THE MICROBIOLOGY OF SALTERN CRYSTALLIZER PONDS AND SALT QUALITY - A SEARCH FOR THE "MISSING LINK"

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Abstract

The size and quality of the salt crystals formed in solar saltern crystallizer ponds worldwide is highly variable. In some places large solid halite crystals precipitate that are easy to process and yield a high-quality product, while elsewhere crystals are soft, have a high content of entrapped mother liquor, and are difficult to harvest and to purify. As the raw material in all cases is seawater of nearly identical composition, it is often assumed that biological processes in the evaporation and/or crystallizer ponds may be responsible for the differences in salt quality. Presence of a community of red halophilic Archaea and the alga *Dunaliella salina* in the crystallizers is generally considered beneficial to the salt production process and excessive production of polysaccharide slime by the cyanobacterium *Aphanothece halophytica* in the intermediate-salinity evaporation ponds may lead to a deterioration of the quality of the salt precipitated in the crystallizers. In a search for the "missing link" between solar salt quality and saltern pond microbiology, I here provide a survey of organic chemicals known to be produced by different members of the microbial communities in saltern ponds. These compounds include "compatible solutes" (small organic molecules synthesized to provide osmotic stabilization of the cells) and different metabolites excreted by the cells in the course of their metabolism. Quantitative estimates of the concentrations of glycerol, glycine betaine, ectoine, dihydroxyacetone, acetate, lactate, and other potentially relevant organic compounds in the ponds show that expected concentrations are probably too low to significantly influence the crystal habit of the precipitating halite. Moreover, laboratory simulation experiments failed to show significant effects of any of these compounds (at concentrations up to 200 ppm) on halite crystallization. A new theory is here proposed, suggesting that proteins released during lysis of pond microorganisms, and especially of the red halophilic Archaea, may at least in part be responsible for the production of poor quality salt.

Introduction

Although the mode of operation of solar salt production plants is very similar worldwide and the composition of the seawater from which the salt is produced is nearly identical, the size and quality of the salt crystals formed in the crystallizer ponds of salt production facilities around the world is highly variable. At some sites large solid halite crystals precipitate with little or no liquid inclusions,

and these can easily be harvested and processed to yield a high-quality product. Elsewhere crystals are soft, contain a high content of mother liquor entrapped in the halite, and are difficult to harvest and to purify.

It is often assumed that biological processes in the evaporation and/or crystallizer ponds may be responsible for the differences in salt quality. Characteristic communities of microorganisms adapted to life at high salt concentrations inhabit the saltern ponds. These biota include planktonic green algae, cyanobacteria, diatoms, a variety of heterotrophic prokaryotes and others, and benthic microbial mats that cover the bottom of the evaporation ponds. The crystallizer brines are generally red due to dense populations of red exteremely halophilic Archaea (family Halobacteriaceae), the red halophilic bacterium *Salinibacter ruber*, and the β-carotene-rich unicellular green flagellate alga *Dunaliella salina*. Differences in the nutritional status of the saltern ponds lead to differences in the extent of the development of the biological communities. Presence of these biological communities is essential for the proper functioning of salterns, and it is well established that the presence of a community of red halophilic Archaea and the alga *Dunaliella* in the crystallizers is generally beneficial to the salt production process (Javor, 1989, 2002; Oren, 2002). However, too extensive development of the biota may lead to the production of poor quality salt (Davis, 1979; Davis and Giordano, 1996). Such problems are often attributed to the excessive production of polysaccharide slime by the cyanobacterium *Aphanothece halophytica* in the intermediate-salinity evaporation ponds. *Aphanothece* is found in the upper layers of the benthic microbial mats at salt concentrations in the range from 100-200 g/l (Javor, 1989; Oren, 2000), and can excrete massive amounts of polysaccharides (De Philippis et al., 1993, 1998), which increase brine viscosity and lead to the production of soft, poor quality salt in the crystallizer ponds downstream (Coleman and White, 1993; Davis and Giordano, 1996; Roux, 1996; Sudo et al., 1995). Salt-stressed *Dunaliella salina* cultures were recently reported to produce extracellular polymeric substances (Mishra and Jha, 2009), but to what extent this phenomenon also occurs in the salterns is yet unknown.

