

EXO BIOLOGY: THE SURVIVAL ABILITY OF HALOPHILES UNDER MARTIAN CONDITIONS

University of California at Berkeley, Earth and Planetary Science Dept.
Berkeley, CA 94720

Students: Huitung Chu and, William Sheng
Advisors: David C. Gan and Lawrence Kuznetz

Abstract

The recently developed Odyssey gamma-ray spectrometer (GRS) has detected high concentrations of hydrogen, which strongly indicates there is permafrost and water ice in the upper meter of soil in the South Pole region of Mars. This finding presents the possibility that halophilic (salt loving) Archea might be present in its ice. It is possible that there may be areas of saline ice on Mars, since saline is found in Arctic ice. Halophiles are known to survive well under adverse conditions and have possibly lain dormant since the Upper Permian (250 million years) in salt deposits (2, 3, 7, 9).

Consequently, two halophiles isolated from San Francisco Bay salt ponds were selected to determine if they could survive the severe Martian conditions. To date, they have survived at least 8 months under experimental conditions. Future experiments will include dormant halophilic isolates from Lake Searles red salt crystals and Upper Permian Berchtesgaden rock salt.

Introduction

Martian surface conditions are not favorable for living organisms. The low pressures of 6 to 10 millibars of 95% carbon dioxide, a mean temperature of -80°C and the presence of superoxides formed by intense ultra-violet (UV) light irradiation, destroy organic compounds. However, some adverse terrestrial conditions did not deter organisms known as Extremophiles from surviving. In fact, the Martian conditions are very similar to the lyophilization process (freeze/dry by vacuum) used for preserving microbial cultures in the laboratory. Water is considered an essential element and appears to be lacking on the Martian surface. However, the presence of liquid water appears feasible and its presence has been demonstrated by experimentation under certain conditions. (12, 13, 14). It most likely exists as surface liquid water for a short time and as subsurface water or permafrost, which may support life forms.

A brief survey of Extremophiles: Among them are the Archea group which include: thermophiles from thermal hot springs and sub oceanic thermal "smokers"; barophiles, bacteria surviving in deep oceans under extremely high pressures; methane bacteria found in swamps, and sulfur hot springs; and psychrophiles (cold loving) found in the Lake Vostok Ice Shelf. Highly radioactive environments such as the Oakridge Atomic and Three Mile Reactors, showed the presence of Deinococcus radiodurans, whose ability to survive extremely radioactive environments is accomplished by constantly repairing damaged DNA with numerous copies of DNA. Another Extremophile is the arthropod-like tardigrades (21). They are so unique that a separate phylum, known as Tardigrada, was created for them. Commonly known as "water bears" they can survive desiccation for a hundred years, in vacuum, while subjected to extreme temperatures and high radiation.

Other Extremophiles include Bacillus infernus, a strict anaerobe isolated from a Virginia mine basalt at depth of 2.8 km. Cryptoendoliths were found to form a unique biological niche in an Antarctica Dry Valley in porous sandstones despite the harsh conditions of low moisture, near freezing temperatures, high winds and high UV flux (16,17). Halophilic (salt loving) archea are found universally in salt ponds and lakes and even as dormant cells or as biopolymers in rock, salt crystals (figures 1, 2 3,4)) and in evaporates and desert varnish. There are also recent findings of evaporates on Mars (21).

Halobacteria are facultative anaerobes, which use rhodopsin that produces ATP from ADP; they survive lysis in a high NaCl environment by maintaining a high concentration of cellular potassium ions. Another halophile uses halorhodopsin as a chloride pump (22). In addition to its role in metabolism, the pigments serve as a shield

against UV light and help to raise the temperature by absorbing sunlight. Sodium chloride also protects the cells from UV light. Other survival mechanisms include cellular potassium and sodium, which are also essential for enzyme function and for using glycine-betaine (18,19).

In our laboratory experiment, dormant Archea halophiles were recovered from Searles Lake red salt crystals and Upper Permian (250 million years) rock salt from the Berchtesgaden salt mines in Germany. There have been others instances of recovered halophiles from the Berchtesgaden and English salt mines in addition to New Mexico caves and even 650 million year old salt deposits (1, 2, 3, 4, 5). The claims for some of these recoveries are under debate. Some reasons for this skepticism include possible contamination and intrusion of more recent halophiles in water into the permeable salt crystals (23). It is common knowledge that recent age salt crystals harbor viable halophiles and their recovery is not difficult (1,16). We subsequently selected halophiles because of their ability to survive long periods under adverse conditions, and their dormancy period. Culturing them in a high salt concentration media excludes most contaminants. They are also readily available. For these reasons, they are a good candidate for this exobiology experiment (5,21). Moreover, since the Mars subsurface may have salt deposits and saline, the likelihood of finding halophiles is promising (6).

