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Effects of atmospheric pressure on the survival of photosynthetic microorganisms during simulations of ecopoesis

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Abstract: Three cyanobacteria (*Anabaena* sp., *Plectonema boryanum* and *Chroococcidiopsis* CCMEE171) and an alga (*Chlorella ellipsoidea*) were grown under simulated martian ecopoesis conditions. A xenon arc lamp with a solar filter provided simulated martian sunlight, and temperature cycled diurnally from −80 °C to 26 °C. A Mars-like atmosphere of 100% CO_2 was provided at 50, 100, 300, 500 and 1000 mbar. The cyanobacteria and alga were inoculated into JSC Mars-1 soil simulant and exposed to each atmospheric pressure for five weeks. Survival and growth were determined via extractable chlorophyll *a* and total esterase (fluorescein diacetate hydrolysis) activity. Maximum survival occurred at 100 and 300 mbar. At 50, 500 and 1000 mbar, esterase activity was near zero, and extractable chlorophyll *a* was less than 10% of control samples. Overall, the cyanobacteria survived better than the alga. Low survival at 50 mbar was probably due to desiccation. Low survival at 500 and 1000 mbar may have been due to CO_2 toxicity.

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Key words: algae, cyanobacteria, ecopoesis, esterase activity, hypobaria, Mars, planetary engineering, terraformation.

Introduction

Currently, life as we know it cannot inhabit the surface of Mars. The combination of low temperature, low pressure, high ultraviolet flux and extreme aridity would kill the hardiest organisms on Earth. However, in the future, humanity might alter Mars' environment to make it more habitable (Averner & Macelroy 1976; McKay 1982; McKay *et al.* 1991; Haynes & McKay 1992; Thomas 1995; McKay 1998; McKay 1999; Graham 2004). Briefly summarized, after initial geophysical modifications that would allow liquid water to exist on the surface of Mars, microorganisms could be introduced (ecopoesis). Photosynthesis and denitrification would slowly convert Mars' CO_2 atmosphere to an O_2/N_2 atmosphere similar to that of Earth (terraformation). Assuming that sufficient reserves of N and other necessary nutrients are available, more complex organisms – plants, animals, etc. – could be introduced to Mars.

Initial experiments of 1 to 14 days duration indicated that a variety of autotrophic and heterotrophic bacteria could survive under simulated ecopoesis conditions of pure CO_2 at 100 mbar pressure and a diurnal temperature cycle of −80 °C to 26 °C (Thomas *et al.* 2006a; Thomas *et al.*, accepted). Here we report on the survival of cyanobacteria and algae during simulations of ecopoesis where the atmospheric pressure was varied from 50 mbar to 1000 mbar.

Materials and methods

Mars simulator

All experiments were performed in a Mars environment simulator (Thomas *et al.* 2006b) at the facilities of Techshot, Inc. in Greenville, Indiana, USA (Fig. 1). For logistical reasons, Earth-normal 24-hour days were used in place of Mars days ('sols'). Diurnal temperature ranged between −80 °C and 26 °C (Fig. 2), which was similar to Mars' equatorial climate during the vernal equinox (Carr 1996). Temperature was computer-controlled with a combination of electric heat and gaseous nitrogen refrigeration. Illumination was provided by a xenon arc lamp (Sylvania 69263-0 Short Arc Lamp, XBO, 1000 W/HS OFR) fitted with a solar filter that provided a close approximation of solar radiation. Photosynthetically active radiation (PAR) at sample level ranged from 15 μmol photons m^{-2} s^{-1} in the shaded region to 1000 μmol photons m^{-2} s^{-1} in direct light (Fig. 1). Total ultraviolet radiation (250–400 nm) was 1.7 μmol photons m^{-2} s^{-1} in the shaded region and 50 μmol photons m^{-2} s^{-1} in direct light. An atmosphere of pure CO_2 was used in all experiments. Atmospheric pressure was decreased within the sample container over a period of 2 hours. The same period of time was used at the end of each experiment to bring the pressure back to ambient. Five atmospheric pressures were used: 50, 100, 300, 500 and 1000 mbar. Up to 1 ml of water

