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Erich D. Fleming
NASA Ames Research Center, Moffett Field, CA

Vrad M. Bebout
NASA Ames Research Center, Moffett Field, CA

Ming X. Tan
NASA Ames Research Center, Moffett Field, CA

Florian Selch
Carnegie Mellon University

Antonio J. Ricco
NASA Ames Research Center, Moffett Field, CA

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Biological system development for GraviSat: A new platform for studying photosynthesis and microalgae in space

Erich D. Fleming [a,*,1], Brad M. Bebout [a,*], Ming X. Tan [a], Florian Selch [b], Antonio J. Ricco [a,*]

[a] NASA Ames Research Center, Moffett Field, CA 94035, United States
[b] Carnegie Mellon University Silicon Valley Campus, Moffett Field, CA 94035, United States

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ABSTRACT

Microalgae have great potential to be used as part of a regenerative life support system and to facilitate *in-situ* resource utilization (ISRU) on long-duration human space missions. Little is currently known, however, about microalgal responses to the space environment over long (months) or even short (hours to days) time scales. We describe here the development of biological support subsystems for a prototype "3U" (i.e., three conjoined 10-cm cubes) nanosatellite, called GraviSat, designed to experimentally elucidate the effects of space microgravity and the radiation environment on microalgae and other microorganisms. The GraviSat project comprises the co-development of biological handling-and-support technologies with implementation of integrated measurement hardware for photosynthetic efficiency and physiological activity in support of long-duration (3–12 months) space missions. It supports sample replication in a fully autonomous system that will grow and analyze microalgal cultures in 120 µL wells around the circumference of a microfluidic polymer disc; the cultures will be launched while in stasis, then grown in orbit. The disc spins at different rotational velocities to generate a range of artificial gravity levels in space, from microgravity to multiples of Earth gravity. Development of the biological support technologies for GraviSat comprised the screening of more than twenty microalgal strains for various physical, metabolic and biochemical attributes that support prolonged growth in a microfluidic disc, as well as the capacity for reversible metabolic stasis. Hardware development included that necessary to facilitate accurate and precise measurements of physical parameters by optical methods (pulse amplitude modulated fluorometry) and electrochemical sensors (ion-sensitive microelectrodes). Nearly all microalgal strains were biocompatible with nanosatellite materials; however, microalgal growth was rapidly inhibited (~1 week) within sealed microwells that did not include dissolved bicarbonate due to CO_2 starvation. Additionally, oxygen production by some microalgae resulted in bubble formation within the wells, which interfered with sensor measurements. Our research achieved prolonged growth periods (>10 months) without excess oxygen production using two microalgal strains, *Chlorella vulgaris* UTEX 29 and *Dunaliella bardawil* 30 861, by lowering light intensities (2–10 µmol photons m^{-2} s^{-1}) and temperature (4–12 °C). Although the experiments described here were performed to develop the GraviSat platform, the results of this study should be useful for the incorporation of microalgae in other satellite payloads with low-volume microfluidic systems.

1. Introduction

Microorganisms offer great promise for application in a number of technologies critical to NASA space exploration goals including long-duration human space flight, colonization of asteroids and planets/moons, and *in-situ* resource utilization. In particular, microalgae, which include the Cyanobacteria and the Chlorophyta (i.e., green algae), are critical for future mission success due to their capacity for oxygenic photosynthesis. Cyanobacteria were responsible for the oxygenation of our atmosphere ~two billion years ago. Today, microalgae fulfill critical roles in the world's carbon and nitrogen cycles, produce much of the atmospheric oxygen, convert N_2 gas to ammonia and are responsible for more than half of the CO_2 fixed on Earth (Falkowski, 1994). As humans venture further into space, we will need to bring along contained ecosystems for sustained life support. Current physico-chemical methods

* Corresponding author.
E-mail addresses: erich.fleming@csuci.edu (E.D. Fleming), Brad.M.Bebout@nasa.gov (B.M. Bebout), ajricco@stanford.edu (A.J. Ricco).
[1] Present address: California State University Channel Islands, Camarillo, CA 93012, United States.

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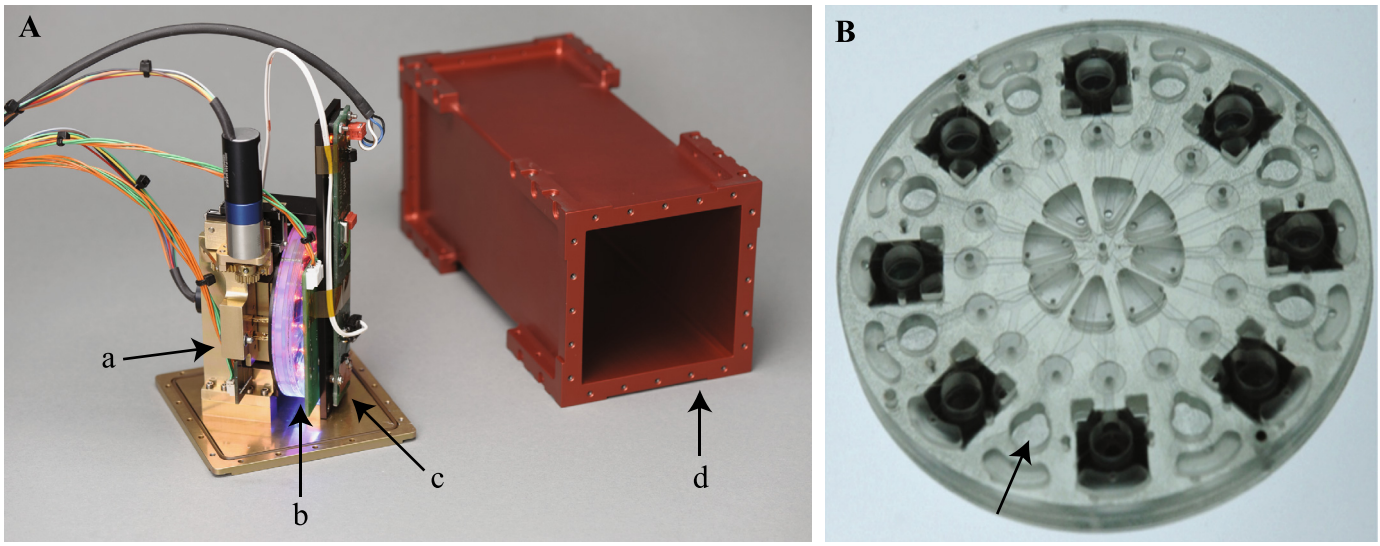

