Space Algae-2 Ground and Lunar Analog Studies in Preparation for Long-Duration Propagation of Cyanobacteria in Spaceflight

Katherine. E. Fisher[1]
KBR, NASA Ames Research Center, Moffett Field, CA 94035, United States

Kristen. E. Saban[2]
University of Arizona, Tucson, AZ 85721, United States

and

Jared T. Broddrick[3] and A. Mark Settles[4]
NASA Ames Research Center, Moffett Field, CA 94035, United States

There are numerous applications for microalgae in spaceflight missions and on Earth, such as, oxygen production, carbon dioxide removal, nutrition, wastewater processing, and biofuel production. Space Algae-2 aims to test the genetic stability of *Arthrospira platensis,* commonly known as spirulina, during six-months of continuous culture in spaceflight on the International Space Station. Long-duration exposure to ionizing radiation and microgravity may impact growth, nutrient composition, and genetic stability. The high protein, vitamin, antioxidant content, and radiation resistance make spirulina a promising candidate for bioregenerative life support systems during long-duration missions. A concept of operations was developed to grow and harvest algal biomass in space. Preflight testing experiments were conducted to optimize conditions for an extended growth period in a gas permeable bioreactor bag. Preflight and post-harvest storage methods were developed in addition to a novel cryopreservation method. After sample return, multi-omics analysis will be conducted to determine the mutation rate, gene expression, and protein and metabolite profile. The concept of operations for Space Algae-2 was tested at HI-SEAS (Hawai'i Space Exploration Analog and Simulation) during a six-day lunar analog mission (EMMIHS23, EuroMoonMars, International MoonBase Alliance, HI-SEAS, 2023). *A. platensis* was grown in the semi-controlled environment using flight-like hardware and solar powered LED lights. Then, the biomass was harvested and used to supplement bread as an example of *A. platensis* utilization. Overall, the data collected from Space Algae-2 will inform potential bioengineering of spirulina for space and terrestrial applications.

Nomenclature

DMSO	=	dimethyl sulfoxide
ESA	=	European Space Agency
FDA	=	US Food and Drug Administration
FEP	=	fluorinated ethylene propylene
Gy	=	gray
HI-SEAS	=	Hawai'i Space Exploration Analog and Simulation
ISS	=	International Space Station
LED	=	light emitting diode

[1] Research Scientist, Bioengineering Branch, Moffett Field, CA 94035
[2] Research Associate, Department of Ecology & Evolutionary Biology, Tucson, AZ 85721
[3] Research Scientist, Space Biosciences Research Branch, Moffett Field, CA 94035
[4] Principal Investigator, Bioengineering Branch, Moffett Field, CA 94035

NIES = National Institute for Environmental Studies Culture Collection, Tsukuba, Japan
OD = optical density
PAR = photosynthetic active region
PBR = photobioreactor
PCC = Pasture Culture collection of Cyanobacteria
rpm = revolution per minute
TF = tangential flow
UTEX = Culture Collection of Algae, University of Texas, Austin
ZM = Zarrouk's medium

I. Introduction

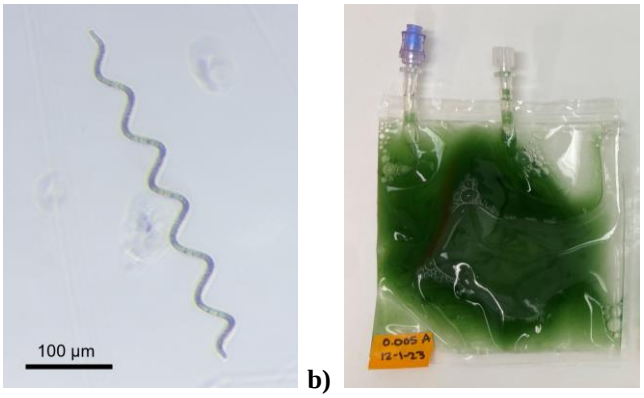
As humanity prepares to make the long-duration mission to Mars, new technologies are needed for nutrition, life-support systems, wastewater treatment, and biofuel production. Microalgae and cyanobacteria can help fill these gaps.[1] Fresh nutrients and reduced mass are essential for missions with little to no re-supply. Since the 1960's there have been many short duration spaceflight experiments with microalgae.[2,3]

The cyanobacteria *Arthrospira platensis*, commonly known as spirulina, is a super food that contains all essential amino acids, antioxidants, vitamins, and minerals.[4] The name spirulina is derived from the spiral shape of the multicellular trichomes (Image 1a). Spirulina has been harvested and used as a protein and nutrition source throughout history in Mesoamerica and central Africa.[5] Today, spirulina is consumed as a food additive and supplement, and is generally regarded as a food-safe microbe (GRAS designation) by the US Food and Drug Administration (FDA). On a small-scale, *A. platensis* is conventionally grown with a range of light intensities in Erlenmeyer flasks or photobioreactors (PBR). Industrial-scale spirulina production occurs in open ponds to produce edible biomass and pigments.[6]

The potential of spirulina in space has been noted by the European Space Agency (ESA), which used spirulina species *Limnospira indica* PCC 8005 as part of the Micro Ecological Life Support System Alternative (MELiSSA) project. This project flew a 50 mL membrane PBR of *L. indica* for four weeks aboard the ISS and found that microgravity had no significant effect on its oxygen production rate.[7] ESA planned a long-duration growth experiment with *Chlorella vulgaris*, but the hardware had an unexplained power failure after two weeks of operation.[8,9] These experiments highlighted the need for simple culture methods and a long-duration experiment to verify that multiple growth cycles in space are possible.

The main sources of abiotic stress in space are microgravity and ionizing radiation. Organisms grown in space have exhibited novel phenotypes in growth cycle, cell morphology and metabolic activity. *E. coli* has shown changes in mutation rate and differentially expressed genes involved in lipid metabolism, cell motility, and cellular transport among others.[10] Li et al. reviewed bacterial growth in spaceflight and suggested that extracellular mass transfer mechanisms play a major role in the effect of microgravity; however, results are highly dependent on experimental design and bacterial strain.[11]

Space Algae-2 is based on the ISS experiment, Space Algae-1, flown in 2018.[12] Briefly, two strains of UV mutagenized *Chlamydomonas reinhardtii*, a single-celled eukaryotic green algae, were cultured for 30 days in liquid media in fluorinated ethylene propylene (FEP) bags. Four growth cycles were conducted, and the cultures were stored live in soft stowage at ambient temperature for sample return. *C. reinhardtii* was susceptible to ionizing radiation and had an approximately 50% increase in mutational load compared to the ground control (manuscript in preparation). A *C. reinhardtii* gene knockout collection was also flown as an investigation in the BioExpt-01 investigations as part of Artemis 1, which is expected to provide additional insights on the genomic



a) **b)**

Image 1. *A. platensis* trichome and FEP culture bag. Trichome image taken through the FEP bag at 400X magnification (a). Culture of *A. platensis* on day 14 grown with flight-like conditions (b).

susceptibility of this species to deep space radiation.[13]

Based on the susceptibility of *C. reinhardtii* to low Earth orbit spaceflight conditions, Space Algae-2 aims to analyze the response of a different organism (photosynthetic cyanobacteria) to six months of continuous batch culture aboard the ISS. *Arthrospira* species have been shown to be radiation resistant in ground studies. Badri et al. found that short doses of gamma radiation up to 6.4 kGy are tolerated by *Arthrospira* sp. PCC 8005.[14] The radiation response included single stranded DNA repair mechanisms, induction of a group of conserved proteins with unknown function, and a dose dependent delay in growth due to reduced expression of genes involved in photosynthesis.[15] Transient high dose radiation experiments may not accurately represent organism responses to long-duration, low dose radiation from either low Earth orbit or deep space.

