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Concentrated brines as habitats for microorganisms

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Summary. Concentrated salt solutions (brines) occur widely in the natural form of coastal lagoons, salt or soda lakes as well as in the man-made form of salterns or saltworks. They are inhabited by a limited number of specialized microorganisms, which use different strategies of haloadaptation. Extremely halophilic archaebacteria (Halobacteriaceae) compensate the high osmotic pressure of brines by high cytoplasmic K^+ion concentrations. This requires appropriate adaptations of both the intracellular and extracellular functional macromolecules. Only some of the halophilic, halotolerant and extremely halophilic eubacteria follow this strategy; while the majority of them, and the halophilic algae, synthesize and accumulate in their cells organic compatible solutes (mono-, disaccharides, glycosylglycerols, sugar alcohols, amino acids, betaines). These compounds have besides their water-binding activity, protective functions for enzymes. In eubacteria the most important compatible solute is glycine betaine. Besides this substance, further new substances have been found, such as ectoine, for which functions as well as biosynthetic pathways have yet to be elucidated.

Key words. Brine microorganisms; saline habitats; halophilic bacteria; compatible solutes; water stress; *Halobacterium*; *Ectothiorhodospira*.

Salt, i.e. sodium chloride, has been in use as an appetizing food additive since very ancient times and by most peoples of the world. Also, in many countries and cultures, salt has been used for thousands of years for preserving meat, fish, raw hides, and – in ancient Egypt – even human bodies[53]. However, this useful and economically very important application has not always been successful; spoilage of salt-preserved foods and hides has been known for a long time, too.

The rise of bacteriology at the beginning of this century brought the explanation for such preservation failures, and the titles of the first bacteriological studies in this field nicely document the particular directions of studies and results: for example Klebahn[29] 'Die Schädlinge des Klippfisches, ein Beitrag zur Kenntnis der salzliebenden Organismen', Harrison and Kennedy[19] 'The red discoloration of cured codfish', Petter[45] 'Over roode en andere bacteriën van gezouten visch', Lochhead[35] 'Bacteriological studies on the red discoloration of salted hides', Gibbons[16] 'Bacteria associated with the reddening of salt fish'.

Spoilage of salted goods was thus found to be frequently accompanied by massive growth of red colored bacteria. As these bacteria were found to require salt for growth they could not have survived in the fish or hides before the salting process. Therefore they must have been associated with the salt itself. It is well known by fishermen, for example in Norway, that salt imported from marine salterns, e.g. those of Portugal or Mediterranean countries, is better suited for salting fish than rock salt, due to its smoothness. On the other hand, salt makers have known

since ancient times that in solar evaporation basins brines turn red, and that this is important for the timing of crystallization sequences of salts from ocean water.

It is therefore surprising that thorough studies of the bacterial flora of such saltworks have only been made very recently. The scanty bacteriological studies of strong brines up to 1925 were summarized briefly by Baas-Becking[2], who also pointed out for the first time the principal difference between brines of concentrated sea water and those of desert lakes, i.e., the influence of the chemical constituents upon the spectrum of organisms to be found in the different kinds of brine. His findings were substantiated by Hof[21]. The first thorough study of a large natural highly saline lake was Volcani's excellent doctoral thesis on the microflora of the Dead Sea[64]. Since then, numerous salt lakes and lake systems have been studied with respect to their microfloras, e.g., Great Salt Lake, Utah[46], again the Dead Sea[40], lakes in the Kulundinskaya Steppe, USSR[26], in the Wadi Natrun, Egypt[23], in the East African Rift Valley[57], in the Great Plains, Canada[33, 41] and in Australia[10].