Fig. 1. A) GraviSat prototype (a) with fluidic disc (b), visible edge-on, illuminated by PAM fluorometry light source (c) and "2U" one-atm containment vessel (d). **B**) Microfluidic disc. 16 circular 120 µL wells are situated near the outer edge of the disc (arrow). Eight clear wells and eight black opaque wells are for optical sensors and electrochemical sensors, respectively.

of life support can support missions up to one year in length (Horneck et al., 2003). In contrast, biologically-based life support systems incorporating microalgae could support multi-year missions including the colonization of other planets, moons or asteroids. Microalgae are well-studied models of photosynthesis, many strains have genomes that are fully sequenced, and microarrays containing all their genes are available (Ikeuchi and Tabata, 2001; Jamers et al., 2009). Microalgal growth can also be controlled very precisely (without moving parts) by varying light intensity. These characteristics make them ideal test subjects for flight experiments designed to determine the effects of the space environment on photosynthesis, which is, arguably, the most important metabolism on Earth and will likely also be important for space travelers venturing away from Earth, even in locales where sunlight is dim and light for plant growth must be generated.

It is well established that the absence or near absence of gravity causes varied and significant deleterious effects in human and plant life (Turner, 2000; Ferl et al., 2002). Microorganisms, however, have been generally regarded as too small and intracellularly homogeneous for gravity to have an impact on their physiology (Pollard, 1965). In recent years, evidence to the contrary has been steadily mounting (Nickerson et al., 2004). *Escherichia coli* (Thévenet et al., 1996; Klaus et al., 1997), *Bacillus subtilis* (Mennigmann and Lange, 1986) and *Salmonella typhimurium* (Mattoni, 1968) cultures have all displayed altered growth characteristics during space flight. These bacteria grew more rapidly (shorter lag time) and reached higher cell densities compared to ground controls. In contrast, the *E. coli* carried aboard GeneSat and the *Saccharomyces cerevisiae* carried aboard PharmaSat grew more slowly in space (Ricco et al., 2011). Although the mechanism(s) through which microgravity impacts microorganism physiology is unknown, the prevailing evidence suggests that microgravity indirectly affects microorganisms through the generation of a quiescent fluid environment which allows microbes to rapidly deplete nutrients (and accumulate waste products) in their immediate vicinity (Klaus et al., 1997), consistent with the slower growth observed aboard nanosatellite experiments (Ricco et al., 2011). Microalgae have been studied less than many other microorganisms with regards to microgravity. However, in the few studies performed to date, microgravity or simulated microgravity induced a number of important changes, including altered growth characteristics (Wang et al., 2004, 2006), greater reactive oxygen production during photosynthesis (Li et al., 2004), increased toxin production (Xiao et al., 2010), and reduced photosynthetic capacity (Wang et al., 2006; Li et al., 2004). In particular, microalgae studied on two separate Chinese spacecraft were exposed to micro-g and 1 xg conditions in space using an onboard centrifuge (Wang et al., 2004, 2006). The microalgae were grown, without replication, in large vessels (120–200 mL) for only one–two weeks, and the results contradicted one another based on growth rate. Microgravity-induced changes in photosynthetic efficiency and growth characteristics are exceedingly important to understand for any bioregenerative life support or *in-situ* resource utilization technology involving the use of microalgae.

Nanosatellites provide a means to conduct experimental manipulations in the space environment on a variety of space-relevant organisms and biological systems including plants, tissue culture lines, and microbes. A fundamental requirement of good experimental design, not readily addressed by many space-borne platforms, is that of replicates and appropriate controls. The difficulty in interpreting the results of biological experiments in the absence of appropriate replication and of (spaceflight as opposed to ground) 1 xg controls for the effects of microgravity has been demonstrated (Mix et al., 2006). The experiments detailed in this paper constitute the scientific payload of a proposed 3U nanosatellite (i.e., a satellite comprised of three conjoined 10-cm cubes), called GraviSat, designed to conduct experiments on microalgae in space. GraviSat is a modification of previously flown GeneSat and PharmaSat platforms (Ricco et al., 2011, 2007). GraviSat incubates microorganisms in multiple growth chambers on a rotating disc, as opposed to a static microfluidic plate, to enable variable artificial gravity control, as shown schematically in Fig. 1A and B. GraviSat also incorporates a flight control, in addition to ground controls, by the inclusion of two fluidic discs, one spinning and one stationary, in the same pressure vessel. Ground controls would also incorporate the two-disc platform: one spinning and one stationary.

