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THOUGHTS ON THE "MISSING LINK" BETWEEN SALTWORKS BIOLOGY AND SOLAR SALT QUALITY

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ABSTRACT
Although solar salterns worldwide use seawater of identical chemical composition as the raw material for salt production, the size and quality of the halite crystals that precipitate in the crystallizer ponds is highly variable. Biological processes have been implicated to be responsible for the differences observed, but the "missing link" between saltworks biology and solar salt quality has never unequivocally been identified. This paper presents an overview of the different organic chemicals that are formed by the members of the microbial communities in saltern evaporation and crystallizer ponds as osmotic stabilizers as well as different compounds formed during further microbial metabolism of those osmotic solutes. Examination of the *in situ* concentrations and the possible role of glycerol, glycine betaine, ectoine, dihydroxyacetone, acetate, lactate, and other organic compounds failed to identify one or more compounds that may accumulate at concentrations high enough to significantly modify the formation of sodium chloride crystals in the salterns and to negatively influence the quality of the salt produced.

KEYWORDS: halite; glycerol; osmotic solutes; salterns; crystallizer ponds; hypersaline; *Dunaliella*; *Aphanothece*; *Haloquadratum*; *Salinibacter*.

1. INTRODUCTION

Solar salterns all over the world use the same raw material for the process of salt making: seawater. The chemistry of the world ocean differs only very little at different geographical locations. However, the quality of the salt produced in saltern operations differs greatly from place to place. Some plants obtain large hard salt crystals of high purity, with little brine entrapped within them; such crystals are easy to wash and yield an excellent quality of salt with little additional purification. Other saltern operations produce small soft crystals that are difficult to harvest and to purify.

The question has often been asked why so different products are obtained from the same starting material while using the same production process. The answer generally given refers to differences in the nutritional status of the evaporation ponds and the crystallizer ponds, leading to differences in the extent of development of biological communities in these ponds. Characteristic biota, adapted to different salt concentrations, inhabit the saltern ponds along the salinity gradient. These biota include both planktonic communities of green algae, cyanobacteria, diatoms, red extremely halophilic Archaea (in the crystallizer ponds), and others, as well as benthic microbial mats that cover the bottom of the primary and secondary evaporation ponds (Javor, 1989, 2002; Oren, 2002). The pioneering studies by Davis (e.g. Davis, 1979; Davis and Giordano, 1996) have shown that active biological communities are essential for the proper functioning of salterns, but that too extensive development of the biota may lead to the production of poor quality salt. Especially sudden fluctuations in salinity and other parameters often lead to a decrease in quality of the salt that precipitates in the crystallizers. One organism considered problematic in this respect is the cyanobacterium *Aphanothece* (also referred to as *Euhalothece*, *Halothece*, *Cyanothece*, *Aphanocapsa*, *Coccochloris* or *Synechococcus* in the literature) (Garcia-Pichel *et al.*, 1998; Margheri

et al., 2008; Oren, 2000). *Aphanothece* is found in salterns worldwide in the upper layers of the benthic microbial mats at salt concentrations in the range from 100-200 g l^{-1} and sometimes also higher. Especially under nutrient stress this organism produces massive amounts of polysaccharide slime (De Philippis *et al.*, 1993, 1998). When this polysaccharide material reaches the crystallizer ponds downstream, the result may be an increase in brine viscosity and the production of soft, poor quality salt (Coleman and Davis and Giordano, 1996; White, 1993; Roux, 1996; Sudo *et al.*, 1995). It was recently reported that salt-stressed culture of *Dunaliella salina*, the unicellular alga that inhabits the crystallizer ponds, can produce complex extracellular polymeric substances that contain different sugars, aromatic residues, aliphatic alkyl groups and amine groups (Mishra and Jha, 2009). To what extent this polymer is also excreted by *Dunaliella* in the crystallizer habitat is yet unknown.

While it is indeed now generally accepted that overflow of cyanobacterial polysaccharides to the crystallizer ponds may be a primary cause of a deterioration in the property of the salt produced, substantial differences in crystal habit and salt quality exist also without any obvious correlation with the properties of the *Aphanothece* communities upstream in the evaporation ponds. Sedivy (2009a, 2009b) clearly formulated the question of the extensive variations in salt quality from solar salterns. He suggested that the cause of the differences in crystallization behavior of halite in the crystallizer ponds may well be connected with biological phenomena and with organic compounds. After all, as stated above, the properties of seawater are the same worldwide, and inorganic nutrients in the water (nitrate, ammonium, phosphate) are to a large extent scavenged by the biological communities. Sedivy therefore speculated about the nature of the "missing link" between saltworks biology and solar salt quality, challenging the scientific community to search for the compound(s) that may be critical in determining the quality of the salt produced.

