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REVIEW

Interrelationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds

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Abstract Thanks to their often very high population densities and their simple community structure, saltern crystallizer ponds form ideal sites to study the behavior of halophilic microorganisms in their natural environment at saturating salt concentrations. The microbial community is dominated by square red halophilic Archaea, recently isolated and described as *Haloquadratum walsbyi*, extremely halophilic red rod-shaped Bacteria of the genus *Salinibacter*, and the unicellular green alga *Dunaliella* as the primary producer. We review here, the information available on the microbial community structure of the saltern crystallizer brines and the interrelationships between the main components of their biota. As *Dunaliella* produces massive amounts of glycerol to provide osmotic stabilization, glycerol is often postulated to be the most important source of organic carbon for the heterotrophic prokaryotes in hypersaline ecosystems. We assess here, the current evidence for the possible importance of glycerol and other carbon sources in the nutrition of the Archaea and the Bacteria, the relative contribution of halophilic Bacteria and Archaea to the heterotrophic activity in the brines, and other factors that determine the nature of the microbial communities that thrive in the salt-saturated brines of saltern crystallizer ponds.

Keywords Hypersaline · Salterns · *Dunaliella* · *Haloquadratum* · *Salinibacter*

Abbreviations
FISH Fluorescent in situ hybridization
PHA Poly-β-hydroxyalkanoate

Introduction

A quarter of a century ago, our view on the biology of salt lakes at or near NaCl saturation was very simple. Borowitzka (1981) summarized the then current knowledge as follows: ''Only two genera of heterotrophic bacteria have been studied and shown to live and grow in extremely saline lakes. They are *Halobacterium* and *Halococcus*, and both live in the water column. They are proteolytic or saccharolytic and probably derive most of their carbon from glycerol and cell protein produced by *Dunaliella*.''

These sentences relate to hypersaline lakes such as the north arm of Great Salt Lake, Utah, the Dead Sea, a few other natural salt-saturated lakes, as well as the crystallizer ponds of multi-pond solar salterns. These saltern brines are colored pink-red due to the presence of dense communities of carotenoid-rich prokaryotes, with cell numbers generally being in the range of 2×10^7–10^8 ml^{-1}, as well as by orange β-carotene-rich

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Three-letter abbreviations for names of genera of Halobacteriaceae conform the recommendations of the ICSP Subcommittee on the Taxonomy of *Halobacteriaceae*.

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cells of the unicellular green alga *Dunaliella salina* (Javor 1989; Oren 2002). Despite minor variations attributable to differences in the availability of nutrients, the general microbial properties of crystallizer brines in salterns worldwide are markedly similar. This makes the saltern crystallizer pond ecosystem one of the most convenient ones to study life at extreme salinities. Information on the interrelationships between the primary producer *Dunaliella* and the different types of heterotrophic prokaryotes can much easier be obtained during research on saltern brines than by study of natural salt lakes, with their often-great seasonal and long-term variability in biological properties.

Now, 25 years after Borowitzka provided the above-cited simple picture of the trophic relationships between the members of the biota in salt-saturated lakes, our views have changed a great deal. Here, we review the information available on the microbial community structure of the saltern crystallizer brines and the interrelationships between the main components of their biota. We will see that the system is indeed simple, but its composition is very different from what we knew then: the two genera *Halobacterium* and *Halococcus* contribute very little, if at all, to the heterotrophic activity. Only in recent years have we learned whom the true principal components of the microbial community in saltern crystallizer ponds and other hypersaline lakes are. Even though we know now the major players, we still poorly understand how they interact. Glycerol may indeed be a key compound for understanding the trophic relationships within the community, but the overall functioning of the simple ecosystem is still poorly understood.

Saltern crystallizer ponds and their biota

Solar salterns have been constructed in many locations in tropic and subtropic areas worldwide to gain common salt—halite from seawater. Seawater is evaporated in stages in shallow ponds maintained at approximately constant salinities, so that each set of ponds harbors a characteristic microbial community adapted to life at the prevailing salt concentration. The first evaporation ponds have salinities close to that of the seawater; accordingly, their biota resemble those of the marine environment. Ponds in which the salt concentration has increased to twice that of seawater generally contain thick benthic microbial mats of unicellular and filamentous cyanobacteria and purple sulfur bacteria. When the salinity has increased to over 3–4 times that of seawater, gypsum precipitates. The gypsum crusts that accumulate on the bottom often show beautifully colored layers of different types of cyanobacteria and photosynthetic purple bacteria. The crystallizer ponds form the last stage in the evaporation process. At total dissolved salt concentrations above 300 g/l, NaCl precipitates as halite crystals, which are harvested whenever a sufficiently thick layer has accumulated on the bottom. In these crystallizer ponds, the most conspicuous microbial communities are not found as benthic mats but as planktonic populations, which bestow a pink to brightly red color to the brines (Javor 1989; Oren 2002).

