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LIFE IN MAGNESIUM- AND CALCIUM-RICH HYPERSALINE ENVIRONMENTS: SALT STRESS BY CHAOTROPIC IONS

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1. Introduction

Most hypersaline environments on Earth are derived from seawater by evaporation. Seawater is dominated by sodium chloride as the main salt. Sodium constitutes 86 % of the cation sum (0.482 M in "standard" seawater of 35 ‰ salinity), with lower concentrations of Mg^{2+} (0.056 M), K^+ (0.011 M), and Ca^{2+} (0.011 M). When seawater evaporates to form hypersaline brines (so-called thalassohaline brines), the ionic composition of seawater is initially preserved. When the salinity increases, sequential precipitation of calcium carbonate (calcite, at total salt concentrations above 6–8 %) and calcium sulfate (as gypsum, $CaSO_4 \cdot 2H_2O$, that starts precipitating when the total dissolved salt concentration has increased to >120–150 g/l) causes minor changes in the ionic rations. Only during the precipitation of NaCl as halite, when the total salt concentration exceeds 300–350 g/l, do we witness a great change in the ratio between monovalent and divalent cations. The bittern brines that remain after most of the sodium ions have been removed from the water are dominated by magnesium as the main cation.

Most halophilic and halotolerant microorganisms prefer to live in near-neutral brines in which Na^+ and Cl^- are the main ions. Some hypersaline environments are highly alkaline. At high pH, the solubility of the divalent cations Mg^{2+} and Ca^{2+} is greatly limited. The microbiology of such haloalkaline environments is discussed elsewhere in this book (Banciu and Sorokin, 2013; Oren, 2013). Microorganisms living in those environments have to cope with the additional stress by the high pH, which causes problems to the bioenergetics of the cells which have to maintain an internal pH below that of the medium, compensating the reverse pH gradient by a high inside-negative membrane potential. Environments at neutral or slightly acidic pH dominated by divalent cations can be even more stressful to microbial life because of the "chaotropic" – destabilizing – nature of calcium and magnesium ions when present at high concentrations. An excess of such chaotropic ions over "kosmotropic" – stabilizing – ions causes severe stress, this in addition to the osmotic stress inherent to solutions with high solute concentrations.

This chapter first discusses the specific effects of chaotropic and kosmotropic ions on microorganisms, as expressed in the "Hofmeister series" in which the different ions are arranged on the basis of their stabilizing or destabilizing effect. This is followed by an evaluation of the intracellular concentrations of chaotropic ions, a topic about which not much is known yet. Then a number of case studies of the microbiology in high divalent cation concentration environments are discussed: the bittern ponds in solar salterns in which brine is stored from which most of the Na^+ has been precipitated as halite, the Dead Sea, a natural salt lake with extremely high – and still increasing – magnesium and calcium concentrations and ever-decreasing concentrations of sodium so that the ratio between chaotropic and kosmotropic ions is rapidly increasing; the brines of Discovery Basin on the bottom of the Mediterranean Sea that contain 5 M $MgCl_2$ and little else; and the $CaCl_2$ brines of Don Juan Pond in Antarctica, an environment in which the boundaries of life are exceeded although claims of the presence of living microorganisms have been made in the past. Finally, a discussion will be devoted to the recently discovered "chaophilic" fungi, organisms that grow better in chaotropic media with extremely high magnesium ion concentrations than in NaCl-dominated media.

An understanding of the limits of the existence of halophilic microorganisms at high concentrations of chaotropic divalent ions is of great importance for the assessment of the possibility of life on Mars. The recent discovery of seasonal flows of liquid, most likely composed of chlorides of magnesium, sodium, and/or calcium, at different locations on Mars (McEwen et al., 2011), makes the issue of life at high divalent cation concentrations particularly relevant today.

2.   Divalent Cation Concentrations and Halophily: The Hofmeister Series

A major reason why divalent cations such as calcium and magnesium at high concentrations are toxic even to the best salt-adapted microorganisms is their destabilizing action on biological structures (McGenity and Oren, 2012). The relative stabilizing/destabilizing action of different cations on proteins was first documented at the end of the nineteenth century when Hofmeister (1888) showed that different salts have different efficiencies at salting out egg-white protein and that some salts do not cause salting out at all. The phenomenon is not fully understood even today, but the conventional view is that competition between dissolved salt and dissolved protein for water of hydration results in a loss or gain in solubility. The so-called Hofmeister series or lyotropic series reflects the order of effectiveness of ions in precipitating or "salting out" hydrophilic sols. The lyotropic series for the common monovalent cations, from stabilizing to destabilizing, is $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. For inorganic monovalent anions, the order is $Cl^- > NO_3^- > ClO_3^- > I^- > CNS^-$ (Hofmeister, 1888; Brown, 1990). The stabilizing ions are named kosmotropic, the destabilizing ones chaotropic. Chaotropes weaken electrostatic interactions and