Sedivy (2009a, 2009b) discussed the question why the quality of the salt produced in solar saltern worldwide is so variable. He suggested that the differences in crystallization behavior of halite may well be connected with biological processes causing the accumulation of organic compounds, and he speculated about the nature of the "missing link" between saltworks biology and solar salt quality. Thus far the identity of the compound(s) involved has not been elucidated.

Compatible solutes and other microbial metabolites as candidates for the "missing link" between saltern microbiology and salt quality

A search for specific organic compounds as candidates for the above-mentioned "missing link" should be based first of all on a thorough understanding of the properties of the different types of microorganisms that inhabit saltern ponds (Oren, 2009). Red halophilic Archaea commonly occur in crystallizers in numbers between 10^7 and 10^8 cells/ml and sometimes even higher, and *Dunaliella* can often be found from 10^3 to 10^5 cells/ml (Javor, 1989; Oren, 2002). Also the earlier evaporation ponds contain dense microbial communities, especially in the benthic mats and gypsum crusts. Therefore a survey of those organic molecules that are produced by the cells in high concentrations or excreted as part of their metabolism may well lead to the identification of the "missing link". Only such compounds can be expected to accumulate in the brines at concentrations sufficiently high to significantly influence the habit of the halite crystals. Table 1 presents a (not necessarily exhaustive) list of organic compounds that belong to the categories defined above.

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

Compound	*Produced by*	*Site of production*	*Used as*
Glucosylglycerol	*Microcoleus* and other cyanobacteria	Low salinity evaporation ponfd	Osmotic solute
Glycine betaine	*Aphanothece* and other cyanobacteria; purple sulfur bacteria	Medium salinity evaporation ponds	Osmotic solute
Polysaccharide slime	Cyanobacteria		Overflow of excess carbon fixed in photosynthesis
Trimethylamine	Breakdown of glycine betaine		-
Glycerol	*Dunaliella*	Crystallizer ponds	Osmotic solute
Polysaccharide slime			Overflow of excess carbon fixed in photosynthesis
D-Lactate	Incomplete oxidation of glycerol by halophilic Archaea		-
Pyruvate			-
Acetate			-
Dihydroxyacetone	Incomplete oxidation of glycerol by *Salinibacter*		-

Some of the compounds listed are so-called "compatible solutes": small organic molecules synthesized by microorganisms to provide osmotic stabilization of the cells. *Dunaliella* in the crystallizer ponds produces glycerol for this purpose, while cyanobacteria and other prokaryotes in the microbial mats and in the plankton of the evaporation ponds accumulate compounds such as glycine betaine (*N*,*N*,*N*-trimethylglycine), glucosylglycerol, and ectoine (Oren, 1999, 2000, 2002). The halophilic Archaea (*Haloquadratum* and others) and Bacteria (*Salinibacter*) in the crystallizer ponds do not produce organic osmotic solutes but accumulate KCl instead. Other compounds in the list are degradation products formed from the different organic osmotic solutes. Anaerobic breakdown of glycine betaine yields trimethylamine (Oren, 1990); *Salinibacter* partially oxidizes glycerol to dihydroxyacetone (Elevi-Bardavid and Oren, 2008), and different halophilic Archaea excrete lactate, pyruvate and acetate when exposed to the presence of glycerol, also when present at low concentrations (Oren and Gurevich, 1994). Figure 1 summarizes the source and further transformations of representative organic compounds expected to be found in the brines of saltern crystallizer ponds.