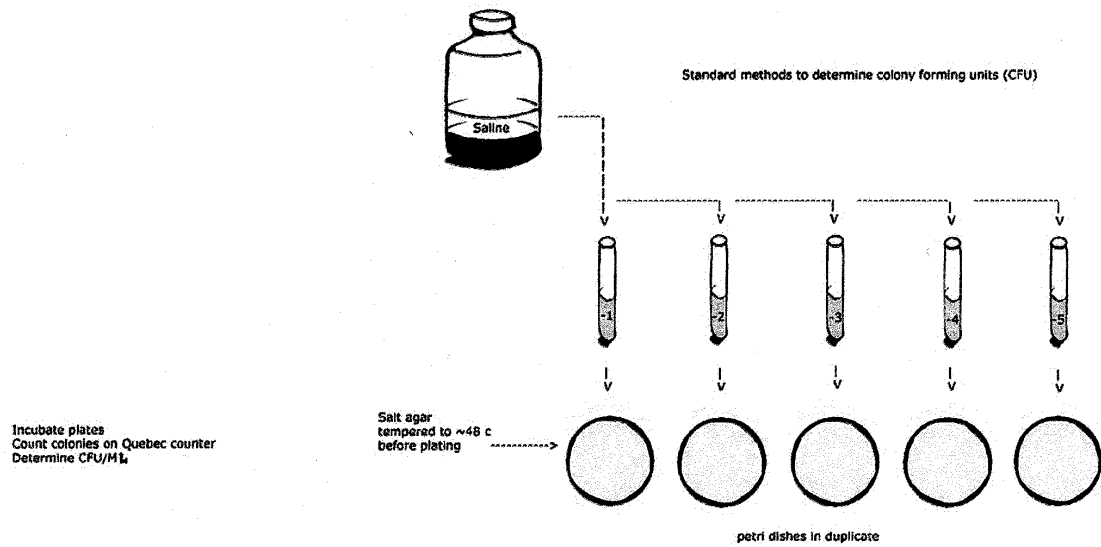
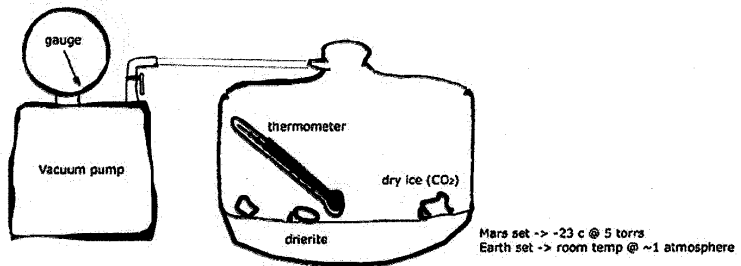
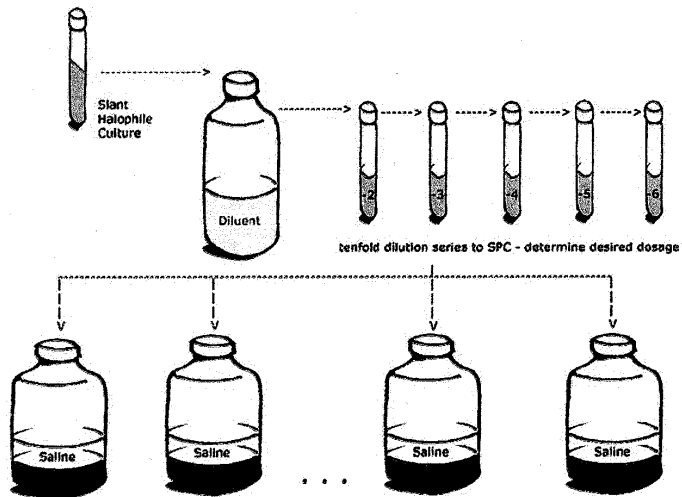
For the first experiment, we tested two fresh halophilic isolates from the San Francisco Bay to determine if they were able to survive under Martian conditions: Halobacterium (Haloferax) volcani (24), originally isolated from the Dead Sea, is mainly gram positive, nonmotile, disk shaped; with colonial morphology – smooth, round edges and displays orange pigment. The second isolate, designated as Halophile 1W is gram variable, non-motile, having short rods, with colonial morphology - smooth, round edges, and displays a yellow pigment (25). Both can tolerate 30% and 20% NaCl concentrations, respectively.

Method

Two halophiles, Haloferax volcani, and one designated as Halophile 1W, were isolated from the San Francisco Bay salt ponds. They were cultured in ATCC 1270 medium: NaCl-194 g, MgCl₂ 16 g, MgSO₄ 24 g, CaCl₂ 1.0 g., NaHCO₂ 0.2 g, KCl 5 g, NaBr 0.5 g, Yeast Extract 5.0 g, 1 L water, PH 7.3.

Plating was done with Salt Tryptose Glucose Yeast Extract Agar (STGYA): Tryptone-5.0 g, Yeast Extract-2.5 g, Glucose-1.0 g, NaCl –10g, Distilled Water- 1 L, pH 7.2. High Salt Concentration Agar was used to determine the degree of salt tolerance in cultures, a series of 30, 60, 120 and 150g NaCl g/ L was added to the STGYA medium. It was found to be impractical to culture halophiles on 25% NaCl agar because the salt crystallizes on the agar surface before the slow growing halophiles show growth of colonies.