A. platensis NIES-39 was chosen for Space Algae-2 due to nutritional quality, photosynthetic efficiency, and GRAS organism status in addition to its radiation resistance. Its relatively small genome size of 6.8 mega base pairs allows for deep sequencing of single nucleotide polymorphisms.[16] Past flight experiments have focused mostly on transcriptomics. NASA GeneLab reported in 2021 that by assay type their data included 70% transcriptomic, 7% whole genome sequencing, 8% proteomic, and <2% metabolomic.[17] Space Algae-2 will include whole genome sequencing, transcriptomics, proteomics, and metabolomics to understand the complex effects of spaceflight. Recent advances in cyanobacterial metabolomics and proteomics allow for the analysis of multi-omics data from Space Algae-2 to help fill the gap for future genetic and metabolic engineering efforts for space and Earth production.[18,19]

The goals of Space Algae-2 are: 1) identify the mutational load on *A. platensis* during a long-duration spaceflight on the ISS; 2) complete multi-omics profiling upon sample return throughout the six-month experiment; and 3) determine the spaceflight impact on *A. platensis* nutritional composition. This experiment will also determine the feasibility of continuous culture of microbes for long-duration missions. Here, we report results from pre-flight ground studies to develop batch culture, cryopreservation, and biomass de-watering methods compatible with spaceflight. A culture system was validated using FEP bags that are optically clear and gas permeable. The growth cycle was extended to limit the number of passages and crew time for the five-to-six-month experiment. As desiccation has been shown to cause viability loss, metabolic changes, and a long reactivation phase;[20] a pre-flight storage method was optimized to provide viable algae at the experiment start. Additionally, a novel cryopreservation method was developed for viable cell return. Our multi-omics analysis will be completed on dewatered biomass. While filter concentration is one of the industry standards for spirulina harvesting, it is a major bottleneck of production.[21] We show an optimized filter concentration method suitable for small volumes in microgravity.

Based on the ground test results, a concept of operations (ConOps) for the mission was developed and tested in the laboratory. In addition, the ConOps was validated during a lunar analog mission at the Hawai'i Space Exploration Analog and Simulation (HI-SEAS). During the six-day mission, the Space Algae-2 methods were adapted and used to grow spirulina in the analog habitat. The resulting *A. platensis* biomass was used to increase the nutritional value of a food source and show a practical application for future space nutrition.

II. Materials and Methods

A. Strain and Culture Conditions

Arthrospira platensis NIES-39 (UTEX 3086, Culture Collection of Algae, University of Texas at Austin) was selected for the Space Algae-2 payload. *A. platensis* was batch cultured with two different methods. The conventional culture method was 50-100 mL cultures grown in 150-250 mL Erlenmeyer flasks at 75 ± 4 µmol m^{-2} s^{-1} continuous illumination, 35 °C, and 100 rpm in a Multitron incubator with warm white LEDs (Infors HT, Laurel, MD). Flight-like cultures were grown with continuous light at 20 ± 4 µmol m^{-2} s^{-1} at 24 °C and static conditions in the Multitron, or at ambient temperature (24 ± 2 °C) under white LED lights (Cat# C421510, Home Depot, Atlanta, GA). Light intensity was measured with a quantum light meter (Apogee Instruments, Logan, UT). The bioreactor bag was a FEP Seamless Intubated Bag (American Durafilm, Holliston, MA) 0.127 mm (0.005") thick with a cross-sectional surface area of approximately 220 cm^2 (14.5×15.2 cm). Cells were cultured in Zarrouk's medium (ZM) without vitamin B-12 at pH 9.[22] Table 1 shows the final concentrations of each component in 1x medium. The ZM was prepared by combining equal volumes of 2x Sol.1 and 2x Sol. 2 and adjusting the pH with NaOH tablets. The media was filter sterilized to avoid precipitation when autoclaved.

B. Culture and cell growth metrics

Optical density at 750 nm (OD_{750}) was measured with a GENESYS™ 180 UV-Vis Spectrophotometer (Thermo Fisher, Waltham, MA). The pH of the *A. platensis* cultures was measured with a FiveEasy pH meter (Mettler-Toledo, Columbus, OH). Microscopy of trichomes through the FEP bag was conducted on an Diaphot inverted microscope

(Nikon) at 40-400X magnification with a 5 MP High-Speed Color CMOS C-Mount Microscope Camera (AmScope, Irvine, CA).

Table 1. Zarrouk's medium composition

Component	Concentration (g/L)
Solution 1:	
$NaHCO_3$	18.0
K_2HPO_4	0.50
Solution 2:	
$NaNO_3$	2.50
K_2SO_4	1.00
NaCl	1.00
$MgSO_4 \cdot 7\ H_2O$	0.20
Na_2 EDTA	0.08
$CaCl_2$	0.04
$FeSO_4 \cdot 7$ H2O	0.01
Microelement solution	1.0 mL
Microelement solution:	
H_3BO_3	2.86
$MnCl_2 \cdot 4\ H_2O$	1.80
$ZnSO_4 \cdot 7\ H_2O$	0.22
Cu_2SO_4	0.08
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02

C. Pre-flight Dark Storage

Heterotrophic dark conditions were tested to preserve *A. platensis* cell viability during pre-launch and for an extended period on orbit. The optimal organic carbon source was determined by testing ZM supplemented with 2 g/L acetate,[23] 4.6 g/L glycerol,[24] and 1 g/L glucose.[25,26] *A. platensis* was grown with the conventional flask culture method spiked with the carbon sources above to assess the effect on growth in lighted conditions. Next, three 70 mL Falcon™ Nontreated Tissue Culture Flasks were filled with 10 mL of ZM or ZM plus acetate or glucose and inoculated with 0.015 mg/mL estimated dry weight from a stock *A. platensis* culture. The flasks were stored in the dark with the lids cracked at ambient temperature for four weeks. The flasks were removed from dark storage and illuminated at 50 µmol m^{-2} s^{-1} at ambient temperature and the OD_{750} was recorded for 23 days.

ZM supplemented with 1 g/L glucose was tested for 13 weeks with the tissue culture flask method above. After 6-13 weeks in dark storage, three flasks per timepoint were illuminated at 20 µmol m^{-2} s^{-1} and ambient temperature. A glucose supplemented positive control with no dark incubation was run in parallel.

Next, flight-like conditions were tested with FEP bags containing 100 mL ZM plus 1 g/L glucose inoculated with 1.0 mL *A. platensis* culture at an OD_{750} of 0.69 (0.01 mg/mL estimated dry weight) and were stored in the dark for 10 weeks. Three bags were analyzed weekly by culturing 1 mL aliquots in 10 mL round bottom polyethylene tubes at 24 °C and 20 µmol m^{-2} s^{-1} light for 7-days. Biomass of the inoculum and final culture was estimated by measuring OD_{750}. To minimize light exposure, another set of three bags were kept undisturbed in the dark and sampled as above at 8 and 10 weeks. At 10 weeks, all six bags were illuminated at 20 µmol m^{-2} s^{-1} at 24 °C, and the growth was measured at OD_{750} in comparison to a positive control.

D. Culture Media Stability

The stability of liquid ZM pH 9 and lyophilized ZM was tested for nine months at room temperature and at 37 °C for an accelerated stability study. To lyophilize ZM, 22.5 mL of sterile, concentrated ZM (20 mL of 5x solution 1, 2.5 mL of 40x Solution 2, pH 9, Table 1) was added to an FEP bag to result in 1x concentration when hydrated with 100 mL of sterile Milli-Q water at the time of inoculation. The bags were frozen at -80 °C and then lyophilized with both ports open for approximately 24 h until fully dehydrated in a FreeZone18 freeze dryer (Labconco, Kansas City, MO). The lyophilized bags were vacuum packaged into mylar bags and the liquid media bags were placed into a cardboard box. All bags were stored light protected at room temperature or in a 37 °C incubator with three replicates per timepoint. At time-zero, three bags from the same media lots, liquid and lyophilized, were inoculated with 0.004 mg/mL *A. platensis* and the growth was monitored at OD_{750} as the control group. At nine months, fresh ZM in FEP bags and each set of nine-month stored media were inoculated with the same quantity of *A. platensis*.

