

appeared in May on 50% sugar medium. CFU numbers were lowest on 70% sugar medium (up to 3×10^2 l^{-1}) due to the extreme water activity of the medium (Table 2).

On selective media containing different concentrations of salt, melanized fungi appeared in high numbers, with two distinct peaks: first at the beginning of the sampling season (May) and the second during the process of crystallization (August), prior to the highest salt concentration in September (Table 2). A growing proportion of melanized fungi was noted with increasing salt concentrations in the selective media. The appearance of melanized fungi on salt-containing media was rapid (5–7 days) in August and significantly slower in May (12–16 days).

3.3. Population composition

Strains obtained using all isolation methods were identified to the species level (except genus *Cladosporium*) for melanized fungi and to the genus level for non-melanized fungi. Only data on population composition of melanized fungi are presented. They were found to belong to the following species: *Hortaea werneckii, Phaeotheca triangularis, Trimmatostroma salinum* and *Aureobasidium pullulans*. These taxa were found with all isolation methods used. The composition of melanized fungal populations obtained by filtration changed during the season and was changed according to the isolation medium used.

Medium containing 50% sugar selected almost only *Cladosporium* spp. throughout the whole season. Only at the beginning and at the end of the season *H. werneckii* prevailed. On 70% sugar medium melanized fungi (*Cladosporium* spp.) were detected in very low numbers (Fig. 1).

On combined salt and sugar medium again mainly strains belonging to the genus *Cladosporium* were found. *P. triangularis* appeared at the beginning of the season with very low CFU and again in September, while *H. werneckii* appeared on this medium sporadically throughout the season (Fig. 1).

On media containing salt, melanized fungi were represented with *Cladosporium* spp. appearing only occasionally with low CFU numbers and three species: *H. werneckii*, *P. triangularis* and *T. salinum*. *H. werneckii* dominated throughout the whole season, with the exception of early September, when *T. salinum* and *P. triangularis* prevailed. A concentration of 32% NaCl selected only *H. werneckii* while other melanized fungi were completely absent (Fig. 2).

4. Discussion

For the enumeration of fungi, two media were chosen, which are otherwise used for the detection of food-borne fungi, capable of growing at reduced water activity. As expected the CFU l^{-1} values were consistently higher on DG-18 than on DRBC, since the natural selective pressure

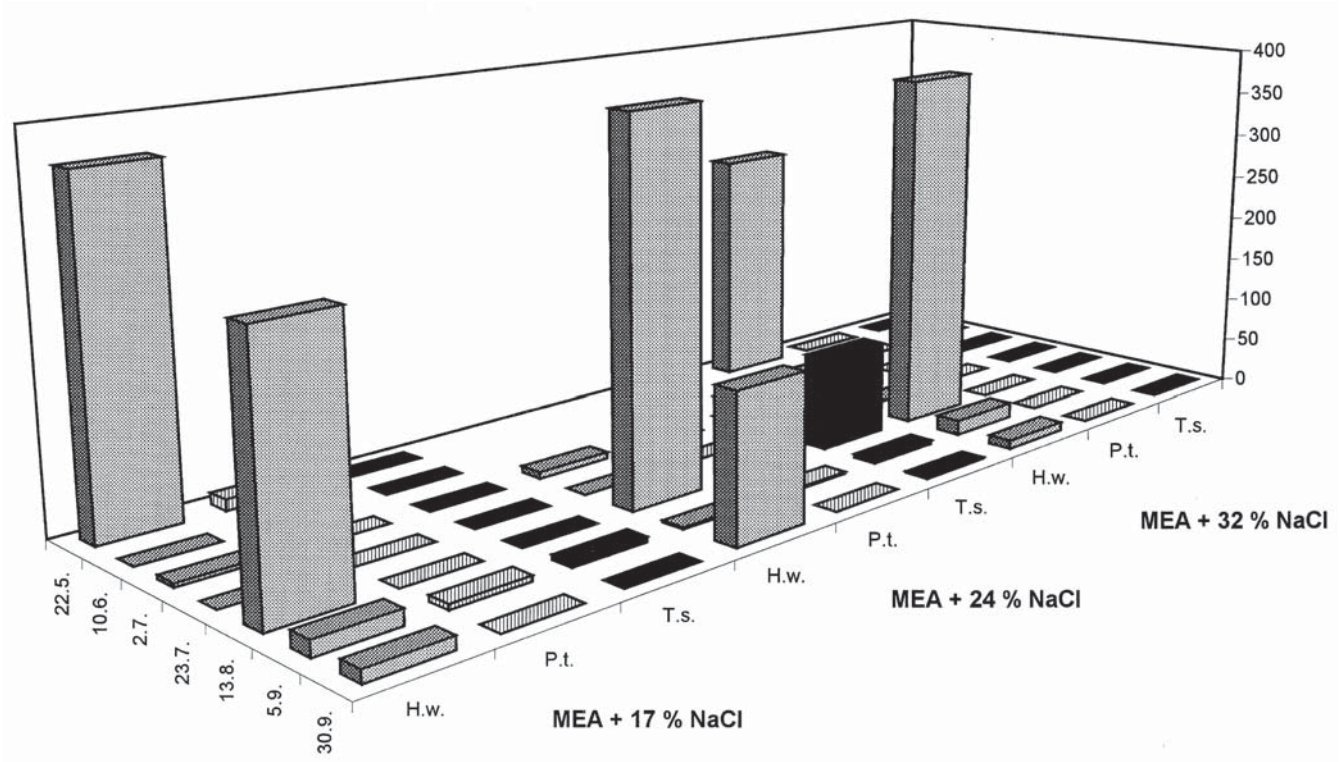

Fig. 2. CFU of prevailing species of melanized fungi in 100 ml of water, detected on selective salt-based media. Abbreviations: H.w. = *Hortaea werneckii*, P.t. = *Phaeotheca triangularis*, T.s. = *Trimmatostroma salinum*.