Titered suspensions of each species in 1% saline, were added to Mars Soil Simulant JSC-1 Mars (10), cultured in 200 mL medicine bottles, and then placed into a bell jar containing dry ice to create a CO₂ atmosphere, using Drierite to remove water vapor. The internal pressure was adjusted to 5 torrs pressure and the assembly was frozen to -23° C in a freezer for the test (fig 7). A duplicate set was also run to serve as the Control set using ambient temperatures and atmosphere conditions in a bell jar that was exposed to light. A non-halophilic spore former, Bacillus mycoides, was selected for comparison. Periodic determinations of survival ability were performed by the heterotrophic plate count (HPC) method found in Standard Methods (7). Ten fold dilution series were made with 1% saline for plating. Plating for each dilution was done in duplicate. After incubation at 37°C for 72 hours, enumeration of colonies was done visually with a Quebec Colony Counter; Colony Forming Units (CFU) were then determined using Standard Methods (7). See Flow Chart.



Results

Survival Ability of Halophiles under Martian Conditions

Experiment Duration: Nov 2001 to October 2002

		<u>Nov 30 '01</u>	<u>Jan 4 '02</u>	<u>Jul 17 '02</u>
		<u>Colony Forming Units (CFU) per milliliter</u>		
<u>Mars Set</u> <u>-23°C</u> <u>5 torrs CO₂</u>	<u>Haloferax</u>	<u>2,200,000</u>	<u>1,200,000</u>	<u>420,000</u>
	<u>volcani</u>			
	<u>Halophile</u>	<u>2,900,000</u>	<u>1,800,000</u>	<u>270,000</u>
	<u>1W</u>			
	<u>Bacillus</u>	<u>1,800,000</u>	<u>not done</u>	<u>240,000</u>
	<u>Mycoides</u>			
<hr/>				
<u>Earth Set</u> <u>Room Temperature</u> <u>Ambient pressure</u>	<u>Haloferax</u>	<u>3,500,000</u>	<u>2,200,000</u>	<u>2,100,000</u>
	<u>volcanii</u>			
	<u>Halophile</u>	<u>2,500,000</u>	<u>1,800,000</u>	<u>2,400,000</u>
	<u>1W</u>			
	<u>Bacillus</u>	<u>3,500,000</u>	<u>not done</u>	<u>290,000</u>
	<u>Mycoides</u>			

Discussion

The Mars Set showed a decrease in numbers of Colony Forming Units (CFU) for both halophiles and Bacillus mycoides, a non-halophilic spore former. In the Earth Control set, halophiles showed less decrease in CFU than the Mars Set. Bacillus mycoides showed a similar decrease in numbers; this decrease may be possibly attributed to freeze/thaw damage of cells incurred by periodic removal from the bell jar for testing. The Earth Control colonies were orange while the Mars Set colonies were white (fig 8). This difference may be attributed to the pigments of the rhodopsin family, which are photosynthetic (5); subsequently, future experiments will be conducted to determine the role of light on colonial color.

Concerning the recovery of dormant halophiles from salt, the findings are under debate because of permeation of more recent halophiles in water by a temperature gradient (15). However, the molecular biological signatures showed that the ancient halophiles are significantly different from the modern species (2).

Future Experiments

1. To determine if the decrease in numbers of the Mars Test Set was due to freeze/thaw effect, aliquots of halophile/soil will be distributed to multiple containers to avoid freeze/thawing. Only one container from each set will be removed periodically for testing, thus ensuring the integrity of the remaining containers.
2. Dormant halophilic isolates recovered from Searles Lake and Upper Permian Berchtesgaden rock salt will be tested by the previously described method, except that the Mars Set will be subjected to -80° Celsius (average

Martian temperature) with CO₂. These isolates were cultured from sterilized salt crystals or rock salt. Sterilization was achieved by flaming and exposure to a UV germicidal lamp in a Sterilgardhood (Baker Co) for one to two hours, with frequent rotation of the crystals to assure complete surface sterilization. The weighed crystals were then dissolved in 1% saline to a final concentration of ~10 to 15% NaCl (1.5 to 2.5 molar) and then cultured in the ATCC Medium 1270 for several weeks at room temperature. A control UV sterilization run was done to determine the efficacy of our UV surface sterilization technique. A Control UV sterilization run was completed to determine the efficacy of the sterilization technique by testing < 2m diameter crystal. No growth of halophiles were observed with this run.

3. Koch Postulates Experiment: To prove that an etiological agent causes a disease, a pure isolate culture of the suspect agent will be tested in a susceptible host. Recovery of the agent in question demonstrates that the agent is responsible for the disease. This procedure is known as Koch's Postulates. In this future experiment, a pure culture of halophile will be allowed to crystallize in a sterile saturated salt solution for dormancy. Successful recovery of the dormant halophile will satisfy Koch's Postulates requirements.