destabilize biological macromolecules. Kosmotropes such as compatible solutes strengthen electrostatic interactions. The series for the monovalent ions shows the order of increasing ionic radii and increasing free energy of hydration. Di- and multivalent ions can be fitted into the series but their radii and hydration energies do not usually conform to their place in the series. Calcium and to a lesser extent magnesium behave as chaotropic ions. High concentrations of chaotropic ions can be compensated to some extent by stabilizing kosmotropic ions (Hallsworth et al., 2003).

A recent reevaluation showed the Hofmeister series to be a sequence in water-ordering power of ions. Ions in solution may disturb the characteristic tetrahedral structure of water. To compare the effects of applied pressure and high salt concentrations on the hydrogen-bonded network of water, neutron diffraction was used, a technique that gives a microscopic measure of the Hofmeister effect. Ions induce a change in water structure equivalent to the application of high pressures, the size of the effect being ion-specific. These changes may be understood in terms of the partial molar volume of the ions relative to those of water molecules (Leberman and Soper, 1995; Parsegian, 1995). The equivalent induced pressure of a particular ion species is correlated with its efficacy in precipitating, or salting out, proteins from solution. But the factors that determine whether an ion stabilizes or destabilizes a molecular surface are still incompletely understood (Parsegian, 1995).

Early investigations on the interactions between chaotropic (destabilizing) and kosmotropic (stabilizing) ions determining growth of halophilic microorganisms were performed already in the 1930s when Lourens Baas Becking devoted extensive studies to the physiology of the unicellular flagellated green algae *Dunaliella viridis* and *D. salina* (Baas Becking, 1930, 1934; Oren, 2011). Much of the behavior of *Dunaliella* and other halophilic microorganisms in their natural habitats was explained by Baas Becking on the basis of antagonism between the different ions in the brines. Calcium proved particularly toxic, especially in slightly acidic media, but magnesium could relieve the toxicity of calcium to some extent. The higher the NaCl concentrations, the more magnesium was required to detoxify calcium to enable growth of *Dunaliella*. For example, in 1 M NaCl, the antagonistic relation Mg:Ca is 4:5, while in 4 M NaCl the proportion becomes many times as great (20:1). The red Archaea of the family *Halobacteriaceae* were found to require high salinity, pH, and a certain amount of magnesium, but they did not develop in cultures containing high calcium concentrations. Cyanobacteria were found to grow best in lower NaCl and low Mg:Ca ratios. The summary statement by Baas Becking (1931) was: "Thus, during the evaporation of sea water, we may predict the environment will become suitable, in succession for: *blue-green algae and fungus → Dunaliella viridis*, first swarmers and zygotes, then palmella → *D. salina → red bacteria*."

Another set of observations by Baas Becking related to the influence of chaotropic anions on the viability of *Dunaliella*. *Dunaliella* proved resistant to very high concentrations of toxic anions such as chromate, cyanide, and

thiocyanate: "Anions have little or no influence on *Dunaliella*. It is intriguing to watch this alga swimming for hours in concentrations of cyanide, thiocyanate, or chromate" (Baas-Becking, 1931). The true physiological basis for the survival of *Dunaliella* in molar concentrations of chromate, cyanide, and other unusual anions is yet to be elucidated, but Baas Becking's explanation for the phenomenon is an interesting one. He attributed the phenomenon to the presence of a strongly negative charge of the cell membrane and the flagella, which can be neutralized by cations in a lyotropic series, rendering the membrane impermeable to anions and selectively permeable to cations.

A study of the ionic composition of the media supporting growth of different strains of halophilic Archaea, comparing *Halobacterium salinarum* which prefers monovalent cations with *Haloferax volcanii*, an isolate from the divalent-rich Dead Sea, was published by Edgerton and Brimblecombe (1981). *Hbt. salinarum* is a true extreme halophile that grows optimally at water activities of 0.78–0.79, values close to the water activity of NaCl-saturated brines ($a_w \sim 0.75$; Grant, 2004). *Hfx. volcanii* should be termed moderately halophilic, with best growth at $a_w = 0.925$. Calculations of the activities of the individual ions in such media, using the Pitzer equations (Pitzer, 1973; Pitzer and Kim, 1974), showed that in brines of increasing salinity, activity coefficients often first decrease, then increase with concentration. Sulfate has a low activity coefficient (<0.1), even at moderate ionic strengths (Edgerton and Brimblecombe, 1981).