GraviSat is designed to facilitate low-cost studies on microalgae in the space environment by autonomously maintaining growing cultures and monitoring their physiological and metabolic processes under different levels of (micro)gravity for periods of time up to and potentially exceeding 6 months. GraviSat's 2U payload (the remaining "U" of the 3U nanosatellite being the "bus", which includes power, data handling, communications, command, and

control functionalities) contains two fluidic discs, one of which rotates to provide artificial gravity, and the other of which is stationary, thus providing an onboard record of the effects of microgravity. The effects of gravity (as provided by the spinning disc) can be statistically evaluated in isolation of various confounding variables (i.e., pre-launch conditions, hypergravity during launch, variable radiation environment). In addition, thin-film heaters located on both faces of the discs will result in near-zero temperature gradients in the radial direction of the rotating disc, eliminating the possibility of centrifugally driven thermal convective mixing within the wells and importantly removing any confounding effects of mechanical mixing. For the terrestrial control discs, the same arrangement of heaters typically results in a small (<0.2 °C once temperatures have stabilized) gradient between the outer faces and the center of the polymer disc, so the magnitude of any gravity-driven thermal convection would be very minimal.

GraviSat supports microbial physiology monitoring using optical and electrochemical sensors that measure chlorophyll *a* fluorescence (pulse amplitude modulated, or PAM fluorometry), pH, dissolved oxygen, and dissolved inorganic carbon dioxide. PAM fluorometry is a non-invasive technique for measuring photosynthetic efficiency. Since it is light based, measurements can be taken every few seconds providing a real-time assessment of photosynthesis while in orbit. Optical and electrochemical sensors are situated in two separate wells that are identical except for the sensor configuration. Eight pairs of wells (16 total) are located evenly around the outer edge of the disc. GraviSat will provide an unprecedented capability to conduct biological experiments capable of discriminating between multiple components of the space environment likely to be important to survivability and photosynthesis, including radiation and gravity level. The development of the systems embodied in the GraviSat design represents a completely new capacity for truly replicated biological experimentation in space with appropriate controls, thus addressing oft-cited critical roadblocks to interpretation of microbiological space experiments. Additionally, the GraviSat platform is highly flexible, and could be used on balloon flights, manned suborbital and interplanetary vehicles, and as a primary or secondary payload incorporated into unmanned lunar and planetary landers.

Here we detail the screening and testing process performed on microalgae during satellite design and construction. This process involved development and implementation of various biological analyses and experimental manipulations to optimize GraviSat system performance, ensure experiment integrity, and reduce the risk for a potential mission. Testing included: 1) A multi-step screening process for microalgae compatible with experimental goals and system hardware (i.e., hardware design, microfluidics, sensors), 2) biocompatibility testing with payload internal materials, particularly the fluidic disc, 3) manipulation of microalgal physiology to reduce growth and gas production, 4) reversible induction of physiological stasis for pre-launch through deployment and 5) performing a several-month ground-based mission simulation.

2. Materials and methods

2.1. Strains and culture conditions

The cyanobacteria and algal cultures used in this study were obtained from culture collections and/or isolated by our group from a variety of environments. *Nostoc* sp. ATCC 29 133 (PCC 73 102), *Synechocystis* sp. 27 184 (PCC 6803), *Synechocystis* sp. 27150 (PCC 6308), *Synechococcus* sp. 27194 (PCC 7001) and *Dunaliella bardawil* 30861 were obtained from the American Type Culture Collection (ATCC). *Chlorella vulgaris* UTEX 29, *Microcystis aeruginosa* B 2669, *Synechococcus elongatus* LB 563 and *Cyanothece* sp. ATCC 29 141 (PCC 7425) were obtained from the University of Texas Culture Collection. The PCC number in parentheses indicates the corresponding Pasteur Culture Collection designation. *Chroococcidiopsis* sp. CCMEE 54, 5606, 61, 62, *Calothrix* sp. 5085, *Scytonema* sp. 5099, *Leptolyngbya* sp. 5048 were obtained from the Culture Collection of Microorganisms from Extreme Environments (CCMEE) at the University of Oregon. *Scenedesmus* strains SV3 and SV6 were isolated from the Sunnyvale Water Pollution Control Plant oxidation ponds (Sunnyvale, CA). A *Thalassiosira* strain was isolated from Elkhorn Slough, Monterey Bay, CA.

All microalgae were maintained as batch cultures in liquid medium without air bubbling or agitation at 23 °C under low light conditions (10–50 μmol photons m^{-2} s^{-1} PAR [photosynthetically active radiation]) light, using "cool-white" fluorescent bulbs. Freshwater strains of cyanobacteria and *Scenedesmus* spp. were maintained in BG11 medium (pH 8) (Castenholz, 1988). Saltwater strains of cyanobacteria and the *Thallasiosira* strain were maintained in modified ASN3 medium (pH 8) (Rippka et al., 1979). The modified ASN3 contained 1.7 mM $NaNO_3$, 0.36 mM KH_2PO_4, and no citrate. *Dunaliella* strains were cultured in ATCC: 1174 DA medium (pH 7.5) and *Chlorella vulgaris* was cultured in ATCC: 847 algal proteose medium (pH 7.5).

2.2. PAM fluorometry

Experiments were conducted using a Walz Diving-PAM fluorometer or a Junior PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany) and data recorded on a computer using the WinControl software provided by Heinz Walz (http://www.walz.com). The Junior PAM was modified by the manufacturer to use a white LED instead of a blue LED excitation light and to use a 4 mm diameter fiber optic instead of a 2 mm plastic light guide. The chlorophyll fluorescence analysis followed that reported by Campbell et al. (1998) and Schreiber et al. (1995). The quantum yield, which is a relative measure of photosynthetic efficiency, was measured using the saturation pulse method on light- and dark-adapted cultures (Genty et al., 1989). Microalgae were exposed to a saturating pulse of light, which reduced the electron transport chain (ETC), closing all of the PSII centers and generating a fluorescence intensity maximum. The quantum yield of PSII ($\Phi PSII$) for a light-adapted sample was calculated based on F_S (steady-state fluorescence under actinic light) and F'_M (maximum fluorescence under actinic light) measurements (Eq. (1)).

$$\Phi PSII = \left(F'_M - F_S\right)/F'_M \tag{1}$$

The maximum quantum yield of PSII in the dark-adapted state was calculated as Eq. (2) where F_O represents steady-state fluorescence in darkness.

$$\Phi PSII_{(dark)} = (F_{M(dark)} - F_O)/F_{M(dark)} \tag{2}$$

The quantum yield ($\Phi PSII$) is also referred to as the photosynthetic efficiency and can be used as a proxy for oxygen production under constant light conditions (Campbell et al., 1998; Beer and Axelsson, 2004). Under stable conditions, the quantum yield should remain unchanged. Therefore, a decrease in the quantum yield and maximum quantum yield is indicative of an increase in stress (Parkhill et al., 2001; Fleming et al., 2007). The alleviation of said stress is expected to result in a return of yield values to pre-stress levels.

