MICROBIAL HABITABILITY OF KILIMANJARO’S GLACIERS AND SOILS

Adrian.Ponce@jpl.nasa.gov
http://ponce.caltech.edu
Planetary Science Section, Jet Propulsion Laboratory
Near Sterile Environments
Mars Analogs for Life Detection

• Near sterile soils and ices (<10 CFU/g).
• Found in extreme deserts, glaciers, and volcanoes.
• Above $10^3$ CFU/g, strong CFU to TOC correlation.
• Contain CFU hot spots → patchy microhabitats.
• Culturing is standard for viability assessment, but VBNC population is >90% of environmental microbes.

Atacama Desert Soils  Polar Ices  Kilimanjaro Ice, Periglacial Soils
• Measure germinable spores, including germinable-but-not-culturable population.

• Aerobic species: *Bacillus*
• Anaerobic species: *Clostridium*
Bacterial Spores
The Toughest Microbial Form of Life

Bacterial Spores

Cryptospiriidae
Mycobacteria
Small Viruses
Fungi
Vegetative bacteria
Lipid Viruses

Increasing Resistance

Planetary Protection

Biodefense and Restoration

Survival and Growth in Extreme Environments
Germinable Spore Assay

Fast Validation of Pathogen Inactivation:

Germinable Spores
Germinable Spore Assay Validation Experiments

Germination

Colony Formation
Germinable Spore Assay
Monitoring Inactivation Processes

*Bacillus* spores in aqueous suspension

![Graphs showing heat and UV sterilization](image_url)
Germinable Spore Assay
Samples from Extreme Environments

Environmental Samples
• *Atacama* Desert (Chile)
• Lake Vida (Antarctica)
• Greenland ice core (GISP2)
• Alaskan permafrost (Barrow)


Kilimanjaro
Kilimanjaro, Tanzania
Kilimanjaro Glacial Ice Endospore Concentrations

Culturable population in ice is ~0 spores/L.
Kilimanjaro Glacial Ice Endospore Concentrations

- Germinable-but-not-culturable population in ice is $\sim 10^3$-$10^4$ spores/L.
Supraglacial Habitat
Microbial Growth in Meltwater Ponds

closest relative from a cold water environment

100% 55%
Supraglacial Habitat

• Large fraction of cold-water species suggests microbial activity in a cold-water ecosystem at the time of deposition.
• Discovered a 1-foot deep, mud-rich meltwater pond on glacier.
Growth in Periglacial Soils

- Periglacial soils and ice dust are volcanic ash.
- Biofilm on some particles (Morphology, EDX).
- No pollen.
Conclusions

• Dust layers contain volcanic ash, not soil particles from regions below summit.
• Germinable-but-not-culturable population in ice is \(~10^4\) spores/L.
• At the time of dust layer formation, the glacier surface hosted an active microbial, cold-water ecosystem.
• Radiocarbon dating of near basal dust layers suggest that NIF glacier formed around 12\textsuperscript{th} century AD. Kilimanjaro ice core record should be reevaluated.
• Periglacial volcanic soils contain biofilms, indicating microbial activity.
Further investigate microbial diversity/activity of glacial dust layers, supraglacial pond and periglacial soils.
- Which species are there? How are they making a living?
- How are they interacting? Novel organisms special adaptations?

High-elevation periglacial soils are among the most extreme but least studied soil systems on Earth.
- Volcanic ash Fe, Al-silicate soils
- Low organic carbon soils (<1mg/g)
- Low water activity ($a_w<0.6$)
- Daily extreme freeze thaw cycle (-26 °C to 5 °C)
- Vegetation coverage: None
- High UV flux
- Dynamic system, glaciers disappearing in 20-50 years.
- Gradients in temperature (fumaroles) and water (melting glacier) enables investigations of a continuum of microhabitats.

Better understanding habitability in Mars special regions, where oligotrophic, phylosilicate rich regolith abuts polar ice caps.
$ FUNDING $
NASA Astrobio, PP Programs
DHS Biodefense Programs
### Kilimanjaro as a Mars Analog Characteristics