2. A SEACH FOR THE "MISSING LINK" BETWEEN SALTWORKS BIOLOGY AND SOLAR SALT QUALITY

Whether indeed a single organic compound exists that may be the "missing link" between the biota of the evaporation and crystallizer ponds and the quality of the salt precipitated in those crystallizers is still unknown. A few possible candidates for such compounds have been suggested in the past: except for the polysaccharide slime of *Aphanothece* and other cyanobacteria and possibly also *Dunaliella*, as discussed above, compounds such as glycerol and amino acids have been proposed (Giordano and Beardall, 2009; Giordano *et al.*, 1994).

A rational search for specific organic compounds that may play a role in the salt making process should start with a thorough understanding of the different types of microorganisms that inhabit the ponds – benthic as well as planktonic, and not only in the crystallizers where the salt is produced but also in the evaporation ponds upstream. The search for such compounds should then center on those organic molecules that are either accumulated by the cells in high concentrations or excreted by the cells in the course of their metabolism. Only such compounds may probably be present at times in concentrations sufficiently high to significantly influence the crystal habit of the forming halite. Even when taking into account that the community density of microorganisms in the saltern can be extremely high (e.g., red halophilic Archaea commonly occur in crystallizers in numbers between 10^7 and 10^8 cells ml^{-1} and sometimes even higher; the biomass in the benthic microbial mats that cover the bottom sediments of the evaporation ponds is also very large) (Javor, 1989; Oren, 2002), compounds that play a minor role in the life of the biota are unlikely to get accumulated to concentrations sufficiently to influence the way salt precipitates in the system – unless we deal with a yet unknown and exceptionally potent compound that acts already at very low concentrations.

Table 1 lists a number of compounds that have in the past been shown or suggested to be formed by the saltern biota. These compounds can be divided into different categories. One prominent group is that of the organic compounds accumulated by different microorganisms to cope with the osmotic pressure exerted by the increasingly hypersaline environment of the salterns. The list of these compounds includes glucosylglycerol, glycine betaine (both used by different cyanobacteria to provide osmotic balance) and glycerol, accumulated to very high concentrations by *Dunaliella salina* (Oren, 1999; 2000; 2002).

Table 1. List of organic compounds produced by halophilic microorganisms in saltern evaporation and crystallizer ponds

COMPOUND	PRODUCED BY	APPROX. SALINITY RANGE ($g\ l^{-1}$)	USED AS	FINAL FATE
Glucosylglycerol	*Microcoleus* and other moderately halophilic cyanobacteria	Up to 150	Osmotic solute	Little known; probably degraded in the early evaporation ponds
Glycine betaine	*Aphanothece* and other halophilic cyanobacteria; anoxygenic phototrophic sulfur bacteria	80-250	Osmotic solute	Can be recycled as osmotic solute as many organisms that do not produce betaine can take it up. Aerobic degradation to CO_2 + NH_4^+; anaerobic degradation forms trimethylamine
Polysaccharide slime	*Aphanothece* and other halophilic cyanobacteria	80-250	Overflow of excess carbon fixed in photosynthesis	Poorly degraded in the saltern system; may reach the crystallizer ponds
	Dunaliella salina	>200-250		
Trimethylamine	Anaerobic breakdown product of glycine betaine	80-250	-	Conversion to CH_4 + NH_4^+ (anaerobically), conversion to CO_2 + NH_4^+ (aerobically)
Glycerol	*Dunaliella salina*	>200-250	Osmotic solute	Taken up by halophilic Archaea and by *Salinibacter*
D-Lactate	Incomplete oxidation product of glycerol by halophilic Archaea	>200-250	-	Taken up rapidly by some halophilic Archaea
Pyruvate	Incomplete oxidation product of glycerol by halophilic Archaea	>200-250	-	Taken up rapidly as preferred growth substrate by some halophilic Archaea
Acetate	Incomplete oxidation product of glycerol by halophilic Archaea	>200-250	-	Taken up slowly by halophilic Archaea
Dihydroxyacetone	Incomplete oxidation product of glycerol by *Salinibacter*	>200-250	-	Taken up by certain halophilic Archaea (*Haloquadratum*)

