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MICROSCOPIC EXAMINATION OF MICROBIAL COMMUNITIES ALONG A SALINITY GRADIENT IN SALTERN EVAPORATION PONDS: A 'HALOPHILIC SAFARI'

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1. Introduction

Halophilic microorganisms form a tremendously diverse group. Within the phylogenetic tree of life, halophilic and highly halotolerant microorganisms are found within each of the three domains: Archaea, Bacteria and Eukarya. The halophiles are no less diverse at the level of their physiology, biochemistry, molecular biology, and genetics. An aspect that is little known, even to many scientists who study life at high salt concentrations, is the surprising morphological diversity displayed by these halophiles in their natural environments. Only relatively few have spent time examining water or sediment samples from hypersaline environments in the microscope. This is to be regretted, as high-salt habitats are among the most gratifying objects for microscopic examination (Javor, 1989; Oren, 2002).

Here I show some of the esthetic aspects of halophilic microbial life by presenting pictures taken during a 'halophilic safari' along the salt gradient in the evaporation and crystallizer ponds of the solar salterns in Eilat, Israel, with a few contributions from salterns located elsewhere. Salterns are by no means the only habitats in which halophilic microorganisms are found, but for the microscopist they are one of the most rewarding ones, both because of the varied shapes of the organisms inhabiting them and because of the high densities at which many of these organisms are present. In addition, the saltern ecosystem encompasses environments from seawater salinity to halite saturation and beyond, and it thus provides a habitat for microorganisms adapted to different salt concentrations, all within a small and easily accessible area. Such solar salt works have rightfully been considered as natural laboratories for microbiological and geochemical investigations (Schneider and Herrmann, 1979).

2. The Saltern Environment

The first evaporation ponds of multi-pond salterns have salinities close to that of the seawater used as the raw material for the production of salt. These ponds are typically inhabited by macroalgae as well as by a varied community of photosynthetic microorganisms including benthic cyanobacteria and diatoms, as well as by different kinds of protozoa, types of marine bacteria, as well as some higher organisms: macroalgae, crustaceans, and some other higher animals (Javor, 1989). Ponds in which the salt concentration has increased to twice that of seawater generally contain thick benthic microbial mats dominated by unicellular and filamentous cyanobacteria including *Aphanothece* (*Halothece*), *Oscillatoria*, *Halospirulina*, and others (Clavero et al., 1994; Giani et al., 1989; Javor, 1989; Margheri et al., 1987; Oren, 2000a; Schneider and Herrmann, 1979; Taher et al., 1995; Thomas and Geisler, 1982). Purple sulfur bacteria are found below the cyanobacterial layer. In those ponds in which the salinity has increased to over 3-4 times that of seawater, massive amounts of gypsum ($CaSO_4.2H_2O$) precipitate. The gypsum crusts that accumulate on the bottom of these ponds often show beautifully colored layers of phototrophic microbial communities with different types of cyanobacteria and purple sulfur bacteria (Caumette et al., 1994; Cornée, 1984; Oren, 2000b; Oren et al., 1995; Sørensen et al., 2004) (Fig. 1). This stratification of the communities within this crust is determined by the vertical gradients of light, oxygen, and sulfide.

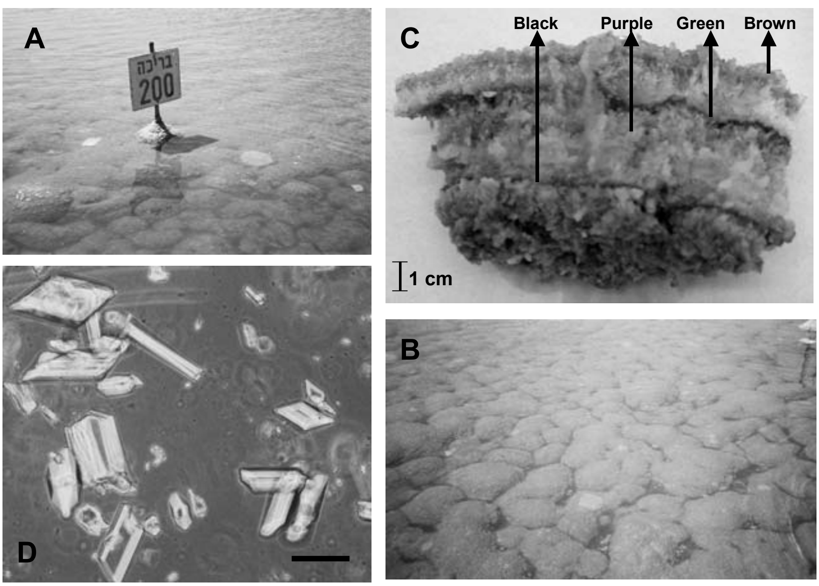

Figure 1. Benthic gypsum deposits in an evaporation pond of the Eilat salterns below a brine of 19.3% salt: photographs of the surface of the crust (A, B) colored brown-orange by dense communities of unicellular *Halothece*-type cyanobacteria (see Fig. 4A-C), a section through the crust (C; photograph courtesy of Tomer Beeri), showing colored bands of unicellular brown-orange (1) and filamentous green cyanobacteria (2), and purple sulfur bacteria (3), and a microscopic view of gypsum crystals from this layer (D; bar = 50 µm).