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Kibo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude, longitude</td>
<td>Latitude: -3.06667, Longitude: 37.35</td>
</tr>
<tr>
<td>Elevation, Areal Extent</td>
<td>5.9 km, 2 km by 2 km</td>
</tr>
<tr>
<td>Prime Science Questions</td>
<td>What is the organic carbon threshold in Fe-, Al- silicate soils for microbial viability?</td>
</tr>
<tr>
<td>Distance of Science Targets from nearest road or airstrip</td>
<td>From nearest road, it is a 6 days hike to reach Kilimanjaro summit.</td>
</tr>
<tr>
<td>Environmental characteristics</td>
<td>Max temp: 5 °C, Min temp: -26 °C, Precipitation: 100 mm, Vegetation coverage: None</td>
</tr>
<tr>
<td>Previous studies</td>
<td>2 manuscript in preparation.</td>
</tr>
<tr>
<td>Primary Landing Site Target</td>
<td>Phylosilicate rich landing sites.</td>
</tr>
</tbody>
</table>
Near Sterile Environments
Mars Analogs for Life Detection

• Near sterile soils (<10 CFU/g) are only found in some extreme arid desert and volcanic soils.
• Culturing is standard method for viability assessment, but VBNC population is >90% of environmental microbes.
• Spores are toughest form of life and most likely interplanetary travelers to survive.
• Germinability assay detections much greater fraction of viable sporeforming microbes.
• In near sterile soils, hot spots due to favorable microhabitats dominate viable microbe distribution.
• Growth vs. dormancy.
• Growth limited by aw, T, Corg and Delta(Eredox).
• Kilimanjaro has gradients in aw, T, Corg across large range to enable systematic studies of microhabitats.
• Diversity on, in and around glacier and near fumaroles.
Mars Astrobiology Questions

- Panspermia
- Natural transfer
- Strongly coupled biospheres
- Interplanetary lithoinoculation

Earth Astrobiology Questions

- Survival/Growth in Extreme Deserts.
- Longevity Ices

Mojave Desert          Atacama Desert          Ice Cores          GISP2 Ice Core
• Northern Ice Field (NIF) Glacier estimated to be 11,700 years old.
• Age based on and $d^{18}O$ minima correlation to $^{14}C$ solar activity.
• $d^{18}O$, Na/F and dust proxies used to determine T and ppt record.
• Three major abrupt climate change events recorded in dust rich layers.
• Kilimanjaro Glaciers are estimated to disappear before 2050.

What is the impact of past climate change on ecosystem?

Measure microbial and pollen diversity as a function of time during past climate change.

Initial Assumptions

• Glaciers are ~12,000 years old.
• During droughts more dust gets carried to glaciers, forming layers.
• Microflora is attached to dust.
Soils from Surrounding Region

Ongoing work: Compare soils to dust from ice layers.
Age of NIF Kilimanjaro Glacier

- This location of NIF does not contain Holocene ice.
NOTICE TO UMBWE ROUTE USERS
THIS ROUTE IS FOR ASCENT ONLY
YOUR DESCENT IS THROUGH MWEKA GATE
Sampling and Data Collection
Sampling and Data Collection
Lifetime Gated Detection

Fluorescein + Tb-DPA

(a) Delay = 0 $\mu$s
(b) Delay = 10 $\mu$s
(c) Delay = 50 $\mu$s
Greenland Ice Cores

Normalized intensity

Wavelength (nm)

Normalized intensity

Germination time (min)
Atacama Desert, Chile

Atacama Desert (taken in Nov 2005)
Kilimanjaro
Location 1
Location 1
## Location 2

<table>
<thead>
<tr>
<th>Elem</th>
<th>1. At %</th>
<th>2. At %</th>
<th>3. At %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>26.63</td>
<td>24.5</td>
<td>23.31</td>
</tr>
<tr>
<td>O K</td>
<td>54.5</td>
<td>55.26</td>
<td>54.67</td>
</tr>
<tr>
<td>NaK</td>
<td>0.98</td>
<td>0.99</td>
<td>1.77</td>
</tr>
<tr>
<td>MgK</td>
<td>0.51</td>
<td>0.37</td>
<td>0.46</td>
</tr>
<tr>
<td>AlK</td>
<td>6.98</td>
<td>8.18</td>
<td>7.46</td>
</tr>
<tr>
<td>SiK</td>
<td>8.64</td>
<td>9.12</td>
<td>10.14</td>
</tr>
<tr>
<td>P K</td>
<td>0.19</td>
<td>0.17</td>
<td>0.2</td>
</tr>
<tr>
<td>S K</td>
<td>0.1</td>
<td>0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>ClK</td>
<td>0.1</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>K K</td>
<td>0.39</td>
<td>0.42</td>
<td>0.88</td>
</tr>
<tr>
<td>CaK</td>
<td>0.33</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>TiK</td>
<td>0.11</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>FeK</td>
<td>0.42</td>
<td>0.45</td>
<td>0.49</td>
</tr>
<tr>
<td>NiK</td>
<td>0.11</td>
<td>0.06</td>
<td>0.07</td>
</tr>
</tbody>
</table>