Although β-carotene derived from *Dunaliella* may be the most abundant carotenoid pigment in the salterns, its dense packaging within granules inside the cell's chloroplast greatly decreases its contribution to the overall light absorbance in the water. As a result, most of the pink-red color of the crystallizer brines is caused by α-bacterioruberin and other 50-carbon bacterioruberin derivatives present in Archaea of the family Halobacteriaceae (Oren et al. 1992; Oren and Dubinsky 1994). However, the genera *Halobacterium* and *Halococcus* mentioned in the quotation from Borowitzka (1981) given above are not the dominant types. Many new genera have since been described within the family, and currently (October 2006) the census stands at 23 genera and 71 species. Many of these have indeed been isolated from saltern crystallizer ponds. Thus, the type strains of the species *Haloferax mediterranei*, *Hfx. gibbonsii*, *Hfx. denitrificans*, *Halorubrum saccharovorum*, *Hrr. coriense*, *Hrr. trapanicum*, *Haloarcula hispanica*, *Har. japonica*, *Natrialba taiwanensis*, *Nab. aegyptiaca*, *Halogeometricum borinquense*, *Halococcus saccharolyticus*, and *Haloterrigena thermotolerans* were all isolated from solar salterns (Oren 2002). This is not at all surprising, as plating of saltern brine samples on agar plates with high-salt, organic-nutrient-rich growth media of the types commonly used to cultivate halophilic Archaea typically gives rise to the growth of colonies belonging to the genera *Halorubrum*, *Haloferax*, and *Haloarcula*. This is not because of their high abundance in the system, but rather because of the ease with which they can be grown. Analysis of the polar lipids extracted from saltern brine biomass shows that *Halobacterium*, which possesses characteristic sulfated triglycosyl and tetraglycosyl glycolipids, is not a dominant component of the community (Oren 1994; Oren et al. 1996), but is present in low numbers only, so that it can be recovered from saltern brines by a selective enrichment procedure (Oren and Litchfield 1999).

Microscopical examination of saltern crystallizer brines already suggests that the above-mentioned species—rod-shaped, flat pleomorphic or coccoid

cells—are not the dominant ones in the community. In crystallizer ponds worldwide, the most abundant type of halophilic Archaea is the flat, square- or rectangular-shaped cell that contains gas vesicles. This type of organism was first recognized in 1980 in a coastal brine pool on the Sinai Peninsula, Egypt (Walsby 1980). The fact that its presence in salterns was never mentioned before is undoubtedly due to its unusual shape, very different from any earlier known prokaryote, and so thin that for the inexperienced observer it is difficult to see even in the phase contrast microscope. Now it is well established that in salterns in Spain, Australia, Israel, and elsewhere, at least half of the total cell numbers, and often much more, belong to this type (Antón et al. 1999; Burns et al. 2004a; Guixa-Boixareu et al. 1996; Oren 1999; Oren et al. 1996, 2005). Thanks to their great abundance in the brines it was possible to obtain much information about these intriguing organisms long before they were brought into culture. Thus, their polar lipid composition could be assessed (Oren et al. 1996), and useful data about the G + C content of their DNA was obtained (Øvreås et al. 2003).

In the mid-1990s, molecular, 16S rRNA targeted methods were first applied to hypersaline brines. A single archaeal phylotype, not closely related to any of the then known genera within the Halobacteriaceae, was detected at the greatest frequency in Spanish and Israeli salterns (Benlloch et al. 1995, 2001; Rodriguez-Valera et al. 1999). When techniques of fluorescence in situ hybridization (FISH) were then applied, using probes targeting this sequence, it was established that the organism that harbors this phylotype is the flat square archaeon present in such high numbers in the brines (Antón et al. 1999).

