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# Microbial communities in salt lakes: Phylogenetic diversity, metabolic diversity, and in situ activities

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# Microbial Communities in Salt Lakes: Phylogenetic Diversity, Metabolic Diversity, and *In Situ* Activities

## Summary of a Roundtable Discussion on our Current Understanding, Limitations to our Knowledge, and Future Approaches, Salt Lake City, 12 May 2008

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**ABSTRACT**

A roundtable discussion was held on Monday, 12 May 2008, following the sessions on 'Microorganisms in Hypersaline Environments' at the 10th Conference on Salt Lake Research & 2008 FRIENDS of Great Salt Lake Issues Forum, University of Utah, Salt Lake City. Among the aspects discussed were: 1. The gap between our knowledge of the microorganisms isolated in culture and the true microbial diversity as apparent when using culture-independent techniques, in most cases based on the characterization of small subunit ribosomal RNA genes; 2. The metabolic diversity of the microorganisms inhabiting saline and hypersaline lakes and the lack of information to what extent the metabolic potential of the microbiota as apparent from culture studies or detection of functional genes is realized in the environment; 3. The limited understanding of the diversity of algae, bacteriophages and protozoa in hypersaline lakes and their relative importance of such microbial predators and grazing animals on the regulation of the microbial community sizes in such lakes; 4. The impact of high throughput "-omics" technologies for assessing the diversity and metabolism of hypersaline environments. In recent years a number of comprehensive studies were performed in selected hypersaline environments by large interdisciplinary teams of scientists. Such studies contribute invaluable information to define the nature and function of the microbial communities in such environments. However, the inability to independently grow specific organisms compared to the genetic diversity revealed by non-cultivation techniques indicates that additional work is needed to develop and define *in vitro* cultivation conditions. More of such studies are needed, with the appropriate funding, to solve the basic questions relating to the importance of microorganisms in saline lakes and other hypersaline ecosystems.

**INTRODUCTION**

During the 10th Conference on Salt Lake Research & 2008 FRIENDS of Great Salt Lake Issues Forum, held at the University of Utah, Salt Lake City, a session of eight talks, accompanied by a number of posters, addressed the microbial diversity of saline and hypersaline lakes and other hypersaline ecosystems. Among the environments discussed were Great Salt Lake (Utah), the Salton Sea (California), the Dead Sea (on the border between Israel and Jordan), Tuz Lake (Turkey), Lonar Lake (India), solar salterns, and Permian halite deposits. Following the oral presentations on Monday, 12 May, a roundtable session was held, in which different aspects relating to the diversity and activities of microorganisms in hypersaline environments were discussed, attempting to place the information presented in the individual talks in a more general context. Below we present a summary of the main items discussed and the conclusions reached during this roundtable session.

### What Microorganisms are the Main Players in Salt Lakes and Other Hypersaline Environments? Culture-Dependent Versus Culture-Independent Approaches

It is surprising at what high rate new species of halophilic and halotolerant bacteria are being described in recent years, not only in absolute numbers (Oren 2008) but also relative to numbers of novel non-halophiles. Out of the 528, 593, and 631 names of species of prokaryotes (Archaea and Bacteria combined) that were validly published in 2005, 2006, and 2007 according to the rules of the International Code of Nomenclature of Prokaryotes (numbers derived from www.bacterio.cict.fr), at least 37, 39 and 74 refer to organisms that have their optimal growth above seawater salinity; thus, over 8.5% of all newly named prokaryotes are halophiles according to this definition. Hence, hypersaline environments and halophilic microorganisms are becoming increasingly popular research targets, especially in Asia.

The number of completed or nearly completed genome sequences of halophiles is also rapidly rising, which provides new information about the genetic diversity and metabolic potential never realized before. The first halophilic archaeal genome to be sequenced was *Halobacterium* strain NRC-1 (Ng et al. 2000), followed by *Haloarcula marismortui* and *Haloferax volcanii* from the Dead Sea, *Halorubrum lacusprofundi* from Deep Lake, Antarctica, the flat square *Haloquadratum walsbyi* from Spanish salterns (Bolhuis et al. 2006), and the alkaliphilic *Natronomonas pharaonis* from the soda lakes of Wadi Natrun, Egypt. To date, genomes of 17 isolates of *Halobacteriales* are completely closed and are ready for use in identifying untold diversity in hypersaline environments (Liolios et al. 2008; www.genomesonline.org). In the domain Bacteria the genome sequence information on the extremely halophilic red *Salinibacter ruber* was published (Mongodin et al. 2005), and the same is true for the moderately halophilic *Chromohalobacter salexigens*, also isolated from solar salterns. Genomic sequences expected to be completed released in the coming year include the anaerobic thermophilic halophile *Halothermothrix orenii* isolated from a salt lake in Tunisia and the eukaryotic brine alga *Dunaliella*.