---

*Image details:*
- **Acc.V:** 20.0 kV
- **Spot Magn:** 3.0
- **Mgn:** 6000x
- **Det WD:** GSE 9.4
- **5 μm**

Location 3
<table>
<thead>
<tr>
<th>Elem</th>
<th>1. At %</th>
<th>2. At %</th>
<th>3. At %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>43.18</td>
<td>40.47</td>
<td>44.44</td>
</tr>
<tr>
<td>O K</td>
<td>44.44</td>
<td>46.12</td>
<td>43.39</td>
</tr>
<tr>
<td>NaK</td>
<td>0.77</td>
<td>0.63</td>
<td>0.49</td>
</tr>
<tr>
<td>MgK</td>
<td>0.3</td>
<td>0.25</td>
<td>0.3</td>
</tr>
<tr>
<td>AlK</td>
<td>2.8</td>
<td>3.44</td>
<td>3.14</td>
</tr>
<tr>
<td>SiK</td>
<td>7.3</td>
<td>8.04</td>
<td>7.34</td>
</tr>
<tr>
<td>P K</td>
<td>0.16</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>S K</td>
<td>0.13</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td>ClK</td>
<td>0.07</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>K K</td>
<td>0.37</td>
<td>0.43</td>
<td>0.25</td>
</tr>
<tr>
<td>CaK</td>
<td>0.17</td>
<td>0.14</td>
<td>0.1</td>
</tr>
<tr>
<td>TiK</td>
<td>0.09</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>FeK</td>
<td>0.22</td>
<td>0.23</td>
<td>0.18</td>
</tr>
<tr>
<td>NiK</td>
<td>0.02</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Application to Monitor Sterilization Processes

Bacillus spores on dry surfaces

![Graphs showing surviving number against time for Oxygen Plasma Sterilization and H₂O₂(vap.) Sterilization](image)
<table>
<thead>
<tr>
<th><strong>PHASE CONTRAST</strong></th>
<th><strong>MALACHITE GREEN</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Refractive index</td>
<td>Uptake of stain</td>
</tr>
<tr>
<td>Spores: phase bright</td>
<td>Spores: Green</td>
</tr>
<tr>
<td>Vegetative cells: phase dark</td>
<td>Vegetative cells: Pink</td>
</tr>
<tr>
<td>$0 - 10^2$ spores/mL</td>
<td>Presence of spores</td>
</tr>
</tbody>
</table>

**Conclusion**

0 – 10² spores/mL
<table>
<thead>
<tr>
<th>Detection Target</th>
<th>Observable</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase Contrast</strong></td>
<td>Refractive index</td>
<td>Spores: phase bright</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vegetative cells: phase dark</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0 - 10^2$ spores/mL</td>
</tr>
<tr>
<td><strong>Malachite Green</strong></td>
<td>Uptake of stain</td>
<td>Spores: Green</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vegetative cells: Pink</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence of spores</td>
</tr>
<tr>
<td><strong>SYTO-9/PI</strong></td>
<td>Membrane integrity</td>
<td>Live cells: Green</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead cells: Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$&lt; 0.01$ live cells/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$&lt; 0.1$ cells/mL</td>
</tr>
</tbody>
</table>
### PHASE CONTRAST

<table>
<thead>
<tr>
<th>Detection Target</th>
<th>Observable</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MALACHITE GREEN</td>
<td>SYTO-9/PI</td>
<td>TETRAZOLIUM CHLORIDE</td>
</tr>
<tr>
<td>Refractive index</td>
<td>Uptake of stain</td>
<td>Membrane integrity</td>
</tr>
<tr>
<td>Spores: phase bright</td>
<td>Spores: Green</td>
<td>Live cells: Green</td>
</tr>
<tr>
<td>Vegetative cells: phase dark</td>
<td>Vegetative cells: Pink</td>
<td>Dead cells: Red</td>
</tr>
<tr>
<td>$0 - 10^2$ spores/mL</td>
<td>Presence of spores</td>
<td>$&lt;0.01$ live cells/mL</td>
</tr>
</tbody>
</table>