It then lasted until 2004 until the cultivation of this elusive organism was reported independently by two groups (Bolhuis et al. 2004; Burns et al. 2004a; see also Bolhuis 2005; Walsby 2005). The isolates from salterns in Spain and in Australia were found to be very similar. A formal description of the organism as a new species belonging to a new genus has now been completed (Burns et al. 2006). It was named *Haloquadratum walsbyi*, honoring Tony Walsby who had been the first to recognize the true nature of these unusual, square flat objects. The G + C percentage of its DNA is 47.9, as already predicted from melting profiles of DNA extracted from saltern crystallizer biomass (Øvreås et al. 2003), far lower than the values of 59–71%, common for the other members of the Halobacteriaceae. The genome of *Haloquadratum* has been sequenced (Bolhuis et al. 2006), and its analysis has yielded much relevant information toward the understanding of its mode of nutrition in situ, as we will see below.

The second quantitatively important component of saltern crystallizer brine biota is the extremely halophilic red, rod-shaped *Salinibacter*, a recently discovered member of the Bacteroidetes. Its presence and abundance was first suggested by the consistent recovery of Bacteroidetes-related 16S rRNA gene sequences following amplification using Bacteria-specific primers. FISH studies then showed that this phylotype belongs to a rod-shaped bacterium that is present in numbers up to 20–25% of the total prokaryote community in Spanish salterns (Antón et al. 1999, 2000). Shortly afterwards, the organism was isolated, and its description as *Salinibacter ruber* was published (Antón et al. 2002). *Salinibacter* resembles in many aspects the properties of *Halobacterium* and other members of the archaeal family Halobacteriaceae: it accumulates KCl to provide osmotic balance, it has a highly acidic proteome (Oren and Mana 2002), and it possesses retinal pigments resembling bacteriorhodopsin, halorhodopsin, and sensory rhodopsins of the Halobacteriaceae. Its red pigment, named salinixanthin, is a novel type of C_{40}-carotenoid acyl glycoside (Lutnæs et al. 2002), and its presence could be detected and quantified in saltern biomass (Oren and Rodriguez-Valera 2001). Also its unique sulfonolipid could be used as a biomarker to directly assess its presence in brines of the salterns of Margherita di Savoia, Italy (Corcelli et al. 2004). Molecular, 16S rRNA gene-based methods have shown its abundance, together with *Haloquadratum*, in Turkish salterns (Guven et al. 2006), and a selective enrichment and isolation procedure enabled its recovery from the salterns of Eilat, Israel (Elevi Bardavid et al. 2006). The genome of *S. ruber* has been sequenced (Mongodin et al. 2005).

With the characterization of *Hqr. walsbyi* and *S. ruber,* we now know the major components of the prokaryotic community of the saltern crystallizer ponds, as confirmed by the characterization of the metagenome of Spanish saltern brine (Legault et al. 2006). Much useful information on the nature of the Bacteria and Archaea present in such brines can still be obtained using culture-dependent techniques: Burns et al. (2004b), convincingly demonstrated that most prokaryotes present can be cultured when applying proper techniques, combined with a lot of patience.

Microbial activities in saltern crystallization ponds: a re-evaluation

In view of our newly acquired understanding of the truly important microbial players in the saltern crystallizer ecosystem, much of the earlier obtained infor-

mation has now to be re-evaluated. The new information may also be used as the basis for the planning of new and better experiments to gain a proper insight into the functioning of the saltern crystallizers as an ecosystem. Examples are given in the following paragraphs.

The relative contribution of Archaea and Bacteria to the heterotrophic activities in the crystallizer ponds: inhibitor studies

One of the fundamental differences between members of the archaeal and the bacterial domains is their different sensitivity to antibiotics and other antibacterial compounds. This difference has been exploited in the past to obtain information about the contribution of either group to the overall activity, based on the assumption that activity of Bacteria or of Archaea can be abolished by use of group-specific inhibitors without significantly affecting the other group. Antibiotics inhibiting growth of halophilic Archaea (anisomycin, bacitracin) could also be used to specifically enrich and isolate *Salinibacter* from saltern ecosystems (Elevi Bardavid et al. 2006).

