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EARLY RESULTS OF ECOPOESIS EXPERIMENTS IN THE SHOT MARTIAN ENVIRONMENT SIMULATOR

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ABSTRACT

Humanity is on the verge of having the capability of constructively directing environmental changes on a planetary scale. One could argue that we are making these changes on Earth today, but in a negative manner. Within the foreseeable future, we will have the technology to modify Mars' environment, and make it a habitable planet. However, we do not have enough information to determine the course of such an event. SHOT has designed and built a test-bed apparatus that can replicate most of Mars' environment conditions (with the notable exceptions of gravity and cosmic radiation) within a 5.6 liter chamber. Here, we present the results of initial experiments to determine the suitability of specific microorganisms as pioneering life-forms for Mars. Included among the potential pioneers were five genera of cyanobacteria (*Anabaena*, *Chroococcidiopsis*, *Plectonema*, *Synechococcus* and *Syenechocystis*), and three partially-characterized eubacterial strains that were isolated from Chile's Atacama Desert (two species of *Bacillus* and *Klebsiella oxytoca*). During these initial trials, we used a present-day mix of martian atmsospheric gases, but at a pressure of 100 mbar (10 times Mars's current atmospheric pressure). Organisms were inoculated into samples of JSC Mars-1 soil stimulant and exposed to full-spectrum simulated martian sunlight. Day/night temperature cycled from 26°C to -80°C and back. Experiments included a 24-hour, brief-exposure trial, a 7-day trial, a14-day trial and a 5-week trial to determine the survival and growth of our potential martian pioneers.

Abbreviations: CCMEE, Culture Collection for Microorganisms from Extreme Environments; PAR, photosynthetically active radiation; PCC, Pasteur Culture Collection; SHOT, Space Hardware Optimization Technology; UTEX, University of Texas Culture Collection.

INTRODUCTION

The idea of planetary engineering—also known as ecopoeisis or terraformation—has been with us for over 25 years. Planetary engineering research has been largely a collection of speculations, hypotheses and mathematical models with little in the way of experimentation. Results from a few experiments that tested very narrow aspects of planetary engineering have been published, but facilities to test ecological relationships were lacking. Earlier this year, SHOT, Inc. completed construction of a Mars simulator (Figure 1) that was designed specifically for ecopoeisis research (described elsewhere in this volume).

Other Mars simulators exist, but they are used primarily for simulating Mars' present-day environment. These have been used to investigate the survival of terrestrial organisms in the context of forward contamination and in potentially favorable martian microenvironments. However, in ecopoesis research, we assume that non-biological processes can be used to make initial changes to Mars environment (e.g., increase the atmospheric pressure to 25 mbar or more). Afterward, pioneer organisms could be "inoculated" onto Mars to continue planetary modification. Pioneer organisms must be able to grow and proliferate—not just survive—in order to bring about additional changes to the environment. In short, the goal of planetary engineering is to compress billions of years of planetary evolution into a few millennia.

The project that we describe here assesses the abilities of potential pioneer organisms to survive and grow in an environment that simulates Mars as it might be after initial engineering efforts. The atmospheric pressure is set at 100 mbar, approximately 10 times Mars' current pressure. A temperature range of -80°C to 26°C mimics Mars' equatorial region (9). Actually, this temperature regime may be more severe than what would be expected with a 100 mbar atmospheric pressure, but it provides a good baseline. In order to overcome personnel difficulties, we elected to use an Earth-standard 24-hour day instead of the slightly longer martian day. Initial results indicate many species of terrestrial bacteria can survive in this environment, but longer experiments will be necessary to determine growth rates and community structures.

MICROORGANISMS

Initial test organisms were chosen for specific phenotypes that allow them to survive in relatively harsh environments—cold tolerance, CO_2 tolerance, desiccation resistance, hypoxia tolerance, spore formation, etc. Additionally, these organisms possessed the ability to change the environment in ways that would be beneficial to planetary engineering efforts: photosynthesis (CO_2 removal and O_2 production), denitrification (N_2 production), pigment production (albedo reduction, UV shielding), etc. None of the organisms tested possessed all of these characteristics, but most possessed at least two.

Atacama Desert Microorganisms: *Bacillus* and *Klebsiella*

The Atacama Desert is one of the driest environments on Earth. As such, it has been proposed as a martian analog environment. Because of the extreme aridity, Atacama soils are often highly enriched in nitrates. On Mars, large nitrate reserves could be denitrified to provide N_2 for the

atmosphere. Viable microorganisms from the Atacama Desert would be resistant to long periods of desiccation and may also possess mechanisms for UV resistance.

We were specifically interested in potential denitrifying bacteria that might survive in the desert environment. Samples from the uppermost 5 cm of soil were aseptically collected during ongoing expeditions from NASA's Ames Research Center during the period between 1998 and 2004. Selected samples were sent to Lyon College for microbiological analysis. 0.5 g to 2.0 g subsamples were suspended in either 0.9% NaCl or denitrification-fluorescence (D-F) broth. After 24 hours at room temperature, aliquots of the suspensions were streaked onto D-F agar and incubated at 25°C for 24-48 hours. Isolated colonies were inoculated onto D-F agar slants for short-term storage. Isolates were Gram stained and subjected to standard physiological tests (21), which identified two of the strains as species of *Bacillus* and one as a member of the Enterobacteriaceae family. DNA extractions were performed (10) for PCR analysis of 5s rRNA gene sequences in order to identify each species.