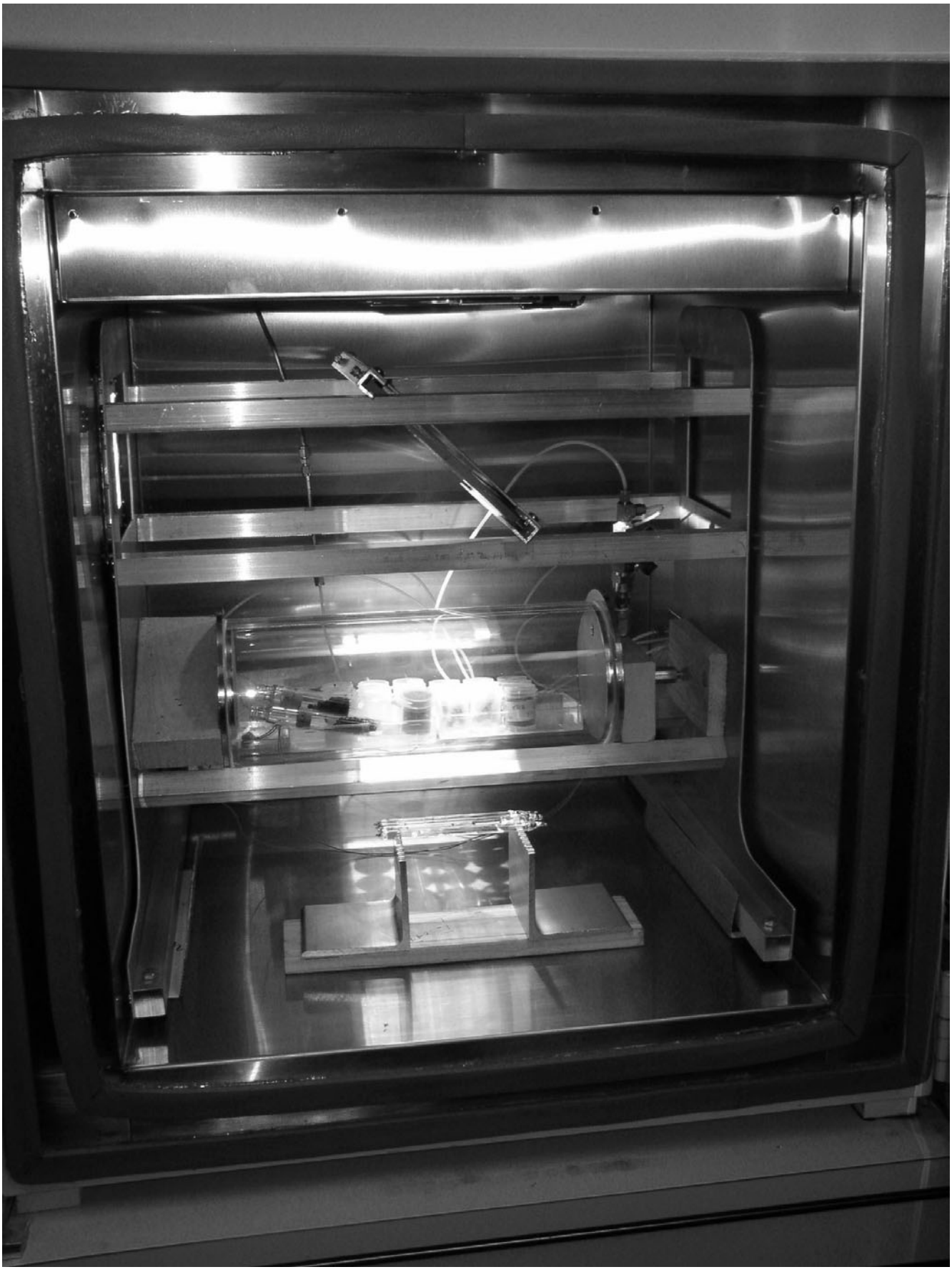

Fig. 1. For legend see opposite page.