Not all halophilic microorganisms use organic compounds for this purpose; KCl is used for osmotic adaptation by the red halophilic Archaea (family Halobacteriaceae) and also by the red extremely halophillic *Salinibacter ruber* (domain Bacteria, Bacteroidetes) that inhabits crystallizer ponds all over the world (Antón *et al.*, 2000, 2002; Oren, 2002; Oren and Rodríguez-Valera, 2001; Oren *et al.*, 2004). A second category of potential interest consists of compounds formed from the above-mentioned osmotic solutes during their microbial degradation. Thus, trimethylamine can be formed

from glycine betaine (Oren, 1990), and dihydroxyacetone, lactate, acetate and pyruvate have been shown to be produced from glycerol by different halophilic prokaryotes (Elevi-Bardavid and Oren, 2008; Oren and Gurevich, 1996). The exopolysaccharide slimes produced by *Aphanothece* and other cyanobacteria, and possibly also *Dunaliella*, as discussed above, form a third category of compounds whose action on halite crystallization should be examined in greater depth.
The sections below provide a short discussion of our current understanding of the presence and the metabolism of the above-mentioned compounds in solar salterns.

3. GLYCEROL AND OTHER ORGANIC OSMOTIC SOLUTES

The possible importance of glycerol as a key compound for the understanding of the biology of saltern crystallizer ponds was stressed for a long time (for a review see Elevi Bardavid *et al.*, 2008). The cytoplasm of *Dunaliella salina*, the primary producer in these ponds, may contain glycerol concentrations as high as 6-7 M. Upon death of the *Dunaliella* this glycerol will leak out of the cells, and some may even leak out of healthy active cells. Many halophilic Archaea readily use glycerol as carbon and energy source. Addition of glycerol to saltern brines led to a stimulation of up to 50% of the community respiration as measured by different techniques, including direct assessment of oxygen uptake (Warkentin *et al.*, 2009) and reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride to the corresponding formazan derivative (Oren, 1995a). Studies in which radiolabeled glycerol was added to crystallizer brines showed rapid uptake and short turnover times, in the order of a number of hours (Oren, 1993). Not all studies have confirmed the ready use of glycerol by the archaeal community in the ponds; thus, attempts to show uptake of glycerol by the Archaea, including the abundant square cells described as *Haloquadatum walsbyi*, using a combination of microautoradiography and fluorescent *in situ* hybridization yielded negative results (Rosselló-Mora *et al.*, 2003). However, a gene annotated as glycerol kinase is present in the *H. walsbyi* genome (Bolhuis *et al.*, 2006). *Salinibacter ruber*, the red representative of the Bacteroidetes that is abundant in many crystallizer ponds (Antón *et al.*, 2000, Oren and Rodríguez-Valera, 2001), also takes up glycerol (Elevi Bardavid and Oren, 2008; Sher *et al.*, 2004), in spite of earlier reports that failed to show use of the compound by this organism (Antón *et al.*, 2002; Rosselló-Mora *et al.*, 2003).
A simple calculation can provide an estimate how much glycerol the *Dunaliella* population in salterns may contain and how much glycerol will thus become available following the death of the cells. *Dunaliella salina* cells measure 5-23 x 4-19 μm (Preisig, 1992). When assuming the cells to be spheres with an average diameter of 12 μm and a volume of 900 μm^3 and with an intracellular concentration of 7 M (which means that by weight the cells contain more glycerol than water), a population of 10^4 cells ml^{-1} (which is more than generally observed in most saltern crystallizer ponds) will contribute no more than 64 μM = 5.9 mg l^{-1} glycerol to the brine. Even an extremely dense population of 10^5 cells ml^{-1} of 20 μm diameter will not contribute more than 2.9 mM = 270 mg l^{-1} glycerol to the brines following cell lysis. Occasionally even denser *Dunaliella* populations have been observed in salterns, and the amount of glycerol contained in the algae will be accordingly higher.
The above calculation does not take the possibility into account that some glycerol may leak out of the intact cells during photosynthesis. There is little information on this topic in the literature. Laboratory studies with *D. salina* showed that generally up to 5% of its photosynthetically fixed carbon is released to the medium (Giordano *et al.*, 1994). To what extent this value represents true excretion or may be a result of breakage of the cells during sample processing remains to be determined. *Dunaliella* cells are fragile, and they easily rupture during filtration used to separate cells from their medium (Goldman and Dennett, 1985). This fact has only seldom been taken into account in studies of *Dunaliella* photosynthesis in the natural environment (see also Oren *et al.*, 1995). The relevance of studies in which filtration was used (e.g. Huntsman, 1972) should therefore be questioned. A short centrifugation procedure as was applied by Giordano *et al.* (1994) is probably better, but proper controls are still needed to ensure that no artifacts are introduced by breakage of fragile cells when compressed into a pellet in the centrifuge tubes. In any case, if indeed the results of such laboratory experiments can be extrapolated to predict the behavior of *Dunaliella* in the saltern brines (which is not necessarily true as in the salterns the cells may be subject to more stressful conditions because of high levels of irradiation, suprapoptimal salinities, etc.), then leakage glycerol from live cells will not greatly increase the overall potential glycerol concentrations calculated above. However, it should be taken into account that, as long as nutrients are present in the brine, *Dunaliella* may divide, grow and die, releasing glycerol. Thus, whereas the cell concentration may be constant, the glycerol concentration may be increasing.