Polymerase chain reaction was performed using primers designed to amplify a portion of the 16S rRNA gene from prokaryotic sources (4). The primers were SSU5'Mtag: CGAGGAAACAGCTATGACCATTAGATACCC and SSU3'Mtag: TGTAAAACGACGGCCAGTTTGTTACGACTT. A two-step PCR protocol using a proof-reading DNA polymerase (Pfusion, Biorad Inc) was utilized. This consisted of 5 cycles of 98° for 30 seconds, 50°C for 30 seconds, and 30 seconds at 72°C followed by 30 cycles of 98° for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Amplification reactions were electrophoresed on a 1.2% agarose gel. Bands of the predicted size (802 base-pairs) were excised from the gel, purified using a commercially available kit (Geneclean Turbo, QBiogene Inc.), and ligated into the pBluescript KS^+ vector which had been cut with restriction enzyme SmaI. The ligation mixture was transformed into *E. coli* DH5α, and resulting white colonies were screened for the presence of inserts by restriction enzyme digestion. DNA sequencing reactions for each independent clone were performed using T3 and T7 primers. The DNA sequences obtained were used to search the Genbank DNA sequence database using the BLAST program (3).

From the sequence data, we were able to identify three of our Atacama isolates. Isolate "Yungay-2" was identified as *Bacillus mojavensis*, *B. subtlis* or *B. licheniformis* (these species are *very* closely related). Isolate "Rock Garden-2" (RG-2) was identified as *Bacillus licheniformis*, and isolate "Atacama 2002-1" was identified as *Klebsiella oxytoca*. Physiologically, the other "Rock Garden" strains (1, 3 and 4) are identical to Rock Garden-2, and are probably also strains of *Bacillus licheniformis*. Due to the shallow depth of the soil samples, we have no way of knowing whether these isolates were preserved "native" bacteria, wind-blown "transient" bacteria or human-derived contaminants. Both *Bacillus* and *Klebsiella* commonly inhabit humans as normal microflora.

Cyanobacteria: *Anabaena*, *Chroococcidiopsis*, *Plectonema*, *Synechococcus* and *Synechocystis*

Members of the genus, *Chroococcidiopsis*, have been isolated from hot and cold deserts (16), and are often the sole autotrophic organisms in these environments. Many strains these

cyanobacteria live in endolithic habitats, which offer increased UV protection, water retention, and thermal moderation. Because of their ability to survive in very arid environments, strains of *Chroococcidiopsis* have been suggested as potential pioneer martian organisms (17). Several strains of *Chroococcidiopsis* were obtained from the Culture Collection of Microorganisms from Extreme Environments (CCMEE, University of Oregon, Eugene), two of which were used in these experiments: CCMEE 171 and CCMEE 662.

In a previous project, four common freshwater cyanobacteria, *Anabaena* sp., *Plectonema Boryanum* UTEX485, *Synechococcus* sp. PCC7942 and *Synechocystis* sp. PCC6803, were grown in very high CO_2 atmospheres (29). Of these, *Anabaena* and *Synechococcus* survived in 100% CO_2, and *Plectonema* actively grew in 100% CO_2. Due to their ranges of CO_2 tolerance (poor to good), all four of these cyanobacteria were tested in the SHOT simulator.

Cave and Desert Varnish Autotrophs

In prior and ongoing work on the geomicrobiological communities in caves, mines, lava tubes and surface rock coatings, we have noticed a wide variety of chemical transformations and other behaviors that might be useful in the ecopoiesis context (7). These include the ability to precipitate minerals on rock surfaces to darken albedo, ability to fix CO_2 as organic carbon using inorganic compounds in the form of gases or mineral sources, and photosynthetic organisms that are adapted to osmotically challenging endolithic (within rocks), chasmolithic (in fractures), sublithic (beneath rocks), or cryptogamic soil environments that hold desert pavements together with algal filaments and fungal hyphae. With these considerations in mind, we have chosen desert microorganisms and cave microorganisms from a variety of sites that we have previously characterized (5, 6, 22, 24, 25, 28). These include Fe/Mn oxidizing bacteria both from speleosols (soils formed in caves, (28)) and surface desert varnish, gypsum fracture-inhabiting cyanobacteria, lavatube wall microorganisms, and organisms that metabolize copper sulfides to copper oxides. Many of these isolates have been partially characterized, but have not been identified (Table I).

The organisms that inhabit desert varnish rock coatings are adapted to extremes of temperature, both diurnally and seasonally, and capable of dealing with high intensity ultraviolet radiation. Of course, the ultraviolet environment of Mars involves shorter wavelengths and higher intensities than anything experienced on Earth (11, 14), nevertheless it is useful to investigate these organisms as potential future Martian candidates. In contrast, the Fe/Mn oxidizing counterparts in caves are likely to be more susceptible to UV than typical surface organisms and we have included them in our experiments as a test of that notion. The gypsophilic or gypsotolerant cyanobacteria from evaporite outcrop fractures have been included because of their relevance to reports of sulfates at the MER Gusev Crater site and from the Mars Express OMEGA experiment (18, 19). Since the Mariner missions of the early 1970's (13, 15), Mars has been known to be well endowed with volcanic landforms of many sorts so that organisms capable of colonizing such surfaces are of significant interest. The actinomycete and streptomycete dominated communities isolated from lava tube surfaces in northwestern NM and manganese rich biotic crusts from andesite flows in central NM have been chosen because of their ability to exist in volcanic terrain and their mineral-precipitating abilities.