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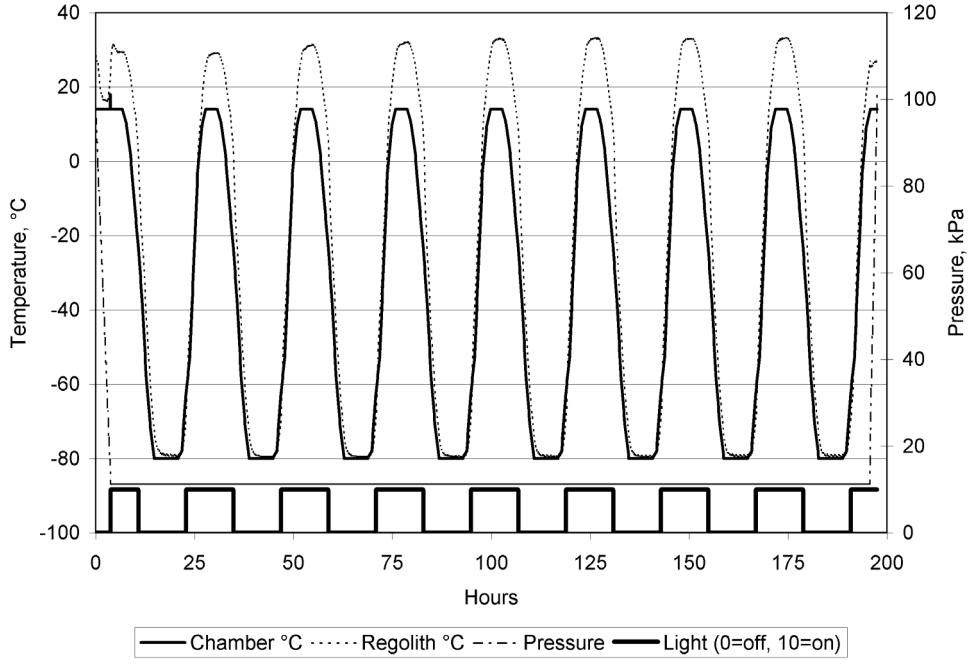

Fig. 2. Environmental conditions within the simulator. This data is from an earlier, 7-day experiment, but it reflects the typical conditions within the sample chamber. In this example, the pressure was held at 100 mbar (~10 kPa).

was added to the chamber daily with a syringe through a valved port to maintain water saturation of the atmosphere. Atmospheric pressure was maintained at experimental levels during the addition of water. Water addition was stopped when standing water appeared in the bottom of the sample chamber. Each simulation lasted for 5 weeks.

Culture conditions

Three cyanobacteria and one alga were used in these experments: *Anabaena* sp. (Carolina Biological Supply), *Chroococcidiopsis* sp. strain CCMEE171 (Culture Collection of Microorganisms from Extreme Environments), *Plectonema boryanum* strain UTEX485 (University of Texas Culture Collection) and *Chlorella ellipsoidea* strain YCC002 (University of Wyoming Culture Collection). Cyanobacterial and algal stock cultures were grown at 25 °C in liquid BG-11 medium (Sigma-Aldrich, St. Louis, MO), pH 7.5, amended with 2.5 mM $NaHCO_3$ and 20 μg l^{-1} vitamin B_{12} (final concentrations). Cultures were continuously illuminated with 50 μmol photons m^{-2} s^{-1} PAR from cool white fluorescent tubes.

Sample preparation

Stock cultures were centrifuged for 20 min at 1000 ***g***. The cell pellets were resuspended in fresh BG-11 medium, without added $NaHCO_3$, to an optical density of 0.15 at 720 nm measured with a Genesys 20 spectrophotometer (Thermo Electron, Waltham, MA). Triplicate samples of each microorganism were prepared in 25 ml polypropylene jars. 10 ml of each culture were added to 7 g of sterilized JSC Mars-1 soil simulant (Allen *et al.* 1998), resulting in a simulated 'mud puddle' with standing water above saturated soil. JSC Mars-1 has been used in many other Mars biology simulations, including preliminary studies for these experiments (Hart *et al.* 2000; Thomas *et al.* 2006a; Thomas *et al.* accepted). Samples were weighed before and after each experiment to determine water loss. Identical triplicate samples were kept in the dark at 4 °C as controls. Samples were transported on ice between Lyon College and Techshot via automobile or overnight courier. Experimental samples were arranged so that each triplicate series received the continuum of available light – shade to full intensity (Fig. 1). After each experiment, the sample jars were re-weighed; sterile water was added

Fig. 1. Techshot's Mars environment simulator (internal view). Samples were contained within the 6 l fused silica (quartz) cylinder, which allowed full-spectrum transmission of light to the samples. Simulated sunlight was reflected onto the samples via a movable, front-surface mirror. The xenon-arc light source was connected at the left-hand side of the simulator (not shown). In this configuration, samples in the middle of the cylinder receive approximately 1000 μmol photons m^{-2} s^{-1} PAR, while the samples in the shaded regions receive approximately 15 μmol photons m^{-2} s^{-1}. Each triplicate sample series had one jar in full light, another in full shade and a third in between the two light extremes. The soil-filled jar at front-centre contained a buried thermistor, which measured soil temperature. Ports on the right end of the cylinder controlled atmospheric pressure and content, and allowed the introduction of water during experiments. The horizontal glass tubes in the bottom of the chamber were used for other experiments not described here.