To my knowledge there has been one attempt to directly measure glycerol concentrations in saltern crystallizer brines. The method was based on the oxidation of glycerol to periodate to yield formaldehyde and formate, followed by colorimetric assay of the formaldehyde with 3-methyl-2-benzothiozolone hydrazone. Other organic compounds (e.g. some sugars) may also release formaldehyde during periodate oxidation, and therefore the protocol can only give an upper limit for the true concentration of glycerol present. The crystallizer brines in Eilat, Israel, at the time populated by 160-2,960 *D. salina* and up to $9x10^7$ prokaryotic cells ml^{-1}, thus contained between 20 and 36 μM glycerol = 1.8-3.3 mg l^{-1} (Oren, 1993), values in the same order of magnitude as the potential concentrations calculated above. It should be noted that these concentrations are several orders of magnitude lower than those used by Sedivy (2009a) to show that glycerol may influence the mode of crystallization of halite from saturated NaCl solutions. To my knowledge this relatively simple procedure to obtain quantitative information on the possible glycerol concentration in the brine has never been applied to salterns elsewhere that produce lower quality crystals and also may contain denser *Dunaliella* populations.

Giordano and Beardall (2009) proposed that *D. salina* may excrete amino acids into the medium when the concentration of ammonium ions in the medium is high. This conclusion was based on results of experiments that showed that ammonium ions when added at the very high concentration of 10 mM caused an increase in the amount of excreted carbon to up to 11% of the $^{14}CO_2$ fixed (Giordano *et al.*, 1994). However, no evidence was presented that indeed this carbon was excreted in the form of amino acids.

Additional organic osmotic solutes are present in the microbial communities in the lower-salinity evaporation ponds. Among the compounds accumulated by cyanobacteria and other prokaryotes in the benthic hypersaline microbial mats are glucosylglycerol (in *Microcoleus* and some other cyanobacteria), glycine betaine (in *Aphanothece* and other halophilic cyanobacteria, as well as in phototrophic purple bacteria) and ectoine, found in a variety of phototrophic and non-phototrophic prokaryotes (Oren, 1999, 2000, 2002). All these compounds can be degraded and recycled at the salinity of the evaporation ponds (Ventosa *et al.*, 1998). Quantitative information about the *in situ* concentrations of these compounds and on their further fate downstream the salt gradient is lacking altogether. Thus far there is no evidence that any of these solutes may accumulate in the crystallizer brines to concentrations sufficiently high to influence the mode of halite crystallization.

4. COMPOUNDS PRODUCED DURING METABOLISM OF OSMOTIC SOLUTES

Among the chemicals listed in Table 1 are five compounds that are released during the microbial degradation of some of the organic osmotic solutes discussed above. These are dihydroxyacetone, lactate, acetate, pyruvate and trimethylamine.