of the hypersaline water gives a competitive advantage to xerophilic fungi. No universal medium is available for the detection of all xerophiles [18]. Two sugar-based media routinely used for isolation of food-borne xerophiles were used [5]. Additionally a combined 12% sugar/10% salt medium and three media with high concentration of salt (17, 25, and 32%) were used for selective isolation of halotolerant and halophilic fungi. Pitt and Hocking [6] characterized fungi growing below a_w 0.85 as xerophilic/halophilic. Therefore we tested all fungi isolated for their ability to grow on 17% NaCl (a_w 0.85).

Fungal counts of about 10 cells l^{-1} are typical for ocean water [19]. The number of CFU, mostly yeasts, can increase to several thousands due to pollution or in the presence of algae [19]. In the hypersaline waters of the salterns, temporal fluctuations in the density of fungi ranged between 10 and 40 000 CFU l^{-1} on enumeration media and 0 to 11 000 CFU l^{-1} for melanized fungi on selective saline media.

Melanized fungi showed their halophilic nature by appearing with the highest numbers on saline media at the beginning (May) and the end of the season (August). The first peak was pronounced on all selective low water activity media. This may be explained by the composition of pond water, which was supplemented in May with hypersaline water of the previous evaporation season, stored in a special pond for approximately 7 months. Due to the prolonged adaptation to highly osmotic solutions, mainly melanized fungi with wider tolerance survived. The second peak in August did not completely correlate with salinity, but rather with maxima of phosphorus and nitrogen. It should also be taken into account that cell counts increase simply due to the evaporation of water in the pools.

The species isolated can be divided into two groups. The non-halophilic fungi (a), isolated mainly on sugar-containing media, belong to non-melanized taxa that are otherwise found on foodstuffs, e.g. *Wallemia sebi, Aspergillus* spp., etc. Some might have their natural niche in the salterns, but it is more likely that they were mainly blown into the water from surrounding soil or from the bottom soil of the pond [10], which is emptied during the winter. After filling the pond with saline water, propagules may have survived osmotic stress, but would not necessarily be able to propagate.

The halotolerant and halophilic fungi (b), isolated on saline media, were almost exclusively melanized fungi belonging into the group of black yeast-like hyphomycetes. They obviously had a selective advantage over the other mycoflora, underlining their preference for a saline environment. Black yeasts were able to survive and propagate in the saline water. This was also shown with baiting in environment and enrichment technique in the laboratory. During August, when environmental nitrogen and phosphorus concentrations were maximal and the O_2 concentration minimal, black yeasts were probably in exponential growth phase, 'demonstrated' by a shortened incubation time.

Black yeast species showed different ecological preferen-

ces. The most frequent one was *H. werneckii*, which was dominant in the environment during the whole season. This fungus was particularly abundant during the halite crystallization period in August and was the only species growing on the whole range of NaCl concentrations from 0% to saturation. On the basis of in vitro eco-physiological studies [20], its ecological niche was suggested to be intermittently drying salty pools. We conclude that the natural ecological niche of *H. werneckii* may be the highly saline water of the crystallization pond.

P. triangularis was detected exclusively on salt-containing media. It shows high adaptability to saline conditions, with the highest frequency of appearance in water between 22 and 28% salt concentration. This species was shown to assimilate carbohydrates and nitrogen sources consistently better in the presence of 5% additional salt [16]. However, in contrast to *H. werneckii*, *P. triangularis* was unable to grow on 32% salt, 26% NaCl being the maximum. It is concluded that *P. triangularis* is a halophilic species with a narrow ecological amplitude.

The same holds true of *T. salinum* [17], which appeared earlier in highly saline media and peaked during salt crystallization.

The genus *Cladosporium*, being detected on moderately saline, but mainly on sugary media throughout the season, was represented by several, hitherto unidentified species. Since they were detected as well in high CFU numbers at 28% NaCl concentration they might nevertheless be metabolically active in water.

A. pullulans can be regarded as a halotolerant species, occurring on combined salt/sugar medium and tolerating 17% salt, though with low CFU numbers. It did not appear on high salt or high sugar media. Otherwise it is a cosmopolitan species on the phyllosphere and in polluted waters [19,21].

The hypersaline water not only contains generally osmotolerant, but also truly halophilic fungi. These taxa are all melanized and belong to a single order of the Ascomycetes, the Dothideales. They all have thick, melanized cell walls, slow, often meristematic growth and proliferation with endoconidiation. A similar morphology is observed with stone-inhabiting fungi [22], and this can thus be regarded as an extremophilic ecotype. None of the known species of marine fungi was encountered; these fungi may therefore not be regarded as extremophilic, and belong to quite different orders of Ascomycota. Consequently the inhabitants of hypersaline waters are unlikely to have evolved from fungi living in sea water.

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