The crystallizer ponds in which NaCl precipitates in the form of halite crystals represent the last stage in the evaporation process. In these ponds the most conspicuous microbial communities are not found in the form of benthic mats as in the lower salinity ponds, but in the form of dense planktonic populations. The brine of the NaCl production ponds is generally colored red as a result of the presence of dense communities of halophilic Archaea of the family *Halobacteriaceae*, as well as β-carotene-rich unicellular green algae (*Dunaliella salina*) (Javor, 1983; Oren, 2002). Pigmented representatives of the domain Bacteria (the genus *Salinibacter*) may also contribute to the red color of the water (Antón et al., 2002; Oren and Rodríguez-Valera, 2001).

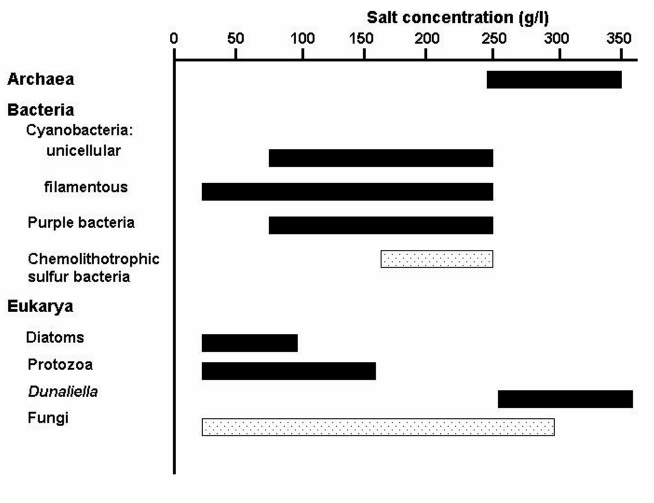

Figure 2. The distribution of the major microscopically distinct types of microorganisms along the salinity gradient in saltern ponds.

The above-described sequence of biological phenomena, as summarized in Fig. 2, is found in solar salt production plants worldwide. Minor variations may occur, caused for example by differences in the nutrient status of the ponds (Javor, 1983) and also by the source of the saline water used to feed the evaporation ponds. Thus, the Eilat saltern that served as the main object of our studies is nowadays mainly fed by reject brine from the reverse osmosis water desalination plant that supplies drinking water to the city of Eilat.

The dense microbial communities that develop in the salterns are not only of scientific interest, as their presence is important for the proper operation of the salt works. The benthic microbial mats in the low- and medium salinity ponds effectively seal the bottom of the ponds, preventing leakage of brine. Both the benthic cyanobacterial mats and the red communities of *Dunaliella*, Archaea and Bacteria in the crystallizer ponds absorb light and thereby raise the water temperature, leading to higher evaporation rates and increased salt production (Davis, 1974; Javor, 1989). In addition, some of the halophilic microorganisms found in these salterns have considerable potential in biotechnology (Margheri et al., 1987; Oren, 2002).

3. Archaea

Microscopic examination of the red crystallizer brines of the Eilat saltern shows an abundance of flat, square to rectangular Archaea (Fig. 3). Together with the orange *Dunaliella salina* cells (see section 5.2) they are the most conspicuous components of the biota of these ponds. Small crystals of celestite ($SrSO_4.2H_2O$) are seen as well (Fig. 3A, 3B). Celestite precipitates at about the same time as halite.