METHODS

Environmental Conditions

The pressure within the simulator was held at 10 kPa (0.1 atm). Depressurization and repressurization at the beginning and end of each experiment occurred over a two hour period. Temperature was cycled from 26° to -80°C and back over a 24-hour period (Figure 2), simulating Mars at low latitudes during the vernal equinox (9). For experiments less than 14 days in duration, a mix of gases proportional to present-day Mars was used: 95% CO_2, 2.7% N_2, 1.6% Ar, 0.13% O_2. 100% CO_2 was used for longer duration experiments. Microbial cultures were inoculated into JSC Mars-1 regolith simulant (2). Full spectrum illumination was provided by a xenon arc lamp fitted with a solar filter. Approximately 50% of the sample chamber received direct illumination (1000 μmol photons m^{-2} s^{-1} PAR), while the remainder received indirect illumination (10-15 μmol photons m^{-2} s^{-1} PAR). Water was added as needed (approximately 1 mL per day) to keep the atmosphere saturated with moisture.

Culture Conditions

Stock cultures of cyanobacteria were maintained on BG-11 agar slants at 25°C, 20-50 μmol photons m^{-2} s^{-1} PAR. Approximately 1-2 weeks before each experiment, subcultures were transferred to liquid BG-11 medium and grown to $A_{720} > 0.25$. Stock cultures of heterotrophic bacteria (Atacama strains) were maintained on trypticase soy agar slants at 25°C. One to three days prior to each experiment, subcultures were transferred to trypticase soy broth, grown to stationary phase, and then diluted to $A_{720} = 0.25$. Individual liquid cultures or mixtures of cultures were added to JSC Mars-1 to the point of saturation. Desert varnish and cave microorganisms were grown on BG-11 agar. Agar cultures were than macerated and mixed with JSC Mars-1.

Bioassays

During experiments, duplicate control samples were kept in the dark at 4ºC. After each experiment, subsamples from control and experimental groups were analyzed for bacterial survival and growth via plate counts (12), trypan-blue live-dead stains (27), fluorescein diacetate (FDA) hydrolysis assays (1, 26) and chlorophyll extractions (8, 23).

RESULTS

Trial 1: Overnight

Six strains of heterotrophic bacteria and five strains of cyanobacteria were inoculated into individual wells of 48-well tissue culture plates. Each well contained one gram of JSC Mars-1 simulant, and enough bacterial suspension was added to saturate the stimulant (approximately on milliliter). Duplicate plates were prepared. One was uncovered and exposed to full spectrum light; the other was covered and placed in the shadowed portion of the simulator.

After approximately 18 hours, the samples were removed and tested for viability. Results of the FDA hydrolysis assays and chlorophyll extractions are shown in Figures 3 and 4, respectively. All of the organisms tested showed increased FDA hydrolysis in direct light. Most of the strains also exhibited increased FDA hydrolysis under indirect light conditions. The chlorophyll extracts showed an increase in chlorophyll production with the notable exception of *Chroococcidiopsis* 171, which lost chlorophyll in the direct exposure sample.

Live-dead microscopic assays and plate counts also showed survival of all of the organisms tested. However, because of the small sizes of the cells tested, the live-dead assays were prone to large errors. Non-cellular material was sometimes counted as cells, and some cells were mis-counted as debris. Plate counts of cyanobacteria took 1-2 weeks in order to grow countable colonies. Also, *Plectonema* and *Anabaena*, being filamentous, gave underestimated results from plate counts. We decided to discontinue both the live-dead stain and plate counts in favor of the chlorophyll extracts and FDA tests.

At the end of this experiment (and in subsequent experiments), we noticed that water condensed inside the quartz cylinder at the shadowed end. In effect, we had a miniature water cycle occurring as moisture evaporated from the directly lit end condensed at the shadowed end. Some of this condensate fell into the sample containers, but the majority pooled at the bottom of the cylinder.

Trial 2: 14 days

As soon as the overnight trial was finished, a new experiment with the same samples was placed into the simulator for a 14-day experiment. This trial also used multi-well plates to hold the samples. During the course of this experiment, we found that while the multi-well plates were good for holding many samples at once, it was difficult to remove samples without cross-contaminating nearby wells.

As Figures 5 and 6 show, the survival rates after two weeks were approximately the same as in the overnight experiment. However, the control groups had higher FDA activities, which make the experimental results appear lower at first glance. All of the samples lost extractable chlorophyll over the 14-day period. Among all of the strains, *Synechocystis* had surprisingly high FDA hydrolysis activity. However, this may be due to contamination (as previously noted). The chlorophyll data do not corroborate the FDA data. Also, *Synechocystis* has previously been shown to have low CO_2 tolerance (29).

Trial 3: 7 days

The purpose of this trial was to determine the suitability of 25 mL polypropylene jars as sample holders (Figure 1). As noted previously, cross-contamination problems limited the usefulness of multi-well sample plates. This trial included mixed cultures of autotrophs and heterotrophs as attempts to form simple, artificial communities. We also included garden soil samples from the grounds of the SHOT facility.

As we observed in the 14-day trial, most of the samples had decreased FDA hydrolytic activity as compared to controls (Figure 7). Notable exceptions were the garden soil from SHOT, and the first three "community" mixtures of autotrophs and heterotrophs. However, the chlorophyll assays indicate that most of the activity in these communities is due to the heterotrophs (Figure 8). From an operational point of view, the polypropylene jars worked much better than the multi-well plates.

Trial 4: 5 weeks

This was the first trial to include desert varnish microorganisms. This experiment also continued the use of artificial communities. In this instance, an artificial soil was created by mixing JSC Mars-1 with approximately equal amounts of *Anabaena* sp., *Bacillus* sp. ("Yungay-2"), *Bacillus licheniformis* ("Rock Garden-2"), *Chroococcidiopsis* strains CCMEE171 and 662, *Klebsiella oxytoca* ("Atacama 2002-1") and *Plectonema boryanum*. The mixture was then spread within the inverted cover of a multi-well plate. After exposure to simulated martian conditions, the "soil" was sampled in multiple areas and analyzed in much the same manner as we would test a terrestrial soil sample. Unlike the previous experiments—in which we used a gas mixture that closely mimicked Mars' atmospheric composition—the atmosphere for this experiment was 100% CO_2.