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Fig. 3. Differences in sample moisture. By the end of each experiment, samples located toward the ends of the chamber (left) contained more water than samples in the middle of the chamber (right). Sample jars were arranged such that each triplicate series experienced the full range of moisture within the simulator chamber. Differences in moisture content during the experiment were probably responsible for the variation about the means in the life assays (Figs 4 and 5). The moister samples showed more esterase activity and contained more chlorophyll than the drier samples. The culture tube at the bottom of the figure was for another experiment running simultaneously.

to return each jar to its original mass. The samples were capped and shaken to resuspend the microorganisms before subsampling for life detection assays. Survival of the microorganisms was determined via extractable chlorophyll *a* and fluorescein diacetate (FDA) hydrolysis (esterase) activity.

Chlorophyll extracts

Chlorophyll *a* extractions were used to determine the relative abundance of photosynthetic organisms (Myers *et al*. 1980; Bowles *et al*. 1985). One milliliter subsamples were taken from each sample and centrifuged for 10 min at 10 000 ***g***. 800 µl of supernatant were removed and discarded. 800 µl of denatured ethanol were added to each subsample (resulting in 80% ethanol solution), which was then vortexed and placed in a −20 °C freezer for 24 hours to extract the chlorophyll. After extraction, the subsamples were centrifuged again for 10 min at 10 000 ***g***. 800 µl of each extract were transferred to a polystyrene semi-micro spectrophotometer cuvette, and their absorbances were measured at 664 nm with an USB2000 diode array spectrophotometer (Ocean Optics, Dunedin, FL). Corrections for residual soil particles were made by subtracting non-specific scattering at 720 nm from the A_{664} measurements. Known solutions of purified chlorophyll *a* (Sigma-Aldrich, St. Louis, MO) were used to produce a standard curve ([chl *a*] µg $ml^{-1} = 188\ A_{664}$, $r^2 = 0.9999$).

FDA hydrolysis assay

Samples were analysed for esterase activity via an assay of FDA hydrolysis (Schnürer & Rosswall 1982; Adam & Duncan 2001) at the beginning and end of each experiment. The FDA hydrolysis assay indicates microbial metabolism across a wide variety of taxa, and correlates well with assays of respiration. Subsamples of 1–2 ml were taken from each sample before and after each experiment and transferred into 15 ml centrifuge tubes. Five milliliters of 60 mM K2PO4 buffer (pH 7.6) were added to each tube, which was then briskly shaken for 10–20 s. Ten microliters of FDA in acetone (5 mg ml^{-1}) were added to each tube, and then all tubes were incubated for 3–5 hours at 25 °C on a rocker table. Following incubation, the samples were extracted by adding 5 ml 2:1 chloroform:methanol. The samples were centrifuged for 10 min at 1000 ***g***, and the supernatant was measured spectrophotometrically at 490 nm with the USB2000 diode array spectrophotometer. Known solutions of fluorescein were extracted in the same manner as the samples, and were used to generate a standard curve ([fluorescein] µg $ml^{-1} = 4.72\ A_{490}$, $r^2 = 0.9225$).

Results and discussion

As was observed in earlier experiments (Thomas *et al*. 2006a; Thomas *et al*. accepted), a 'water cycle' developed within the sample chamber. Water evaporated from the samples and