2.3. Experimental conditions

Unless stated otherwise, the experiments described below were performed in either of two types of standard 96-well plates. Early experiments were performed in commercially available 96-well

optical-bottom plates (black) from Nalge Nunc International (Penfield, NY); well dimensions (diameter × height) – 6.5 × 12.2 mm, well volume – 400 µL. The plates were sealed using Breathe-Easy® sealing membranes (medical-grade polyurethane membrane with acrylic adhesive on one side) from Diversified Biotech (Dedham, MA). Later experiments utilized custom-made polycarbonate 96-well plates with clear acrylic sheets on the top and bottom. These plates had the same footprint and volume as the Nunc 96-well optical plates to facilitate their use in equipment designed for the Nunc 96-well plates. The top covering-sheet had two 1 mm diameter holes for each well so cultures or medium could be loaded into the well while allowing air to be displaced. The top covering-sheet was then covered with a layer of double-sided 3M-467MP acrylic "transfer tape" and an additional thin sheet of clear acrylic plastic to seal the wells. Even though attempts were made to remove all air from each well before sealing, some air was trapped in the loading holes.

Temperature was controlled by placing plates in a benchtop cooler/heater (TropiCooler model 260014, Boekel, Feasterville, PA). Plates and cultures were illuminated from above using "cool-white" fluorescent light. Light intensity was manipulated by altering the distance between the light source and the plate and by using neutral density filters. The light intensity at the bottom of a well was ~30% of that at the top of a well. Plates were gently agitating every day by hand to reduce any effects due to spatial heterogeneity (from differences in light intensity and other causes) in each well. For any future mission, ground controls could be mechanically rotated at a low rate (i.e., rotation of the entire ground-control payload on a "rotisserie" sort of mechanism) to counteract sedimentation (Nicholson et al., 2011). Visible light was measured using a LiCor LI-1000 light meter equipped with a quantum sensor (LiCor, Lincoln, Nebraska). GraviSat will employ light emitting diodes (LED) and not "cool-white" fluorescents for illumination. However, differences in spectral quality between the "cool-white" fluorescent bulbs and the LEDs (which are available with a spectrum very similar to the fluorescent bulbs) should be minimal and have no meaningful affect on microalgal physiology. Temperature control on GraviSat will be the same as that used on the GeneSat, PharmaSat and O/OREOS spacecraft (Ricco et al., 2011, 2007; Nicholson et al., 2011). Importantly, thin film heaters like those used in these missions eliminate thermal gradients along the radial direction of the disc, which could potentially drive convective stirring in the absence of gravity.

Optical density at 750 nm ($OD_{750\,nm}$) of cultures grown in 96-well plates was measured using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). Plates were carried to and from the microplate reader in darkness to maintain a low-light environment and limit alterations of microalgal photochemistry by ambient room light. Additionally, readings by the microplate reader were too brief (<1 s) to induce photochemical processes. GraviSat will employ the same system as O/OREOS to determine light intensity and optical density in each well (Nicholson et al., 2011). Chlorophyll *a* fluorescence and quantum yield were measured using a Junior PAM, the 4-mm diameter optical fiber of which was affixed to an X–Y positioner; Chl *a* fluorescence was measured through the clear bottom of the plate. The positioner was constructed from two linear stages with stepper motors from Newmark Systems, Inc. (Mission Viejo, CA). The stages and Junior PAM were controlled and synchronized using a custom LabVIEW program (National Instruments, Austin, TX).

2.3.1. Biocompatibility tests

Eight strains of microalgae, including *D. bardawil*, *D. salina*, *C. vulgaris*, *Synechocystis* ATCC 27 184, *Chroococcidiopsis* CCMEE 54, *N. punctiforme*, *Synechococcus* ATCC 27 194 and *Thallasiosira*, were tested for biocompatibility with different materials that are incorporated into the GraviSat microfluidics disc. Materials included acrylic (poly(methyl methacrylate)), polycarbonate plastic, and acrylic adhesives (300LSE & 467MP; 3M, St. Paul, MN). Material processing and cleaning protocols were also tested, including conventional machining and laser cutting, sonic cleaning in a water-bath, and material baking in a vacuum oven (off-gassing). As with GeneSat and PharmaSat, materials sensitive to autoclave temperatures (plastics and adhesives) were treated with ethylene oxide to sterilize all surfaces before use in the experiments. The materials were then off-gassed at 55 °C under vacuum for 2 weeks to remove ethylene oxide and other harmful volatiles. The materials were immersed into microalgal culture and incubated at 23 °C under 50 µmol photon $m^{-2}\,s^{-1}$ light for no more than 2 weeks. For comparison, the microalgae were also grown in a PharmaSat microfluidics card (Ricco et al., 2011) that had been previously tested for biocompatibility with yeast (*Saccharomyces cerevisiae*). The PharmaSat card includes 48 wells (4 mm diameter × 7.8 mm deep, ~100 µL volume per well) and is composed of acrylic, acrylic adhesive, poly(ethelene terephyhalate) (PET) and polystyrene – the same materials (except for PET) used for the acrylic-based GraviSat discs. Microalgae were loaded into the 100 µL wells, which were then sealed using a Breathe-Easy® membrane.