After five weeks, all regions of the simulated soil had markedly decreased activity as compared to the controls (Figure 9). However, several of the desert varnish microbes retained substantial FDA hydrolysis activity (Figure 10). (Unfortunately, much of the chlorophyll data were lost when DJT's computer was stolen.)

DISCUSSION

Although we generated some useful data, the primary purpose of these experiments was to work out the methodological and logistical problems with the simulator. Our experiments have shown that many microorganisms survive and grow under short-term exposure, but none of the microbes tested exhibited growth during exposures longer than two weeks. While these results are discouraging, they do not preclude the possibility of ecopoesis.

Throughout these experiments, the only environmental variable that was significantly changed was the exposure time. The temperature regime modeled present-day Mars, not that of Mars with a thicker atmosphere. Also, the organisms tested were not necessarily the most desiccation- or UV-resistant. Screening of additional pioneer microorganisms is ongoing. Additionally, longer-term experiments may allow the artificial selection and directed evolution of better-adapted microorganisms. Genetic manipulation of candidate organisms may improve the survivability of naturally-occurring strains (20). Genetic engineering experiments specifically in the context of ecopoesis are currently underway.

Aside from future planetary engineering efforts, this research has particular significance for planetary protection. We have shown that at least a fraction of the organisms tested can survive under near-martian conditions for at least five weeks. In UV-shielded areas—such as the inside of a lander—could prolong the period of viability. Especially in the context of astrobiology and

the search for life, we must make certain that we don't carry viable microorganisms to Mars (or any other potentially habitable world). Otherwise, we will not be able to discern between native and contaminant microorganisms.

From an educational viewpoint, ecopoeisis provides an exciting opportunity to engage students in science. While many students have heard of terraforming in *Star Trek* and other science fiction stories, they usually don't realize that serious science underlies the fiction. Ecopoesis can be used as a focal point for discussions of geology, environmental science, microbiology, ecology and other disciplines to show the interdisciplinary nature of planetary science—whether that planet is Earth, Mars or one that hasn't been discovered yet.

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FIGURES

Figure 1. The SHOT Mars simulator. Samples are contained within the 6 liter quartz cylinder. Simulated sunlight is reflected onto the samples via a movable, front-surface mirror. The xenon-arc light source is connected at the left side of the simulator (not shown).

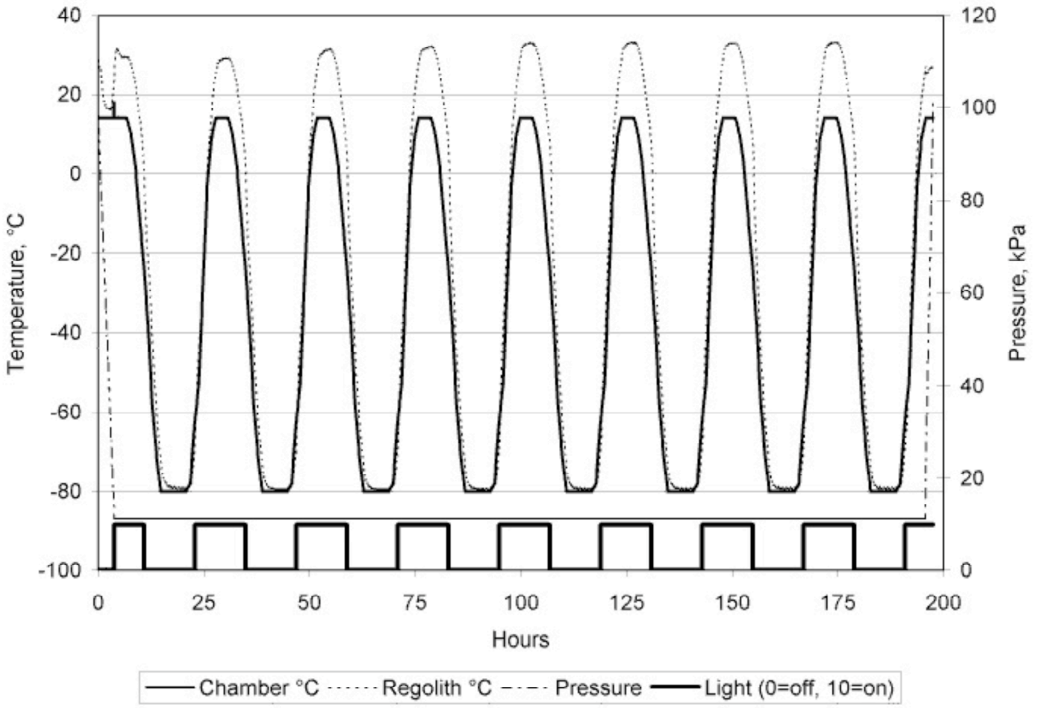

Figure 2. Environmental conditions within the Mars simulator. These data were taken from the 7-day experiment, but are representative of all trials. The simulated environment closely follows the model proposed for Mars during the vernal equinox (9).