The rapid increase in our understanding of the microbial diversity in hypersaline systems is due to the intensive use of genetic tools that provide additional dimensions for assessing diversity without the need to culture organisms. In recent years, DNA isolation techniques from environmental samples have greatly improved and are no longer the limiting factor. The discovery of unique genes and organisms has fueled even more interest in hypersaline environments. Application of molecular methods, in most cases based on the characterization of small subunit ribosomal RNA genes (16S rRNA for prokaryotes, 18S rRNA for eukaryotes) amplified directly from DNA isolated from the biomass shows that most sequences recovered have no equivalent in the database of known species. The results are indeed typical of all such whole-environment genetic studies. The application of 16S rRNA-based culture-independent techniques to hypersaline environments was pioneered by Benlloch et al. (1995) in the salterns of Alicante, Spain, and has since been applied to a variety of environments: saltern ponds (Benlloch et al. 2002; Legault et al. 2006), Mono Lake, California (Humayoun et al. 2002), Great Salt Lake, Utah (Baxter et al. 2005), and the Great Salt Plains, Oklahoma (Kirkwood et al. 2008). This was a key discussion point of the symposium that indicated additional work is needed to bring concordance to the organisms found thus far. The diversity detected using such approaches depends to a large extent on the money available for sequencing; if funding is appropriate, very large numbers of novel 16S rRNA sequences can be retrieved, as is shown by the study by Humayoun et al. (2002) in Mono Lake.

Initial use of the Phylochip to detect microbes in air (Brodie et al. 2007), with the potential to detect over 9000 groups of microbes based on the 16S rRNA gene sequence offers a new option to examine the diversity of hypersaline environments. Use of this technology in Great Salt Lake dramatically expanded the view of the level of diversity that may exist independent of culturing. The true value of this approach remains to be realized, but the preliminary data look very exciting and worth pursuing.

The combination of culture-dependent and culture-independent techniques has provided the basis for our current understanding of microbial diversity in high-salt environments (for an overview see e.g. Oren 2002a, 2002b, 2007). Molecular, culture-independent techniques, when properly applied, can provide information on the nature of the often yet uncultured dominant microorganisms in the environment. The information thus obtained can be used to specifically search for conditions to cultivate and study these organisms if enough sequence is acquired to predict some of the nutritional requirements and genetic regulation mechanisms. This approach has recently been successful in two important cases, with the isolation and characterization of both the square, gas-vacuolate archaeon *Haloquadratum walsbyi* (Burns et al. 2007) and the rod-shaped red *Salinibacter ruber* (Antón et al. 2002), both from solar salterns. A study in Australian saltern crystallizer ponds showed that with appropriate techniques and much patience, many members of the *Halobacteriaceae* present in the brines can be cultured (Burns et al. 2004). Extensive subculturing in liquid media is necessary as isolation on solid agar is not possible for some species.

The authors of this roundtable discussion summary suggest that the best approach is a combination of both cultivation-dependent and cultivation-independent methods–to fully characterize the microbial communities in salt lakes and other hypersaline environments. Environmental sequences alone without culture-based characterization will not adequately advance understanding of the causes of diversity, but can direct culture efforts toward the isolation of yet uncultivated microbes.

**Metabolic Potential and *In Situ* Metabolic Activities of Microbial Communities at High Salt Concentrations**

More important than the phylogenetic diversity of the species of halophilic microorganisms present in salt lakes is the function these microorganisms perform in the ecosystem. Here our understanding is still rather limited. One popular experimental approach is to look for the metabolic potential of the community, and this can be done in a number of ways. The potential of the community (or of selected isolates obtained from the community) to degrade any of a large number of carbon sources can provide information on processes that the community can theoretically perform. The Biolog® system enables high-throughput testing of a large number of carbon sources. Some modification of the standard protocol may have to be applied when high salt concentrations are present, but overall this assay has proved successful in a number of hypersaline systems (Litchfield et al. 2001). Alternatively, one may use "metabolomics", a set of exploratory LC/MS/MS techniques to examine the small molecules present in an environmental sample. This approach is littered with pitfalls that are largely due to ensuring accurate identification of the molecular mass with the database of identifiers. Nevertheless, metabolomics is an important method to apply in understanding the metabolic potential of hypersaline environments since relatively few of the organisms are available *in vitro* to serve as models for metabolic study.