E. Biomass harvesting

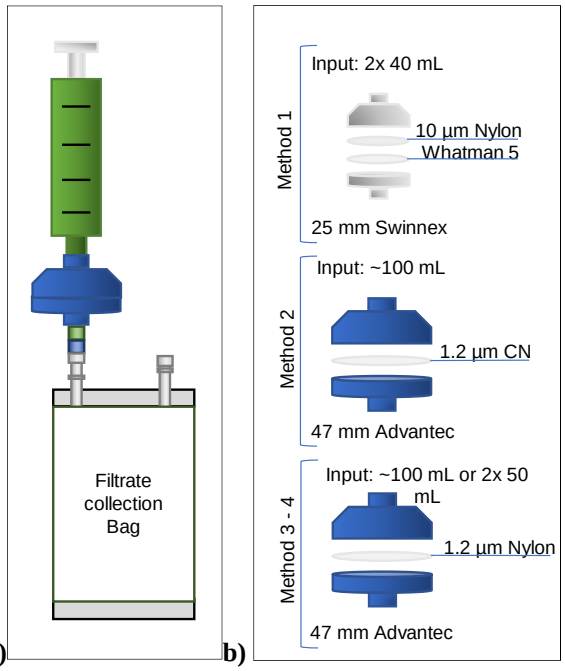
Multiple methods were tested to de-water and concentrate ≥ 80 mL of dense *A. platensis* culture onto a filter membrane with a syringe (Figure 1b). Initially, 25 mm Swinnex filter holders (MilliporeSigma, Burlington, MA) were used to support 5, 10, and 20 µm nylon membranes. A Whatman 5 filter paper was placed under the nylon membrane to retain any trichomes that passed through the nylon filter. A backflow procedure was used to reduce backpressure on the filtration unit. During a 40 mL filtration, 3-5 mL of filtrate was pulled back through the membrane 2-5 times. The 25 mm filter unit method tested for time required to complete a filtration procedure was a 10 µm nylon net membrane with a Whatman 5 filter (Figure 1a, 1b method 1). The filter unit was connected to an empty FEP bag via plastic tubing and Luer lock connectors. Two filtrations of 40 mL were completed for each 100 mL bag tested.

A larger diameter filter unit was tested using an Advantec 47 mm filter holder (Cole-Parmer, Vernon Hills, IL). Cellulose nitrate (CN) 1.2 µm membranes were tested with 100 mL filtrations (Fig. 1b method 2). Nylon 1.2 µm membranes were tested with 50 mL (Fig. 1b method 3) and 100 mL filtrations (Fig. 1b method 4). The filter unit was

connected to an empty FEP bag. Backflow procedures were completed when filtering larger than 50 mL volumes. The filtration procedure was timed for each method to calculate filtration rate (mL/min).

F. Cryopreservation

Cryopreservation of *A. platensis* liquid cultures was modified from the Shiraishi et al. (2016) method.[27] Aliquots of 250 µL dense culture (OD_{750}=1.6) were mixed with 250 µL of 20% dimethyl sulfoxide (DMSO) and 20% trehalose in ZM. The 10% DMSO, 10% trehalose cell culture mixture was incubated at room temperature for 15 min before freezing at -80 °C in a standard cardboard freezer box for microcentrifuge tubes. Stability of the frozen cultures was tested for 35 days. Samples were removed from the freezer and kept frozen until thawed by adding 1 mL of 37 °C ZM and placed into a 37 °C water bath for 60 s. The thawed sample was gently pipetted into a tissue culture flask containing 8.5 mL warm ZM. The flasks were incubated at 24 °C with 20 µmol m^{-2} s^{-1} light for 14 days. At time-zero, 250 µL of the input culture was grown in 9.75 mL of ZM as described in the above conditions as a fresh cell control.



a) **b)**

Figure 1. Schematic of the filtration methods tested. A 50 or 100 mL syringe is used to push algae culture through a filter unit and the filtrate is captured in a waste bag (a). Panel B shows the exploded view of the membrane(s) and filter units for methods 1-4.

G. Flight Procedure Testing

The ISS ConOps includes passaging cultures, cryopreservation of live cells, and de-watering biomass every 14 days. To estimate feasibility and potential crew time, the procedures were tested and timed. Six bags of 100 mL ZM inoculated with 200 µL *A. platensis* culture from the previous bag were continuously cultured for 12 weeks. At each timepoint all six bags were removed from the incubator and photographed. Each culture was passaged by sampling 200 µL of culture with 1 mL Luer lock syringe and inoculating into an FEP bag containing 100 mL fresh ZM media. For the first three timepoints, an additional 250 µL aliquot of each culture was cryopreserved into a tube containing 250 µL of 20% DMSO in ZM. For the first two timepoints 80 mL was filter concentrated using the final 25 mm diameter filtration method described above. At the third timepoint a new filtration method with a 1.2 µm cellulose nitrate membrane and a 50 mm diameter filter unit (Cytiva) was used. The time for each step of the procedure and the total duration was recorded for three timepoints.

H. Lunar Analog Study

An abbreviated growth cycle using the FEP bag bioreactors and filter harvesting system was conducted at the HI-SEAS habitat. Located at 2400 m on the side of the Mauna Loa volcano on the Big Island, Hawai'i, the 11 m diameter dome houses a crew of six analog astronauts, utilizes solar power, and water tanks. The experiment was conducted in the small laboratory on the first floor of the habitat. Four 100 mL FEP bags containing lyophilized ZM were hydrated with laboratory sink water treated with 1 mg/L sodium thiosulfate to neutralize any residual oxidant and subsequently sterile filtered through a 25 mm diameter 0.45 µm nylon membrane (Cat# EW-02915-02, Cole-Parmer). Four biological replicates were inoculated with 5 mL of dense culture at approximately 2.9 x 10^5 trichomes

Image 2. HI-SEAS habitat on Mauna Loa. Two analog astronauts hike back to the habitat on EVA (extravehicular activity).

per milliliter. The culture bags were grown at ~50 µmol m^{-2} s^{-1} under four full spectrum grow lights (Growstar, Los Angeles, CA) at ambient temperature (20-24 °C). Growth was monitored by trichome counts with a Neubauer Improved glass hemocytometer under a dissection microscope (AmScope). Images of the bags were taken, and the pH was measured daily with pH 7-14 indicator strips (Macherey-Nagel, Allentown, PA).

On day four, the amount of gas build-up in each bag was measured. All 105 mL of each culture was filter concentrated onto a 47 mm 1.2 µm nylon filter and the wet mass was measured. The harvested spirulina and the stock bags, were combined, washed, and used to supplement one bread loaf (180 g bread flour, 3.4 g instant yeast, 3.75 g salt, and 133 mL warm water). The wet spirulina biomass (3.08 g) was mixed into the warm water and added to the dry ingredients. The dough was kneaded for 10 minutes, proofed for one hour at room temperature, and baked for 30 minutes at 177 °C until the internal temperature reached 88 °C.

III. Results

A. Validation of Flight Growth System

The FEP bag growth system was compared to a conventional shake-flask culture method using the same inoculum. The lighting and temperature were optimized to extend the FEP growth cycle to 14-17 days to reduce the number of crew activities needed for to grow algae continuously for 5-6 months. Figure 2a shows the flask-grown cultures incubated at 35 °C and 75 µmol m^{-2} s^{-1} continuous illumination stopped growing after eight days with an average maximum OD_{750} of 1.44 (range 1.33-1.60). In the flight growth conditions at 24 °C and 20 µmol m^{-2} s^{-1} continuous illumination, the *A. platensis* reached an average maximum OD_{750} of 1.78 (range 1.65-1.92) between 15-18 days of culture. The average biomass produced in flight-like conditions on day 8, 10, and 15, was 0.28 g/L, 0.60 g/L, and 1.83 g/L, respectively.

Microorganism metabolism can impact the culture pH. Figure 2b shows the relationship between culture pH and OD_{750} in FEP bag cultures. During the exponential growth phase (OD 0.2 to 1.3), the culture pH increased linearly from pH 9 to approximately 10 with a slope of 0.9 pH units per absorbance unit (r^2 = 0.97). As the cultures reached stationary phase (OD >1.5), culture pH increased as high as pH 12.3 but OD was less predictive in this dense culture stage.