Whether the compounds listed in Table 1 and Fig. 1 indeed may influence the quality of the halite formed in saltern ponds depends on their concentrations. Few attempts have thus far been made to estimate the *in situ* concentrations of such compounds, and in most cases no sufficiently sensitive methods have been developed for the quantification of these chemicals at low concentrations in saturated brines. Glycerol may be present in the cytoplasm of *Dunaliella* at concentrations up to 6-7 M. Measurements have shown that the compound, when released to the brine, is rapidly taken up by the heterotrophic archaeal community (Oren, 1993), although indications were obtained that *Haloquadatum*, the dominant type of archaeon in many saltern crystallizer ponds, does not readily incorporate glycerol under *in situ* conditions

(Rosselló-Mora et al., 2003). *Salinibacter* also takes up glycerol (Elevi Bardavid and Oren, 2008; Sher et al., 2004), although not all studies confirmed this observation (Antón et al., 2002; Rosselló-Mora et al., 2003). Although the cell membrane of *Dunaliella* is little permeable to glycerol, some photosynthetically produced glycerol may leak out of the cells (Giordano et al., 1994). The extent of such leakage under the conditions encountered in the saltern environment has never been properly assessed. Accordingly it is yet unknown whether more glycerol is produced by living cells than may be released from the cytoplasm during cell death.

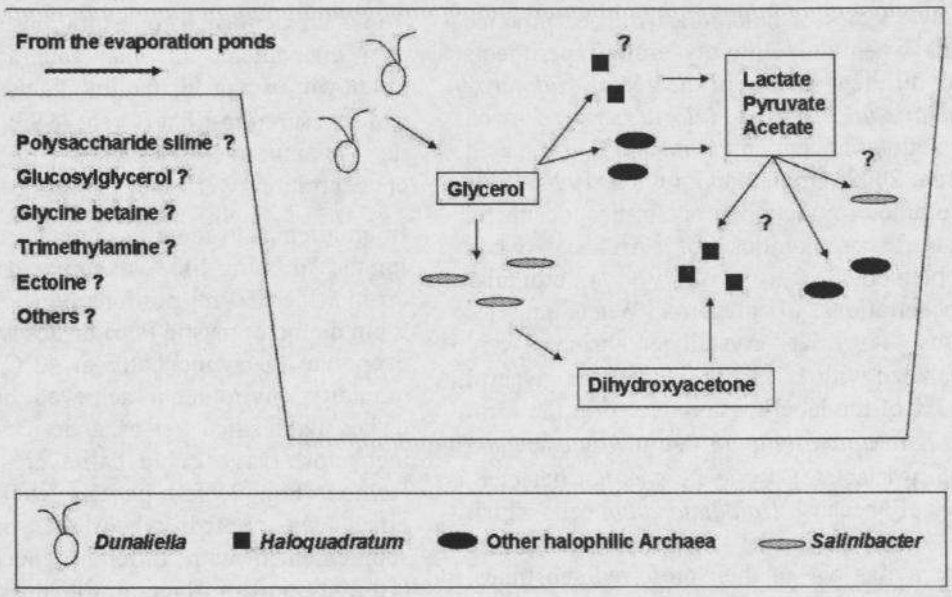

Fig. 1. The origin and fate of different organic compounds of biological origin expected to be formed and/or metabolized by the biological communities in saltern crystallizer ponds.

An attempt was made in the salterns of Eilat, Israel to measure glycerol concentrations in crystallizer brines, based on periodate oxidation of glycerol to formaldehyde and formate, followed by colorimetric assay of the formaldehyde formed. Sugars and some other organic compounds may also produce formaldehyde upon periodate oxidation, and therefore the protocol only enables the calculation of an upper limit for the true concentration of glycerol. The concentrations thus measured were very low: between 20 and 36 μM = 1.8-3.3 mg/l (Oren, 1993), concentrations several orders of magnitude lower than those used by Sedivy (2009a) to test the influence of glycerol on the crystallization of halite from saturated NaCl solutions. At the time of the measurements the brines contained between 160 and 2,960 *Dunaliella* cells/ml. I have not seen any reports showing that this relatively simple analytical procedure has ever been applied to saltern brines elsewhere that yield lower quality crystals and may contain denser *Dunaliella* populations. No attempts appear to have been made to obtain quantitative information about the concentrations of organic osmotic solutes such as glucosylglycerol, glycine betaine and ectoine that are synthesized by different planktonic and benthic members of the microbial communities the evaporation ponds at lower salinities.