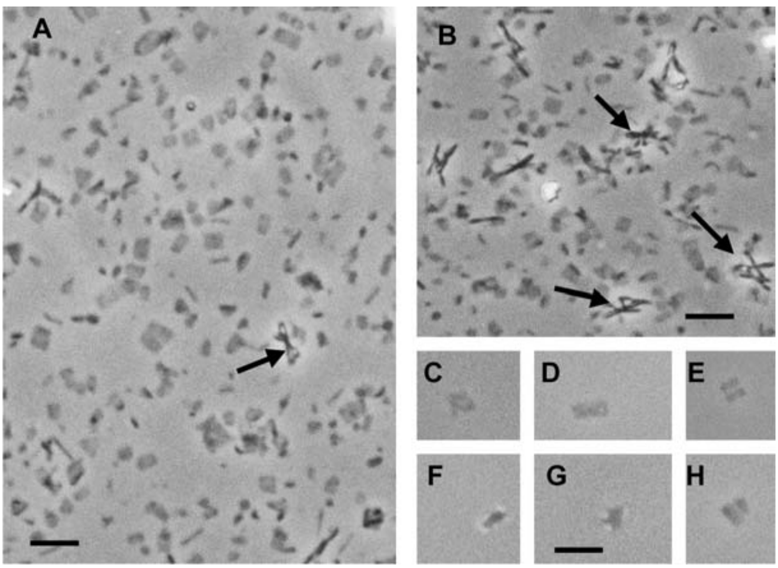

Figure 3. Square, rectangular and trapezoid shaped halophilic Archaea from an NaCl-saturated crystallizer pond of the salterns of Eilat. Cells in panel A and B were concentrated by centrifugation, causing collapse of the gas vesicles. Numerous crystals of celestite ($SrSO_4.2H_2O$) are visible especially in panel B (arrows). Panels C-H show single cells of square halophilic Archaea not subjected to centrifugation. Refractile gas vesicles are visible in panel C, F, and G. Panel E and H show dividing cells. Scale bar = 10 µm.

The flat square Archaea were first described by Walsby (1980) from a brine pool in Sinai, Egypt. After their biological nature was recognized, it rapidly appeared that they are abundant in saltern crystallizer ponds worldwide. The organism contains refractile gas vesicles (visible in Fig. 3C, 3F, and 3G, not visible in Fig. 3A and 3B as these show cells concentrated by high-speed centrifugation, which had caused the collapse of the gas vesicles). A number of electron microscopic studies have been devoted to the characterization of these fascinating organisms (Kessel and Cohen, 1982; Parkes and Walsby, 1981; Stoeckenius, 1981). Our understanding of these unusually shaped prokaryotes has been reviewed a few years ago (Oren, 1999).

The square Archaea belong to the *Halobacteriaceae*, and are phylogenetically remotely related to the genus *Haloferax*. Until recently no cultures were available, and therefore little was known about the physiology of the organism. However, the square archaeon has now been brought into culture (Bolhuis et al., 2004; Burns, 2004), and it may therefore be expected that we will soon learn much more about this fascinating organism.

Other species of the family *Halobacteriaceae* have been isolated from saltern ponds worldwide (Oren, 2001). Numerically these are less abundant than the square archaeon, and they are less distinctive morphologically, so that they cannot be easily recognized in micrographs of the crystallizer pond community.

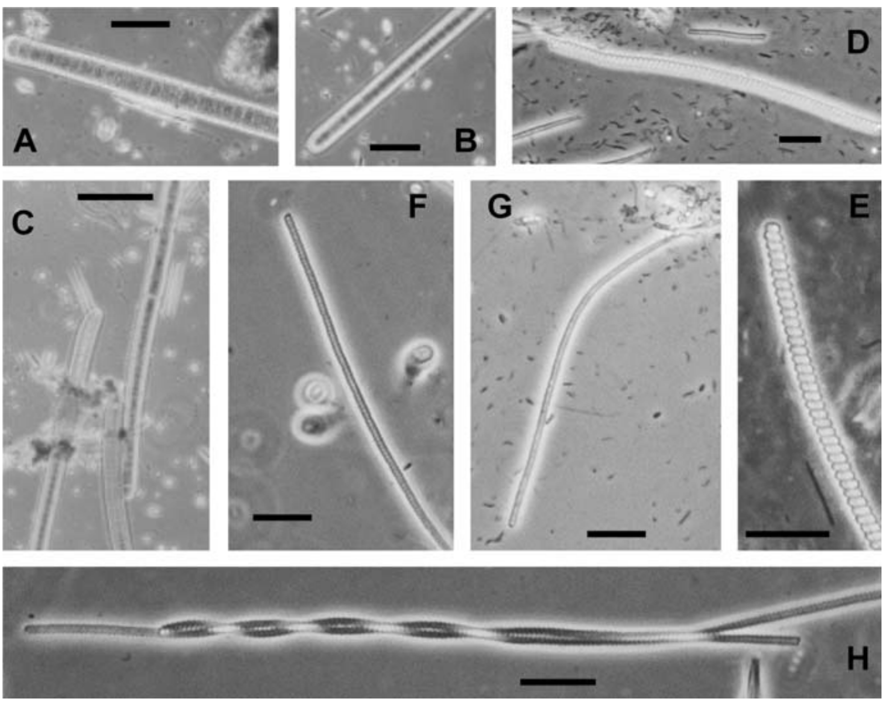

Figure 4. Filamentous benthic cyanobacteria from low-salinity concentrating ponds of the Eilat saltern: large *Oscillatoria* filaments from a pond containing approximately 4% salt (A-C), *Halospirulina tapeticola* from a pond with about 8% salt (D,E), and another type of *Halospirulina* with very narrow filaments from the same pond (F-H). Scale bars represent 50 µm (A-C) or 20 µm (D-H).

4. Bacteria

In many of the recognized phyla within the domain Bacteria we find halophilic or halotolerant representatives. Certain types of halophilic Bacteria are found at high densities in the saltern ecosystem, and many of these have conspicuous morphologies so that they can easily been recognized microscopically. Two groups, the cyanobacteria and the purple sulfur bacteria, are especially prominent because the colors they impart to the sediments.