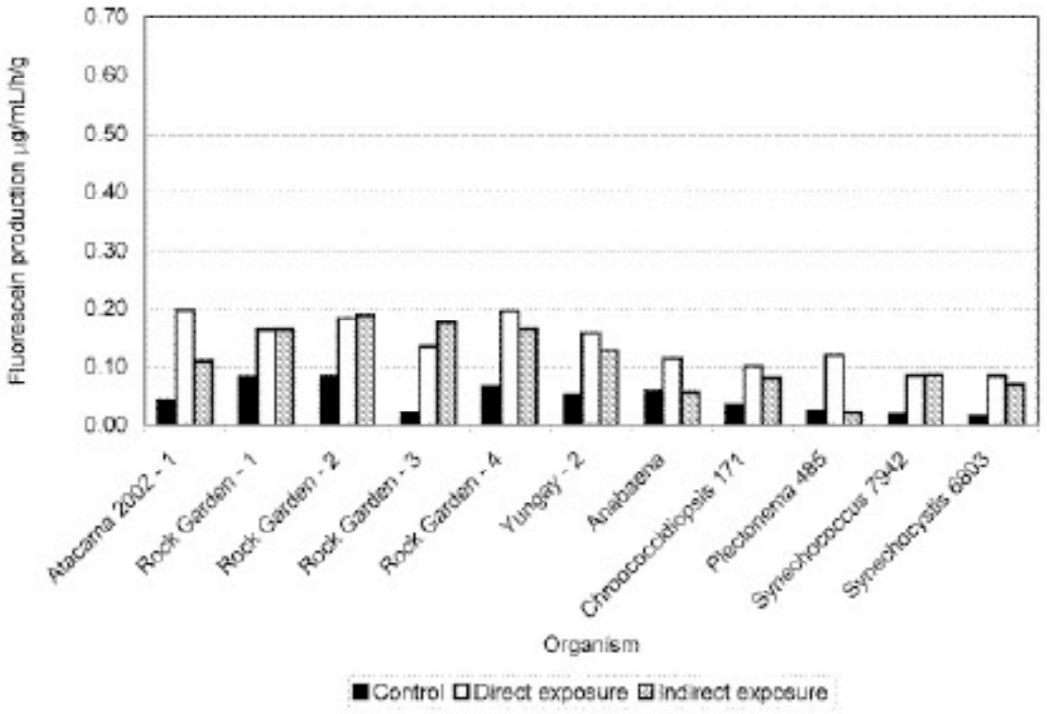

Figure 3. FDA hydrolysis, overnight trial. Survival was generally good, and most organisms showed increased activity (presumably due to growth). Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

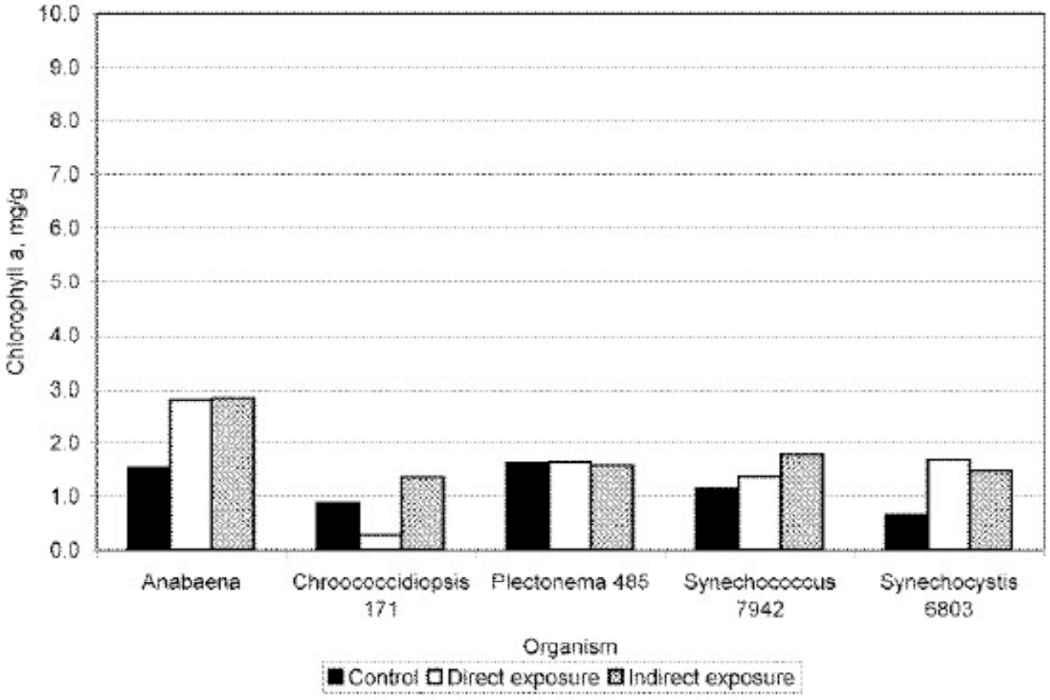

Figure 4. Chlorophyll extraction, overnight trial. Chlorophyll assay results are roughly correlated with FDA hydrolysis. The notable exception was *Chroococcidiopsis* CCMEE171, which had low extractable chlorophyll, but high FDA hydrolysis activity. Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