Many of these natural salt lakes are also used as mineral sources for the chemical industry of the countries where they are situated. The chemical composition of brines depends on the minerals dissolved in the water before evaporation. As seawater has a rather constant composition worldwide, brines in marine salterns and coastal lagoons confirm this uniformity. Ecologists thus speak of 'thalassohaline' environments in contrast to 'athalassohaline' ones that show typically different chemical compositions and are usually landlocked lakes such as 'desert

Table 1. Major ions of highly saline lakes and brines (in g/l); n, not given

	Cl⁻	Br⁻	SO₄²⁻	HCO₃⁻ CO₃²⁻	Na⁺	K⁺	Mg²⁺	Ca²⁺	Total salinity	pH
Great Salt Lake[46] (Utah, USA)	181	0.2	27.0	0.72	105.4	6.7	11.1	0.3	332.5	7.7
Dead Sea[40] (Israel, Jordan)	212.4	5.1	0.5	0.2	39.2	7.3	40.7	16.9	322.6	5.9–6.3
Wadi Natrun, Zugm[23]	154.6	n	22.6	67.2	142.0	2.3	0	0	393.9	11
Wadi Natrun, Gaar[23] (Egypt)	173.7	n	48.0	6.6	137.0	1.4	0	0	374.2	10.9
Hot Lake[6] (Wash., USA)	1.9	n	243.0	3.1	16.8	1.5	53.6	0.7	392	n
Marine saltern[32] (Puerto Rico)	144.0	n	19.0	n	65.4	5.2	20.1	0.2	253.9	n
Basque Lake No. 1[6] (B.C., Canada)	1.7	n	195.0	0.3	13.6	1.6	42.4	n	> 258	n

lakes'. Table 1 gives a few examples of the chemical composition of different highly saline environments. There is a marked difference between alkaline ('soda lakes') and 'neutral' salt lakes with respect to the concentration of carbonate, magnesium and calcium: High concentrations of carbonate lead to magnesium- and calcium-free brines; this has severe consequences for the microflora[58].

The variety of microorganisms found in saline environments is mainly determined by the three parameters salinity, ion composition and pH. Seawater has an average NaCl concentration of 0.5 mol/kg and an osmolality of about 1.0 (corresponding to a water activity of 0.981). A saturated NaCl brine contains about 6 mol/kg (water activity: 0.75). The span between these limits is the environment of halophilic and halotolerant microbes.

Definitions of 'halophilic', 'moderate halophilic' etc. have been changing during the years. A recent and acceptable list of definitions is given in table 2. The decisive difference between moderately halophilic microbes on the one hand, and halophiles sensu stricto plus extreme halophiles on the other hand lies in the typical broad salinity optimum for the growth of the former, as compared with a vital minimal salinity requirement of the latter. This lower limit is 1.5 M NaCl in halophiles sensu stricto and 3.0 M NaCl in extreme halophiles.

The spectrum of species in saline biotopes is – at least for eukaryotes – drastically restricted. In neutral pH chloride lakes the only higher organisms found are the brine shrimp (Artemia salina) and the brine fly (Ephydra sp.).[23] Protozoa have been mentioned in very few cases[15, 23, 47], and algal species in highly saline environments are restricted mainly to red or green colored Dunaliella species[23, 46] and Asteromonas gracilis[4].

Thus the strongly saline environment is primarily a domain of prokaryotes with tightly limited biogeochemical cycles. The primary producers are – besides the algae (if they are present) – a number of halophilic cyanobacteria, e.g. Aphanothece halophytica, Dactylococcopsis salina, Spirulina platensis, Synechococcus and Synechocystis species[37, 49], and anoxygenic anaerobic phototrophic bacteria, e.g. Ectothiorhodospira halophila[48], E. halochloris[24], E. abdelmalekii[25], Rhodospirillum salexigens[11] and R. salinarum[39]. Bacteria involved in the sulfur cycle (sulfate reducers[1] and sulfur oxidizers) and in the nitrogen cycle (nitrate reducers and ammonia oxidizers) have been proven to exist in extreme salinities[23].