Description of isolates to be used for future experiments:

<u>Source</u>	<u>Colonial Morphology</u>	<u>Gram Stain</u>	<u>Growth in % NaCl Medium</u>				<u>ATCC 1270</u>
			<u>6</u>	<u>9</u>	<u>12</u>	<u>15</u>	
Searles Lake	yellow, smooth, regular	var. cocci ~1 µm	+	+	+	+	+
Searles Lake	white, smooth, regular	var cocci~1µm	+	+	+	+	+
Searles Lake	yeast, white, smooth	pos. ~ 5 um	+				
Berchtesgaden	yellow-orange, smooth	var. cocci	+	+	+		+

Conclusion

The halophiles, Haloferax volcani and Halophile 1W, were able to survive under Martian conditions; however their decrease in numbers may be attributed to the freeze/thaw effect. The Martian halophilic archae species, better adapted to their harsher environment, may survive in greater numbers than the terrestrial strains used in this experiment.

Outreach Activities

Cal Day Open House UCB Mars Missions Projects Booth and Posters 2001 and 2002

City of Oakley School District Science Fair Booth Display and Posters

Acknowledgements

University of California at Berkeley:

Professors William Dietrich, Jillian Banfield and Michael Zach of the Earth and Planetary Science Dept.
Professor Antje Hofmeister, Plant and Microbial Biology Dept., Professor Frank Morrison, Material Science Dept. and UCB students: Grace Lee and Eric Lin.

NASA

Dr. Michael Hecht, Jet Propulsion Laboratory, Pasadena, Drs. Richard Quinn and Robert Haberle, Chris McKay and Marc Cohen of Ames Research Center

California State Department of Health Services

Dr. Daniel Mills, Microbial Diseases Laboratory (now with USDA Western Regional Laboratories)

Biovir Laboratories

Dr. Richard Danielsen and Professor Robert Cooper

Ms. Monica Gan for manuscript review

References

1. Armstrong, W.P. 1981 "The Pink Playas of Owens Valley", *Fremontia* 93-10
<http://waynesword.Palomar.edu/plsept98.htm>
2. Vreeland, R, Rosenzweig, W, Powers, D., 2000 "Isolation of a 250 Million-year old Halotolerant Bacterium from a Primary Salt Crystal" *Nature* 407: p897-900
3. Stan-Lotter, H., McGenity, T. J., Legat, A., Denner, E., Glaser, K, Stetter, K. O., Wanner, G 1999 "Very Similar Strains of *Halococcus salifodinae* are found in geographically separated Permo-Triassic salt deposits" *Microbiology* 145: p3565-3374
4. Dyall-Smith, M., Danson, M 2001 "Life of Brine: Halophiles in 2001" *Biology* 2(12) Reports 4033.1-4033.3
5. Landis, G. 2001 "Martian Water: Are There Extant Halobacteria on Mars?" *Astrobiology* 1(2): p161-164
6. Spears, J, Levy, R. "Life Within Rock Salt: A Surprising Diverse Neighborhood" 102nd Meeting of American Society for Microbiology paper N 145. May 19-23, 2002 Salt Lake City UT
7. <http://www.uib.no/ums/magazine/updates/Halophile/halophile.htm>: "Life at the Extreme"
8. "Standard Methods for the Examination of Water and Wastewater" APHA, AWWA and WEF 20th edition , 1999
9. Grant, W. D. "Halobacteria: the Evidence for Longevity" *Extremophiles*, v, 270-287, 1998
10. Allen, CC, Jager, K.M., Morris, R V, Lindstrom, D. J., Lindstrom, M.M. and Lockwood, J.P. "JSC Mars-1: A Martian Soil Simulant, Space 98, American Society of Civil Engineers: April 1998
11. Boone, D.R., Lin, Z., Zahn, D.L, Ballowill, Drake, G R., Stevens, O.T., Aldrich, H.C. " *Bacillus infernus* sp. nov, an Fe (III)- and Mn (IV)-reducing Anaerobe From the Deep Terrestrial Subsurface" *Int. Journal Systematic Bacteriology*, 45: 441-448
12. Kuznetz, L and Gan, D "On the Existence and Stability of Liquid Water on Mars Today", *Astrobiology*, 2: 183-195, 2002
13. Haberle, R.M., McKay, C.P., Schaeffer, J, Joshi, M., Cabrol, N.A. and Grin, E.A. Meteorology Control on the Formation of Paleolakes on Mars (Abstract 509) Proceedings of the Lunar and Planetary Science Conference. Available at: <http://www.lpi.usra.edu/meetings/lkp2000/pdf/1509.pdf>. 2002
14. Hecht, M. "Metastability of Liquid Water on Mars" *Icarus*, 156, 373-387 2002