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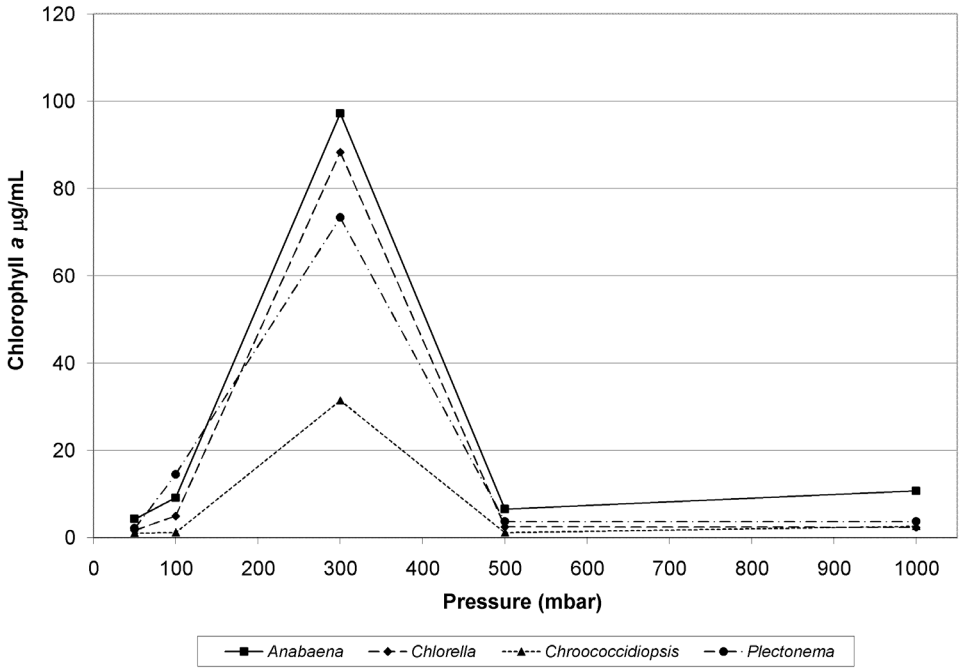

Fig. 4. Chlorophyll extracts. 1 ml of liquid was removed from each sample, centrifuged and extracted in 80% ethanol in the dark for 24 hours at −20 °C. Extracts were measured spectrophotometrically at 664 nm. Controls were combined from all experiments. Error bars equal standard deviations ($n = 10$–15 for controls, $n = 3$ for experimental groups). For all samples, the largest amount of extractable chlorophyll *a* was found in the 300 mbar experiment.

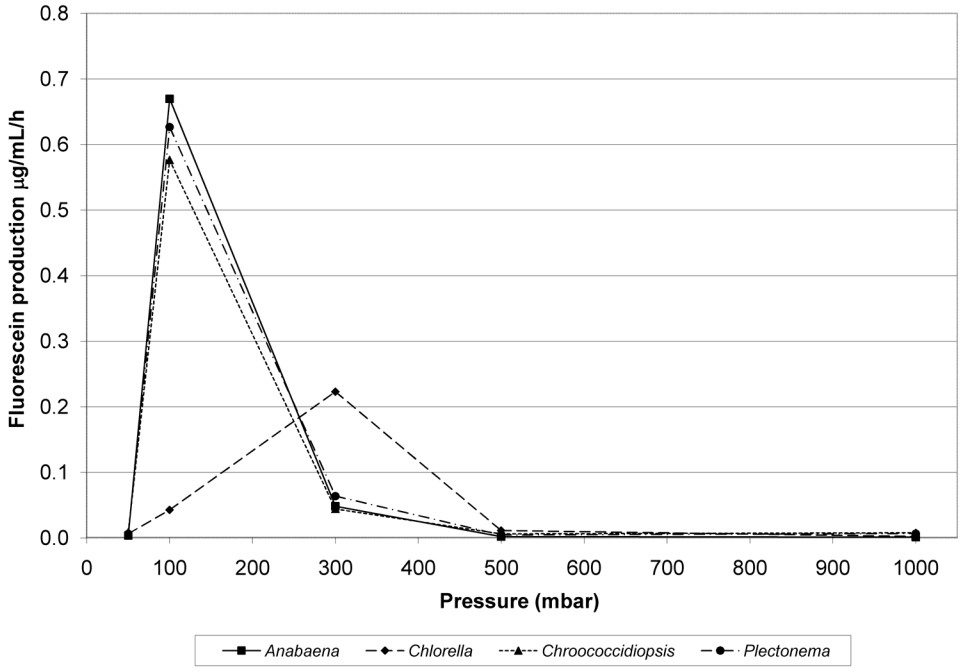

Fig. 5. FDA hydrolysis assays. 1–2 ml of liquid were removed from each sample and brought to 5 ml with the addition of pH 7.6 phosphate buffer. 10 ml of 5 mg ml^{-1} FDA solution were added to each sample, followed by incubation for 2–6 hours at 25 °C. Samples were extracted in 2:1 chloroform:methanol. The methanol:buffer fractions were measured spectrophotometrically at 490 nm. Controls were combined from all experiments. Error bars equal standard deviations ($n = 10$–15 for controls, $n = 3$ for experimental groups). For the three cyanobacteria, the highest amount of FDA hydrolysis activity was found in the 100 mbar experiment, but the alga, *Chlorella ellipsoidea*, had more activity in the 300 mbar experiment.