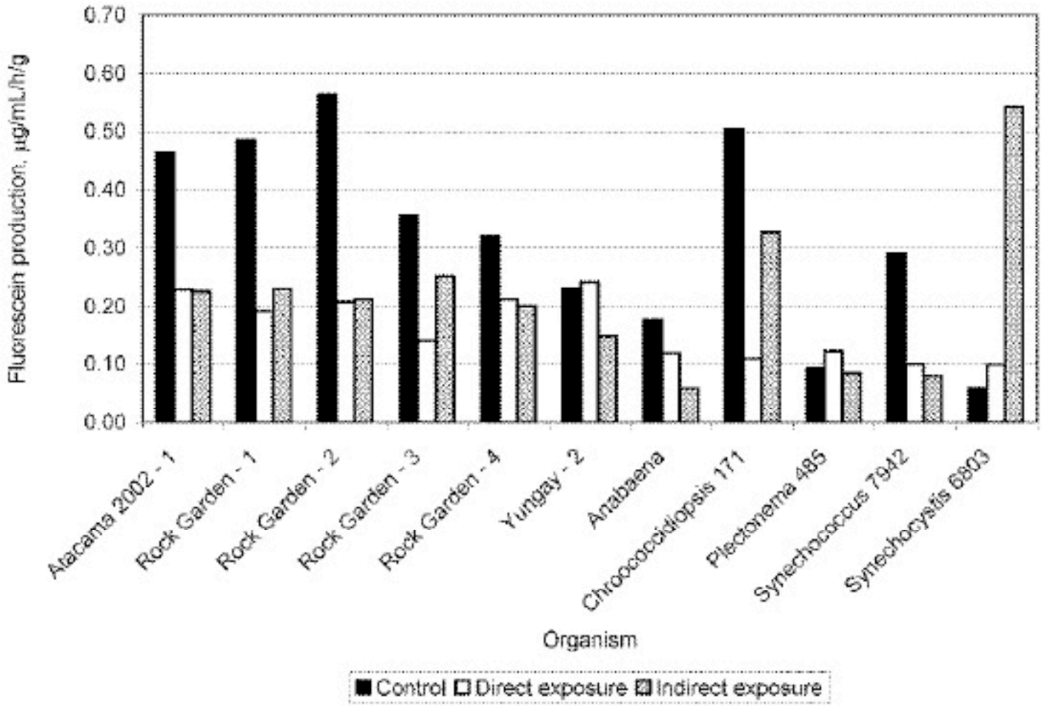

Figure 5. FDA hydrolysis, 14-day trial. With the exception of *Plectonema* and *Synechocystis*, none of the microorganisms showed as much activity as the control groups. *Plectonema* has the highest known CO_2 tolerance of the cyanobacteria tested. Previous experiments have shown that *Synechocystis* has low tolerance to CO_2, which casts some doubt on the high activity shown here. Because of difficulties with removing the samples from their containers, the *Synechocystis* results may be due to cross-contamination with other samples. Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

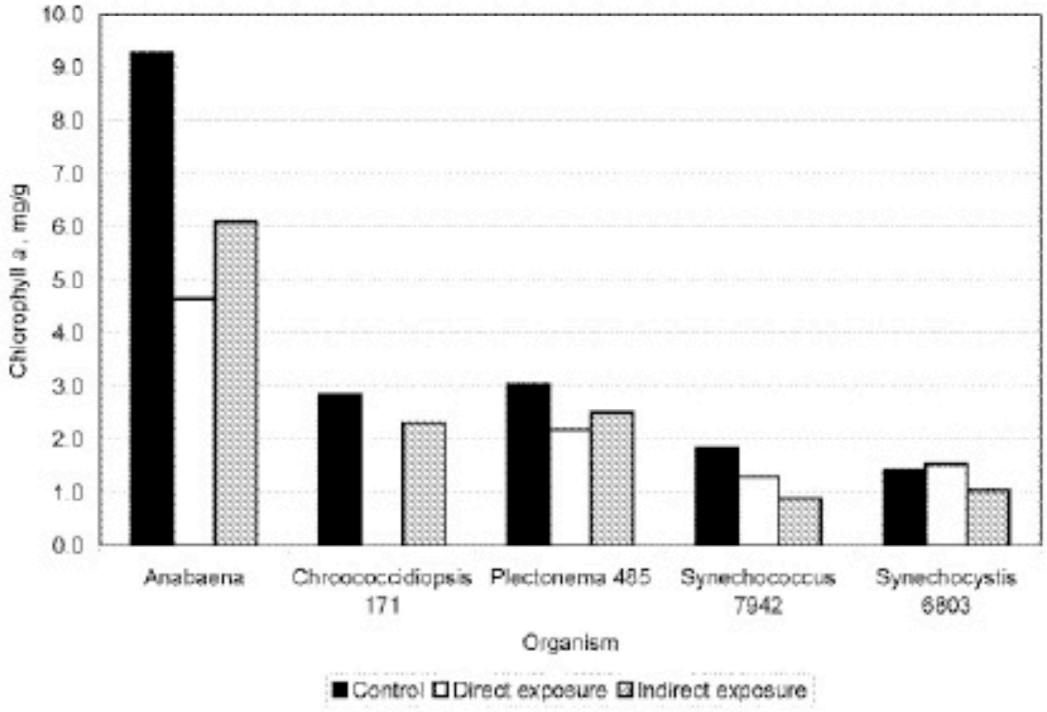

Figure 6. Chlorophyll extraction, 14-day trial. Extractable chlorophyll results are generally consistent with FDA hydrolysis data. These data for *Synechocystis* are more consistent with previous experiments than FDA hydrolysis results. Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