Molecular techniques have in recent years extended our knowledge about the metabolic potential of microbial communities. Detection of "functional" genes specific for certain metabolic processes can be used to assess the presence or absence of certain groups of microorganisms or of certain biochemical pathways. The water column of Mono Lake, California has been a popular environment for testing this approach. Genes used for this purpose include ribulose bisphosphate carboxylase/oxygenase involved in autotrophic fixation of carbon dioxide (Giri et al. 2004), ammonia monooxygenase, the key enzyme of autotrophic oxidation of ammonia to nitrite (Ward et al. 2000), bisulfite reductase as an indicator for the presence of bacteria performing dissimilatory sulfate reduction, and genes involved in the methanogenic pathway (Scholten et al. 2005).

Analysis of genome libraries generated from DNA isolated from the environment can provide much more extensive information, and the recently developed "Geochip" which contains probes for over 4000 functional genes from (non-halophilic) Bacteria, is currently being tested to study of the potential metabolic diversity in hypersaline environments. The chips were designed for use in soil, specifically for bioremediation applications. Their use in hypersaline ecosystems may necessitate redesign. Future Geochips may need to include probes specific for archaeal and more diverse biochemical pathways that may play roles in hypersaline metabolism. Many halophiles (notably the *Halobacteriaceae*, but also *Salinibacter* and possibly the anaerobic fermentative *Halanaerobiales* as well) contain a large excess of acidic amino acids in their proteins, and this should of course be reflected in the nucleotide sequence and codon usage of the encoding genes, so that gene probes based on relatively well-conserved genes from non-halophiles may not always react with the homologous halophile gene. A conclusion of the session was that it may be time to redesign some of the high-throughput techniques to include more probes for Archaea and for halophilic and halotolerant algae in addition to the eubacterial probes that are well known and used extensively on the Geochip.

In evaluating the function of metabolic activity in a microbial community, one must assess to what extent the potential, as evaluated using DNA-based techniques or from culture-dependent laboratory studies, is indeed realized in the environment. It is sometimes stated that "If you've got it, you're using it", thus assuming that the possession of one or more of the key genes for a certain metabolic pathway is sufficient to prove that that metabolic pathway indeed is operative in the organisms or in the community *in situ*. There is no *a priori* justification for such a statement in environments that are not extreme. However, in extreme environments, the parameters are narrower, and this statement may be insightful in realizing that specific metabolites are hallmarks of that location and they can only arise via a single or a limited number of mechanisms. Testing for the microbial conversions to occur in salt lakes under field conditions is always much more difficult than demonstrating presence of the genes necessary for such conversions. Studies that go all the way to prove that the genetic potential is realized in the environment are unfortunately very rare.

An interesting case in this respect is the recent finding of *amoA* genes, encoding the key enzyme of autotrophic nitrification, in the nearly salt-saturated North Arm of Great Salt Lake, as reported at the conference. Theoretical considerations based on thermodynamic calculations make it improbable that autotrophic nitrification can function at

such high salt concentrations (Oren 1999, 2001). If indeed it can be proven that ammonia is oxidized to nitrite at measurable rates in Great Salt Lake brines, this would necessitate a change in our concepts on how nitrifying bacteria function or indicate that new biochemical mechanisms are yet to be discovered in these environments. In that case special efforts should be made to isolate the organism(s) responsible for the process (which is not easy, as even non-halophilic nitrifiers are notoriously difficult to grow), but only in that way can we extend our understanding and assess why the earlier theoretical models may not apply for Great Salt Lake nitrifiers.

Molecular biology techniques may help here in the future as well. For example, if we can assess the expression of *amoA* genes within the microbial community by quantifying mRNA levels, then we will have additional evidence that the genes are being transcribed. Still, the final proof will be the direct measurement of the enzyme activity itself. Techniques of metabolomics have not yet been extensively applied to hypersaline environments, but such techniques which assay specific metabolites in the field do have a considerable potential for revealing processes performed by the microbial community present. The potential impact of lateral gene transfer from one organism to another (or from phage) is hard to calculate, but in environmental metabolomics or genomics, where one is examining the broad view of processes occurring within the whole microbial community, this is not a concern.

**Bacteria in the Food Chain of Hypersaline Lakes**

It is clear that prokaryotes–Bacteria as well as Archaea–form an essential link in the food chain in saline and hypersaline environments. While we have a reasonably good understanding of the factors that promote development of halophilic and halotolerant prokaryotes, we know much less about the factors that may cause their decline: formation of non-culturable states, predation by protozoa, predation by higher animals, and lysis by bacteriophages.