15. Parkes, J. Microbiology: "A Case for Bacterial Immortality" *Nature*, 407, 844-848, 2000
16. National Academy of Space Bulletin. "Limits of Life on Earth" chap. 4 and Web Page:
www.nas.edu/ssb/bcmarsch4.htm
17. Vestal, J.R. "Cryptoendolithic Microbiota from the Antarctic Cold Desert" *Applied Environmental Microbiology*, 54, 960-965 1991
18. Litchfield, C. "Survival Strategies for Microorganisms in Hypersaline Environment and the Case for Life on Mars", *Meteoritics and Planetary Science*, 33, 813-819 1998
19. Landis, G.A. "Searching for Life: The Case for Halobacteria on Mars" STAIF Conference on Space Exploration Technology, AIP Conference Proceedings, 552, 393-396 2001
20. Landis, G.A. "Water, Pink Salt and Cold Dry Gullies: Halobacteria and the Case for Life on Mars" *Analog* (April) 2001
21. Rothchild, L. "Life in Extreme Environments" *Ad Astra* Jan/Feb 32-41, 2002
22. Mukohata Y, Matsuno-Yag, A, Kaji, Y. "Light Induces Proton Uptake and ATP Synthesis by Bacteriorhodopsin depleted Halobacterium" in Morishita, H. and Masui, M. (eds) *Saline Environment*, *Bus. Ctr. Acad. Sci Japan*, 31-37
23. McKay, C. (personal communication, Aug 2002)
24. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins. 1989

Table 1. Chemical Compositions

	VL-1	VL-2	Pathfinder	JSC Mars-1	
Oxide	Wt%*	Wt%*	Wt%**	Wt%***	Wt%****
SiO ₂	43	43	44.0	34.5	43.5
Al ₂ O ₃	7.3	7	7.5	18.5	23.3
TiO ₂	0.66	0.56	1.1	3.0	3.8
Fe ₂ O ₃	18.5	17.8	16.5	12.4	15.6
MnO	n.a.	n.a.	n.a.	0.2	0.3
CaO	5.9	5.7	5.6	4.9	6.2
MgO	6	6	7.0	2.7	3.4
K ₂ O	<0.15	<0.15	0.3	0.5	0.6
Na ₂ O	n.a.	n.a.	2.1	1.9	2.4
P ₂ O ₅	n.a.	n.a.	n.a.	0.7	0.9
SO ₃	6.6	8.1	4.9	n.a.	n.a.
Cl	0.7	0.5	0.5	n.a.	n.a.
LOI	n.a.	n.a.	n.a.	21.8	n.a.
Total	89	89	89.5	101.1	100.0

n.a. not analyzed; all iron calculated as Fe₂O₃

LOI (loss on ignition) weight loss after 2 hrs at 900°C; includes H₂O and SO₂

* Viking landers 1 and 2 XRF (mean of 3; Banin et al, 1992)

** Pathfinder APXS (mean of 5, norm. to 44 wt% SiO₂; Rieder et al, 1997)

*** XRF (Hooper et al, 1993)

**** XRF (volatile-free, normalized; Hooper et al, 1993)

Volatile Content Martian soil simulant JSC Mars-1 contains considerable water. Heating experiments in flowing argon demonstrate weight losses after one hour ranging from 7.8 wt% at 100°C to 21.1 wt% at 600°C. These numbers represent total volatile loss, which is probably dominated by H₂O but would also include SO₂ if sulfates are present.

The Martian surface soil, by contrast, is extremely dry. Viking experiments released 0.1 to 1.0 wt% water from soil samples heated to 500°C (Biemann et al, 1977).

Halobacterium media (ATCC medium 1270)

NaCl	194 grams
MgCl ₂	16 grams
MgSO ₄	24 grams
CaCl ₂	1 gram
KCl	5 grams
NaHCO ₃	0.2 grams
NaBr	0.5 grams
Yeast extract	5 grams
distilled water	up to 1 liter

adjust pH to 7.3

(add 15g agar for solid media)

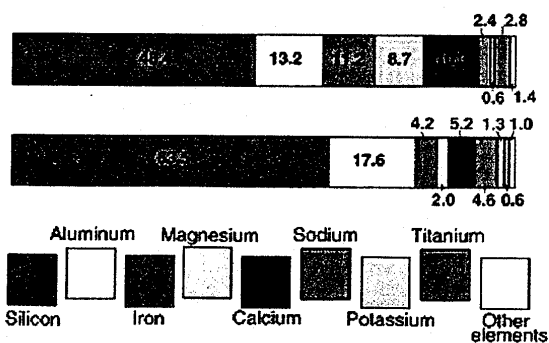
Mars Pathfinder

Analysis of Martian Samples by the Alpha Proton X-Ray Spectrometer: Preliminary Results Comparison with SNCs and the Earth