The compounds trimethylamine, lactate, acetate, pyruvate and dihydroxyacetone, listed in Table 1, are all degradation products of the above-mentioned organic osmotic solutes by halophilic prokaryotes found in the salterns, and therefore their presence and possible *in situ* concentrations in the crystallizer ponds deserve to be discussed as well. Anaerobic breakdown of glycine betaine,

the main compatible solute accumulated by halophilic cyanobacteria, yields trimethylamine (Oren, 1990). Trimethylamine cannot be expected to accumulate in the salterns to high concentrations as it is efficiently converted to methane and carbon dioxide in the anaerobic sediments of the evaporation ponds (Canfield et al., 2004; Sørensen et al., 2009). Its concentration in crystallizer brines has never yet been assessed.

Many species of halophilic Archaea produce acids when fed with glycerol. Experiments with different species of the genera *Haloferax*, *Haloarcula*, and *Halorubrum* showed formation of acetic, pyruvic, and D-lactic acid (Oren, 2002; Oren and Gurevich, 1994). This incomplete oxidation process also occurs in natural communities of Archaea when supplemented with micromolar concentrations of glycerol. When samples from the Eilat crystallizer brines were incubated with 1.5-3 μM ^{14}C-labeled glycerol, 8-11% of the label was recovered in the form of organic acids (up to 0.4 μM acetate and 0.05 μM lactate). Pyruvate was not detected, probably because *Haloquadratum* cells which are abundant in the brines efficiently use pyruvate as one of their preferred substrates. The lactate formed disappeared within a day after the glycerol was depleted. However, the amount of labeled acetate decreased only slowly (Oren and Gurevich, 1994). Estimated turnover times in the crystallizer brines being between 127 and 730 h (Oren, 1995), this in spite of the apparent efficient uptake of acetate by *Haloquadratum* observed in crystallizer ponds of the salterns of Santa Pola, Spain (Rosselló-Mora et al., 2003). A sensitive and specific enzyme-based assay was adapted for the quantitative determination of acetate in saltern brines; application of the assay to Eilat crystallizer brines yielded concentrations between 8.1 and 11.4 μM (Oren, 1995).

A recent addition to the list of metabolites of interest produced by the microbial community in the saltern crystallizers is dihydroxyacetone. Dihydroxyacetone is a product of incomplete oxidation of glycerol by *Salinibacter ruber*, an extremely halophilic representative of the Bacteria (Sher et al., 2004; Elevi Bardavid and Oren, 2008). No quantitative estimates are yet available on the distribution of this compound in the salterns, but *Haloquadratum* is known to efficiently take up the compound (Elevi Bardavid and Oren, 2008).

Experiments toward the identification of the possible "missing link" between saltern microbiology and salt quality

Glycerol, when added to concentrated NaCl solutions at concentrations of 0.5-5 g/l (5.4-54 mM), visibly influenced the crystallization habit of halite (Sedivy, 2009a). To what extent such concentrations are indeed ever encountered in solar saltern ponds is unknown; except for the low values of 20-36 μM measured in Eilat (Oren, 1993; see above) no quantitative estimates of glycerol concentrations were ever published.