4.1. CYANOBACTERIA

Cyanobacteria are the main components of the soft gelatinous benthic microbial mats that cover the lower salinity evaporation ponds, and their presence is also conspicuous in the gypsum crusts on the bottom of the ponds of intermediate salinity (see also Fig. 1).

Figure 4 shows a variety of filamentous cyanobacteria present in the benthic microbial mats that cover the bottom of the lower salinity evaporation ponds of the Eilat saltern. Several morphological types can be seen in these mats: thick filaments of *Oscillatoria* sp. (Fig. 4A-C) and tightly wound spirals of *Halospirulina* or *Spirulina* spp. of different filament diameter (Fig. 4D-H). Similar spirally wound filaments and other types of filamentous cyanobacteria have been recorded from salterns in Spain, France, and elsewhere (Clavero et al., 1994; Giani et al., 1989; Nübel et al., 2000; Thomas and Geisler, 1982).

The gypsum crust that develops on the bottom of the intermediate salinity ponds contains brightly colored layers, of which the upper brown-orange layer and the green layer below derive their coloration from the dense communities of cyanobacteria inhabiting them. The upper 0.5-2 cm thick brown layer is populated by carotenoid-rich unicellular cyanobacteria (*Halothece*-*Aphanothece* type) (Fig. 5A-C). These cells have a low chlorophyll and phycocyanin content. A *Phormidium*-type filamentous cyanobacterium is the dominant organism in the green layer (Fig. 5D-F). An olive-green layer is sometimes found below the red-purple layer of phototrophic sulfur bacteria (see section 4.2). Slender filamentous, yet unidentified microorganisms are found in this layer (Fig. 5G-H). The biology of the Eilat gypsum crust has been documented before (Oren, 2000a, 2000b; Oren et al., 1995; Sørensen et al., 2004). Similar layered cyanobacterial communities have been described from gypsum deposits in Spanish and French salterns (Caumette et al., 1994; Cornée, 1984; Thomas, 1984) and from a mixed gypsum-halite crust from salt pools of Baja California, Mexico (Rothschild et al., 1994).

4.2. PHOTOSYNTHETIC PURPLE BACTERIA

Red-purple layers of photosynthetic sulfur bacteria are often found below the cyanobacterial layers in the benthic microbial mats that develop in saltern evaporation ponds. The organisms responsible for the red color belong to genera such as *Halochromatium*, *Thiohalococcus* and *Ectothiorhodospira* or *Halorhodospira*, all members of the Proteobacteria branch of the domain Bacteria. These are anaerobes that obtain their energy from light, especially from the near-infrared radiation (800-900 nm) that is not absorbed by the cyanobacterial layers above. Sulfide, produced by dissimilatory sulfate reduction in the layers below, serves as electron donor for the photoautotrophic fixation of CO_2. Such red layers have been reported from salterns in France, Spain, Slovenia, and elsewhere (Caumette et al., 1994; Cornée, 1984; Schneider and Herrmann, 1979).

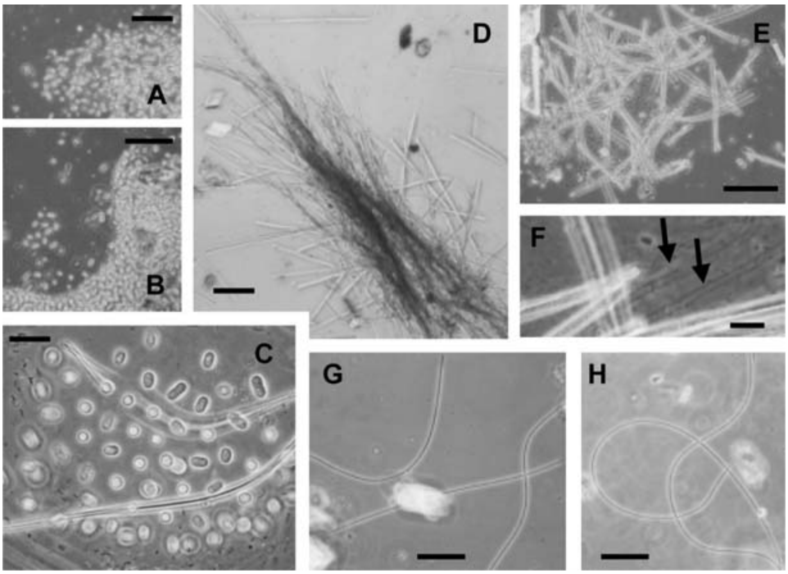

Figure 5. Unicellular *Halothece* (*Aphanothece*)-like cells from the upper brown layer of a gypsum crust found on the bottom of a saltern crystallizer pond with 19.3% salt (A-C), *Phormidium*-type filamentous cyanobacteria from the green layer in this crust (D-F) (compare also Fig. 1C), with empty sheaths seen in panel F; arrows), and unidentified slender cyanobacteria (?) from an olive-green layer below the red-purple layer (G,H). The bars represent 100 μm (A,B,D), 50 μm (E,G) or 20 μm (C,F).