	Mars			Earth		
	A-3, Rock "Barnacle Bill"	A-5, Soil	SNCs (Mars Meteorites)	Continental Crust		Oceanic Crust
	weight %	weight %	weight %	Average	Sediments	weight %
				weight %	weight %	
MgO	3.1	8.6	9.3 - 31.6	3.1	3.1	7.7
Al ₂ O ₃	12.4	10.1	0.7 - 12.0	15.2	13.0	15.6
SiO ₂	55.0	43.8	38.2 - 52.7	60.2	50.0	50.7
K ₂ O*	1.4	0.7	0.022 - 0.19	2.9	2.0	0.17
CaO	4.6	5.3	0.6 - 15.8	5.5	8.4	11.4
TiO ₂	0.7	0.7	0.1 - 1.8	0.7	0.7	1.5
MnO*	0.9	0.6	0.44 - 0.55	0.1	0.1	0.16
FeO	12.7	17.5	17.6 - 27.1	6.05	5.5	9.9
FeO/MnO	14.1	29.2	37.0 - 51.5	-	-	-

*Values for potassium (K) and manganese (Mn) are probably too high and subject to revision after further analysis.

What is the composition of the lava?



Chemical composition of lavas erupted by Hawaiian volcanoes and Mount St. Helens. Numbers are weight percent oxide.
Courtesy of U.S. Geological Survey.

The lava is basalt. Hawaiian basalts contain about 50% silica, 10% each of iron, magnesium, calcium, about 15% aluminum, 2% titanium and 2% sodium.

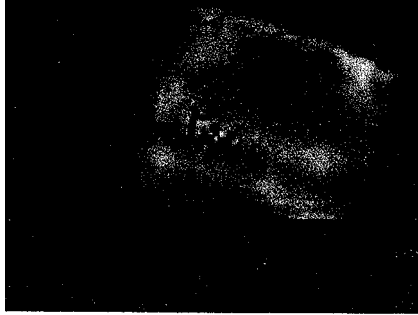


Figure 1. Photomicrograph (100x oil immersion) of six bacteria-shaped particles in primary fluid inclusion from 97 kyr old salt in Badwater salt core (85 meter depth).



Figure 2. Time sequence photomicrograph (40x) of rod-shaped particle that is moving with a fluid inclusion from a 9 kyr salt in Badwater salt core (8 meter depth).

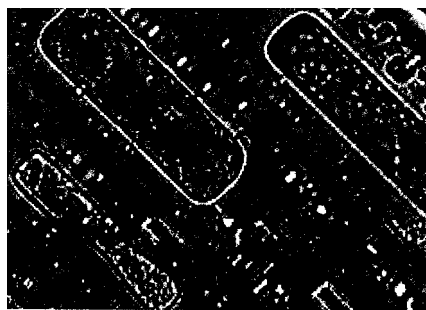


Figure 3. Halophiles trapped within salt crystal grown in laboratory media. Photomicrograph (40x) taken one week after crystal formed. The halophiles are mobile and tend not to be fixed to crystal wall (<http://geoweb.princeton.edu/research/geomicrobio/halite.html>).

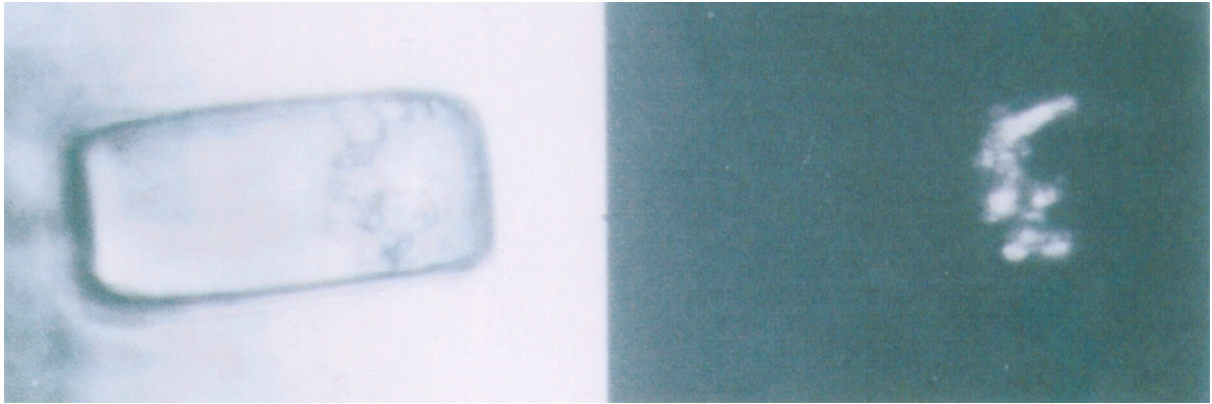


Figure 4. Fluid inclusion in a 20-million-year-old salt crystal, in visible (left) and ultraviolet light. Fluorescence under ultraviolet is consistent with the presence of organic matter. But it is not conclusive evidence, nor does it provide information about the type of material — which might be the remains of entombed bacteria or, conceivably, the intact biopolymers of ancient but viable bacteria. The crystal is about 65 micrometers in length. (Image courtesy of W. D. Grant.)