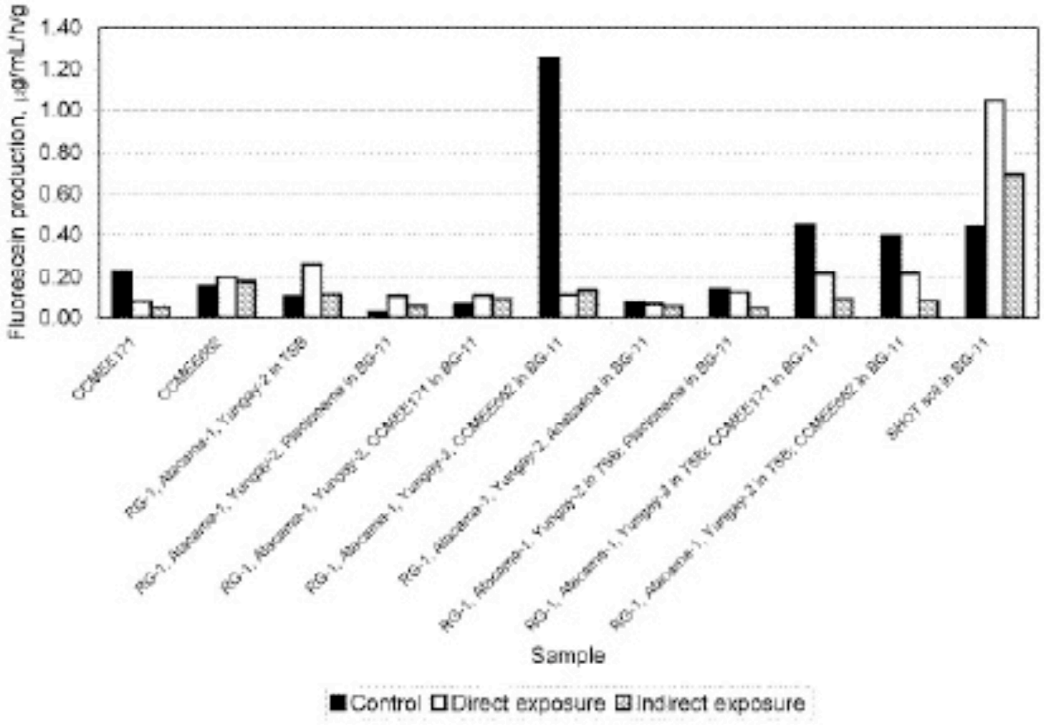

Figure 7. FDA hydrolysis, 7-day trial. This experiment included both single and mixed cultures. Overall, the best results were obtained from the garden soil obtained from around the SHOT facility. Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

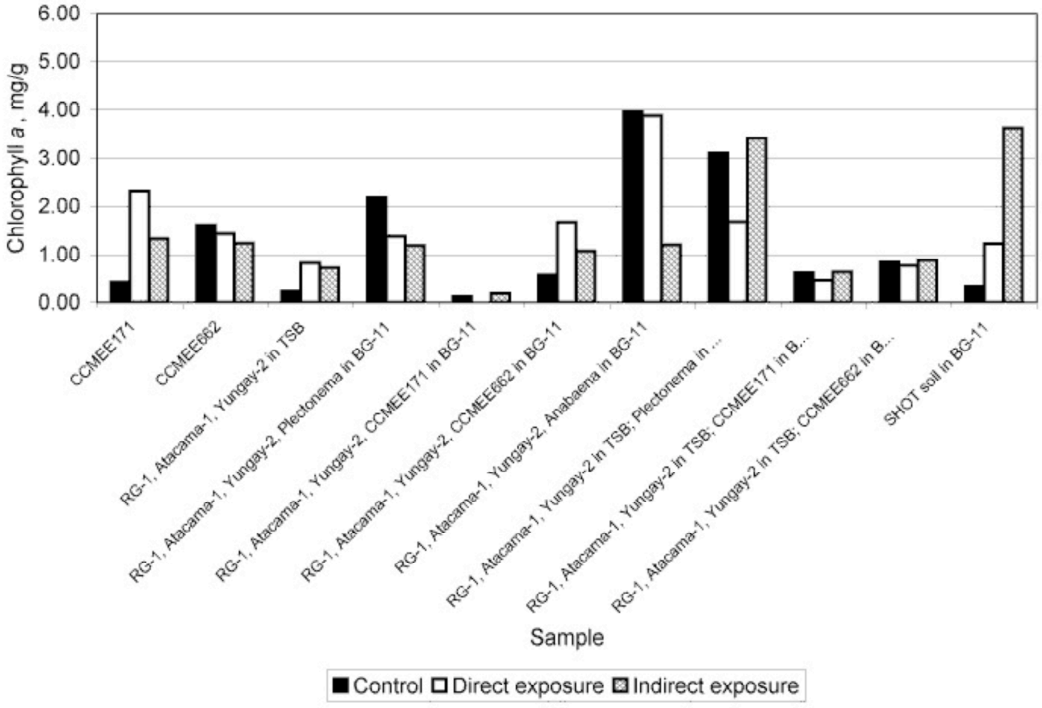

Figure 8. Chlorophyll extraction, 7-day trial. This experiment included both single and mixed cultures. These results provide some indication as to the amount of FDA activity shown in Figure 7 that was due to the cyanobacterial component of the mixed cultures. Each bar represents a single sample; results are normalized to 1 gram of dry soil simulant.