With respect to chemoorganoheterotrophic organisms, highly saline environments are conspicuously dominated by red and pink colored bacteria – as mentioned above – that have been shown to belong in the archaebacterial family of the Halobacteriaceae (present genera: Halobacterium, Halococcus, Natronobacterium, Natronococcus[59]). As these bacteria were first isolated from salted fish and hides they were found to exhibit complex growth requirements (amino acids, vitamins, etc.). Consequently, further isolates were obtained from salt lakes and salterns usually with complex growth media. Thus for a long time bacteriologists considered the Halobacteriaceae as typical aerobic chemoorganoheterotrophic, amino acid-degrading bacteria. Only recently has a much greater physiological variety emerged as a result of more thorough and diversified studies[28, 38, 43, 51, 60]. The ecophysiology and the taxonomy of the Halobacteriaceae has been critically reviewed recently[59]. It was shown that the present taxonomical status – based simply on a few classical properties – is absolutely insufficient and misleading. Therefore a rearrangement was proposed – based on detailed chemotaxonomic data[52] – into nine groups or clusters of species[59].

For a long time the terms 'extremely halophilic bacteria', 'halobacteria', and 'Halobacteriaceae' have been used as synonyms. This is wrong because on the one hand there exist quite a number of extremely halophilic and extremely halotolerant eubacteria, too, although they are less conspicuous as they lack the bright red colors of the Halobacteriaceae, and on the other hand not all species of the Halobacteriaceae are extremely halophilic (e.g. Halobacterium volcanii). Many – especially halotolerant – heterotrophic eubacteria have been described and some of them thoroughly studied, e.g. Vibrio costicola[30], Paracoccus halodenitrificans, Micrococcus halobius, Pseudomonas[22, 61], Beneckea[17] and Bacillus[66] species, Pediococcus halophilus[62], Halomonas elongata[65], and the actinomycete Actinopolyspora halophila[18]. Strictly anaerobic heterotrophic eubacteria have also recently been described from extremely saline habitats, e.g. Clostridium lortetii[42], Haloanaerobium praevalens[68] and Halobacteroides halobius[44].

Table 2. Grouping of microorganisms according to salinity requirements. Modified after MacKay et al.[37]

	NaCl concentration	Approximate percentage (%)
Non-halophilic	0 –0.25 M	0 – 1.5
Marine	0.25–1.0 M	1.5– 6
Moderately halophilic	0.5 –2.5 M	3 –15
Halophilic sensu stricto	1.5 –4.0 M	9 –24
Extremely halophilic	3.0 –5.0 M	18 –30

Mechanisms to overcome water stress

The physiological stress which microorganisms in saline habitats have to cope with is – besides the high ionic strength – the low concentration of freely available water. In this respect, halophilic microorganisms are in a similar situation as osmophilic yeasts, e.g., *Saccharomyces rouxii, Debaryomyces hansenii*[27], and xerotolerant fungi like *Xeromyces bisporus* or *Penicillium janczewskii*[20].

The well-known food-preserving effect of highly concentrated sugar or salt solutions is due to the resulting low water activity, which causes dehydration of infecting organisms and thus a standstill of all their physiological functions. Halophilic and halotolerant microorganisms must therefore possess mechanisms to prevent the osmotically caused loss of cytoplasmic water. Obviously, the cytoplasmic membrane is freely permeable for water. Therefore an active control by water-pumping mechanisms seems unlikely. The osmotic balance across the membrane can only be established when the cytoplasmic water activity is kept as low as that of the surrounding medium without damage to the physiological functions of the cell. The critical point is that the functional proteins would be irreversibly denatured when they lose their hydration shell.

Microorganisms have developed two basically different strategies to survive water stress: Either the cytoplasmic membrane allows free entrance of outer osmotically active substances (salts); then the organism has to possess metabolic systems that are not impaired by salts. Or the cytoplasmic membrane allows only slow or limited entrance of outer salts, which trigger the rapid formation of organic materials which are osmotically active to counterbalance outer osmotic pressure. These cell-made osmoregulants have to be compatible with the cellular enzymatic activities and following Brown[7], are therefore called 'compatible solutes'.

In cases where the cytoplasm has to tolerate high salinities similar to those of the medium, complicated struc-

tural changes of the metabolic and regulatory cellular system are necessary. Halobacteriaceae, for which this type of salt adaptation is characteristic, possess proteins with an excess of acid amino acids and a deficiency of non-polar amino acids. In addition, to stabilize their tertiary structures they specifically require monovalent cations[31, 50, 63]; only the potassium ion is suitable, however, because of its smaller hydration shell, whereas the sodium ion is not. Therefore a concentration gradient must be permanently maintained by expensive, energy-consuming ion pumping mechanisms.