In the Eilat salterns, patches of red *Halochromatium*-type cells are often found below the cyanobacterial layer in the low-salinity evaporation ponds. A prominent red layer is present in the gypsum crust in the ponds of intermediate salinity (Oren et al., 1995; Sørensen et al., 2004; see also Fig. 1C). These layers contain mainly ovoid cells morphologically resembling *Halochromatium* (Fig. 6A-D). Intracellular sulfur granules, formed by partial oxidation of sulfide, are often seen within the cells (Fig. 6D). *Halorhodospira*-like cells have also been reported to occur in the Eilat gypsum crust (Oren et al., 1995).

4.3. CHEMOLITHOTROPHIC SULFUR BACTERIA

Spherical bacteria with intracellular granules, apparently of elemental sulfur, are associated with the green layer of the Eilat gypsum crust, or are found in a whitish layer just below (Fig. 6E and 6F). They may represent a yet-uncultured type of chemolithotrophic sulfur oxidizing bacteria, and as such ('*Achromatium*'-like cells) they were described earlier (Oren et al., 1995). A chemoautotrophic way of life is suggested for these organisms, as no prominent coloration is found in accumulations of these cells. However, presence of photosynthetic pigments and the possibility of a phototrophic way of life cannot be strictly excluded.

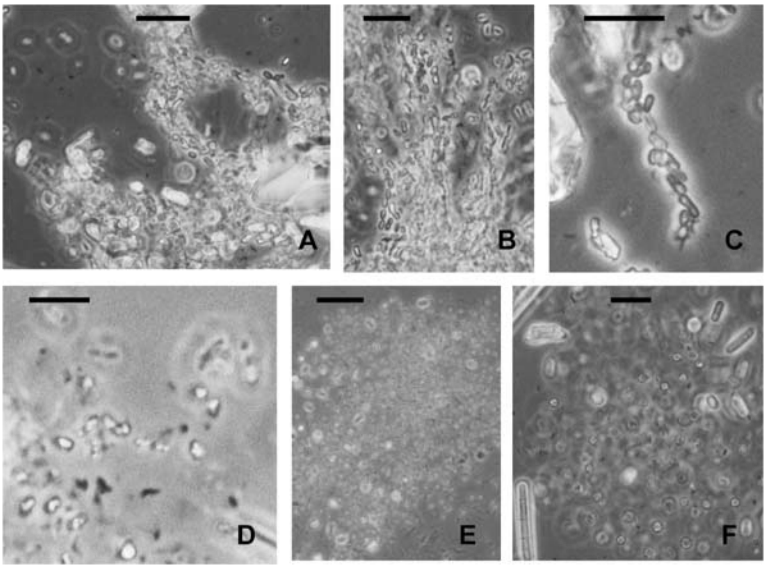

Figure 6. Red *Halochromatium*-type photosynthetic sulfur bacteria from the purple layer of the Eilat saltern gypsum crust in a pond of 19.3% salt (A-D); see also Fig. 1C. Intracellular sulfur granules are visible in panel D. Panels E and F show small spherical bacteria with intracellular granules, apparently of elemental sulfur, associated with the green layer of the Eilat gypsum crust. These yet unidentified and uncultured organisms may be chemolithotrophic sulfur oxidizing bacteria, but presence of photosynthetic pigments and the possibility of phototrophic life cannot be excluded. The bars represent 20 µm (A-C,F), 10 µm (D) or 20 µm (F).

4.4. HETEROTROPHIC BACTERIA

A variety of heterotrophic bacteria, both benthic and pelagic, are associated with the autotrophic communities in the saltern evaporation ponds, and these live at the expense of organic material produced by the photosynthetic microbial communities present. Morphologically these organisms are generally little distinctive, but their presence is conspicuous especially where large amounts of organic material accumulate, for example in the form of the polysaccharide slime excreted by the unicellular cyanobacteria in the upper layer of the gypsum crust in the Eilat saltern (Fig. 7).