Figure 5. The vivid red brine (teeming with halophilic archaeobacteria) of Owens Lake contrasts sharply with the gleaming white deposits of soda ash (sodium carbonate). The picturesque Inyo Range can be seen in the distance.

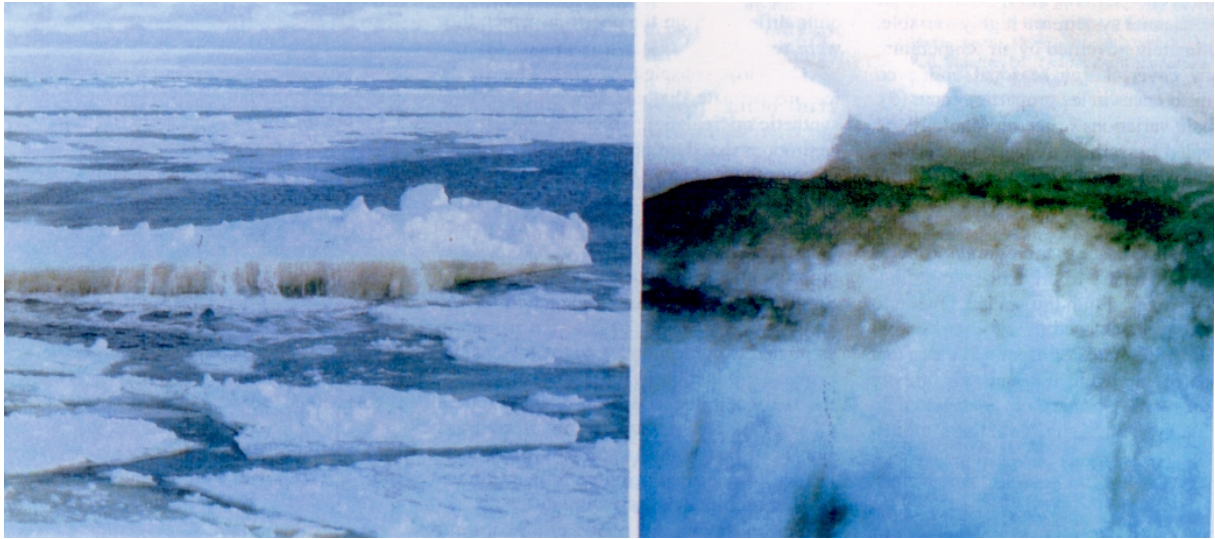


Figure 6. Sea-ice diatoms can reach such densities that their photosynthetic pigments color the underside of ice floes brown. The sea-ice organisms grow throughout the ice in brine channels, the brown vertical lines in the image on the right, as well as in porous ice which can be seen as dark brown horizontal layers.
(Photograph by D. N. Thomas.)

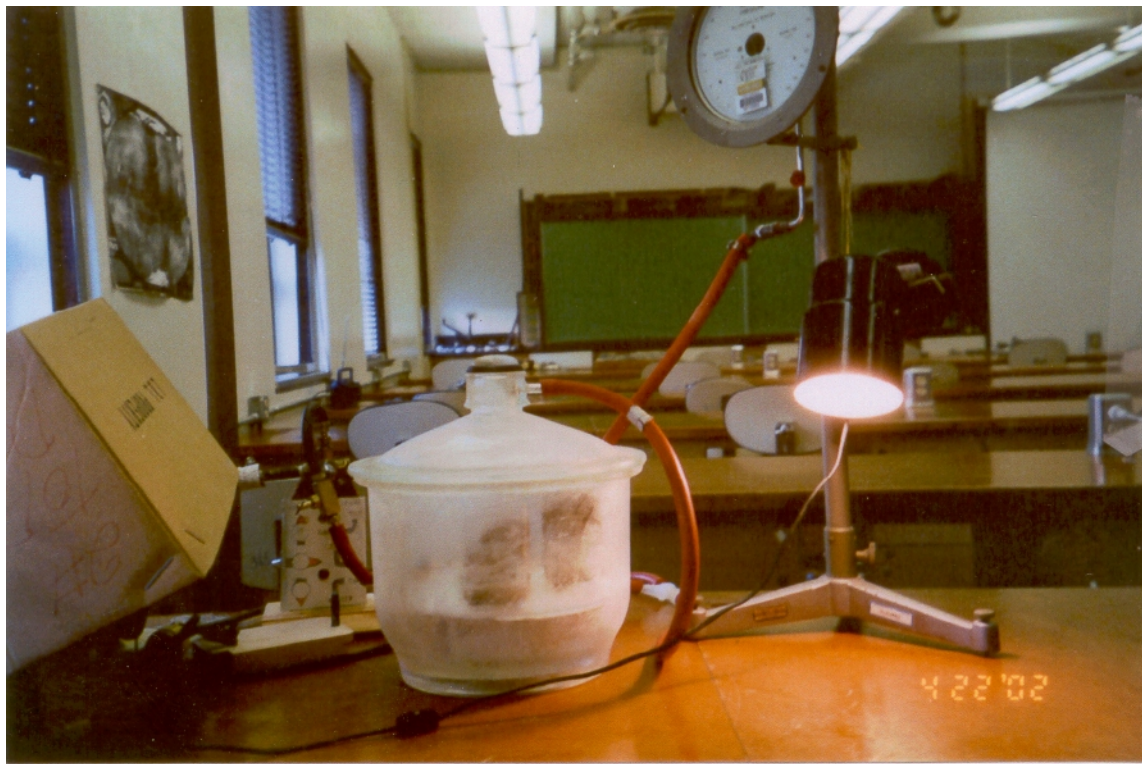


Figure 7. Halophile survival test using bell jars.