A number of characteristic properties found in Halobacteriaceae, and first ascribed to their extremely halophilic nature, have been found to be common to other, non-halophilic archaebacteria, too.

Compatible solutes

In a certain way one could consider the cytoplasmic ions of Halobacteriaceae as compatible solutes, too, because they do not substantially inhibit the enzymatic mechanisms in these bacteria. Generally, however, the term compatible solute describes organic osmotically active substances that do not impede normal, i.e., salt-sensitive enzymes.

With a few possible exceptions found only very recently[18, 42, 68], enzymes of eubacteria exhibit maximal activities at low ionic strength and are more or less inactivated by high salinities. In a few cases, where the salt sensitivity of metabolic enzymes is undisputable, but high cytoplasmic ion concentrations have been described[8, 55, 66], it still has to be substantiated whether enzymes work at suboptimal velocities or perhaps other mechanisms of adaptation are involved.

For the majority of halophilic and halotolerant eubacteria, however, it has been proven that they accumulate non-ionic organic molecules and as far as possible keep salts out of the cytoplasm. Besides their osmotic function,
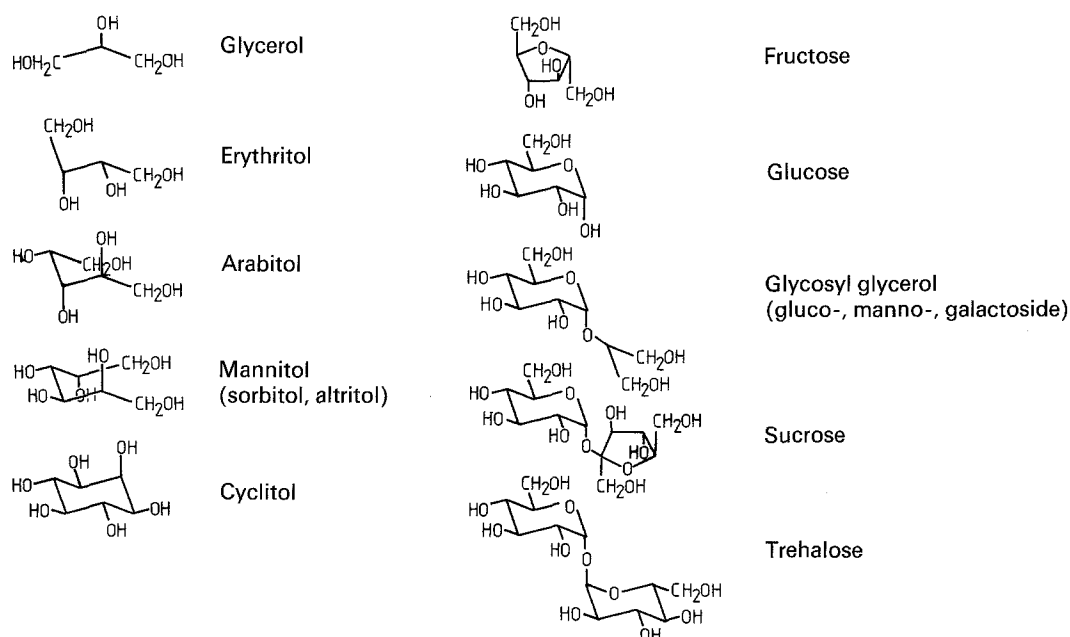