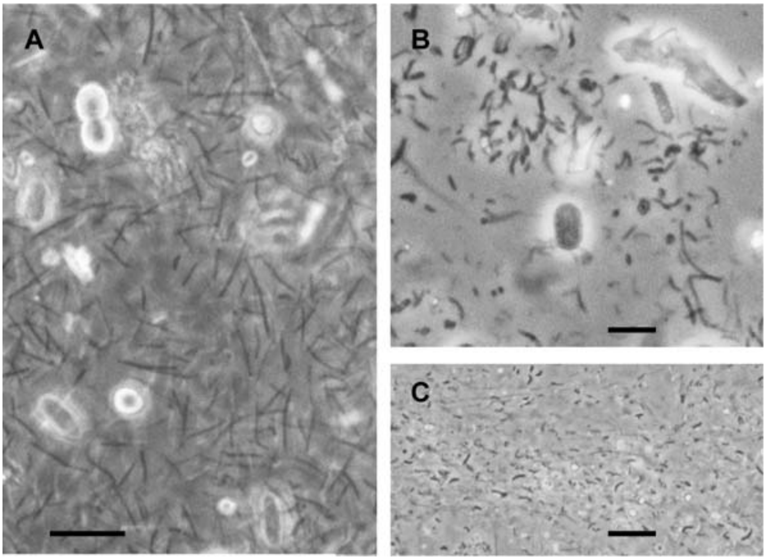

Figure 7. Different morphological types of probably heterotrophic bacteria found within the polysaccharide matrix excreted by the unicellular cyanobacteria in the upper brown-orange layer of the Eilat saltern gypsum crust below brine of 19.3% salt (A-C). The bars represent 20 µm (A,C) or 10 µm (B).

Representatives of the domain Bacteria also occur at the highest salinity in the crystallizer ponds. The halophilic Archaea described in section 3 (Fig. 3) share their habitat with *Salinibacter*, a recently discovered member of the Bacteria (*Cytophaga*/*Flavobacterium* branch). *Salinibacter* is a red pigmented heterotrophic bacterium that physiologically resembles the halophilic Archaea to a large extent. In Spanish saltern crystallizer ponds it may represent up to 5-25% of the total prokaryotic community in the brines (Antón et al., 1999, 2002; Oren and Rodríguez-Valera, 2001; Oren et al., 2004). In the Eilat salterns *Salinibacter* is present as well, but it is less is abundant (Elevi and Oren, 2004).

5. Eukarya

Generally spoken the biota of the saltern evaporation ponds are dominated by prokaryotes. Eukaryotic microorganisms (and macroorganisms as well) still abound in the first concentrating ponds, whose salinity does not greatly differ from that of seawater. When conditions become hypersaline, eukaryotes become rarer. A few types of diatoms and of protozoa are found along with the cyanobacteria and other prokaryotic organisms in the microbial mats that cover the bottom of the evaporation ponds along the salinity gradient, but they are present in low numbers. However, when halite saturation is reached in the crystallizer ponds, a eukaryotic unicellular alga, *Dunaliella salina*, becomes one of the dominant components of the biota, and is responsible for most or all of the primary productivity in the salt-saturated brines.

5.1. DIATOMS

Diatoms are found in the Eilat saltern ponds especially in the initial stages of evaporation (Fig. 8A-D). They become rarer with the increasing salinity of the ponds, but some diatoms can be found at salt concentrations up to about 20% (Fig. 8E-G).

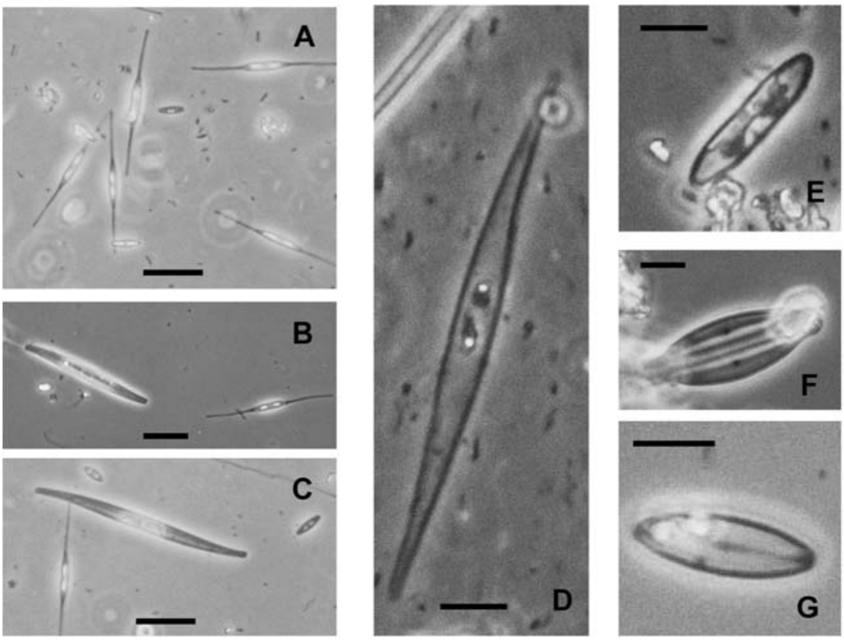

Figure 8. Different types of diatoms encountered in the Eilat salterns in ponds with 4% (A-C), 8% (D), and 19.3% salt (E-G). They can tentatively be identified as *Nitzschia* sp. (A-C), *Gyrosigma* or *Pleurosigma* sp. (B-D), *Navicula* sp. (E,G), and *Amphora* sp. (F). The bars represent 20 µm (A-C) or 10 µm (D-G).