After it was discovered that cells of *Halobacterium* and other members of the Halobacteriaceae are lysed by low concentrations of taurocholic acid, deoxycholic acid and other bile acids—concentrations that do not affect the growth of many Bacteria adapted to life at high salinities, bile acids were used to differentiate between heterotrophic activity (incorporation of radiolabeled amino acids or glycerol) of Archaea and Bacteria. In the salt-saturated ponds, nearly all heterotrophic activity was abolished by low concentrations of bile acids, while only little inhibition was measured in evaporation ponds containing less than 200 g/l salts (Oren 1990a, 1991). Anisomycin, a potent inhibitor of protein synthesis by ribosomes of eukaryotes as well as Archaea of the family Halobacteriaceae, was added to inhibit amino acids incorporation by Archaea, while chloramphenicol or erythromycin was used to examine the extent of the contribution by Bacteria to the overall heterotrophic activity in saltern ponds in Israel (Oren 1990b, 1991). Aphidicolin was used to probe thymidine incorporation into DNA by Archaea (Oren 1990c). Pedrós-Alió et al. (2000) similarly exploited erythromycin and taurocholate to selectively inhibit leucine incorporation by Bacteria and Archaea, respectively, in their studies of the biota along the salt gradient in Spanish saltern ponds.

All the above studies were performed before the true nature of the dominant organisms in the ponds was known. The formal species description of *Hqr. walsbyi* (Burns et al. 2006) states that the species is not only sensitive to anisomycin, as expected for members of the Halobacteriaceae, but also to chloramphenicol and erythromycin, which are typical inhibitors of protein synthesis in Bacteria. The isolate further proved resistant to bacitracin, an antibiotic that effectively inhibits growth of most other members of the family. Laboratory cultures of *S. ruber* are sensitive to chloramphenicol and streptomycin, and resistant to anisomycin and aphidicolin, as expected (Antón et al. 2002). The sensitivity of either organism to different bile acids remains to be assessed. It should be noted that the sensitivity of these organisms to the different inhibitors mentioned might also depend on the salinity of their medium and their physiological state. Therefore, renewed studies of the relevant organisms suspended in saltern brines are necessary to reassess the results of earlier inhibitor studies under field conditions (Oren 1990a, b, c, 1991; Pedrós-Alió et al. 2000) and to determine better experimental conditions to use such inhibitors to obtain reliable information on the relative contribution of each group of organisms to the activities observed.

Availability and turnover of glycerol in the saltern crystallizer pond ecosystem

The assumption by Borowitzka (1981) that glycerol produced by *Dunaliella* may be one of the main carbon and energy sources for the heterotrophic community in the salterns, is based on the fact that the alga produces copious amounts of glycerol to serve as osmotic solute. Intracellular concentrations of glycerol of up to 6–7 M have been reported. The question is therefore, how much of the glycerol produced becomes available to the prokaryotic communities, either due to leakage of some of the solute through the membrane of intact algal cells, or by their death and lysis.

Biological membranes are generally highly permeable to glycerol. It is clear that an organism such as *Dunaliella*, which relies on glycerol to provide osmotic balance to the cell—thus abolishing the need to accumulate toxic salts, cannot afford losing its glycerol through a permeable membrane. It has been ascertained that indeed its cytoplasmic membrane is several orders of magnitude less permeable to glycerol than other membranes tested (Brown et al. 1982b; Gimmler and Hartung 1988). Healthy, exponentially growing cultures of different *Dunaliella* species at temperatures not exceeding 40°C appear to release very little glycerol to the medium, but glycerol leakage becomes

significant above 45°C (Wegmann et al. 1980). Such temperatures are not commonly found in the salterns: in Eilat, Israel, we measured up to 35°C in mid-summer, but brine temperatures up to 42°C were reported from Spanish salterns (Rodriguez-Valera et al. 1985). Some *Dunaliella* strains, however, consistently leak smaller or larger amounts of glycerol, without any noticeable decrease in growth rate. This was first documented in a mutant of *D. parva* CCAP 19/9 (Hart and Gilmour 1991), but recent experiments showed that leakage of glycerol from healthy *Dunaliella* cells may be much more widespread (Al Harbi and Gilmour 2006). Release of intracellular glycerol may also be induced by hypotonic stress, at least in the marine species *D. tertiolecta* (Fujii and Hellebust 1992). However, it cannot be expected that *D. salina* in the saltern ponds with their rather constant salinity, only increasing during the salt precipitation process, is commonly subjected to hypotonic stress.