2.3.2. Physiological manipulations

Microalgal growth and photosynthetic efficiency were assayed under varying photoperiods, light intensities, temperatures and levels of bicarbonate availability. All experiments were performed in 96-well sealed polycarbonate plates. Microalgal cultures used in these experiments included *D. bardawil*, *D. salina*, *C. vulgaris*, *Synechocystis* ATCC 27 184, *Chroococcidiopsis* sp. CCMEE 54, *N. punctiforme*, *Synechococcus* sp. ATCC 27 194, *Synechococcus elongatus* LB 563, *Synechocystis* sp. ATCC 27 150, *Cyanothece* sp. ATCC 29 141, *M. aeruginosa*, *Scenedesmus* sp. SV6 and *Thallasiosira* sp.

Photoperiods comprising 15:30 and 15:60 light:dark cycles (L:D; min:min) were generated with "cool-white" fluorescent lamps controlled by an electrical timer. Relatively short light:dark cycles were chosen in an effort to limit the buildup of oxygen beyond its solubility (i.e., bubble formation) during periods of illumination. The lamps generated visible light (400–700 nm) at a photon flux density of 75 µmol photons $m^{-2}\,s^{-1}$. Plates were incubated at 23 °C.

For experiments using continuous light (without a photoperiod), microalgae (*C. vulgaris* and *D. bardawil*) were exposed to photon flux densities of 120, 78, 51, 33, 21, and 13 µmol photons $m^{-2}\,s^{-1}$ and temperatures of 4, 12 and 23 °C. Multiple plate-incubators were used to simultaneously maintain the different temperatures. Light intensity gradients were created within each 96-well plate by layering neutral density filters on top of the plate, providing 16 wells per light intensity. No added sodium bicarbonate was used in this particular experiment.

In a separate series of experiments, bicarbonate manipulations were performed by adding sodium bicarbonate to media at a final concentration of 1.0, 0.1 and 0.001 M. All media was buffered with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulonic acid) at a pH of 8. Experiments were run at 4, 12 and 23 °C under constant light intensities of 120, 60 and 30 µmol photons $m^{-2}\,s^{-1}$.

Oxygen production was calculated in some experiments. The diameters of trapped bubbles that rose to the top of each well were used to calculate the volume of gas. Gas bubble shape was assumed to be that of a half sphere.

2.3.3. Sensitivity to radiation

Chlorella vulgaris sensitivity to ultraviolet A (UVA) radiation (320–400 nm; 3 $W\,m^{-2}$) was tested at 4 and 23 °C under constant photon flux densities of 120, 78, 51, 33, 21, and 13 µmol photons $m^{-2}\,s^{-1}$. No added sodium bicarbonate was used in the

medium for this particular experiment. Cultures were placed in a 96-well, clear plastic Nunc plates to increase UVA exposure. UVA radiation was generated by black-light fluorescent lamps (Sylvania) with a peak output at 350 nm. UVA intensity was measured using an IL-1700 radiometer (International Light, Newburyport, MA).

2.3.4. Reversible induction of stasis

Microalgal cultures *Chroococcidiopsis* CCMEE 54, *C. vulgaris*, *Synechococcus* ATCC 27 194, *Synechocystis* ATCC 27 184, *Synechococcus elongatus* LB 563, and *Scenedesmus* SV6 were sealed in 96-well polycarbonate plates and kept in darkness at 4 or 23 °C for 4 or 6 weeks. After this period the plates were incubated at 23 °C and illuminated at 75 µmol photons $m^{-2}\,s^{-1}$ for 2 to 7 days. Microalgal cultures were treated using various methods prior to stasis including: suspension in water, suspension in nutrient medium (BG-11 or 847 algal proteose medium), suspension in dilute nutrient medium (10^{-1} dilution), oxic conditions, anoxic conditions, and the addition of 1 $g\,L^{-1}$ glucose. For treatment with different media, cells were initially grown in nutrient medium and then centrifuged at 3000 rcf (relative centrifugal force to 1 xg) for 5 min. The medium supernatant was removed and the cells were suspended in water, nutrient medium or 0.1 concentration nutrient medium. Anoxic conditions were generated by bubbling cultures with N_2 gas. Comparable oxic conditions were generated by bubbling cultures with air. Bubbling was performed aseptically by passing the gas through a 0.2 µm filter.

2.3.5. Full-length mission simulation

Microalgal cultures of *C. vulgaris* and *D. bardawil* were sealed in a 96-well polycarbonate plate (45 wells of each species) and incubated at 23 °C in darkness for 6 weeks. The plate was then transferred to a plate incubator and incubated at 12 °C under 3 µmol photons $m^{-2}\,s^{-1}$ visible light (continuous illumination) for 10 months. Control wells (6 total) contained only water. Each well was assayed for optical density and photosynthetic efficiency. No added sodium bicarbonate was used in media for this particular experiment.

2.4. Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics Program Version 21 (IBM corp., Armonk, NY). One-way analyses of variance (ANOVA) were performed on data pertaining to microalgal biocompatibility with nanosatellite materials and data pertaining to growth rates of *C. vulgaris* and *D. bardawil* over a range of light intensities. Repeated measures analyses were performed on data pertaining to experiments involving variable bicarbonate, light:dark cycling, ultraviolet stress, and recovery from stasis. For the experiment on UVA sensitivity, only overlapping data during UVA exposure were compared between temperature treatments. Sphericity of data was assumed in all analyses.