There are, however, also specific stabilizing effects of divalent cations on the cell envelope of halophilic Archaea. Cells of many pleomorphic types such as *Haloferax* and *Haloarcula* species become spherical when suspended in media devoid of divalent cations. Differences in Mg^{2+} requirements of halophilic Archaea are likely to be reflected in peculiarities of protein chemistry of the cell envelope (Cohen et al., 1983).

3. Intracellular Concentrations of Divalent Cations in Halophilic Microorganisms

There are only very few data on the intracellular concentrations of divalent cations within microorganisms grown in media rich in magnesium or calcium. Such measurements are not straightforward as the calculations depend on very exact estimates of the volumes of intracellular and extracellular water in cell pellets. Presence of a periplasmic volume complicates the calculations. Moreover, it is necessary to discriminate between free ions and ions bound to cellular structures – DNA, RNA, and proteins. Only few attempts have therefore been made to estimate the intracellular Mg^{2+} and Ca^{2+} concentrations in halophilic microorganisms.

In a comparative study, intracellular concentrations of different ions were measured in the non-halophilic *Escherichia coli*, the moderately halophilic bacterium *Salinivibrio costicola*, the moderately halophilic, high divalent cation-requiring archaeon *Haloferax volcanii*, and the extremely halophilic archaeon *Halobacterium*

salinarum (*cutirubrum*). Intracellular magnesium concentrations in *E. coli*, *S. costicola*, and *Hbt. salinarum* were estimated at 29.7, 39.8, and 102.4 mmol/kg of cell water, respectively (de Médicis et al., 1986). How much of this magnesium is free in solution and how much is bound to cellular structures is unknown. Unfortunately, no data were presented on the magnesium concentrations measured inside *Hfx. volcanii*, possibly due to the analytical problems connected with the high magnesium concentrations in the growth medium.

Attempts to estimate the intracellular magnesium and calcium concentrations in *Halorubrum sodomense*, a halophilic archaeon with an unusually high magnesium requirement and magnesium tolerance, isolated from the Dead Sea, showed apparent intracellular Mg concentrations to be lower than those in the outside medium, but still the estimated values were surprisingly high (Table 1). It must be remembered that part of the "intracellular" magnesium and calcium may be bound to the cell wall, membranes, and macromolecules in the cytoplasm. Artifacts are possible as well due to the difficulty to estimate lower intracellular concentrations in the presence of high concentrations in the surrounding medium, the uncertainty in the determination of the exact contribution of intracellular volume within cell pellets, etc.

Little is known about the mechanism of regulation of intracellular Mg^{2+} concentrations in halophilic Archaea. In *Halobacterium salinarum*, an outward calcium transport mechanism was identified mediated by a Na^+/Ca^{2+} antiporter (Belliveau and Lanyi, 1978).

4. Case Studies

4.1. SOLAR SALTERNS

The brines in the evaporation ponds of solar saltern brines up to the stage of halite crystallization are typical thalassohaline solutions, dominated by NaCl. However, during the precipitation of halite in the crystallizer ponds, the relative concentrations of Mg^{2+} and Ca^{2+} increase sharply, so that organisms growing in the salt-saturated ponds need to be able to tolerate high divalent cation concentrations as well, in addition to the general salt tolerance required (Javor, 1989; Oren, 2002). Few systematic studies have been performed on the upper limit of divalent ion tolerance in halophilic Archaea and other organisms that make up the biota of saltern crystallizer ponds. Javor (1984) described a strain GNM-3 from the Guerrero Negro, Mexico, salterns, with an unusually high magnesium requirement and tolerance. This red-pink, motile strain containing gas vesicles and bacteriorhodopsin, probably belonging to the genus *Halobacterium*, grows optimally in the presence of 1.2 M $MgCl_2$ in the presence of 3 M NaCl.

A prominent member of the biota of saltern crystallizer ponds worldwide is the flat square archaeon *Haloquadratum walsbyi*. Strain C23, the type strain of *Hqr. walsbyi*, had no specific optimum for $MgCl_2$, but concentrations above 1 M

Table 1. Apparent intracellular cation concentrations of *Halorubrum sodomense* ATCC 33755^T, grown at different concentrations of magnesium and calcium.