In an attempt to identify potential candidates for the "missing link", experiments were set up in which 50-ml portions of 25% NaCl in 9-cm diameter plastic Petri dishes were left to evaporate in an incubator at 42°C in a low humidity environment achieved by placing dishes with silica gel as a desiccant in the incubator. Large cubic halite crystals were formed (Fig. 2, left panel). No significant effect was noted when the brine was supplemented with different concentrations (10-200 ppm) of glycerol, dihydroxyacetone, glycine betaine, ectoine, Na-acetate, Na-lactate, Na-pyruvate, or trimethylamine. However, a profound effect was obtained in the presence of low concentrations of protein. The right panel of Fig. 2 shows an experiment in which addition of bovine serum albumin caused the formation of small soft crystals formed near the upper edge of the wall of the Petri dish. A similar effect was seen with a number of other proteins, but not with equivalent concentrations of amino acids (added as Difco Casamino acids). A cell lysate of the halophilic archaeon *Halobacterium salinarum* R1, obtained by sonication, had a similar effect when added to a concentration of 15 ppm protein and higher. A lysate of *Dunaliella* was less effective in modifying the mode of salt crystallization, and addition of intact *Halobacterium* or *Dunaliella* cells representing the same amount of protein had no effect. Further experiments are currently in progress to elucidate the nature of the effect of different proteins on halite crystallization.

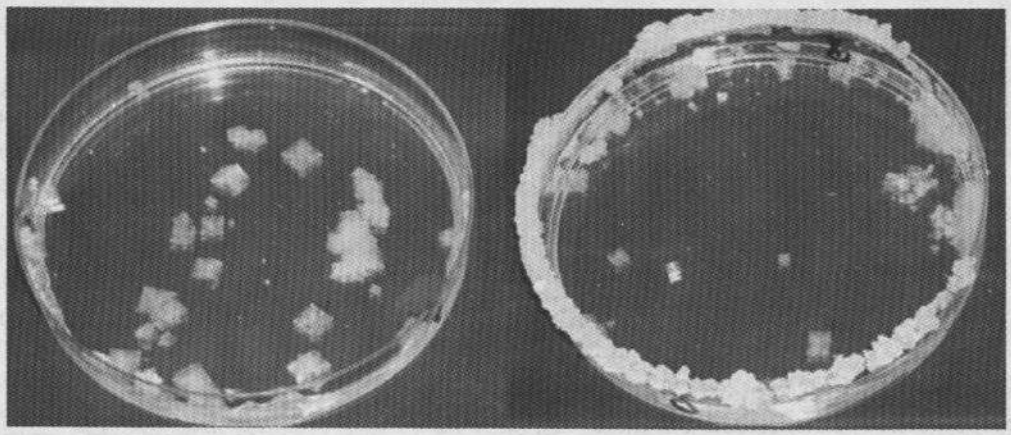

Fig. 2. Halite crystals formed by evaporation of a 25% NaCl solution without additives (left) and in the presence of 20 ppm bovine serum albumin (right).

Is protein indeed the "missing link" between saltern microbiology and salt quality?

As far as I have been able to ascertain, the possible link between protein dissolved in the brine or adsorbed to surfaces and the quality of the halite crystals precipitating in solar salterns has not been proposed earlier. However, protein was used in earlier times to improve the process of salt boiling in pans. In his famous treatise on mining "De Re Metallica", published in 1556, Georgius Agricola (Georg Bauer, 1494-1555) wrote:

> "In order to accelerate the condensation of the brine, when the master has poured in two casks and as many dippersful of brine, he adds about a Roman *cyathus* and a half of bullock's blood, or of calf's blood, or buck's blood, or else he mixes into the nineteenth dipperful of brine, in order that it may be dissolved and distributed into all the corners of the caldron; in other places the blood is dissolved in beer". (Translation: Hoover and Hoover)

More than a hundred years later this practice still existed, as described by William Jackson in 1669:

> "... then they put into their Panns amongst their Brine a certain mixture, made of about 20. Gallons of Brine, and 2. quarts of Calves Cows or chiefly Sheeps bloud, mixt into a Claret-Colour: of this mixture they put about 2. quarts into a Pann that holds about 360. quarts of Brine ..."