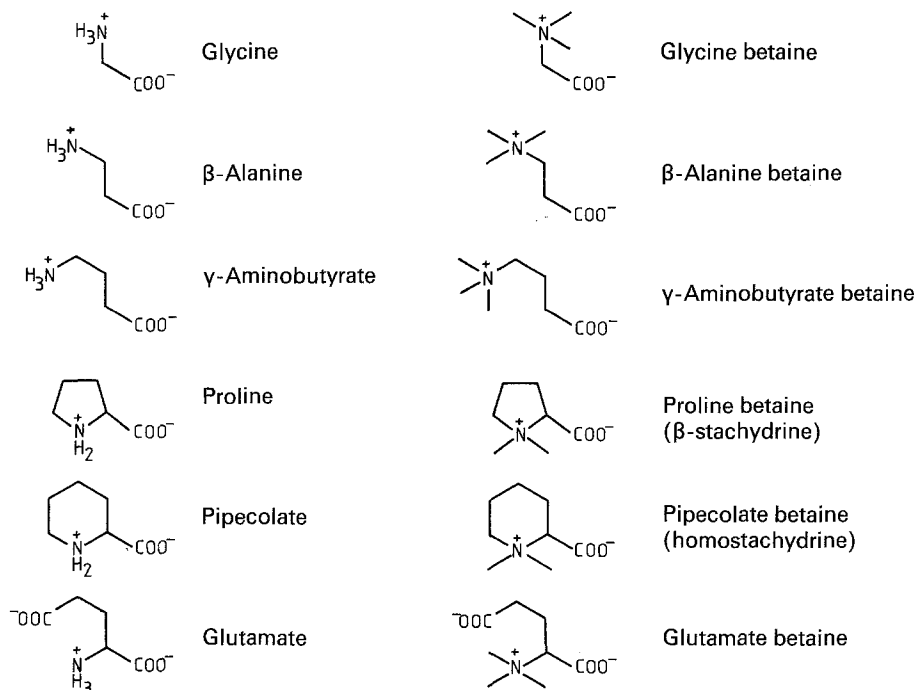Figure 1. Compatible solutes of polyol and sugar type.

Figure 2. Compatible solutes of amino acid and betaine type.

compatible solutes have a protective one in the cytoplasm, as they have to secure hydration and solubility of the enzymatic apparatus.

The compatible solutes known up to now belong into few typical categories of chemical compounds. In the majority of cases they are small, highly water soluble molecules that are uncharged at physiological pH values. The classes of compounds they belong to are polyols and sugars (plus derivatives), amino acids and betaines (figs 1 and 2). Polyols are mainly found in halophilic algae[4], osmophilic yeasts[27] and xerotolerant fungi[20], but appear to be less important in bacteria. Mono- and disaccharides are, however, not restricted to specific groups of organisms; their qualification as protective agents has been questioned[7,49]. The osmoadaptive function of glycosyl glycerol in marine red algae (Rhodophyta)[4], as well as in a number of marine cyanobacteria, has been definitely proven[5,36,37,49].

The suitability of amino acids as compatible solutes is problematic because only few of them are present in their zwitterionic form at physiological pH, and even fewer show the high water solubility required. One of the few suitable candidates among the amino acids is glycine, with a water solubility of 3.3 mol/kg, a value that is markedly surpassed by its β- and γ-homologues (β-alanine: 6.1; γ-aminobutyrate: 12.6 mol/kg). Only the cyclic amino acids proline and pipecolate possess solubility values similar to that of γ-aminobutyrate, whereas the N-methylated amino acids, the betaines, are all highly soluble in water.

As betaines carry a permanent positive charge at the N atom they form zwitter ions over a wide pH range. Although the molecular mechanisms are not yet understood, one can say that the betaines known so far are excellent compatible solutes. The importance of the sulfur analogs of betaines, the thetines (e.g., dimeth-

ylpropiothetine) as osmotically active substances and compatible solutes in algae is at present still disputed[67].

Compatible solutes in extremely halophilic phototrophic eubacteria

Extremely halophilic phototrophic sulfur bacteria have been isolated and newly described in our laboratory[24,25]. The isolation was followed by detailed studies of their mechanisms of haloadaptation.

By the use of ^{13}C-NMR spectroscopy, gas chromatography, and high pressure liquid chromatography we found that the main compatible solute in Ectothiorhodospira halochloris, E. abdelmalekii and E. halophila is glycine betaine, reaching a cytoplasmic concentration of maximal 2.5 mol/kg water. Together with the inorganic ions in the cells the concentration of cytoplasmic solutes is sufficient to counterbalance the high extracellular brine concentration and maintain osmotic equilibrium across the membrane[12,14].