	Na^+_{out}	Na^+_{in}	K^+_{out}	K^+_{in}	Mg^{2+}_{out}	Mg^{2+}_{in}	Ca^{2+}_{out}	Ca^+_{in}	$Total_{in}$	$Total_{out}$
High magnesium, low calcium medium	2.14	0.66	0.01	3.10	0.95	0.33	0.05	0.035	3.15	4.13
Intermediate magnesium, intermediate calcium medium	2.14	1.00	0.01	3.14	0.95	0.42	0.25	0.19	3.15	4.75
Low magnesium, high calcium medium	2.14	0.52	0.01	3.47	0.50	0.18	0.50	0.19	3.15	4.36

Modified from Oren (1986a).
Concentrations are given in molar units. The general outline of the experiments, estimation of intracellular and extracellular volumes in cell pellets, etc. were as given by Oren (1986b). Na$^+$ and K$^+$ were assessed by flame photometry. Intracellular Ca^{2+} concentrations were estimated by measuring the distribution of ^{45}Ca in cell pellets and culture supernatants of cells grown in medium supplemented with ^{45}CaCl$_2$. Magnesium was estimated colorimetrically using a modification of the procedure described by Taras (1948).

yielded higher cell densities than lower concentrations, while growth peaked at 0.4–0.6 M MgSO$_4$. Strain HBSQ001 grew optimally at 0.2 M MgCl$_2$ but required 0.6 M MgSO$_4$ to reach the same density. In both strains, growth declined markedly at high MgCl$_2$ concentrations compared to MgSO$_4$, suggesting that the high chloride ion concentration (>5 M) may have been inhibitory (Burns et al., 2007). In spite of the presence of energy-demanding cation efflux systems, the high external magnesium concentrations may lead to an increase in the internal magnesium concentration that is higher than in other organisms. Bolhuis et al. (2006), who analyzed the genome of *Hqr. walsbyi*, argued that magnesium ions have a stabilizing effect on the DNA duplex, the secondary structure of RNA, and DNA-RNA heteroduplexes. In case of an already stable high-GC genome, as present in most members of the *Halobacteriaceae*, the additional stabilizing effect of magnesium might therefore result in DNA rigidity that may interfere with essential processes like DNA replication and transcription. *Hqr. walsbyi* is a genome with an unusually low G+C content (47.9 mol %). It was proposed that the drift to an AT-rich genome might be induced as a long-term evolutionary adaptation to this over-stabilization by magnesium.

Divalent cations dominate in the bittern brines that remain after most of the common salt has precipitated. Such bittern ponds are often considered to be devoid of life. No bacteria or archaea grew in the enrichment cultures or on agar plates inoculated with bittern brines (Javor, 1983, 1984). Still the possibility exists that viable cells remain in the bitterns. In a culture-independent molecular (16S rRNA sequence-based) community analysis of magnesium-rich bittern brine from a Tunisian saltern at 380–400 g/l total salt concentration, viability tests based on the staining behavior with acridine orange suggested 46 % of the cells to be viable but not detectable by culturability tests (Baati et al., 2011). This brine contained only 2.7 g/l Na but had 96.7 g/l Mg and 256.9 g/l chloride, had a measured water activity of 0.68, and harbored 1.4×10^7 prokaryotes/ml (by microscopic count). It remains to be ascertained to what extent the staining procedure employed can really be used to reliable information on the presence of viable cells. Unfortunately, the authors did not employ the LIVE/DEAD BacLight strain adapted for use in hypersaline environments by Leuko et al. (2004). Sequences retrieved by Baati et al. (2011) include *Salinibacter*-related ones (*Bacteroidetes*), *Haloquadratum* (58 % of the archaeal sequences), and *Halorubrum* spp. The bitterns of the La Trinitat saltern in Spain yielded many isolates of yeasts, showing that there also are eukaryotic microorganisms adapted to low water activity and high divalent ion concentrations (Butinar et al., 2005; see also Sect. 5).

4.2.   THE DEAD SEA

The Dead Sea is a unique hypersaline lake that is not only dominated by divalent cations but in which the concentrations of magnesium and calcium are constantly

Table 2. Dead Sea ionic composition, salt concentrations.