Dihydroxyacetone is the most recent addition to the list of compounds of interest to the understanding of the microbial community metabolism in the saltern ecosystem. When glycerol was added to cultures of the extremely halophilic bacterium *Salinibacter ruber*, up to 20% of the glycerol taken up was converted to a not previously characterized overflow product (Sher *et al.*, 2004). When later the genome sequence of the square archaeon *Haloquadratum walsbyi*, the most abundant prokaryote in many salterns, suggested presence of a well-developed uptake system for dihydroxyacetone (Bolhuis *et al.*, 2006), the compound excreted by *Salinibacter* was rapidly identified as dihydroxyacetone (Elevi Bardavid and Oren, 2008). No specific and sensitive chemical assay for dihydroxyacetone has yet been developed that may detect the compound in micromolar concentrations or below in hypersaline brines, and thus no information exists on its true concentrations and turnover *in situ*.

Use of glycerol and carbohydrates by halophilic Archaea is often accompanied by the production of acids. Excretion of acetate, pyruvate, and D-lactate was observed in many species of *Haloferax*, *Haloarcula*, and *Halorubrum* (Oren, 2002; Oren and Gurevich, 1994). Pyruvate is a compound of special interest as it is a key component in the growth media used for cultivation of *Haloquadratum*. Pyruvate also simulated oxygen update by the Eilat crystallizer brine heterotrophic community (Warkentin *et al.*, 2009). The question was therefore asked whether excretion of acids occurs only in the presence of very high concentrations of the carbon sources or also at the very low concentrations of glycerol expected to be found in natural brines inhabited by *Dunaliella*. When samples from the Eilat saltern crystallizers were incubated with 1.5-3 μM ^{14}C-labeled glycerol, a substantial fraction (8-11%) of the label added was recovered not as cell material or as CO_2 but in the form of organic acids. D-Lactate and acetate were produced at a molar ratio of 4.3-4.8:1, with the formation of up to 0.4 μM acetate and 0.05 μM lactate (Oren and Gurevich, 1994). When similar

experiments were performed in Dead Sea water, pyruvate was also detected. It is well possible that pyruvate was initially formed also in the saltern brines, but was rapidly taken up by *Haloquadratum* so that it did not accumulate. The lactate formed was degraded within a day after depletion of the glycerol, but the amount of labeled acetate that had accumulated decreased only very slowly (Oren and Gurevich, 1994). Acetate turnover in the crystallizer brines was very slow, with turnover times estimated between 127 and 730 h (Oren, 1995b). It is not clear how this observation can be explained in view of the apparently efficient uptake of acetate by *Haloquadratum* in Spanish saltern ponds, based on fluorescence *in situ* hybridization experiments combined with microautoradiography (Rosselló-Mora *et al.*, 2003). Presence of acetate at concentrations between 8.1 and 11.4 μM was reported in the Eilat crystallizer ponds (Oren, 1995b), based on the use of a specific, enzymatic method measuring the amount of adenosine monophosphate formed from adenosine triphosphate in the presence of acetate, coenzyme A and S-acetyl-CoA synthetase (King, 1991). These concentrations were in the same order of magnitude as those estimated from acetate uptake kinetics (Oren, 1995b).