Detailed studies have been made of the taxonomic distribution of diatoms in saltern ponds of different salinities worldwide, including Spain, France, Italy, Mexico, and South Africa (Campbell and Davis, 2000; Clavero et al., 1994, 2000; Javor, 1989; Margheri et al., 1987; Noël, 1982, 1984; Rincé and Robert, 1983). Among the genera most widespread in salterns are *Nitzschia*, *Amphora*, *Navicula*, *Entomoneis*, and *Pleurosigma*. It has been suggested that diatoms may be useful indicators to monitor the physical and chemical status of saltern evaporation ponds (Campbell and Davis, 2000).

5.2. THE GREEN ALGA *DUNALIELLA*

Dunaliella salina (*Chlorophyceae*, *Volvocales*) is a characteristic inhabitant of saltern crystallizer ponds. Microscopic examination of the brines shows red carotenoid-rich flagellated cells (Fig. 9). These contribute to the coloration of the water, together with the halophilic Archaea and possibly with Bacteria of the genus *Salinibacter* (see sections 3 and 4.4). Biotechnological operations have been set up in several places in the world to exploit the alga for the production of β-carotene (Oren, 2002).

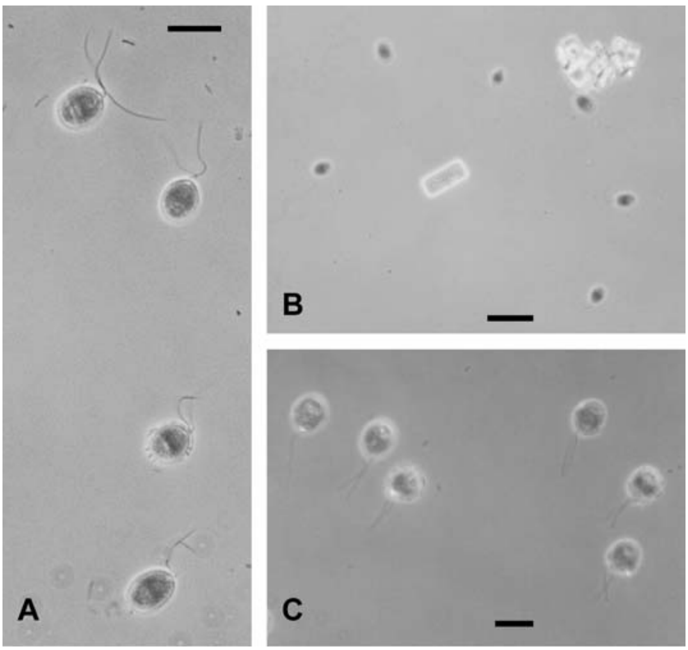

Figure 9. Red-orange cells of *Dunaliella salina* from the NaCl-saturated crystallizer ponds of the Eilat salterns. Small halite crystals are seen in panel B. The bars represent 20 μm (A,C) or 50 μm (B).

5.3. PROTOZOA

The cyanobacteria-dominated mats on the bottom of the lower and intermediate salinity evaporation ponds are grazed by different types of ciliate, flagellate and amoeboid protozoa. Some are found at salt concentrations as high as 20%. Figure 10 shows a selection of types of protozoa found in the Eilat salterns. Occurrence of protozoa at high salt concentrations has been documented in the past (Javor, 1989; Oren, 1989; Post et al., 1983), but little is know about the biology of these organisms.

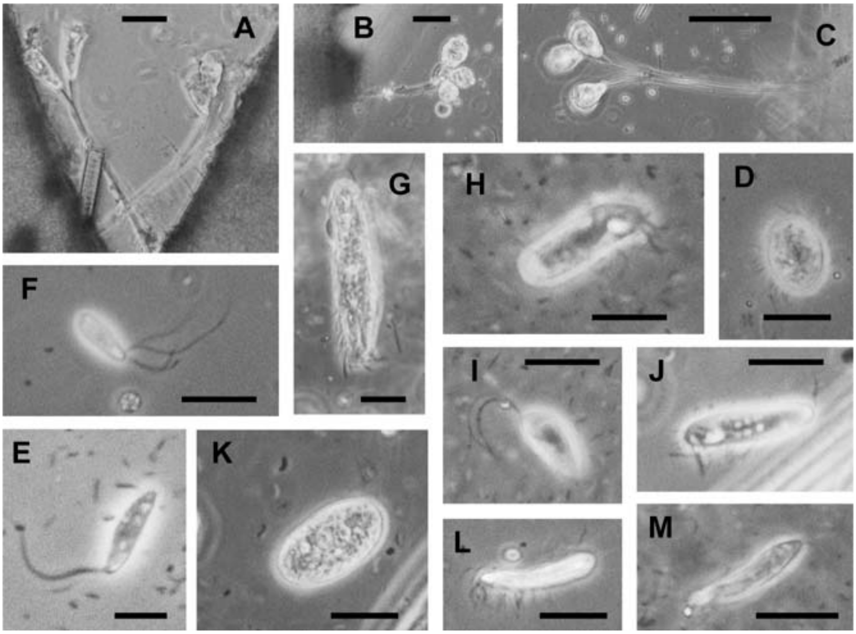

Figure 10. Different types of ciliate and flagellate protozoa encountered in the Eilat salterns in ponds with 4% (A-D), 8% (E), and 19.3% salt (F-M). The sessile stalked ciliate protozoa shown in panels A-C attached to leaves of *Zostera* (?) probably belong to the genus *Zoothamnium*. The bars represent 50 µm (A-C), 20 µm (D,F-H,J-M), or 10 µm (E,I).