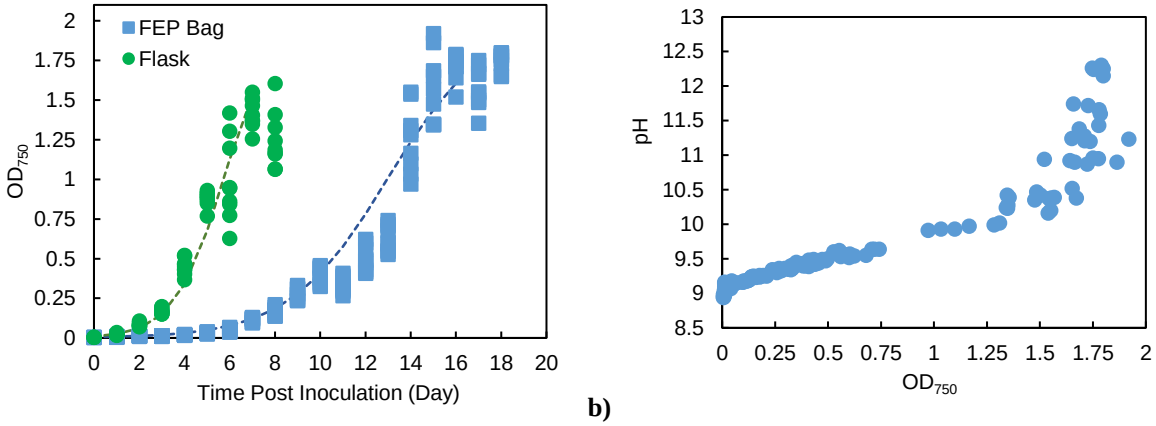


Figure 2. Flask grown vs. FEP bag cultures**:** two combined data sets of three biological replicates for each method. Three technical replicates of three bags/flasks were measured per time point, n=9. Dotted lines show estimated logarithmic growth curves (a). Scatterplot of optical density and pH of bag grown *A. platensis*, three technical replicates of six biological replicates n=18 (b).

B. Pre-flight dark storage

Live culture storage conditions need to be defined to establish requirements for launch and initiating the spaceflight growth experiment. Glucose, acetate, and glycerol were added to ZM as carbon sources that could potentially be useful to extend dark storage of *A. platensis* cultures. Glycerol supplementation resulted in cell death and was not included in longer term testing. Figure 3a shows growth responses of inoculated cultures after four weeks of dark storage with glucose, acetate, or no supplemental carbon source. The glucose supplemented culture grew immediately upon exposure to continuous light and reached peak OD_{750} in 14 days. By contrast, acetate-supplemented and non-

supplemented cultures reached mid-log phase in 21 and 23 days, respectively. One of three non-supplemented ZM cultures showed no growth.

Longer-term storage of glucose supplemented cultures was tested in tissue culture flasks for up to 13 weeks of dark storage (data not shown). After nine weeks of dark storage the cultures grew at a comparable or faster rate than the control when returned to light. After 11 weeks, the cultures showed delays in growth. Ten weeks was selected as the maximum dark storage period for tests using six FEP bag cultures supplemented with glucose. Three of the bags were stored for ten weeks in the dark and then reactivated with continuous light (ZM+Glucose A), while three other cultures were monitored on a weekly basis for turbidity and viability (ZM+Glucose B). The average OD for the ZM+Glucose B condition throughout dark storage was 0.041 ± 0.016 standard deviation, and viability was tested weekly by growing 1 mL of the culture in continuous light for seven days. The ZM+Glucose B group was protected from light by aluminum foil during sampling. Bacterial contamination was found in all six bags, likely due to the glucose supplement. However, the contamination did not seem to impact the growth of the ZM+Glucose B cultures. Figure 3b compares growth curves of the dark-stored FEP bag cultures relative to a freshly inoculated ZM media

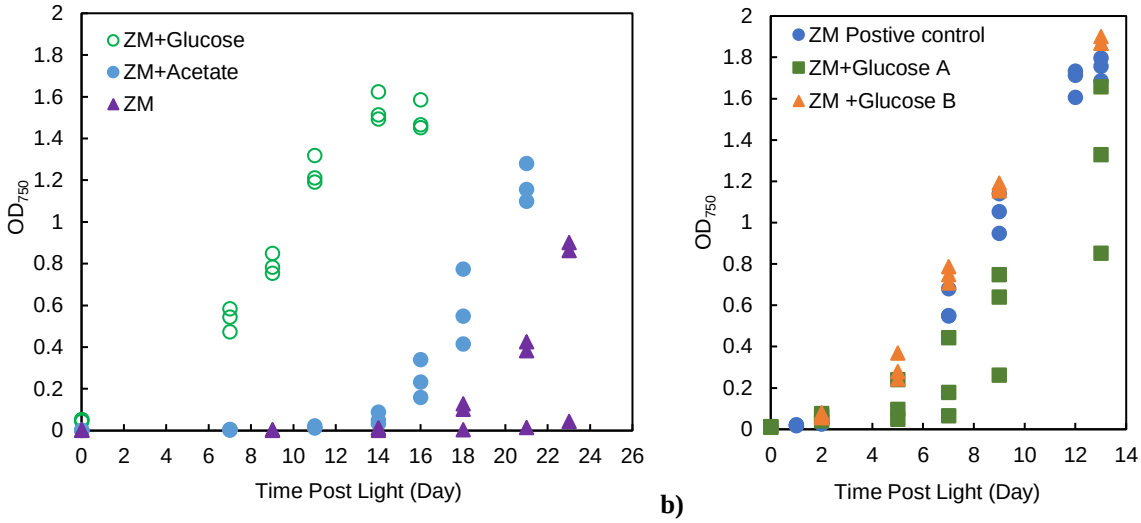


Figure 3. Growth curves of *A. platensis* after dark storage. Growth curves comparing glucose, acetate, and non-supplemented ZM after a four-week dark period, n=3 (a). Growth curves post 10-week dark storage comparing no sampling (ZM+Glucose A) to weekly sampling (ZM+Glucose B); ZM positive control was not stored in the dark, n=3 (b).

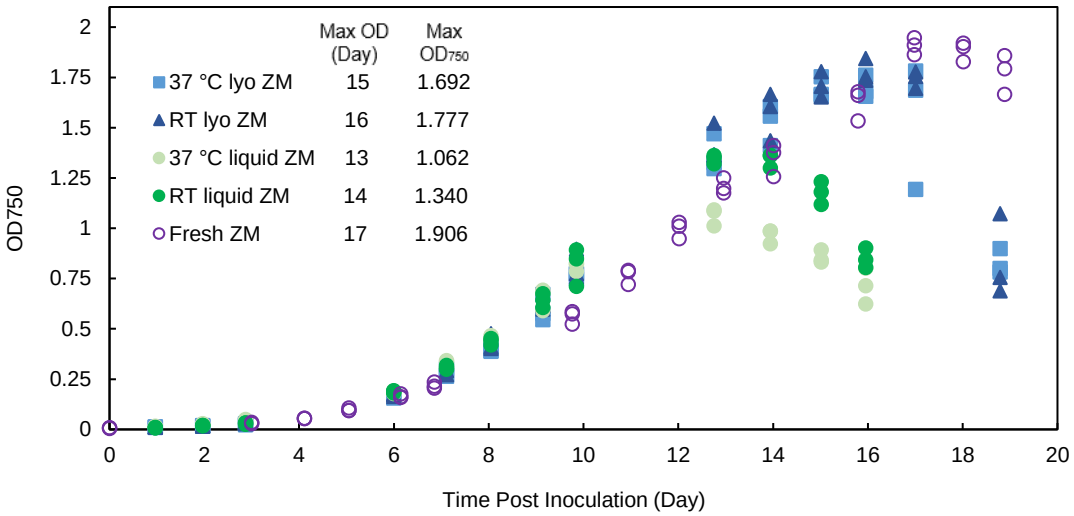


Figure 4. Growth curves after nine-month shelf-life test of ZM. After storage at ambient temperature (RT) or 37 °C, FEP bags containing 100 mL liquid or lyophilized ZM were inoculated with *A. platensis*, n=3. Fresh ZM was inoculated at the same density as the control. The inset table shows the maximum OD and the day it was observed.

control. The ZM+Glucose A cultures without weekly sampling had greater variation in growth but all cultures grew to a sufficient density for passaging.

C. Culture Media Stability

A ten-week dark storage period for cultures supplemented with glucose allows for a timeline with media prepared and stored in FEP bags up to 9 months prior to the final culture of the experiment. Nine months of storage at ambient temperature (RT) increased the media pH from 9.0 to 9.7 and reduced the bag mass by an average of 1.28 g. Media stored for nine months at 37 °C had a pH of 9.9 and lost an average of 6.66 g. The stored FEP bags also accumulated gas during storage with an average 16 mL or 394 mL at RT and 37 °C temperatures, respectively. Stored lyophilized media had no change in pH regardless of storage temperature. Figure 4 shows growth curves of *A. platensis* with the stored media types compared to fresh media. The stored liquid media resulted in cultures with a lower maximum OD than freshly prepared or stored, lyophilized media. However, only the liquid media stored at 37 °C resulted in a maximum culture density lower than the ranges observed in Figure 1 suggesting RT storage of liquid media will be stable enough to support algae growth at the latter passages in the flight experiment timeline.