Year	1959–1960		1977	1996	2007
	Upper water mass	Lower water mass			
Na^+ (M)	1.57	1.84	1.73	1.59	1.54
K^+ (M)	0.17	0.19	0.18	0.20	0.21
Mg^{2+} (M)	1.49	1.75	1.81	1.89	1.98
Ca^{2+} (M)	0.41	0.43	0.43	0.44	0.47
Cl^- (M)	5.56	6.18	6.34	6.34	6.48
Br^- (M)	0.06	0.07	0.07	0.07	0.08
SO_4^{2-} (M)	NR	NR	0.005	0.005	0.005
$(Na^++K^+)/(Mg^{2+}+Ca^{2+})$[a]	0.92	0.93	0.85	0.77	0.71
Total dissolved salts (g/l)	298	335	339	339	347

Data were derived from Beyth (1980) and unpublished data (I. Gavrieli, the Geological Survey of Israel, personal communication).

NR not reported.

[a]Molar ratio.

increasing: since the early 1980s, the water column is saturated with respect to NaCl, and massive amounts of halite precipitate to the bottom, increasing the salt layer by about 10 cm per year. As a result, the ratio between monovalent and divalent cations has sharply decreased in the past decades (Table 2). Activity coefficients of the major ions, calculated by Krumgalz and Millero (1982) for the Dead Sea for 1979, as based on the equations of Pitzer (1973), were Na^+, 0.961; K^+, 0.548; Mg^{2+}, 2.276; Ca^{2+}, 1.034; and Cl^-, 3.37; Edgerton and Briblecombe (1981) gave estimated activity coefficients of 0.821, 0.527, 1.072, 0.579, and 2.298, respectively.

A variety of microorganisms have been isolated from the Dead Sea over the years; these include Archaea of the family *Halobacteriaceae*, bacteria, unicellular algae, and fungi, and there also is evidence for the presence of different types of viruses. With respect to their adaptation of life at high salt concentrations and to the specific conditions prevailing in the divalent cation-dominated brines, most research effort has been devoted to the halophilic Archaea. Species isolated from the lake and described include *Haloferax volcanii* (Mullakhanbhai and Larsen, 1975), *Haloarcula marismortui*, *Halorubrum sodomense* (Oren, 1983), and *Halobaculum gomorrense* (Oren et al., 1995). *Hfx. volcanii* is less salt demanding than most other representatives of the *Halobacteriaceae* but is markedly tolerant toward the presence of high magnesium concentrations, some growth still being possible at 2 M Na^+ and 1.4 M Mg^{2+} (Mullakhanbhai and Larsen, 1975). The optimal medium for growth of *Hrr. sodomense* contains 0.8 M $MgCl_2$ + 12.5 % NaCl. In the presence of 2.1 M NaCl, 0.6–1.2 M Mg^{2+} is needed for optimal growth. When grown at lower magnesium concentrations, growth rates were reduced and cells became irregular spheres. Calcium can at least partially replace

magnesium, and good growth was found in medium containing 1 M $CaCl_2$, 15 mM $MgCl_2$, and 2.1 M NaCl. Relatively good growth was even observed with as little as 0.5 M NaCl when the $MgCl_2$ was raised to 1.5 or 2.0 M (Oren, 1983). *Hbc. gomorrense* is no less magnesium tolerant, with optimal growth at 0.6–1 M Mg^{2+} in the presence of 2.1 M NaCl. At least 1 M Na^+ was needed in the presence of 0.8 M $MgCl_2$, and media with less than 0.2 M Mg^{2+} supported poor or no growth (Oren et al., 1995).

Based on this information, it is clear that the salt concentrations in the Dead Sea with its current >2 M Mg^{2+} and about 1.5 M Na^+ are far above the optimum even for the best salt-adapted and chaotropic ion-adapted Archaea. Also species of the unicellular green algal genus *Dunaliella*, the sole primary producer in the Dead Sea, cannot grow under these conditions. Therefore, the development of massive blooms of microorganisms in the lake is currently possible only following exceptionally rainy winters when the upper meters of the water column become diluted by fresh water and the salinity drops significantly. Such blooming events were witnessed in 1980 and in 1992, when up to 2×10^7 and 3.5×10^7 red halophilic Archaea were found per ml in the surface layers (5–10 m, the maximum depth being about 300 m). In other periods when no density stratification existed, some viable Archaea remained still present, but their metabolic activity was minimal. Thus, the uptake rates of glycerol and amino acids in 1991 (following a number of dry years) were in the order of 0.02–0.05 nmol/l h, as compared to 10–40 nmol/l h during the bloom in the summer of 1980 (Oren, 1992). A metagenomic study of the Dead Sea microbial community in 2007, more than 10 years after the termination of the 1992–1995 bloom, showed the presence of a small but highly diverse community of Archaea in the surface waters, phylogenetically affiliated with genera such as *Halorhabdus*, *Halosimplex*, *Halomicrobium*, *Halogeometricum*, *Haloplanus*, *Natronomonas*, and *Halalkalicoccus* with no closely related cultured representatives. This in contrast to the metagenome of the 1992 bloom material which was essentially composed of a single lineage remotely affiliated with the genus *Halobacterium* on the basis of 16S rRNA sequences, but showing an entirely different polar lipid pattern (Bodaker et al., 2009, 2010).