Diel measurements of oxygen concentrations in a mesocosm simulation setup filled with 45 l of brine from the Eilat crystallizer ponds showed high photosynthetic activity of *D. salina*: up to 0.8–1.5 μmol oxygen/l h was evolved during daytime by the 1,300–2,100 algal cells/l present (Khristo, Elevi Bardavid, and Oren, unpublished results). If all photosynthetically fixed carbon were converted to glycerol, this would be equivalent to up to 0.27–0.50 μmol glycerol/l h. Attempts to directly measure steady-state concentrations in the brines (using a highly sensitive but not very specific colorimetric assay that quantifies formaldehyde following periodate oxidation) yielded low values, in the range of 20–36 μM (Oren 1993 and unpublished results, showing no significant differences throughout day–night cycles), and radiotracer experiments suggested a high turnover rate of glycerol in the brines (Oren 1993). Evidence that glycerol is indeed leaking out of healthy *Dunaliella* cells in the saltern ponds was obtained in an experiment, in which a brine sample was enriched with additional *D. salina* cells collected from the brine by flotation: in a system that contained 57,000 *D. salina* per ml, incubated in light–dark cycles under conditions identical to those present in the salterns, an increase in glycerol concentration in the medium of 80 μmol/l day was measured, this time using a specific, enzymatic assay (Khristo, Elevi Bardavid, and Oren, unpublished results).

A key question is, whether and to what extent glycerol is metabolized by the two dominant components of the heterotrophic community in the salterns. Most halophilic Archaea of the family Halobacteriaceae readily use glycerol, and many convert it in part to acidic products—acetate, pyruvate and lactate (Oren and Gurevich 1994; see also the following paragraph). Addition of glycerol to brines from the Eilat saltern ponds gave rise to a stimulation in respiratory activity as measured by the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride to the corresponding formazan derivative, but the stimulation (46%) was less pronounced than that obtained with samples from the Dead Sea (Oren 1995a). It is interesting to note that FISH experiments in Spanish saltern crystallizer ponds, using 16S rRNA-directed probes as markers for *Haloquadratum*, combined with microautoradiography following incubation with different radiolabeled substrates, indicated that glycerol was not used by the square cells (Rosselló-Mora et al. 2003). However, a gene annotated as *glpK*—glycerol kinase is present in the *Hqr. walsbyi* genome (Bolhuis et al. 2006). The species description of *S. ruber* suggested that the organism is unable to grow on glycerol (Antón et al. 2002); however, later experiments have shown that glycerol is used, mediated by an inducible glycerol kinase (Sher et al. 2004). Experiments to determine whether glycerol is taken up by both organisms when suspended in saltern crystallizer brine are in progress.

Formation and breakdown of small organic acids in saltern ponds

Although the Halobacteriaceae are aerobes that respire most of their organic carbon used as energy source to carbon dioxide, some substrates, including simple sugars, sugar alcohols and glycerol, are oxidized incompletely, and products such as acetic acid, lactic acid and pyruvic acid are excreted into the medium, causing its acidification. Formation of these acids was first reported in a study of *Hrr. saccharovorum* (Tomlinson and Hochstein 1972), but it is much more widespread: representatives of the genera *Haloferax*, *Haloarcula*, and others produce such acids (Oren and Gurevich 1994). While acetate is known as an excretion product in many aerobic prokaryotes that incompletely oxidize sugars, the finding of pyruvate was less expected. A study using radiolabeled glycerol added in micromolar concentrations to saltern crystallizer brines showed that acetate and lactate were formed also under natural conditions, so that this phenomenon is not restricted to cells flooded with exceedingly high substrate concentrations. Pyruvate was detected in addition in similar experiments with Dead Sea brines, also dominated by halophilic Archaea, but was not found in the salterns (Oren and Gurevich 1994). It is noteworthy that pyruvate is now a key component for the media used to grow *Haloquadratum* cells (Bolhuis et al. 2004; Burns et al. 2004a).

Our new understanding of the community structure in the saltern brines calls for a renewed evaluation of the phenomenon of incomplete oxidation of glycerol and possibly of other compounds as well. Whether lactate and acetate are excreted during incomplete oxidation of glycerol by *Haloquadratum*, in culture as well as in its natural environment, remains to be assessed. It was earlier reported that the lactate formed in saltern crystallizer brine from labeled glycerol was turned over rapidly, but that the concentration of labeled acetate decreased only very slowly (Oren 1995b; Oren and Gurevich 1994). On the other hand, FISH experiments combined with microautoradiography in Spanish saltern crystallizer ponds suggested that acetate is readily used by *Haloquadratum* (Rosselló-Mora et al. 2003).