D. Biomass Harvesting

Biomass harvesting methods were tested using filter assemblies as shown in Figure 1b. These methods varied the filter area, filter pore size, filter material, and volume of culture filtered. Figure 5 is a box plot comparing four methods of biomass de-watering tested for average flow rate. A single factor analysis of variance (ANOVA) was significant for differences between methods ($p = 1.5 \times 10^{-6}$). A Tukey's HSD test found that method 3 was significantly faster than methods 1 and 4. Method 3 filtered 45 mL of culture with a 47 mm diameter, 1.2 µm nylon filter and had an average flow rate of 35.6 mL/min. Method 1 filtered 40 mL with a smaller filter area, while method 4 filtered 95 mL of culture with the same filter assembly as method 3. In addition, the nylon membranes are more durable than CN membranes.

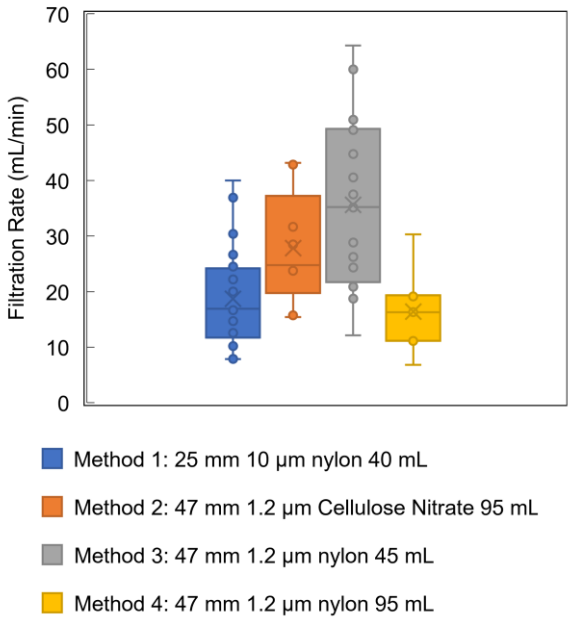


Figure 5. Comparison of filtration rate for four biomass harvesting methods. Filter diameter, pore size, and volume filtered are shown in the legend. Boxplot of the filtration rate for the four filtering methods tested. Plots show all data points with the standard deviation (whiskers), average (line), and interquartile range (boxes) for each method. Sample sizes for methods 1, 2, 3, and 4 are 24, 9, 26, and 7, respectively.

E. Cryopreservation

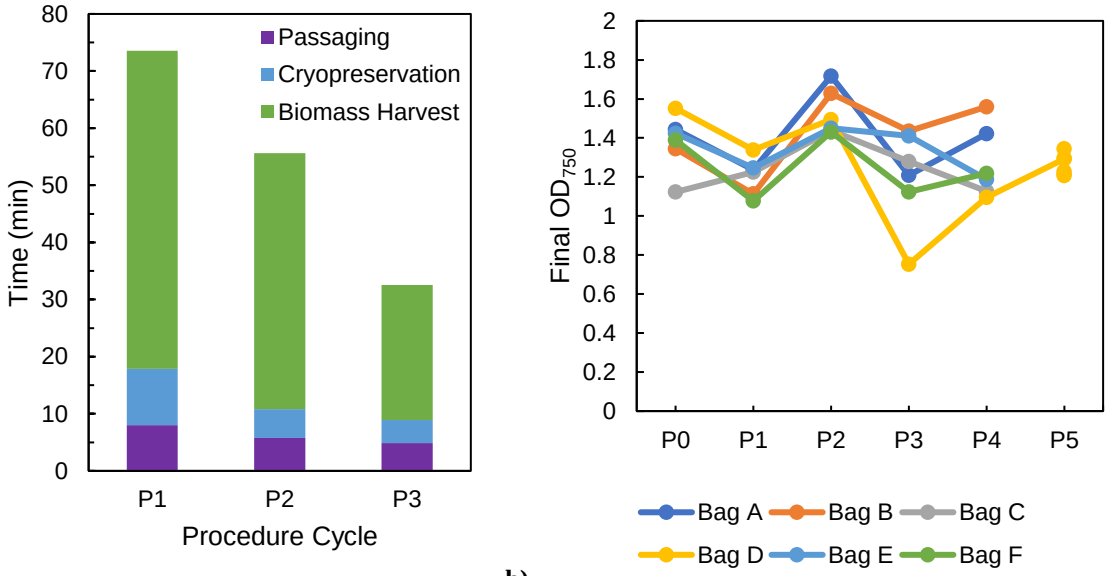
The modified cryopreservation method was tested for cell viability in comparison to fresh inoculum in ZM media. The *A. platensis* cells were stored frozen at -80 °C for 20 and 35 days. At each storage time point, three biological replicates were thawed and inoculated into ZM media at an average OD_{750} of 0.04. Three control cultures with fresh cells were inoculated at the same density in parallel. After 7 days, the fresh, 20-day, and 35-day stored cells grew to an OD_{750} of 0.77 ± 0.02, 0.82 ± 0.14 and 0.70 ± 0.04, respectively. There was no statistical difference between the three groups based on single factor ANOVA ($p = 0.23$).

F. Flight Procedure Testing

Preliminary crew procedures were tested for reliability and the time required to complete culture passaging, cryopreservation of cells, and biomass harvesting by a scientist unfamiliar with the procedures. Six cultures per cycle were processed in three cycles of procedures (P1-3). The total hands-on time ranged from 74 to 33 minutes and was reduced with practice and procedure optimization (Figure 6a). Microscopy was not included in these tests. Passaging and cryopreservation for six cultures individually took ~6 minutes during each procedure cycle (Figure 6a). P1 and P2 used the 25 mm filter assembly from de-watering method 1. Harvesting the biomass was the longest component of

the procedures, taking 55.7 and 44.8 min in P1 and P2, respectively. The filter assembly for P3 used 50 mm Cytiva filter units, which decreased the de-watering time to 23.6 minutes. However, the Cytiva filter units were prone to leakage, and the Advantec 47 mm filter unit was selected for the filtration tests in Figure 5.

Space Algae-2 requires reliable growth with serial passages occurring at 14- or 15-day intervals. Six continuous growth cycles over 12 weeks were tested for production levels based on culture optical density at passage. Figure 6b shows the final OD_{750} of each cycle. The average OD_{750} on day 14 or 15 was 1.3 ± 0.2 standard deviation. There was significant variance in growth ($p = 0.007$, single factor ANOVA), but the final ODs were consistent with previous variation in culture endpoints. Bag D at P3 had an OD < 0.8 but returned to an expected level at P4. To simulate a scenario where multiple cultures become contaminated, Bag D was grown for three additional days and used to seed all six culture bags for P5.



a) **b)**

Figure 6. Flight procedure timing and continuous culture results. The timing for passaging, cryopreservation, and filtration for three rounds (a). Twelve-week continuous culture of *A. platensis* in 100 mL FEP bags at 24 ± 2 °C and 20 µmol m^{-2} s^{-1} light (b). The bags were passaged at day 14 or 15 and the final OD_{750} is shown for 6 culture cycles (P0-P5), n=6.

G. Lunar Analog Study

Four cultures of *A. platensis* were successfully grown inside the HI-SEAS analog habitat (Image 3a-b). The treated laboratory water source used for the hydration of the lyophilized media functioned as expected and there was no initial lag in growth. The cultures were insulated inside a non-functioning incubator, to help mitigate low night temperatures (Image 3b). Figure 7 shows the number of trichomes per microliter increased by two to three doublings in the four days of growth. On average, the FEP bags accumulated 22 mL of gas during the growth cycle. Important for processing in microgravity, the syringe filter concentration method retained the liquids and the biomass from each bag was successfully collected onto a filter membrane. The amount of biomass grown in each bag was 0.48, 0.49, 0.45, and 0.39 g wet mass. The time for one crewmember to complete the culture set up was approximately two hours. During the experiment, microscopy, photography, and pH measurements took 30

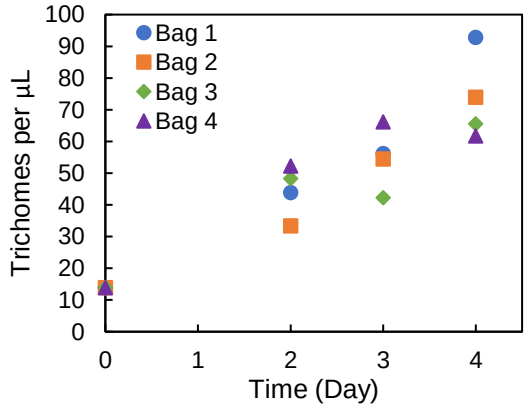


Figure 7. Growth curve of *A. platensis* cultures grown at HI-SEAS.

minutes per day. Biomass harvesting and bread making took two hours. Total crew time was five hours distributed over five days.