The metagenome of the 1992 community showed enrichment in COGs (Clusters of Orthologous Groups) of Mg^{2+} and Co^{2+} channels. Enrichment in putative archaeal CorA magnesium channels in the 1992 library (up to 11-fold for COG 0598) points to a potential, yet unknown, resistance mechanism used by the Dead Sea halophiles. Although CorA is usually associated with Mg^{2+} influx activity, when exposed to high extracellular Mg^{2+} concentrations, CorA can mediate Mg^{2+} efflux (Gibson et al., 1991; Papp-Wallace and Maguire, 2008).

The magnesium concentration in the Dead Sea now rapidly approaches the value of 2.3 M, considered as the upper limit for life when there is no significant concentration of a kosmotropic ion to offset the inhibition by chaotropic ions (the limit determined by Hallsworth et al., 2007; see Sect. 4.3). Thus, when the current trend of decreasing water levels and halite precipitation will continue,

conditions may soon become too extreme for life of even those microorganisms best adapted to the adverse conditions presented by the Dead Sea brine.

4.3.   THE LIMITS OF LIFE AT HIGH MAGNESIUM CHLORIDE: STUDIES ON DISCOVERY BRINE

A natural environment with magnesium chloride concentrations far higher than those presently encountered in the Dead Sea is Discovery Basin, located at a depth of 3.58 km below the surface of the Mediterranean Sea, 200 km off the western coast of Crete. This deep-sea brine pool originated about 2,000 years ago by dissolution of bischofite ($MgCl_2.6H_2O$) that had formed during the desiccation of the Mediterranean Sea around 5.5 million years ago. The existence of a large-scale bischofite formation deposited during the Messinian salinity crisis demonstrates that the eastern Mediterranean had become evaporated to near dryness during that time. The undersea brine pool was discovered in December 1993–January 1994. It has a surface area of about 7.5 km^2, a temperature of 35–38 °C, and a total dissolved salts concentration of about 470 g/l, and it contains almost pure magnesium chloride (concentrations in mol/kg H_2O: Mg^{2+}, 5.15; Cl^-, 10.15; Na^+, 0.084; K^+, 0.090; Ca^{2+}, 0.001; SO_4^{2-}, 0.110; Br^-, 0.110) (Wallmann et al., 1997, 2002).

The existence of this magnesium chloride brine and the salinity gradient formed at the interface between the Mediterranean Sea water and the concentrated brine provided a unique opportunity to explore the limits of life at increasing concentrations of a chaotropic salt, not compensated by significant concentrations of stabilizing kosmotropic cations. The first microbiological exploration of Discovery brine suggested the presence of a significant microbial community down to the bottom of the brine pool. At the interface, the microscopic counts (DAPI stain) were 6.1×10^4 cells/ml, decreasing to 1.9×10^4/ml at the bottom. Analysis of clone libraries and enzymatic assays provided evidence for the occurrence of dissimilatory sulfate reduction, methanogenesis, as well as heterotrophic activity (glutamate uptake) (van der Wielen et al., 2005). 16S rRNA gene clone libraries prepared from different depths in the brine along the salt gradient showed a high abundance of sequences affiliated with the genus *Halorhabdus* (*Halobacteriaceae*), a genus that contains at least one anaerobic representative, *Hrd. tiamatea*, which was isolated from a deep-sea brine in the Red Sea (Antunes et al., 2008, 2011). *Halorhabdus* clones constituted 11 % of the interface clone libraries and 33 % of the clone libraries from the brine itself (van der Wielen et al., 2005). Further analyses of gene libraries prepared from DNA isolated from the brines uncovered *cbbL* and *cbbM* genes coding for the ribulose-1,5-bisphosphate carboxylase/oxygenase both in the brine and in the brine/seawater interface but not in the overlying seawater. Diversity of these genes was low. The *cbbL* sequences were remotely affiliated with a *Thiobacillus* or with one of the RuBisCo genes of

Hydrogenovibrio marinus. The *cbbM* genes clustered with thiobacilli and formed a new group. This was presented as evidence for the existence of a potential for autotrophic CO_2 fixation in the 5 M $MgCl_2$ brines. However, attempts to amplify genes for ammonia monooxygenase, methane monooxygenase, and diheme cytochrome *c* (involved in bacterial thiosulfate oxidation) remained unsuccessful (van der Wielen, 2006).