Under certain conditions the extremely halophilic Ectothiorhodospira species form two additional compatible solutes, which were identified as the disaccharide trehalose[12] and as the new, so far unknown cyclic amino acid 1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carbonic acid, to which we gave the trivial name 'ectoine'[13,54]. A comparison of the structure-related properties of ectoine with betaines and amino acids showed that formally it is an amino acid derivative, but on the basis of charge distances and of the charge distribution in the zwitterionic molecule it is structurally comparable to betaines. A new HPLC and gas chromatography system was developed that allowed rapid screening for organic solutes[12]. With this method it was show that a large number of bacterial species belonging to most diverse phylogenetic branches accumulate glycine betaine as a reaction to wa-

ter stress[22]. It may be said that glycine betaine appears to be the most common bacterial compatible solute. Our present scanning program shows that more, so far unknown, compatible solutes are to be expected.

Clearly not all bacterial species that accumulate betaine are also able to synthesize this compound. For the autotrophic cyanobacteria and anoxygenic phototrophic bacteria there is, however, no doubt about their betaine-synthesizing capacity. Many heterotrophic bacteria accumulate betaines from complex medium ingredients[34, 56].

The high content of dissolved organic matter found in Wadi Natrun brines[23] may to a substantial part be due to betaine losses from the massive red blooms of *Ectothiorhodospira halophila* upon dilution stress or other nutritional shortcomings. Glycine betaine has been frequently found in brine and interstitial water of sediments in Solar Lake, Sinai Peninsula (Dr Jaap Boon, Amsterdam, pers. comm.), where abundant growth of cyanobacteria and anoxygenic phototrophic bacteria have been shown to occur[9].

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Life above the boiling point of water?

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Summary. Various extremely thermophilic archaebacteria exhibit optimum growth at above 80 °C. *Pyrodictium* is the most thermophilic of these organisms, growing at temperatures of up to 110 °C and exhibiting optimum growth at about 105 °C. All of these organisms grow by diverse types of anaerobic and aerobic metabolism.
Key words. Archaebacteria; thermophilic bacteria; *Pyrodictium.*

1. Introduction

For a long time, thermophilic bacteria with temperature optima above 45 °C have been recognized to be widely distributed in soils, self-heated hay, and geothermally heated areas. Most of them show an upper temperature limit of growth between 60 and 80 °C and are members of genera also containing mesophiles, such as *Bacillus* and *Clostridium.* About 15 years ago, bacteria living in the hot springs of Yellowstone National Park were observed[3] and the first extremely thermophilic organism with a temperature maximum at 85 °C was isolated[4]. Since that time, various extremely thermophilic bacteria with temperature optima well above 80 °C were obtained which, as a rule, do not grow at 60 °C or below. *Pyrodictium,* the most extreme thermophilic organism existing in pure culture does not even grow at 82 °C or below[17]. Almost all of these organisms (one exception[10]) belong to the methanogenic and S°-metabolizing archaebacteria[23], the properties of which are reviewed here.

2. Habitats

All the extremely thermophilic, methanogenic and S°-dependent archaebacteria isolated have been found in geothermal areas. Sulfur is formed there by the oxidation of H_2S and by the reaction of H_2S with SO_2. Both of these gases are often present in volcanic exhalations[22]. Liquid water is one important requirement for life[3]. The maximum temperatures for liquid water are pressure-dependent, and in deep-sea hydrothermal areas 2500 m below the surface water temperatures may exceed 300 °C[5]. Terrestrial solfataric springs and mud holes exhibit temperatures of up to 100 °C. They include neutral to weakly alkaline (pH 7–9) springs rich in Cl⁻ as well as acidic sulfate-rich water- or mudholes[3,19]. The examination of soil profiles within solfatara fields in Iceland, Italy and the Azores showed that these water-containing soils typically consist of two layers which have quite different properties; there is an oxidized, strongly acidic ochre-colored upper layer of about 15–30 cm in thickness over-