The total wet mass of spirulina, including stock cultures, baked into the bread was 3.08 g. The raw dough produced by this method had a slight green tint, but the baked loaf did not. Image 3c-d shows the bread supplemented with spirulina baked in the lunar analog habitat.

IV. Discussion

Through these ground studies, we developed a culture system and ConOps for Space Algae-2. FEP bags are simple, reliable bioreactors for *A. platensis* and will provide containment during spaceflight with sufficient gas exchange. Though not applicable for Space Algae-2, FEP bags can be washed and reused many times. In our pre-flight testing, the bags have survived over five rounds of autoclaving with no leaks detected as long as one port is open to avoid over pressurization. They are also compatible with sodium hypochlorite sterilization. According to the Teflon™ FEP Properties Bulletin, this material has long-term weatherability with "no measurable change after 20 years in Florida" outdoors. Optical clarity is expected to reduce over time due to small scratches. More experimentation is needed to quantify the light that passes through aged FEP bags. Overall, there is potential to scale up the size of the FEP bioreactors and reuse them for long-duration missions.

Morphology changes and contamination will need to be monitored for during the ISS experiment. Cultures can be directly observed or analyzed with microscopy through the FEP material. As demonstrated in the HI-SEAS analog habitat, the optical clarity of the FEP bags allowed crew to check on the culture health and cell density. Timing of passage procedures can be adjusted in response to changes in growth rate. Tests for growth stability over multiple passages showed that fluctuations in the OD at passage did not impact the final OD of the next cycle of growth (Figure 6). On P5, one culture bag was used to seed all six of the new bags. This scenario demonstrates the flexibility of *A. platensis* for flight in the rare case that multiple bags become contaminated or grow fast enough to reach cell death phase prior to a crew passage activity. In these cases, one healthy bag can be used to seed others. If contamination is extensive enough to impact all bioreactors simultaneously, cryopreserved samples from previous passages could be thawed and used to reseed new bags. Spirulina is continuously cultured in industry and laboratories and is not prone to contamination in PBRs due to the high pH of the media (9-11.5). The pH of the culture can be an indicator of biomass concentration; as cyanobacterial cells metabolize the bicarbonate to CO_2 and OH^- ions the pH increases.[28]

The growth cycle of *A. platensis* is flexible and can be tailored to meet the needs of the mission by adjusting light intensity and temperature. To limit crew time and achieve a sustained culture duration of 5-6 months, the culture period for Space Algae-2 was lengthened to 14 days. The low temperature and light allowed cultures to grow slowly and reach high density; a similar trend is expected in spaceflight. Due to the lack of convective mixing, mass transfer of nutrients and waste products occurs through diffusion, which can slow the growth rate and lead to higher yields.[11] Healthy trichomes are positively buoyant and motile. Non-motile organisms often have improved growth in space because the lack of sedimentation improves nutrient uptake. This improvement is less common in motile organisms.[11] Fahrion et al. (2023) studied temperature and light conditions for *L. indica* for future growth in space. Their results were similar to this study and found higher final biomass with low light (36 µmol m^{-2} s^{-1}). Low temperature (23 °C) resulted in extended growth periods, but reduced pigment concentrations. With their specific strain, the optimal culture conditions for high biomass, pigment and photosynthetic efficiency, and low glycogen content were 30 °C and 35-75 µmol m^{-2} s^{-1} light.[28]

In this ground study, glucose supplementation was found to be the optimal organic carbon source to maintain *A. platensis* viability in soft stowage conditions for pre-launch stability. Mixotrophic growth can speed up propagation time, but for the flight experiment non-supplemented ZM will be used after the initial culture. Adding sugar to the medium also increases the chance for contamination, as observed in this study. During preliminary dark storage tests, samples were tested routinely resulting in some exposure to light. The flight-like test of dark stored cultures used two sets of cultures, one sampled weekly and another kept light protected for eight weeks and sampled again at week 10. Our results suggest that exposure to low levels of light throughout storage is beneficial to the cultures.

Liquid ZM stored for nine months successfully grew *A. platensis* cultures but resulted in a lower final OD compared to a fresh media control and lyophilized ZM. The lyophilized media was stable for nine months at room temperature and 37 °C and could be an option to stabilize the pH of the medium for longer term growth systems. Sterilized ISS potable water has been used to hydrate media in FEP bags to grow yeast and yogurt cultures in the BioNutrients-2 flight experiments.[29] Alternatively, ultra-pure water could be sent up with the experiment to hydrate the ZM. For Space Algae-2, liquid media is preferred to limit crew time needed to complete procedures for passaging and sample harvest. Further studies are being performed to investigate the stability of liquid ZM in FEP bags sealed in mylar zip-lock bags to limit gas exchange and stabilize the pH.

The dewatering and concentration of spirulina biomass is a challenging bottleneck of production on earth, and in space it will be more difficult without the help of gravity. When the flight procedures were tested, the passaging and cryopreservation steps were efficient, but the filtration step was too slow with the 25 mm diameter filter unit even with the included backflow steps. There was a large variation in timing between the first and second passage due to viscosity; it is unknown why cultures sometimes have more exopolysaccharides making it more viscous and difficult to filter. Rossi et al. also experienced membrane fouling when researching crossflow micro- and ultrafiltration methods for *A. platensis*.[30] We have optimized a membrane filtration method for batch culture in spaceflight. It is faster overall to split the 100 mL samples and filter through two 47 mm diameter units with 1.2 µm nylon membranes that collect 99-100% of trichomes. Each of the six 100 mL culture bags will produce approximately 180 mg of dry mass, which is projected to be sufficient for two technical replicate omics and nutritional analysis for each culture. With the current FEP bag production system, 1.6 - 2.7 L of *A. platensis* culture is needed to produce a 3-5 g serving of dry biomass. For future oxygen production and CO_2 removal in spaceflight, an optimized 20 L bioreactor could support one crew member.[31] Other estimates are upwards of 500 L and liquid gas phase separation in microgravity remains a challenge.[32] It should be possible to recycle the water used to culture microalgae back into the culture system or into the environmental control life support system (ECLSS) as gray water. However, chemical analysis of the spent media would be required to ensure compatibility with the current ISS water recovery system, and there are no plans to recycle spent media during Space Algae-2.

Tallec et al. developed a two-stage, automated biomass harvester for *Limnospira indica* for spaceflight applications.[21] The first stage separates biomass from liquid media through double-sided, dead-end filtration with stainless steel membranes, vibration, and backwashing. The second stage removes organic matter from the media and allows for nutrient recycling. In this study, it was difficult to remove the biomass from the filters after continuous operation due to caking. More research is needed to understand adhesion properties and to develop alternative dewatering methods. For example, automated tangential flow (TF) filtration would improve efficiency in harvesting from large continuous cultures by concentrating biomass using crossflow feed stream that runs in parallel to the filter