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from the bottom of the chamber during the day, and then condensed as dew and frost during the night. Most of the condensation occurred at the ends of the chamber, out of the direct light. Thus, the samples in the middle of the chamber were more desiccated than the samples at the ends (Fig. 3). All samples were re-hydrated to their original states before the FDA hydrolysis and chlorophyll extraction assays were performed.

The results of the life detection assays for the five experiments are summarized in Figs 4 and 5. Since the control samples for each experiment were stored in the dark at 4 °C, and were otherwise treated identically, the data were combined for all five experiments. Among the three cyanobacteria, the highest FDA hydrolysis activity was found at 100 mbar, but the highest chlorophyll content was found at 300 mbar. The alga, *Chlorella*, had its highest FDA activity and chlorophyll content at 300 mbar. Outside of the 100–300 mbar range, both extractable chlorophyll and FDA hydrolysis levels were very low. Previous research with cyanobacteria in high CO_2 atmospheres and ambient pressure (Thomas *et al*. 2005) has shown significant growth inhibition of *Anabaena* and *Plectonema* at CO_2 concentrations of 40% or more. Another cyanobacterium, *Synechocystis*, was inhibited by 20% CO_2. At ambient pressure, this corresponds to a partial pressure range of CO_2 (pCO_2) of 200–400 mbar – which overlaps the survival range shown in Figs 4 and 5. The low survival rates in the 500 and 1000 mbar experiments may have been due to CO_2 toxicity. High concentrations of CO_2 in aqueous systems can result in detrimental intracellular changes associated with decreased pH, anaerobiosis and random carboxylation/carbamylation of biomolecules (Thomas *et al*. 2005). While *Anabaena* and *Plectonema* can survive in 100% CO_2 under culture conditions, the added stresses of other parameters of the ecopoeitic conditions resulted in inhibition and death.

Besides the inhibitory effects of CO_2, atmospheric pressures below 100 mbar may have both physically and physiologically inhibitory effects. Since the diurnal temperature varied from −80 °C to 26 °C, the microbial cultures (especially those in direct light) lost water and were then deep-frozen every day. Our results are consistent with research on *Bacillus* spp. that showed little or no growth at 25 mbar (Schuerger & Nicholson 2005; Schuerger *et al*. 2006a).

Ultraviolet radiation was the other major stress factor for our test organisms. Previous work has shown that simulated Martian UV levels quickly inhibit several *Bacillus* spp. and a strain of *Chroococcidiopsis* (Cockell *et al*. 2005; Schuerger *et al*. 2006b). Desiccation such as that experienced by the organisms in this project increased the rate of inhibition produced by UV radiation. The cyanobacteria and alga used in this project require light to survive, but also require a certain amount of shielding from UV radiation. This means that they probably only grow within a thin layer that is deep enough within the water/regolith column to be shielded from excess UV, but shallow enough to receive adequate amounts of PAR.

These experiments only begin to address the issues and problems associated with ecopoesis. At the beginning of these experiments, we wanted to discover which of these photoautotrophs would grow under Martian conditions at various stages of ecopoesis. However, our results indicate that, under most of the conditions tested, growth did not occur and survival declined. Although the FDA assay showed increased activity in the cyanobacteria at 300 mbar, it is not significantly higher than the controls. Additional experiments in which surviving microbes are re-cultured and then put back under Martian conditions may allow for the selection of hardier strains. A multitude of other potential pioneer Martian organisms in culture collections and natural settings also awaits possible testing and selection. As interest in this area of research increases, and additional test facilities become available, we will be able to answer more of the questions pertaining to the establishment and development of a new biosphere on Mars. At the same time, we will further our understanding of the functions and evolution of Earth's earliest ecosystems.

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