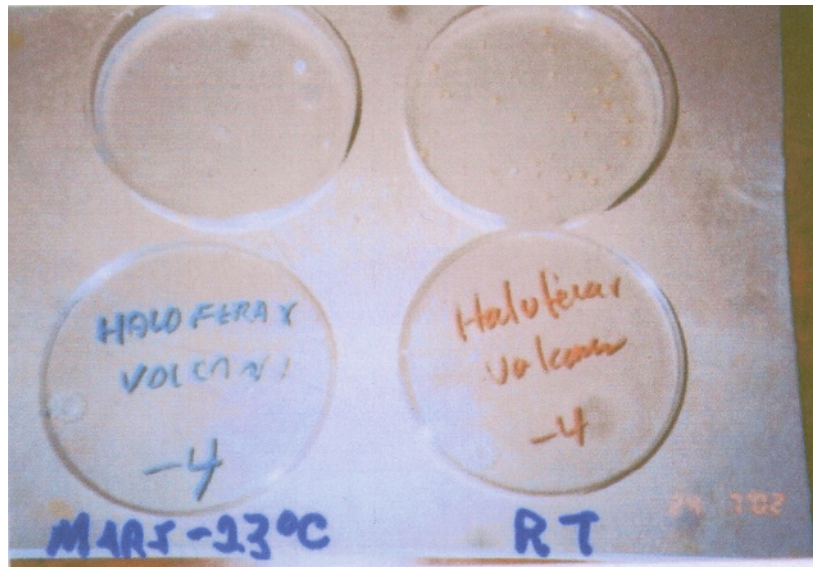


Figure 8. Mars Set and Earth Control Set – Haloferax volcani colonial color differences.

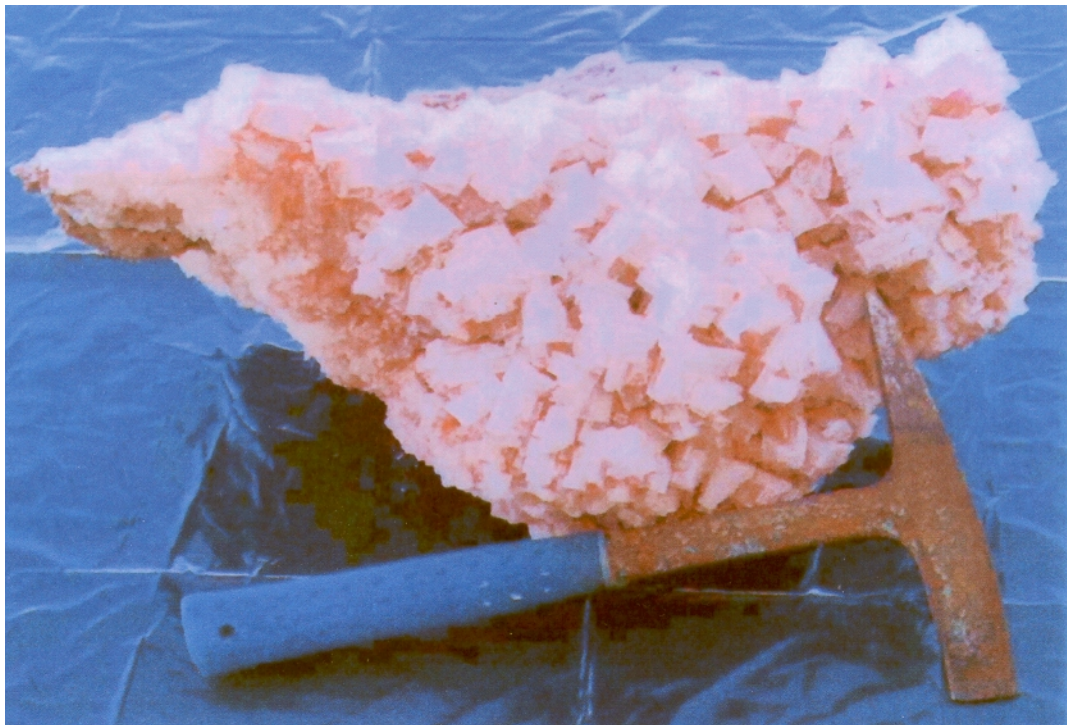


Figure 9. Searles Lake red salt crystals containing dormant halophiles.