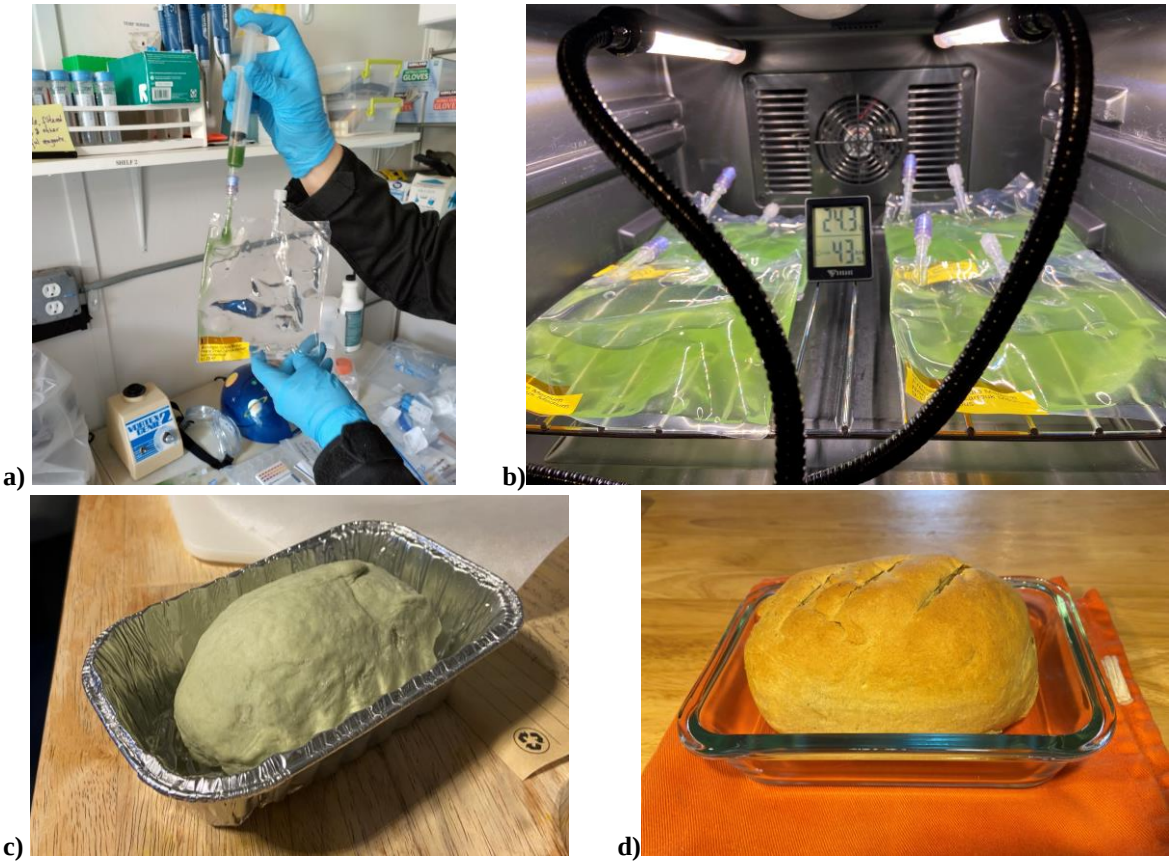


Image 3. *A. platensis* growth and bread supplementation at HI-SEAS. Inoculation of hydrated lyophilized ZM in the HI-SEAS laboratory (a). Flight-like cultures on day four of growth (b). Pre-risen bread dough (c) and baked loaf supplemented with fresh *A. platensis* biomass (d).

removing spent media to reduce caking. TF filtration is amenable to continuous flow systems and automating the filtration would reduce hands-on crew time needed for harvesting. For our batch culture experiment there was not a small enough TF unit that provided enough liquid removal. TF filtration is also time consuming and requires more materials than syringe filtration.

Spirulina cultures are usually continuously grown to maintain stocks. The cryopreservation method described in this study is the result of a significant optimization experiment campaign (data not shown, manuscript in preparation). In addition, a six-month cryopreservation storage test is in progress. This novel preservation method can impact the spirulina production industry because it is challenging to store viable *A. platensis* cells frozen or dried. For Space Algae-2, the cryopreservation method will enable the return of viable cells from the ISS that can be used to verify mutations identified by whole genome sequencing. In addition, cellular morphology can be assessed upon thawing to confirm any morphology changes aboard the ISS. We have observed that pre- and post-freeze morphology is similar with this method.

The HI-SEAS Space Algae-2 experiment showed that algae can be grown in a semi-controlled environment with minimal resources and used to make a nutritional product. Little crew time was needed because the cultures do not require continuous maintenance. A total of five hours of crew time over five days was used for this experiment. This time included extra tasks like daily trichome counting, pH measurement, and bag hydration that are not anticipated for the ISS flight. Slow sustainable growth with minimal power draw (20 W) was achieved. The lyophilized ZM reduced mass and allowed for resource utilization in the habitat. A higher light level was used to increase growth rate for this six-day mission, though incubation at a higher temperature would have increased growth further. HI-SEAS is powered by a solar charged battery, which can drain during long stretches of cloudy weather and certain power saving procedures must be put in place. On most days during this mission the battery achieved full charge. The cultures were successfully harvested using the optimized syringe filtration method. Approximately 0.16 g dry mass was baked into bread, which equated to 5.3% of a 3 g serving. Rahimah et al. found the optimal concentration of spirulina to dry ingredients to be 2-4%.[33] The baking temperature pasteurizes the culture and reduces the risk of ingesting other live microbes that might contaminate a spirulina culture. However, contamination is unlikely due to the high pH and lack of sugar in the medium. Spirulina has also been added to many other foods to increase nutritional quality, e.g., kefir [34,35] and pasta.[36] Although qualitative and quantitative taste sensory panels are outside the scope of Space Algae-2, future sensory experiments would help determine palatability and potential dietary supplement uses within a flight-like environment.

V. Conclusion

We have determined the culturing parameters, storage conditions, and harvesting procedure for spirulina growth on the ISS. The multi-omics data collected from Space Algae-2 will uncover the effects of continuous culturing in microgravity as well as exposure to space radiation levels found in the ISS cabin. These data are expected to identify strain variants or genes that improve growth in low Earth orbit. This basic research study will set the groundwork for future studies on cyanobacterial growth and utilization for bioregenerative life support and nutrition for long-duration space travel.

Acknowledgments

We thank Adam Williams (University of Minnesota) and Kato Claeys (Director and Commander of HI-SEAS) for their help and support during the HI-SEAS analog experiment. Special thanks to Kevin Sims and Sandra Vu of KBR for feedback and edits on this manuscript. Funding for the Space Algae-2 ground studies was provided by NASA Space Biology, grant number 18-FG_2-0059. Funding for Kristen Saban, an undergraduate research assistant who conducted the ConOps testing, was provided by the Space Life Sciences Training Program (SLSTP), NASA Ames Research Center. Funding for the HI-SEAS research experiment was provided in part by: International MoonBase Alliance (IMA), EuroMoonMars (EMM), and EuroSpaceHub Academy.

References

[1] E.D. Revellame, R. Aguda, K.M. Gatdula, W. Holmes, D.L. Fortela, W. Sharp, D. Gang, A. Chistoserdov, R. Hernandez, and M.E. Zappi, "Microalgae in bioregenerative life support systems for space applications," *Algal Research*, Vol. 77, January 2024, pp. 103332.

[2] D. Häder, "On the Way to Mars—Flagellated Algae in Bioregenerative Life Support Systems Under Microgravity Conditions," *Frontiers in Plant Science*, Vol. 10, No. 1621, 2020.

[3] T. Niederwieser, P. Kociolek, and D. Klaus, "A review of algal research in space," *Acta Astronautica*, Vol. 146, March 2018, pp. 359–367.

[4] R.A. Soni, K. Sudhakar, and R.S. Rana, "Spirulina – From growth to nutritional product: A review," *Trends in Food Science Technology,* Vol. 69, September 2017, pp. 157–171.

[5] G. Usharani, P. Saranraj, and D. Kanchana, "Spirulina Cultivation: A Review," *International Journal of Pharmaceutical & Biological Archives,* Vol. 3, No. 6, 2012, pp. 1327–1341.

[6] F. Delrue, E. Alaux, L. Moudjaoui, C. Gaignard, G. Fleury, A. Perilhou, P. Richaud, M. Petitjean, and J.F. Sassi, "Optimization of Arthrospira platensis (Spirulina) growth: From laboratory scale to pilot scale," *Fermentation*, Vol. 3, No. 4, 2017.

[7] L. Poughon, C. Creuly, F. Godia, N. Leys, and C.G. Dussap, "Photobioreactor Limnospira indica Growth Model: Application From the MELiSSA Plant Pilot Scale to ISS Flight Experiment," *Frontiers in Astronomy and Space Sciences,* Vol. 8, No. 700277, 2021.