3. Results

3.1. Strain selection

The initial pool of microalgae consisted of 20 strains of different genera including the cyanobacteria *Synechococcus, Synechocystis, Nostoc*, and *Microcystis* as well as the eukaryotic green algae *Chlorella, Dunaliella* and *Scenedesmus*. *Synechococcus, Synechocystis, Chlorella, and Dunaliella* exist as single cells. *Nostoc*, *Microcystis* and *Scenedesmus* exist as small chains of cells, aggregates or clumps. Nonetheless, all strains have near neutral buoyancy. Only *Dunaliella* possess flagella and are capable of "swimming." The goal was to generate a collection that contained one or two model organisms

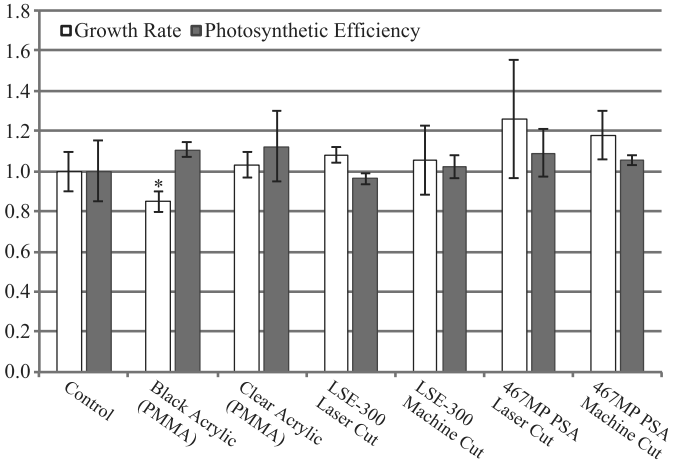

Fig. 2. Normalized growth rate (measured by OD_{750}; open bars) and normalized photosynthetic yield (measured by PAM fluorometry, shaded bars) for cultures of *Dunaliella bardawil* grown in 1174 DA medium with various materials of construction from the GraviSat disc, including black acrylic (PMMA), clear PMMA, 3M LSE-300 pressure-sensitive adhesive (PSA; laser cut and machine cut), 3M 467MP PSA (laser cut and machine cut). The cultures were placed into contact with these materials at 23 °C and grown for a total duration of 14 days. Values are based on measurements taken while cultures were in exponential phase. An asterisk indicates the average is significantly different from that of the control. Error bars represent ± one standard deviation of 4 replicates.

with a set of physiological characteristics that satisfy all experimental criteria. Two strains of the 20 examined, *C. vulgaris* and *D. bardawil*, were determined to satisfy all experimental criteria, as detailed below.

3.2. Biocompatibility

3.2.1. Materials

Based on visual inspection and PAM fluorometry, all cultures remained viable and photosynthetically active for about one week in the previously flight-qualified PharmaSat microfluidics card. However, *ΦPSII* steadily decreased over the course of the experiment suggesting a mild deleterious effect of the PharmaSat card environment (data not shown). A subset of microalgal cultures (*C. vulgaris*, *D. salina* and *Synechocystis* ATCC 27 184) were incubated in glass tubes and exposed to materials that made up the PharmaSat card and the GraviSat disc. All component materials individually had little to no deleterious effect on growth or photosynthetic efficiency. *D. bardawil* growth rates were slightly reduced by the use of black acrylic ($F_{6,21} = 33$; $p < 0.0001$), but photosynthetic efficiency was not affected ($F_{6,21} = 1.7$; $p = 0.17$) (Fig. 2). In addition, *Synechocystis* ATCC 27 184 when exposed to untreated acrylic adhesive (3M 300LSE and 467MP) showed slight decreases (~10%) in growth rate and photosynthetic efficiency ($F_{5,17} = 7.6$; $p = 0.002$). Only the sealing of *C. vulgaris* cells in ethylene-oxide-treated discs that were not subsequently degassed led to cell death, and then only after 5 days.

3.2.2. System design

The rather short growth periods recorded in the PharmaSat card were attributed to carbon limitation brought about by a low overall rate of supply of atmospheric CO_2 to the liquid medium. CO_2 must first diffuse through the 50-µm-thick polystyrene membrane, then over varying distances in the liquid medium to reach the algae. To delay the onset of carbon limitation, strains of microalgae were grown in media supplemented with sodium bicarbonate as a source of inorganic carbon. The highest levels of bicarbonate tested (1.0 mM) were inhibitory to some microalgal strains. However, all cyanobacteria in media supplemented with 0.1 mM