Our knowledge of protozoa preying on prokaryotes and algae at the highest salinity is very limited. Comprehensive studies of the microbial food web along the salinity gradient in a Spanish saltern showed that the higher the salinity, the less important protozoa become in regulating prokaryote community densities (Pedrós-Alió et al. 2000; Joint et al. 2002). In spite of this, there is a long list of heterotrophic ciliate, flagellate and amoeboid protozoa that have been observed to occur at high salinities (Hauer & Rogerson 2005). The recent finding of flagellate protozoa preying on prokaryotes in solar saltern crystallizer brines in Korea (Park et al. 2003) shows that a reevaluation of the role of protozoa in saline and hypersaline lakes is warranted.

In spite of the abundance of brine shrimp species (*Artemia* spp.) in many salt lakes worldwide, we know little about the importance of these brine shrimps in regulating prokaryote community densities. Recently a number of studies have been initiated to examine the nature of the prokaryotes associated with *Artemia* and its intestinal system.

At the highest salinities bacteriophages appear to be more important in regulating community densities of halophilic Archaea and Bacteria than are protozoa (Pedrós-Alió et al. 2000). Only few studies have thus far been devoted to the diversity of bacteriophages in saline and hypersaline lakes. Following a bloom of red halophilic Archaea in the Dead Sea in 1992–1993, virus-like particles abounded in the water column, outnumbering prokaryote cells by an order of magnitude (Oren et al. 1997). Some characterization of bacteriophage numbers and properties has also been performed in Mono Lake (Jiang et al. 2004), in the Salton Sea (Wood et al. 2002), and in Spanish solar salterns (Diez et al. 2000). However, our understanding of the biology of these phages and their interrelationships with the prokaryotes they infect is in most cases very limited.

**The Importance of Interdisciplinary Studies to Advance our Understanding of the Microbial Ecology of Salt Lakes**

In recent years there have been a number of comprehensive studies in which single hypersaline environments were studied simultaneously by large interdisciplinary teams of scientists. Such studies, which applied a wide range of complementary approaches on the same environment and the same samples, have contributed invaluable information on the nature and functioning of the microbial communities in such environments much more than a large number of isolated studies could have done.

The MIDAS ('Microbial Diversity in Aquatic Systems') workshop, held at the salterns of Santa Pola, Alicante, Spain in May 1999 and sponsored by the European Union, presents a beautiful example of what can be achieved when a number of international research teams, each with its own expertise, work together to learn about the microbiology of a hypersaline environment. Publications emerging from that

workshop have dealt with the prokaryotic genetic diversity along the salinity gradient using different approaches (Benlloch et al. 2002; Casamajor et al. 2002), viral diversity along the salt gradient (Diez et al. 2000), the diversity of planktonic photoautotrophic microorganisms (Estrada et al. 2004), assessment of primary production, nutrient assimilation and microzooplankton grazing (Joint et al. 2002), and evaluation of the factors that control heterotrophic prokaryotic abundance (Gasol et al. 2004). Moreover, the samples collected from the crystallizer ponds during that workshop have yielded some of the first isolates of *Salinibacter ruber*, the novel type of extremely halophilic bacteria phylogenetically affiliated with the *Bacteroidetes* but physiologically closely resembling the archaeal family *Halobacteriaceae* (Antón et al. 2002).

The 'Microbial Observatories' program, initiated by the U.S. National Science Foundation, has enabled more such studies to be made in hypersaline environments. Studies in the Great Salt Plains, Oklahoma in recent years have shown how much novel information can be obtained when properly funded. The scientific output thus far includes papers on the characterization of halotolerant aerobic heterotrophic bacteria from the Great Salt Plains (Caton et al. 2004), carbon substrate utilization, antibiotic sensitivity, and numerical taxonomy of bacterial isolates (Litzner et al. 2006), aerobic biodegradation of aromatic compounds by the halophilic communities (Nicholson & Fathepure 2005), DNA-repair potential of *Halomonas* spp. isolated from the from the ecosystem (Wilson et al. 2004), a novel bacteriophage (Seaman & Day 2007), and diversity of diatoms (Potter et al. 2006), cyanobacteria (Kirkwood et al. 2008) and other algae (Henley et al. 2006).

More such studies are needed in other salt lakes and other salt-stressed ecosystem, with appropriate funding, to solve the basic questions relating to the importance of prokaryotic and eukaryotic microorganisms in saline lakes and other hypersaline ecosystems. Studies of interdependence between photoautotrophs and heterotrophs could be of particular value.

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