Studies on glycerol metabolism by *Salinibacter* showed that some degree of incomplete oxidation occurs there also, at least in laboratory cultures fed with relatively high (millimolar range) concentrations of glycerol (Sher et al. 2004). The nature of the products formed remains to be assessed. Furthermore, it is still unknown whether such incomplete oxidation of glycerol may also occur under low nutrient conditions in the natural saltern brine.

Summarizing: while the occurrence of incomplete oxidation of glycerol by members of the Halobacteriaceae and by *Salinibacter* has been well established, we still do not know which components of the community in the salterns are responsible for the formation and subsequent disappearance of lactate and acetate following addition of glycerol: *Haloquadratum*, *Salinibacter*, or some other, yet to be characterized microorganisms.

The possible importance of dihydroxyacetone as a key substrate in the saltern ecosystem

Genomics studies of *Hqr. walsbyi* yielded an interesting new insight into its potential in situ metabolism, when it was found that this organism possesses a well-developed uptake system for dihydroxyacetone. It is based on concentration gradient-driven facilitated diffusion of dihydroxyacetone through the cell membrane, followed by its phosphorylation by a unique cytoplasmatic phosphoenolpyruvate-dependent phosphotransferase system (Bolhuis et al. 2006). No such systems for dihydroxyacetone utilization have been found in the genomes of other halophilic Archaea sequenced (*Halobacterium* NRC1, *Har. marismortui*, *Natronomonas pharaonis* and other yet unpublished genomes). We can confirm that dihydroxyacetone, when added to brines from the saltern crystallizer ponds in Eilat, is consumed by the community even more rapidly than glycerol (Khristo, Elevi Bardavid, and Oren, unpublished results).

It was suggested that dihydroxyacetone might be a possible overflow compound of carbon metabolism by *Salinibacter* (Bolhuis et al. 2006), although no experimental evidence was presented to substantiate such a claim. The water-soluble compound excreted into the medium by *S. ruber* during glycerol metabolism (Sher et al. 2004) cannot be dihydroxyacetone: the rather unspecific colorimetric assay used in those experiments to assess the disappearance of glycerol would have detected dihydroxyacetone as well. However, the possibility that dihydroxyacetone may be derived from the metabolism of *Dunaliella* deserves to be examined. Dihydroxyacetone is an intermediate in the so-called ''glycerol cycle'' used by the alga during the synthesis and degradation of glycerol used as its osmotic solute. Glycerol is synthesized by reduction of dihydroxyacetone phosphate to glycerol phosphate, which is then dephosphorylated to glycerol; excess glycerol can be returned to the cellular metabolism by its oxidation to dihydroxyacetone and subsequent phosphorylation to dihydroxyacetone phosphate. Studies of the spatial distribution of the enzymatic activities within *Dunaliella* cells showed that the enzymes producing and degrading dihydroxyacetone are located in the cytoplasm rather than in the chloroplast (Brown et al. 1982a; Gimmler and Lotter 1982). To what extent the cytoplasmic membrane of *Dunaliella* is permeable to dihydroxyacetone is unknown, but the possibility should be taken into account that this compound may leak out of the cells, especially when excess glycerol has to be returned to the cell metabolism after osmotic downshock or when for other reasons more glycerol has been produced photosynthetically, than required. Preliminary experiments by our group have as yet failed to detect significant amounts of dihydroxacetone in saltern brines following different incubation conditions. However, the colorimetric assay used (Burton 1957), while free of interference by glycerol, has limited sensitivity.

Poly-β-alkanoate in the saltern crystallizer brine and its possible use to assess the nutritional status of the prokaryotic community

One of the prominent features of *Haloquadratum* cells is the presence of granules of the storage polymer poly-β-hydroxyalkanoate, a polymer of β-hydroxybutyrate

with variable amounts of β-hydroxyvalerate. These granules can be easily recognized in electron micrographs of cells collected from natural brines (Kessel and Cohen 1982; Oren et al. 1996; Stoeckenius 1981) and cultured cells (Bolhuis et al. 2006; Burns et al. 2004a), as well in the light microscope after application of specific staining methods (Burns et al. 2004a). PHA is found widespread in the prokaryote world in cells grown in the presence of an excess of a carbon source in combination with limitation of another essential nutrient such as N, P, S, or O_2, and has also been detected in a few other members of the Halobacteriaceae (Fernandez-Castillo et al. 1986; Lillo and Rodriguez-Valera 1990; Oren 2002; Rodriguez-Valera and Lillo 1992).