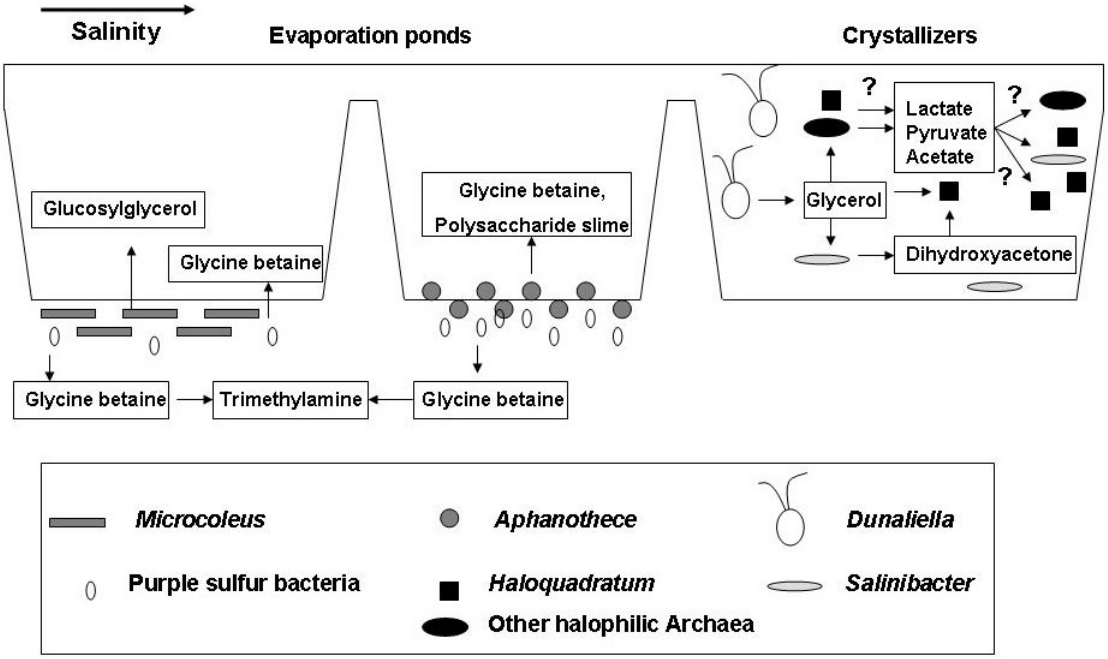

Figure 1. The origin and fate of different organic compounds formed by the biological communities in saltern evaporation and crystallizer ponds

Another group of compounds of potential interest formed during microbial degradation of osmotic solutes consists of trimethylamine and other methylated amines that may be derived from the breakdown of glycine betaine. Glycine betaine, produced in large quantities in hypersaline microbial mats by *Aphanothece* and other cyanobacteria as well as by anoxygenic phototrophic prokaryotes can be degraded both aerobically and anaerobically. Methylated amines are typical anaerobic degradation products of glycine betaine (Oren, 1990). In the anaerobic sediments of the microbial mats these methylated amines are further degraded to yield methane (Canfield *et al.*, 2004; Sørensen *et al.*, 2009). To what extent methylated amines may also reach the crystallizer ponds is unknown.

Figure 1 illustrates the formation and further conversions in saltern evaporation and crystallizer ponds of the compounds mentioned in the above sections.

5. FINAL COMMENTS

Examination of the nature of the diverse organic chemicals synthesized by the members of the microbial communities in saltern evaporation and crystallizer ponds as osmotic stabilizers, as well as different compounds formed during microbial metabolism of those osmotic solutes shows that a wide variety of organic compounds can be expected to occur in saltern brines. Compounds of potential interest include the osmotic solutes glycerol produced by *Dunaliella*, glycine betaine, ectoine, and other osmotically active compounds formed by phototrophic and non-phototrophic bacteria, and

metabolic products that may be excreted by different halophilic prokaryotes such as dihydroxyacetone, acetate, and lactate.

For a compound to be identified as the "missing link" between saltworks biology and solar salt quality it should be present in the saltern crystallizer brines at concentrations sufficiently high to influence the mode of crystallization of halite. Earlier laboratory experiments have shown that glycerol, when added to concentrated NaCl solutions at a concentration of 500 mg l^{-1} (5.4 mM) visibly influenced the crystallization habit of halite; a far more dramatic effect was observed at a concentration ten times as high (Sedivy, 2009a). However, as discussed above, such glycerol concentrations are several orders of magnitude higher than those actually measured in saltern crystallizer brines (Oren, 1993), and therefore the relevance of these observations is not immediately clear.

One recurring problem in studies of the occurrence of small organic molecules in saltern brines is the lack of sufficiently sensitive analytical assays to detect the compounds and to provide a reliable quantitative estimate of their presence. All relevant compounds are hydrophilic and there are no selective extraction procedures to separate them from the saltern waters. The high salinity of the brines also interferes with many common analytical procedures. Only in two cases have the concentrations of relevant compounds been successfully estimated in salterns (Eilat, Israel): for acetate (Oren, 1995b) following dilution of the sample and application of a sensitive enzymatic assay (King, 1991), and for glycerol, using a very sensitive chemical assay that unfortunately has a limited specificity and can therefore only estimate the upper concentration limit (Oren, 1993). In both cases the calculated concentrations were in the range of a few micromolars to a few tens of micromolars, i.e. far lower than the millimolar concentrations apparently needed to significantly influence halite crystallization.

In conclusion, our current understanding of the metabolism of the different types of halophilic microorganisms that inhabit saltern ponds all over the world has enabled the recognition of possible candidates for the "missing link" between saltworks biology and solar salt, but has not yet led to the identification of that missing link.

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