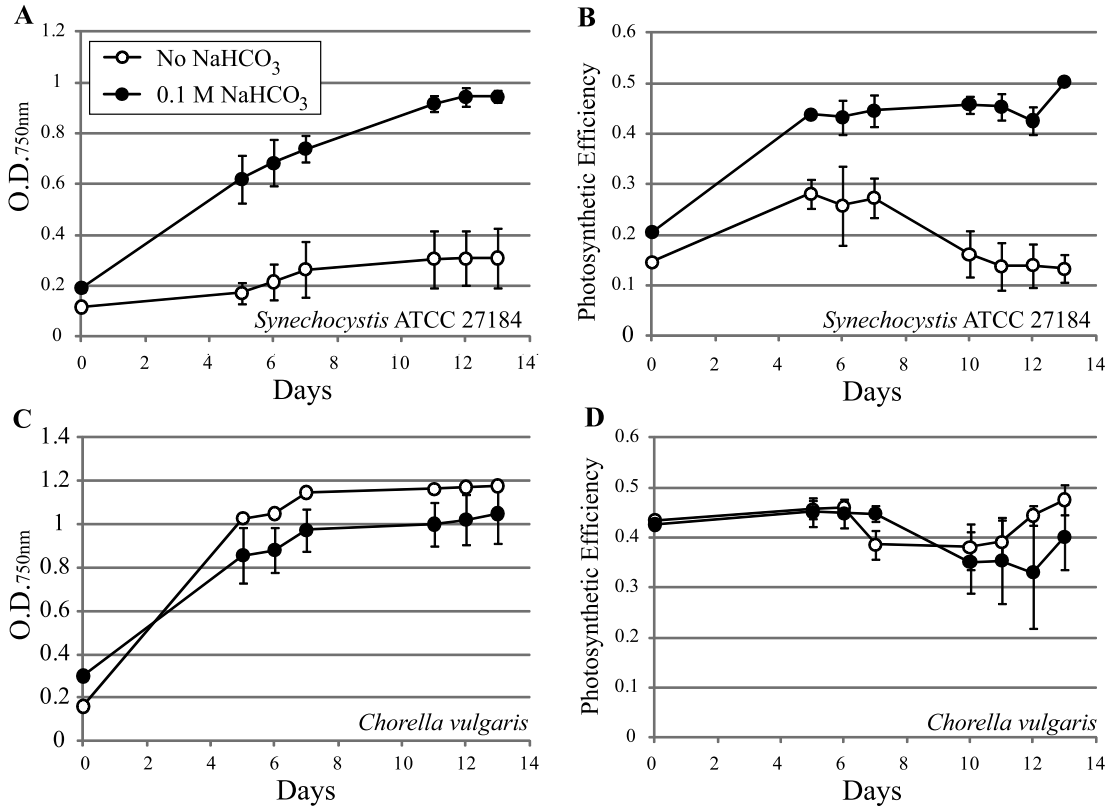

Fig. 3. Growth and viability parameters for cultures of the cyanobacterium *Synechocystis* ATCC 27 184 (**A** & **B**) and the green alga *Chlorella vulgaris* UTEX 29 (**C** & **D**) grown at 23 °C under 60 µmol photons $m^{-2}\,s^{-1}$ light in BG11 or ATCC 847 medium, respectively, without added sodium bicarbonate (open circles) or supplemented with 0.1 M sodium bicarbonate (solid circles). **A** & **C**) Photosynthetic efficiency measured via PAM fluorometry. **B** & **D**) Optical density at 750 nm. Error bars represent the standard deviations of 5 replicates.

sodium bicarbonate displayed higher growth rates, higher photosynthetic efficiencies and longer growth periods compared to the non-bicarbonate-supplemented controls. Additional bicarbonate also resulted in higher gas production and bubble formation. Similar gas production was not observed in control wells with only 0.1 mM sodium bicarbonate media suggesting that the gas production was from photosynthesis and not due to dissolved gas nucleating out of the media. Bicarbonate concentrations had little or no effect on the growth or photosynthetic efficiency of green algal strains (*C. vulgaris* and *D. salina*), although gas production was greater when bicarbonate was added to media. Fig. 3 illustrates the differences between cyanobacteria (*Synechocystis* ATCC 27 184) and algae (*C. vulgaris*) with regard to the effects of bicarbonate supplementation on growth ($F_{7,112} = 59$; $p < 0.001$) and photosynthetic efficiency ($F_{7,112} = 4.1$; $p < 0.001$).

3.3. Physiological manipulations to extend experimental duration in GraviSat cards

3.3.1. Photoperiod manipulation

As a possible alternative to bicarbonate supplementation, six strains of cyanobacteria (*Synechocystis* ATCC 27 184, *Synechococcus* ATCC 27 194, *Cyanothece* ATCC 29 141, *Synechocystis* ATCC 27 150, *S. elongatus* LB 563, *Microcystis aeruginosa* B 2669) and three strains of green algae (*C. vulgaris*, *D. bardawil*, *Scenedesmus* SV3) were exposed to cycles of 15:30 L:D (min:min) and 15:60 L:D at 23 °C and 10 µmol photons $m^{-2}\,s^{-1}$ PAR for 40 days. Fig. 4 illustrates the growth and photosynthetic efficiency curves for the representative species *Synechocystis* ATCC 27 184 and *C. vulgaris*. Microalgal cultures grew more rapidly under the 15:30 L:D regime, likely because they received a greater average light intensity ($F_{32,1152} = 29$; $p < 0.001$). Doubling the dark period slowed growth and, in some cases, quadrupled the growth period from 2 weeks to 2 months. Photosynthetic efficiency steadily declined over the 40-day experiment for all cyanobacteria under both L:D regimes, although the rate of decline was less under a 15:60 L:D regime. Unlike cyanobacteria, photosynthetic efficiencies in the green algal strains were stable for the entire 40-day experiment and were largely unaffected by photoperiod length ($F_{5,180} = 86$; $p < 0.001$). For most microalgae studied, prolonging the dark period slowed growth and reduced gas production. Only *C. vulgaris* showed no net oxygen production compared to control wells (no algae). For cyanobacterial strains, bubbles were at least 2–3 times larger by volume after 40 days than bubbles in control wells, although bubble volume was less under 15:60 L:D than for 15:30 L:D. Bubbles in the control wells formed within the first 1–3 days of the experiment and did not increase in number or size thereafter. These bubbles were most likely the result of dissolved gases nucleating from the aqueous buffer.