Still, viable microorganisms could not be recovered from the brines below the interface, with the possible exception of *Bacillus* and relatives that may have survived in situ as endospores. These cannot be subcultured on media containing molar concentrations of $MgCl_2$ (Sass et al., 2008; Antunes et al., 2011). Cells of different species of non-halophiles and slight halophiles (*Alteromonas marina*, *Bacillus firmus*) survive in Discovery Basin brine for no longer than a few hours (Borin et al., 2008).

It is now clear that no active cells are present in the 5 M $MgCl_2$ brines. It was calculated that 5 M $MgCl_2$ would have a chaotropic effect of 212 kJ/g, more than twice that of a saturated phenol solution. And the Discovery brine has an a_w of <0.4, far below the value of ~0.6 considered as the lower limit to support life. The highest permissible $MgCl_2$ concentration in culture media with growth scored after 18 months of cultivation was 1.26 M, equivalent to a water activity (a_w) of 0.916. The fact that 16S rRNA genes and genes for different metabolic functions (*dsrAB* of sulfate reducers, *mcrA* of methanogens) could be amplified from the most saline layers is due to the fact that DNA is preserved well in the concentrated magnesium chloride solutions. When, however, a search was made for specific mRNA molecules, which are much more labile than DNA, it became clear that active cells were present only up to about 2.3 M $MgCl_2$. Messenger RNA for *drsAB* was found up to 1.88 M, *mcrA* mRNA up to 2.23 M, concentrations equivalent to a_w 0.801 and 0.845, respectively. mRNA of *dsrAB* of *Desulfohalobiaceae* (a family of halophilic sulfate reducers) was restricted to the zone of 1.60–2.23 M $MgCl_2$. The upper limit of $MgCl_2$ in the absence of compensating stabilizing ions appears to be about 2.3 M (Hallsworth et al., 2007). The rapid degradation of mRNA and the stability of bacterial DNA in the high-magnesium brines were confirmed in a simulation experiment in which cells of a *Marinobacter* isolated from the seawater/Discovery brine interphase were incubated in the 5 M $MgCl_2$ brine. DNA was only moderately degraded after 45 days and both 16S rRNA and *gyrB* gene sequences could be amplified. *gyrB* mRNA could be recovered only in the first hour of exposure of the cells to the brine. In the absence of compensating kosmotropic ions, a concentration of 2.3 M $MgCl_2$ appears to be the upper limit for life (Hallsworth et al., 2007). DNA preserved in such strong brines could constitute a genetic reservoir of traits acquirable by horizontal gene transfer (Borin et al., 2008). Despite the presence of energy-yielding redox couplings in the $MgCl_2$-rich chemocline (sulfate reduction, methanogenesis), the macromolecule-destabilizing effects of $MgCl_2$ above a concentration of 2.3 M must be considered to be incompatible with life (McGenity and Oren, 2012).

4.4.   THE MAGNESIUM SULFATE BRINES OF HOT LAKE, WASHINGTON

An intriguing but poorly investigated magnesium sulfate-dominated environment is Hot Lake, Washington. This is a stratified terminal lake with a salinity gradient from about 100 g/l salts in the surface waters to 400 g/l salts at the bottom. Bottom brine contained (g/l) Mg^{2+}, 53.6; SO_4^{2-}, 243; Na^+, 16.8; Cl^-, 1.9; K^+, 1.5; Ca^{2+}, 0.7; and HCO_3^-/CO_3^{2-}, 3.1 (Brock, 1979; Trüper and Galinski, 1986). The pH ranged from 8.7 at the surface to 7.0 at the bottom. Heliothermal heating caused the waters at 1.5–2.2 m depth to heat up to 40 °C. Oxygen was depleted at depths below 1.5 m.

Organisms detected in the lake include the green alga *Chara* and different types of cyanobacteria found at depths below 1 m (*Plectonema*, *Oscillatoria*, *Anacystis*, *Gomphosphaeria*), and a dense population of *Chlorobium*-like green sulfur bacteria was recorded in the upper part of the monimolimnion, slightly below 2 m depth at >200 g/l total salts. Higher organisms were found as well: the brine shrimp *Artemia salina* and the rotifer *Brachionus angularis* (Anderson, 1958). This intriguing ecosystem deserves a more in-depth study, also with respect to the adaptation of the organisms present to life at high magnesium concentrations.