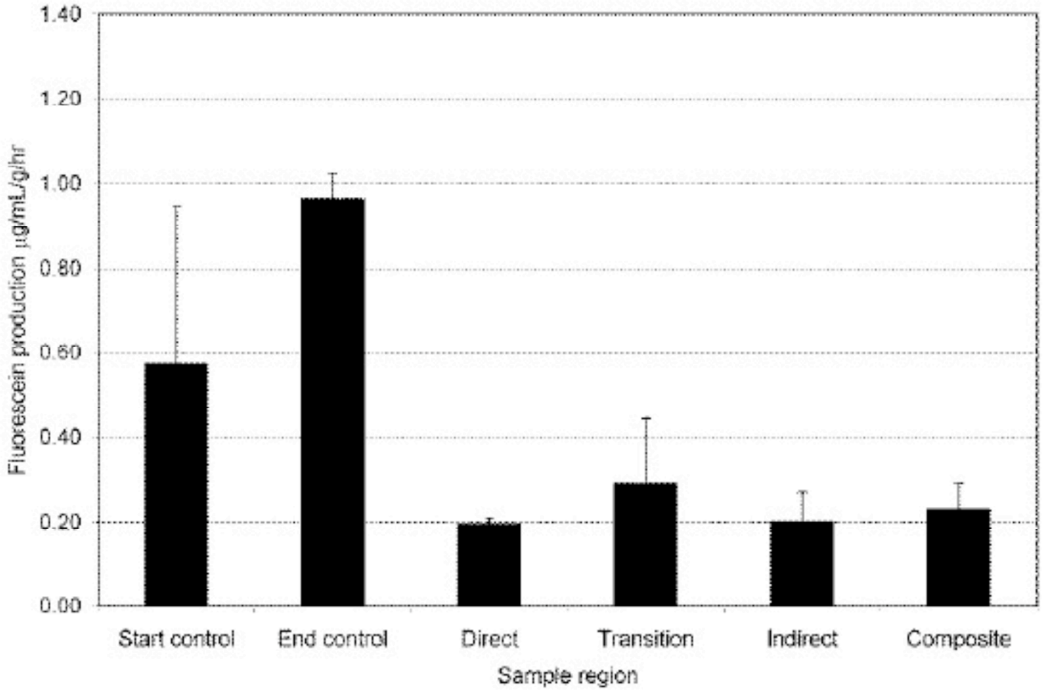

Figure 9. FDA hydrolysis, 5-week trial, cyanobacteria and Atacama strains. Unlike the previous experiments, this was a single tray of soil stimulant with a mixture of cyanobacteria and heterotrophic bacteria. Triplicate samples were taken from each region (bars = s.d.). "Composite" refers leftover material within the tray that was mixed before testing. Results are normalized to 1 gram of dry soil stimulant.

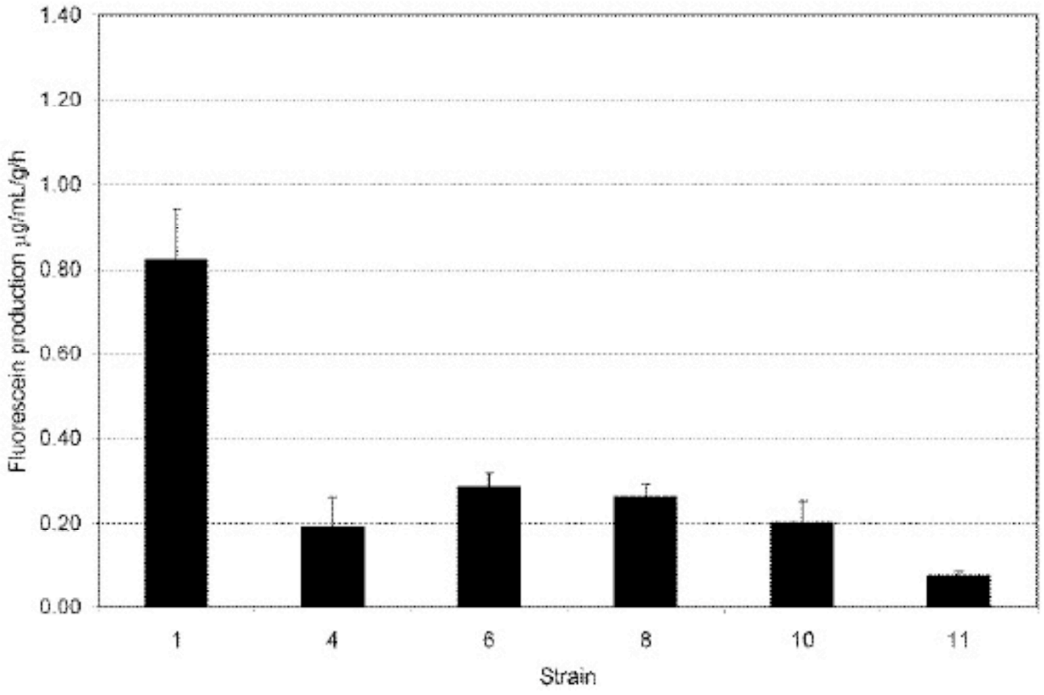

Figure 10. FDA hydrolysis, 5-week trial, cave and desert varnish strains. These samples were exposed in individual containers alongside the simulated soil of Figure 9. Most of these organisms have not been identified. Descriptions are provided in Table I. Triplicate samples were taken from each container (bars = s.d.); results are normalized to 1 gram of dry soil stimulant.

Table I. Partially-characterized microorganisms from caves and desert varnishes.

Strain #, Organism	Origin	Media	Comments
1, *Pedomicrobium manganicum*	Desert varnish, Hanksville, UT	Mn/Fe acetate Mn low carbon Mn no carbon	Mn oxidizer, also other metals are oxidized (e.g., gold, iron).
4, DVLL 04-02-02	Luis Lopez Desert varnish, Socorro, NM	Mn/Fe acetate Mn low carbon Mn no carbon	Produces black surface crystals.
6, EF 06-12-14	Lechuguilla Cave, Carlsbad Caverns National Park, NM	1/2 R2A *Hyphomicrobium* medium *Pseudomonas* medium	Small cells with long filamentous processes found in the de-cemented, punky bedrock.
8, FW 06-02-09	Four Windows Lavatube, El Malpais National Monument, NM	Actinomycete medium Oatmeal agar Basalt medium	Actinomycete that precipitates calcite crystals on basaltic lava tube surfaces.
10, PA 01-01-01	Pahoehoe Lavatube, El Malpais National Monument, NM	Actinomycete medium Oatmeal agar Basalt medium	Possibly involved in precipitation of cave "moonmilk" calcite deposits.
11, Chalc-2	Native on copper sulfides	Chalcocite rock dust agar with or without carbon source (1/2 R2A or other)	Live on sulfides and sequester Cu as oxides.