The prominent presence of PHA within the *Haloquadratum* cells in the natural community suggests that a certain extent of unbalanced growth occurs, in which the growth of the cells is restricted by nutrient limitation, and that carbon and energy resources are therefore diverted to the production of the storage polymer. If this is indeed true, the community's content of PHA can be used to assess the nutritional status of the ponds: excess carbon source and available energy, combined with the lack of key inorganic nutrients would lead to massive accumulation of the compound, while carbon- and energy depletion is expected to lead to degradation of the intracellular reserves. *Salinibacter* does not produce PHA, so that experiments quantifying PHA in the salterns will provide information on the archaeal component only. Thanks to the high density of the Archaea in saltern crystallizer ponds, only small volumes of sample are needed to assay the PHA content, using a simple colorimetric assay based on digestion with concentrated sulfuric acid to convert β-hydroxybutyrate to crotonate. In some brine samples the PHA content varied along the diel cycle, with accumulation during daytime and breakdown during the night; in other samples, little day–night variation was observed. Addition of glycerol, dihydroxyacetone and pyruvate all caused a rapid increase in PHA content of the community. However, acetate, a substrate suggested to be available to *Haloquadratum* as based on microautoradiography—FISH experiments (Rosselló-Mora et al. 2003), and also a known precursor for PHA biosynthesis in many prokaryotes, failed to stimulate PHA formation in Eilat saltern brine samples (Khristo, Elevi Bardavid, and Oren, unpublished). In view of the ease of the assay, we hope to extend these experiments in the future to increase our understanding of the nutritional status of the biota and the range of carbon sources that can be used by *Haloquadratum*.

Why is *Haloquadratum* so successful in colonizing saltern crystallizer brines?

The square archaeon now known as *Hqr. walsbyi* was first observed in 1980 (Walsby 1980), but in spite of numerous attempts by many scientists, using different approaches, its successful cultivation was first achieved only 24 years later (Bolhuis et al. 2004; Burns et al. 2004a). It is much less simple to grow than most other cultured members of the Halobacteriaceae, and growth in the laboratory is relatively slow. Therefore, the question should be asked why this organism, in particular, is so successful in competing with other, faster growing members of the group.

The recently published genome sequence of the isolated organism and its annotation (Bolhuis et al. 2006), as well as the broader picture of the inter-strain variation within the population, obtained using methods of environmental genomics (Legault et al. 2006), undoubtedly provide some important clues here. The organism appears to be highly versatile in its metabolic potential, but so are many other species within the Halobacteriaceae, notably the members of the genera *Haloferax* and *Haloarcula*. These are far less fastidious than *Haloquadratum*, grow much faster (at least at their optimal salt concentration, which is generally far lower than that encountered in the saltern brines), and—especially in the case of *Haloferax*, may excrete halocins—protein antibiotics that specifically inhibit growth of other members of the family. Whether such halocins may indeed play a role in the interspecies competition in saltern ponds, remains to be shown. The first attempts to demonstrate presence of halocins in salterns gave negative results (Kis-Papo and Oren 2000), but *Haloquadratum* had not yet been isolated at the time and was therefore not considered as a potential halocin-producing strain or as a strain that may be sensitive to halocins produced by other halophilic Archaea. Whether *Hqr. walsbyi* produces any halocins is still unknown. No putative halocin-encoding genes were annotated in the *Hqr. walsbyi* genome (Bolhuis et al. 2006), but this is not too surprising in view of the small number of halocin protein sequences known and the lack of homology between them (O'Connor and Shand 2002).

One possible advantage of *Haloquadratum* may be its tolerance toward high concentrations of magnesium. During the evaporation of seawater the magnesium concentration rises, and it keeps increasing while NaCl precipitates from the crystallizer brines as halite. A high level of tolerance toward magnesium is therefore, a prerequisite for a successful existence in the saltern crystallizer ecosystem. However, other genera of

Halobacteriaceae such as *Halorubrum* and *Halobaculum* include members adapted to life at magnesium concentrations exceeding 1.5 M, such as occur in the Dead Sea (Oren 1983; Oren et al. 1995).