4.5.   THE CONCENTRATED CALCIUM CHLORIDE BRINES OF DON JUAN POND, ANTARCTICA: THE SUPREME CHALLENGE TO LIFE

Since it was discovered in 1961, Don Juan Pond, located in the Wright Valley, Victoria Land, Antarctica, has intrigued biologists searching for the most extreme environments on Earth supporting life. Don Juan Pond is an unfrozen shallow (average depth 11 cm) lake about 200×700 m in size. During the summer, two small streams drain the moraine to the west. The temperature of the water reaches −24 °C to −3 °C in October–December. The water is a concentrated calcium chloride brine with up to 474 g/l total dissolved salts. The freezing point of the brine was determined at about −48 °C, and the pH was around 5.4 (Meyer et al., 1962). The ionic concentrations vary during the seasons: in December 1968, the water density was 1.386 g/ml, and it contained 137.1 g/kg Ca^{2+} and 251 g/kg Cl^-; in July 1974, a density of 1.208 g/ml was measured, with 74.1 g/kg Ca^{2+} and 148 g/kg Cl^-. Siegel et al. (1979) reported even higher Ca^{2+} and Cl^- concentrations. Concentrations of Na^+, K^+, and Mg^{2+} are very low (Matsubaya et al., 1979).

Early studies suggested that living microorganisms may be present in Don Juan Pond. Meyer et al. (1962) reported bacterial rods and cocci growing in colonies. Different bacteria (*Bacillus megaterium*, *Micrococcus* sp., *Corynebacterium*

sp.) and a yeast (*Sporobolomyces* sp.) were isolated from the brine. During a field study in the Austral summer of 1978–1979, a 3–5 mm thick microbial mat was observed, extending 500–600 m^2 over much of the western part of Don Juan Pond. It contained *Oscillatoria*-like filaments, *Chlorella*-like cells, *Dunaliella*-like flagellates, as well as nonmotile colorless and red bacteria. The mat contained 327,000 ppm dissolved solid material, compared to 485,000 ppm in the brine of pond. Chlorophyll *a* and other photosynthetic pigments were also detected, and photosynthetic activity (oxygen evolution) could be measured upon incubation of mat material at +4 °C (Siegel et al., 1979).

   In spite of these old observations, there is no conclusive evidence of microbes to grow in $CaCl_2$-dominated Don Juan Pond, presumably because concentrated $CaCl_2$ brines, in addition to the extremely low a_w, destabilize biological macromolecules just as $MgCl_2$. A $CaCl_2$ brine of >470 g/l has a water activity below 0.45 (Grant, 2004; Horowitz et al., 1972; Oren, 1993; Siegel et al., 1979). The recently reported N_2O evolution from the pond is due to abiotic processes and not, e.g., by bacterial dissimilatory reduction of oxidized nitrogen compounds (Samarkin et al., 2010). Biological activity is possible on the shore of the lake, where salinities are reduced and active algal mats containing *Oscillatoria*-like filaments, unicellular cyanobacteria, diatoms, fungi, and other organisms can develop (Siegel et al., 1979, 1983).

5. Do "Chaophilic" Microorganisms Exist?

The organisms able to grow at the lowest water activities are not prokaryotes but fungi. Some fungi such as *Xeromyces bisporus* can grow at a_w 0.61 (Grant, 2004; Hocking and Pitt, 1999). Surprisingly, some fungi grow optimally under chaotropic conditions. Glycerol at high concentrations is chaotropic, and it disrupts and permeabilizes biological membranes. However, a strain of *Xeromyces bisporus* was recently described that grew fastest in a highly chaotropic condition containing 6.84 M glycerol ($a_w = 0.714$) and could even grow in 7.60 M glycerol ($a_w = 0.653$) in the absence of any compensating kosmotropic compounds. It apparently has a preference for conditions that disorder macromolecular and membrane structures. Thus, we may have here a representative of a previously uncharacterized class of extremophiles: "chaotolerant" or even "chaophilic" microbes (Williams and Hallsworth, 2009).

   More such "chaotolerant" fungi were recovered from the $MgCl_2$-rich bittern ponds of the coastal salterns of Sečovlje, on the border between Slovenia and Croatia. Isolates of *Cladosporium*, other filamentous fungi, yeasts, and black yeasts could grow on media with up to 1.5 M $MgCl_2$ and higher without a kosmotropic salt such as NaCl to compensate the chaotropic effect of the medium (Sonjak et al., 2010).

6.   References

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