[8] G. Detrell, H. Helisch, J. Keppler, J. Martin, N. Henn, S. Fasoulas, and R. Ewald, "PBR@LSR: the Algae-based Photobioreactor Experiment at the ISS - Operations and Results," *49th International Conference on Environmental Systems,* July, 2020.

[9] H. Helisch, J. Keppler, G. Detrell, S. Belz, R. Ewald, S. Fasoulas, and A.G. Heyer, "High density long-term cultivation of Chlorella vulgaris SAG 211-12 in a novel microgravity-capable membrane raceway photobioreactor for future bioregenerative life support in SPACE," *Life Sciences in Space Research*, Vol. 24, 2020, pp. 91–107.

[10] G. Senatore, F. Mastroleo, N. Leys, and G. Mauriello, "Effect of microgravity & space radiation on microbes," *Future Microbiology*, Vol. 13, No. 7, 2018, pp. 831–847.

[11] W. Li, A.M. Diaz, T. Irwin, A.E. Orourke, and L.M. Calle, "Microgravity Effect on Bacterial Growth: A Literature Review," *51st International Conference on Environmental Systems*, July, 2022.

[12] J. Zhang, B.S.F. Müller, K.N. Tyre, H.L. Hersh, F. Bai, Y. Hu, M.F.R. Resende, B. Rathinasabapathi, and A.M. Settles, "Competitive Growth Assay of Mutagenized Chlamydomonas reinhardtii Compatible With the International Space Station Veggie Plant Growth Chamber," *Frontiers in Plant Science,* Vol. 11, No. 631, 2020.

[13] T. G. Hammond, P. L. Allen, H. W. Wells, J. M. Russick, C. Nislow, G. Giaever, and H. H. Birdsall, "Moonshot: Affordable, Simple, Flight Hardware for the Artemis-1 Mission and Beyond," *Frontiers in Space Technologies*, Vol. 1, No. 593523, 2020.

[14] H. Badri, P. Monsieurs, I. Coninx, R. Wattiez, and N. Leys, "Molecular investigation of the radiation resistance of edible cyanobacterium Arthrospira sp. PCC 8005," *Microbiology*, Vol. 4, No. 2, 2015, pp. 187–207.

[15] H. Badri, P. Monsieurs, I. Coninx, R. Nauts, R. Wattiez, and N. Leys, "Temporal gene expression of the cyanobacterium arthrospira in response to gamma rays," *PLoS One*, Vol. 10, No. 8, 2015.

[16] H. Shiraishi, and H. Nishida, "Complete Genome Sequence of the Edible Filamentous Cyanobacterium Arthrospira platensis NIES-39, Based on Long-Read Sequencing," *Microbiology Resource Announcements*, December 2022.

[17] D.C. Berrios, J. Galazka, K. Grigorev, S. Gebre, and S. V. Costes, "NASA GeneLab: Interfaces for the exploration of space omics data," *Nucleic Acids Research*, Vol. 49, Database Issue, 2021, pp. D1515–D1522.

[18] R. Pathania, A. Srivastava, S. Srivastava, and P. Shukla, "Metabolic systems biology and multi-omics of cyanobacteria: Perspectives and future directions," *Bioresource Technology,* Vol. 343, No. 126007, 2022.

[19] N. Battchikova, D. Muth-Pawlak, and E.-M. Aro, "Proteomics of cyanobacteria: current horizons," *Current Opinion in Biotechnology,* Vol. 54, December 2018, pp. 65–71.

[20] I.A. Kharchuk, "Changes in Morphometric Indices of Arthrospira (Spirulina) platensis Cells and Trichomes during Dehydration and the Reactivation Period," *Russian Journal of Plant Physiology,* Vol. 67, No. 4, 2020, pp. 680–689.

[21] J. Tallec, M. Vandermies, C. Coene, B. Lamaze-Lefebvre, D. Demey, M. Frappart, and E. Couallier, "Implementation of an automated process for Limnospira indica harvesting and culture medium recycling for space applications," *Frontiers in Astronomy and Space Sciences,* Vol. 10, December 2023.

[22] T. Tarko, A. DuDa-ChoDak, and M. Kobus, "Influence of Growth Medium Composition on Synthesis of Bioactive Compounds and Antioxidant Properties of Selected Strains of Arthrospira Cyanobacteria," *Czech Journal of Food Sciences*, Vol. 30, No. 3, 2012, pp. 258–267.

[23] F. Chen, Y. Zhang, and S. Guo, "Growth and Phycocyanin formation of Spirulina platensis in Photoheterotrophic Culture," *Biotechnology Letters,* Vol. 18, No. 5, 1996, pp. 603–608.

[24] E.G. de Morais, J.I. Druzian, I.L. Nunes, M.G. de Morais, and J.A.V. Costa, "Glycerol increases growth, protein production and alters the fatty acids profile of Spirulina (Arthrospira) sp LEB 18," *Process Biochemistry,* Vol. 76, 2019, pp. 40–45.

[25] F.J. Marquez, K. Sasaki, T. Kakizono, N. Nishio, I. And, and S. Nagap, "Growth Characteristics of Spirulina platens& in Mixotrophic and Heterotrophic Conditions," *Journal of Fermentation and Bioengineering,* Vol. 76, No. 5, 1993, pp. 408–410.

[26] K. Chojnacka, and A. Noworyta, "Evaluation of Spirulina sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures," *Enzyme and Microbial Technology*, Vol. 34, No. 5, 2004, pp. 461–465.

[27] H. Shiraishi, "Cryopreservation of the edible alkalophilic cyanobacterium Arthrospira platensis," *Bioscience, Biotechnology and Biochemistry*, Vol. 80, No. 10, 2016, pp. 2051–2057.

[28] J. Fahrion, C.G. Dussap, and N. Leys, "Assessment of batch culture conditions for cyanobacterial propagation for a bioreactor in space," *Frontiers in Astronomy and Space Sciences*, Vol. 10, April 2023.

[29] N. Ball, A. Hindupur, H. Kagawa, A. Kostakis, A.L. Gresser, K. Sims, S. Sharif, A.G. Villanueva, F. Donovan, A. Mark Settles, and J.A. Hogan, "BioNutrients-2: Improvements to the BioNutrients-1 Nutrient Production System," *50th International Conference on Environmental Systems*, July 2021.

[30] N. Rossi, M. Derouiniot-Chaplain, P. Jaouen, P. Legentilhomme, and I. Petit, "Arthrospira platensis harvesting with membranes: Fouling phenomenon with limiting and critical flux," *Bioresource Technology*, Vol. 99, No. 14, 2008, pp. 6162–6167.

[31] J. Fahrion, F. Mastroleo, C.G. Dussap, and N. Leys, "Use of Photobioreactors in Regenerative Life Support Systems for Human Space Exploration," *Frontiers in Microbiology*, Vol. 12, No. 699525, 2021.

[32] R. Keller, K. Goli, W. Porter, A. Alrabaa, and J.A. Jones, "Cyanobacteria and Algal-Based Biological Life Support System (BLSS) and Planetary Surface Atmospheric Revitalizing Bioreactor Brief Concept Review," *Life*, Vol. 13, No. 3, 2023, pp. 816.

[33] S. Rahimah, M.F. Sonjaya, R. Andoyo, A. Satya, S. Nurhasanah, T. Chrismadha, "Physical and Organoleptic Characteristic of Bread Substituted with Spirulina Platensis," *Biomass Conversion and Sustainable Biorefinery*, edited by Lubis, M.A.R., et al., Green Energy and Technology, Springer, Singapore, 2024, pp. 295–306.

[34] D.S. Atik, B. Gürbüz, E. Bölük, and İ. Palabıyık, "Development of vegan kefir fortified with Spirulina platensis," *Food Bioscience*, Vol. 42, No. 101050, 2021.

[35] Ö. Kahraman Ilıkkan, and E.Ş. Bağdat, "The effect of kefir enrichment with Arthrospira platensis and Chlorella vulgaris on kefir microbiota, antioxidant, and physicochemical properties," *Journal of Applied Phycology*, December 2022.

[36] A. Hussein, G. Ibrahim, M. Kamil, M. El-Shamarka, S. Mostafa, and D. Mohamed, "Spirulina-enriched pasta as functional food rich in protein and antioxidant," *Biointerface Research in Applied Chemistry*, Vol. 11, No. 6, 2021, pp. 14736–14750.