5.4. FUNGI

Direct microscopic examination of saltern brine and sediment samples does not show conspicuous communities of yeasts and filamentous fungi to be present. However, it is now well established that halophilic yeasts and fungi are present in the saltern ecosystem (Gunde-Cimerman et al., 2000; Zalar et al., 1999a, 1999b). Figure 11 shows micrographs of representative isolates obtained from the Sečovlje salterns on the Adriatic coast at the border between Slovenia and Croatia.

6. Epilogue

Although microorganisms, and especially the prokaryotic types, show only a limited morphological diversity, a microscopic examination of microbial communities in their natural environment can be an esthetically highly rewarding experience. The beauty of the marine microflora was convincingly shown by Sieburth (1975, 1979) in his two books of electron micrographs of marine microorganisms. The hypersaline system presented by the salterns, with their series of evaporation ponds of increasing salinity –

each harboring its characteristic microbial communities, presents us with an even greater morphological variety, which is no less fascinating than that in the marine environment.

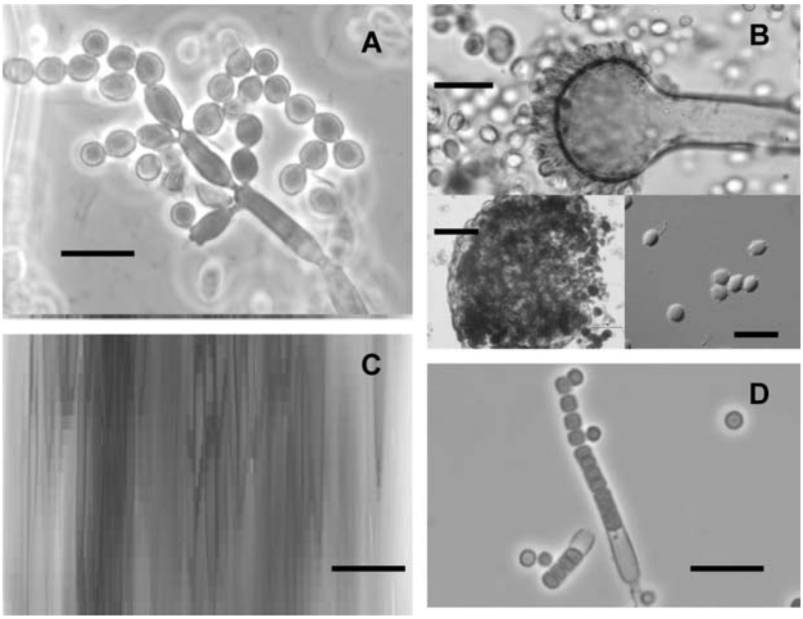

Figure 11. Halophilic fungi isolated from Mediterranean salterns: conidia and conidiophore of *Cladosporium halotolerans* (A) (The bar represents 10 µm), conidiophore with conidia, cleistothecium, and ascospores of *Eurotium amstelodami* (anamorph: *Aspergillus hollandicus*) (B) (The bars represent 10, 25, and 10 µm, respectively), endoconidia in hyphae of *Phaeotheca triangularis* (C) (The bar represents 20 µm), and conidia of *Wallemia sebi* (D) (The bar represents 10 µm). Photographs by P. Zalar and L. Butinar.

The survey presented here should in no way be considered as a complete inventory of the microbiota of the saltern system. It only shows the morphologically most conspicuous organisms, and many less conspicuous organisms that may play an important role in the functioning of the biological system of the salterns thus remain undetected. Although there is a large extent of similarity between the biological properties of salterns worldwide, local variations do exist, and a microscopical study of the planktonic and benthic microflora of saltern ponds from other geographic locations will undoubtedly show different features and yield additional types of microorganisms not prominent in the Eilat salterns described here.

Each of the microorganisms shown in the photographs presented above is interesting in its own right, and deserves in-depth studies of its mode of life and its function in the saltern ecosystem. Many of these organisms are yet to be isolated in culture and studied in the laboratory. Such studies will add much to our understanding of the biology of the salterns and the functioning of microorganisms at high salt concentrations. Meanwhile, microscopic examination of the organisms in their natural environment remains a rewarding pastime, not only scientifically but from an esthetic point of view as well.

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