Haloquadratum has bacteriorhodopsin- and halorhodopsin-like retinal proteins, and can therefore be expected to derive part of its energy from light (Bolhuis et al. 2006). However, it shares this property with many other representatives of the Halobacteriaceae, including *Halobacterium*, a genus that, in spite of its high salt requirement and tolerance, is not at all successful in colonizing the saltern environment (Oren and Litchfield 1999). It remains to be assessed what portion of its energy *Haloquadratum* may obtain from photons of sunlight. The gas vesicles present in these flat square cells may function to increase their light harvesting potential. In spite of earlier assumptions that the cells may use their gas vesicles to float toward the surface of the water and will thus be able to obtain more oxygen, which may be in short supply in salt-saturated brines, measurements of their flotation rate did not support this theory (Oren et al. 2005). Therefore, a possible alternative function of the gas vesicles, which are located mainly at the edges of the flat square or rectangular cells, may be to keep the cells oriented in a horizontal plane, so that light harvesting can be optimal (Bolhuis et al. 2006; Oren et al. 2005). It is further obvious that the flat shape of the cells greatly increases the surface-to-volume ratio, enabling more efficient transport of nutrients and possibly of oxygen as well. To what extent oxygen may become a limiting factor in the saltern brines is unknown. We have found large diel variations in oxygen concentrations in the experimental Eilat saltern brine mesocosms described above, with maxima at mid-day due to photosynthesis by *Dunaliella*—up to 63, 46, and 36 μM in May, June, and August, decreasing during the night to values as low as 46, 35, and 28 μM, respectively (Khristo, Elevi Bardavid, and Oren, unpublished results).

The finding by Bolhuis et al. (2004) that *Haloquadratum* grows much better on agar plates in the direct neighborhood of *Salinibacter* colonies suggests the existence of some kind of positive interaction between the two organisms. The true nature of this positive interaction remains to be ascertained, and its efficacy under field conditions should be assessed further.

What is the role of phages and protozoa as prokaryotic loss factors in saltern crystallizer ponds?

A study of viral lysis and bacterivory along the salinity gradient in a Spanish saltern system showed high activities of protozoa predating on bacteria in the lower salinity evaporation ponds, but lysis by phages was found to be the major prokaryotic loss factor in the crystallizers (Guixa-Boixareu et al. 1996). Electron micrographs of *Haloquadratum* cells collected from these ponds showed an abundance of phage-like particles in some of the cells (Guixa-Boixareu et al. 1996), and phage-related genes were annotated in the *Hqr. walsbyi* genome (Bolhuis et al. 2006) and in the metagenome of saltern crystallizer biomass (Legault et al. 2006). Whether also *Salinibacter* is attacked by specific phages in the salterns remains to be assessed.

The recent finding of large numbers of up to 2.8×10^4 heterotrophic nanoflagellates actively grazing on prokaryotes in Korean saltern ponds up to the highest salinites (Cho 2005; Park et al. 2003) will require a reassessment of the role that protozoa play in regulating the community density of halophilic Archaea and Bacteria in crystallizer ponds of salterns worldwide.

Conclusion and outlook

Today, 25 years after Borowitzka wrote the sentences quoted at the beginning of this paper, we can reformulate these words as follows, taking into account all that we have learned since: ''Only two genera of heterotrophic prokaryotes have been shown to live and grow in large numbers in extremely saline lakes. They are *Haloquadratum* and *Salinibacter*, and both live in the water column. They are proteolytic or saccharolytic and probably derive most of their carbon from glycerol and cell protein produced by *Dunaliella*.''

The view that extremely hypersaline, salt-saturated lakes such as saltern crystallizer ponds form simple ecosystems, dominated by a few organisms only, has not changed. However, today, we realize that the main players are not those known a quarter of a century ago; instead, they are completely different organisms whose true nature is only now becoming clear. In spite of the apparent simplicity of the ecosystem, the functioning of the different trophic levels, in particular, the nature of the carbon and energy sources used in situ by the different heterotrophic prokaryotes present, is still far from clear. The old idea that glycerol produced by *Dunaliella* may be one of the key compounds for the understanding of the system is still valid today, but the new information reviewed above clearly shows that the heterotrophic activities are undoubtedly far more complex than a simple aerobic oxidation of glycerol to carbon dioxide.

As outlined above, the proper questions about the functioning of hypersaline lake ecosystems can be

asked, now we have obtained a more or less complete insight in the nature of the main organisms present. Saltern ecosystems worldwide are the perfect environment to serve as model systems for research aiming to answer these questions.

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