Kilimanjaro samples incubated with 1/5 TSB at 37°C
# Detection Target

<table>
<thead>
<tr>
<th>Phase Contrast</th>
<th>Malachite Green</th>
<th>SYTO-9/PI</th>
<th>Tetrazolium Chloride</th>
<th>DiBAC$_4$(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refractive index</td>
<td>Uptake of stain</td>
<td>Membrane integrity</td>
<td>Respiratory potential</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>Spores: phase bright</td>
<td>Spores: Green</td>
<td>Live cells: Green</td>
<td>Dehydrogenase activity: Red</td>
<td>Active cells: Green</td>
</tr>
<tr>
<td>Vegetative cells: phase dark</td>
<td>Vegetative cells: Pink</td>
<td>Dead cells: Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 10$^2$ spores/mL</td>
<td>Presence of spores</td>
<td>&lt;0.01 live cells/mL</td>
<td>Presence of dehydrogenase activity and membrane potential upon incubation with TSB</td>
<td></td>
</tr>
</tbody>
</table>

### Conclusion

Kilimanjaro samples incubated with 1/5 TSB at 37°C
DiBAC$_4$(3) and MicroEVA results show changes in spore parameters over time.

**DiBAC$_4$(3)**
- Intensity of DiBAC$_4$(3)-positive spores (%)
- Time (min) from 0 to 40

**MicroEVA**
- % Emission intensity
- Time (min) from 0 to 50
Microbial Diversity

Evaluate rate of microbial diversity decline and recovery with specific past climate change events.
• Chemistry, Methods, Instrumentation for viability assessment
  • Bacillus, Clostridium as biological indicators for sterility assurance.
  • Air (MEM), water, surface hygiene monitoring.
  • Bacterial spore resistance, longevity, and characteristics of ancient viable microbes
  • Microbial survival and growth in extreme environments
  • Applications in Planetary Protection, Astrobiology and Biological Defense
  • Microbial survival and growth in extreme environments
  • Atacama, Lake Vida, Greenland, Kilimanjaro
• Lessons for life detection
  • Limit for life vs limit of detection
  • Spatial and temporal heterogeneity
• Kilimanjaro Story
  • Add new carbon dates,
  • Add description of nearest clone matches
  • Add new pictures, popular culture Hemingway, Gore, Thompson
  • Impact of climate change on ecosystem,
  • Closest analog to ancient Martian lakes and Europa ocean on earth
    • Cold, dry, windy, and protected by only a thin atmosphere.
    • Catalog the bacteria and algae that live there
    • Look for extremophiles that can survive the harsh ultraviolet light at 20,000 feet
  • Science in situ versus return
    • Design science mission strategies for planetary exploration and search for life in the Solar Sy
• Add references to slides
• Add context of Mars exploration
  • Follow the water, special regions and habitability
  • Biomarkers to cells
  • Second origin or panspermia (lithoinoculation, discuss feasibility data for each step of transit)
### Analysis of the closest relatives on the basis of 16S rDNA sequences