Sometimes egg white was used instead of cattle blood:

> "... then they put into the Pann, 2. quarts of the Mixture following: They take a quart of Whites of Eggs, beat them thoroughly with as much Brine, till they are well broken; then mix them with 20. Gallons of brine, as before was done with the Bloud;"

Baas Becking (1931) calculated that Agricola and Jackson respectively recommended using 1 part of blood per 1500 and 1300 parts of brine. With cattle blood that per 100 ml contains approximately 11 g hemoglobin and 4 g of plasma proteins, this would mean an addition of no less than 100 ppm protein. Most of this protein undoubtedly denatured during the boiling process, and was removed as foam. How exactly addition of blood improved the salt boiling process is not clear.

The often dense communities of halophilic microorganisms in the crystallizers represent large amounts of protein; values reported range between <0.4 mg/l in oligotrophic salterns to 20-35 mg/l in eutrophic systems (Javor, 2002). Such values agree well with a ratio of 0.75-0.85 mg particulate protein per 10^{10} cells of halophilic Archaea in a bloom in the Dead Sea (Oren and Gurevich, 1995).

The above-documented effect of selected proteins and of *Halobacterium* cell lysate on the crystallization of halite was not observed

with equivalent concentrations of amino acids. Thus, the assumption of Giordano and Beardall (2009), based on earlier radiolabeling experiments suggesting that *Dunaliella salina* excretes amino acids into the medium in the presence of high concentrations of ammonium ions (Giordano et al., 1994), is probably of little relevance to the search for the "missing link".

A lysate of halophilic Archaea influenced crystal habit much more than a lysate of *Dunaliella*. Whether the strongly acidic nature of the archaeal proteins (Oren, 2002) may be the factor that determines their efficacy is not known. Bovine serum albumin, with an isoelectric point of 4.7, is an acidic protein as well. More experiments are required to test the possible correlation between the isoelectric point of proteins and their effect on halite crystallization.

If indeed the proteins released during lysis of halophilic Archaea may be responsible for the formation of poor quality salt in the crystallizers, then the study of the factors that lead to cell lysis in the salterns becomes highly relevant. Dense communities of intact archaeal cells do not appear to disturb the formation of large good-quality halite crystals, and their presence is generally considered beneficial to salt production.

Most species of the Halobacteriaceae lyse when the salinity decreases to values below 150 g/l, which is far lower than the salinities ever encountered in crystallizer ponds. Another far more relevant option is cell lysis by halophages – viruses that attack halophilic Archaea. Presence of such viruses is known for a long time, and most types characterized in the past were isolated from saltern brines (Oren, 2002). Unfortunately, hardly anything is known about the dynamics of halophages in natural halophilic Archaea communities. Electron microscopical examination of brines from Spanish saltern crystallizer ponds showed phage-like particles to be present at numbers one to two orders higher than the number of prokaryotes, with numbers up to 10^9 phage-like particles per ml in NaCl-saturated brines. Between one and ten percent of the flat square, *Haloquadratum*-like Archaea in the crystallizer ponds had visible phages inside the cells, often in high numbers. However, it was calculated that viruses did not exert a strong control over the prokaryotic abundance and growth rate, and at the highest salinities the percentage of cells lost daily by viral lysis was estimated to be lower than 5% (Guixa-Boixareu et al., 1996). Pulsed-field gel electrophoresis analysis of viral nucleic acids extracted from Spanish saltern ponds gave some indication of the viral diversity encountered in the ecosystem (Diez et al., 2000). A study of the dynamics of halophages in saltern ponds in Jamaica showed that viruses may become active and initiate a lytic cycle following dilution of brines by rain water (Daniels and Wais, 1990; Wais and Daniels, 1985). The dynamics of the Archaea – halophage equilibrium in the saltern crystallizer ponds now deserves a far more thorough investigation in view of the possibility that massive phage-induced cell lysis may negatively influence salt quality.

The ideas presented above are as yet no more than a theory, to be proven or refuted by further experiments and by observations in the field. If indeed halophilic Archaea-derived proteins are responsible for the formation of low quality salt, the theory can provide a framework for the proper diagnosis of the problem. A full understanding of the factors that cause lysis of microbial cells in the salterns may then form the basis for improved pond management in the future.

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