<table>
<thead>
<tr>
<th>Phylogenetic affiliation</th>
<th>Clone</th>
<th>Top matches, source</th>
<th>GenBank accession no.</th>
<th>Nucleotide identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria, Γ-subclass</td>
<td>K323G02</td>
<td>un culturated gamma, Lake Tanganyika anoxic hypolimnion, Tanzania</td>
<td>DQ463708</td>
<td>98.69%</td>
</tr>
<tr>
<td>Proteobacteria, B-subclass</td>
<td>K711B09</td>
<td>un culturated bacterium, fresh snow at 6350m in the Everest region</td>
<td>EF190150</td>
<td>97.15%</td>
</tr>
<tr>
<td>Proteobacteria, A-subclass</td>
<td>K712B08</td>
<td>un culturated bacterium, indoor dust</td>
<td>AM697086</td>
<td>95.18%</td>
</tr>
<tr>
<td>Proteobacteria, A-subclass</td>
<td>K713B07</td>
<td>un culturated bacterium, surface sample from Gulkana glacier, Alaska</td>
<td>AB464938</td>
<td>97.88%</td>
</tr>
<tr>
<td>Proteobacteria, A-subclass</td>
<td>K713G11</td>
<td>un culturated bacterium, mattress dust</td>
<td>FM874375</td>
<td>99.23%</td>
</tr>
<tr>
<td>Proteobacteria, A-subclass</td>
<td>K713A05</td>
<td>Roseomonas aquatica, drinking water</td>
<td>AM231587</td>
<td>98.85%</td>
</tr>
<tr>
<td>Proteobacteria, A-subclass</td>
<td>K711A11</td>
<td>alphaproteobacterium 34619, hospital water</td>
<td>AF298301</td>
<td>99.09%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>K711A10</td>
<td>alphaproteobacterium 34619, hospital water</td>
<td>EU159489</td>
<td>98.63%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>K711A10</td>
<td>alphaproteobacterium 34619, hospital water</td>
<td>DQ404891</td>
<td>94.96%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>K711A10</td>
<td>alphaproteobacterium, contaminated sediments, Tennessee</td>
<td>DQ351728</td>
<td>95.58%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>K711A10</td>
<td>Flexibacteraceae bacterium VUG-C4, Victoria upper glacier, Antarctica</td>
<td>EU159489</td>
<td>95.37%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>K711A10</td>
<td>Bacteroides bacterium, soil from La Gorce mountains, Antarctica</td>
<td>EU159489</td>
<td>95.29%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K322A02/ K711C06</td>
<td>Cryobacterium sp. ZSI-15, marine sediments from the Antarctic Ocean</td>
<td>FJ889650</td>
<td>99.41% / 99.63%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K713E06</td>
<td>Cryobacterium sp. DR8, water and soil from Crater Lake, Antarctica</td>
<td>FJ464884</td>
<td>99.34% / 99.56%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K712C01</td>
<td>Antarctic bacterium, water and soil from Crater Lake, Antarctica</td>
<td>FJ464878</td>
<td>97.52%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K713H01</td>
<td>un culturated bacterium, surface sample from Gulkana glacier, Alaska</td>
<td>AB464893</td>
<td>99.49%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K713H01</td>
<td>Antarctic bacterium, Collins glacier, Antarctica</td>
<td>EU636015</td>
<td>99.53%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K713H01</td>
<td>Cryobacterium sp. ZSI-15, marine sediments from the Antarctic Ocean</td>
<td>FJ889650</td>
<td>97.79%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K712A07</td>
<td>Cryobacterium sp. DR8, water and soil from Crater Lake, Antarctica</td>
<td>FJ464878</td>
<td>97.71%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K712A07</td>
<td>un culturated actinobacterium, vicinity of a uranium mine, Bulgaria</td>
<td>FM877676</td>
<td>97.61%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K712A07</td>
<td>un culturated actinobacterium, soil from the Atacama Desert, Chile</td>
<td>EF016798</td>
<td>95.88%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>K711E08</td>
<td>Aeromicrobium panacelticum, soil from a ginseng field, South Korea</td>
<td>AB245387</td>
<td>97.55%</td>
</tr>
</tbody>
</table>

*Top matches as determined by the BLAST method using the GenBank database [Altschul, S.F., 1990]*

* Nucleotide identity as determined by the sequence similarity function in ARB.
### Phase Contrast
- Refractive index
- Spores: phase bright
- Vegetative cells: phase dark
- $0 - 10^2$ spores/mL

### Malachite Green
- Uptake of stain
- Spores: Green
- Vegetative cells: Pink
- Presence of spores
- Presence of dehydrogenase activity and membrane potential upon incubation with TSB

### SYTO-9/PI
- Membrane integrity
- Live cells: Green
- Dead cells: Red
- $<0.01$ live cells/mL
- $<0.1$ cells/mL

### Tetrazolium Chloride
- Respiratory potential
- Dehydrogenase activity: Red
- Active cells: Green

### DiBAC$_4$(3)
- Membrane potential
- Active cells: Green

---

Kilimanjaro samples incubated with 10 mM L-alanine and AGFK at 37°C
Distance from surface (cm)

Phase bright bodies per mL of glacier ice

Kilimanjaro Glacial Ice Endospore Concentrations
Kilimanjaro Glacial Ice Endospore Concentrations

Distance from surface (cm)

Malachite green stained cells per mL of glacier ice
Summary of Ligand-Induced Effects

• Ligand Enhancement
  – Binding affinity always increases when ligand is bound
  – Independent of ligand/analyte charge, denticity
  – Due to polarizing effect of ligand!

• Induced Gadolinium Break
  – Ionization energy of Ln$^{3+}$ determines extent of Å-environment
  – Effect of ligand on lanthanide is governed by Ln$^{3+}$ polarizability!
  – Terbium is most affected due to half-shell effect!

These effects can be used to optimize lanthanide receptor site design!
Eu(DO2A)(DPA)$^-$ + Tb(DO2A)$^+$ → Eu(DO2A)$^+$ + Tb(DO2A)(DPA)$^-$

The Terbium Advantage
**Tb-DPA Luminescence**

**Anthrax Smoke Detector**