3.3.2. Temperature manipulation with continuous light

To characterize growth under continuous illumination conditions, cultures of *C. vulgaris* and *D. bardawil* were sealed in black polycarbonate 96-well plates having transparent tops and bottoms and exposed to visible light intensities between 10 and 120 µmol photons $m^{-2}\,s^{-1}$ at 12 °C for 45 days. Growth rates were lower at 12 °C compared to 23 °C (previous experiments in this study). For both strains, growth was inhibited at low and high light intensities (Fig. 5). For *C. vulgaris*, growth rates were highest under 33 µmol photons $m^{-2}\,s^{-1}$ light ($F_{5,47} = 20$; $p < 0.001$). For *D. bardawil*, growth rates were highest between 33 and 78 µmol photons $m^{-2}\,s^{-1}$ ($F_{5,47} = 8.5$; $p < 0.001$). For both strains, photosynthetic efficiencies remained relatively stable for the duration of the experiment although they did decrease slightly with time. There was

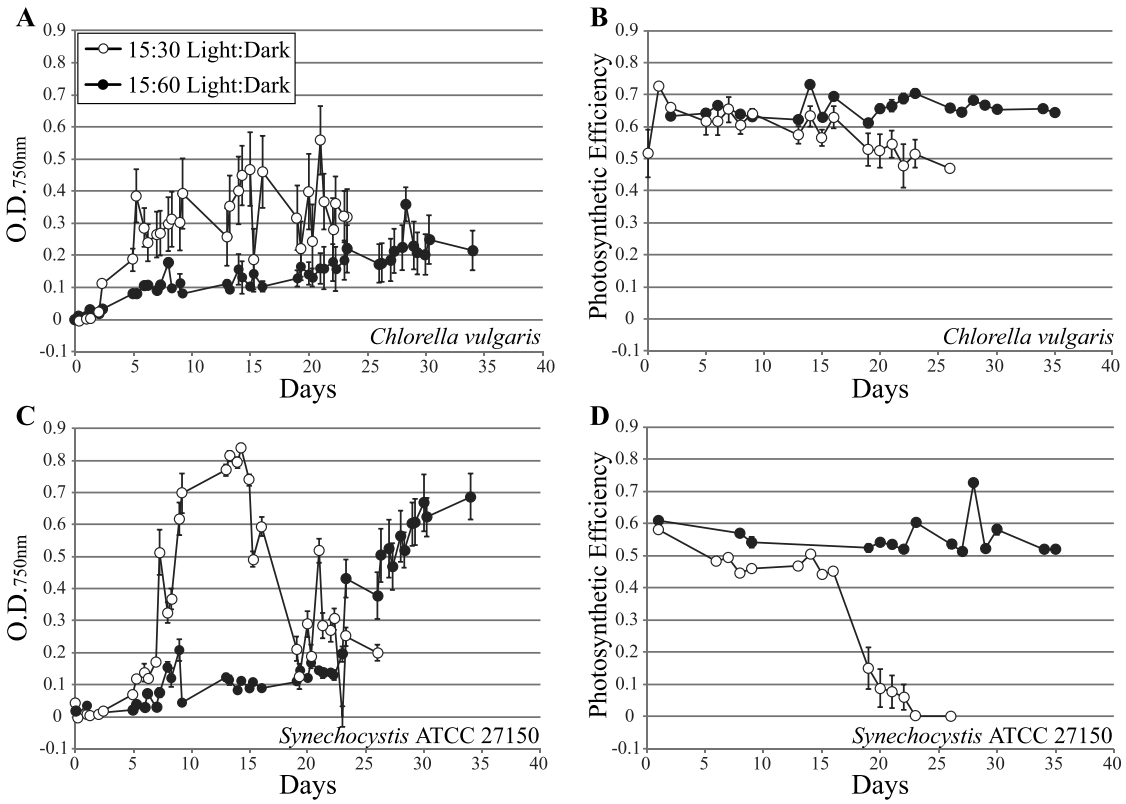

Fig. 4. **A** & **C**) Cell density (OD_{750}) and **B** & **D**) photosynthetic efficiency ($\Phi PSII$) of *Chlorella vulgaris* UTEX 29 (**A** & **B**) and *Synechocystis* ATCC 27 150 (**C** & **D**) grown under light:dark periods of 15:30 min:min (open circles) or 15:60 min:min (closed circles) at 23 °C and 10 µmol photons m^{-2} s^{-1}. Error bars represent ± one standard error for 10 replicates.

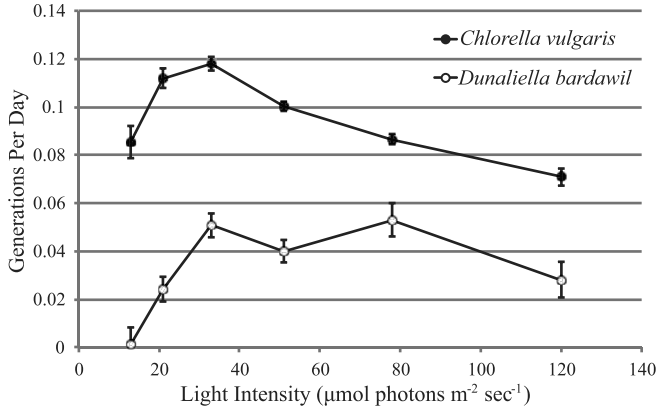

Fig. 5. Growth rates of *Chlorella vulgaris* UTEX 29 and *Dunaliella bardawil* at 12 °C under continuous visible illumination with light intensities of 120, 78, 51, 33, 21, and 13 µmol photons m^{-2} s^{-1}. The number of generations per day is calculated from daily measurements of OD_{750}. Error bars represent ± one standard error for 8 replicates.

also no apparent bubble formation by the two strains at any of the light intensities.

3.3.3. Potential sensitivity to stress

To test stress sensitivity, *C. vulgaris* and *D. bardawil* were exposed to 3 W m^{-2} UVA radiation (about 15% of peak UVA radiation on the Earth surface under clear skies) at 4 and 23 °C and under visible light from 7 to 120 µmol photons m^{-2} s^{-1}. UVA radiation was used as it has an immediate and measurable adverse effect on photosynthesis and, in particular, photosynthetic efficiency in microalgae. At both temperatures and light intensities the photosynthetic efficiency decreased at a similar rate upon exposure to UVA radiation (Fig. 6). Additionally, after constant exposure for 3–5 h all cultures recovered to pre-exposure levels. Although there was slight variation between treatments ($F_{7,56} = 7.9$; $p < 0.001$), sensitivity to UVA radiation did not increase with decreasing temperature or light intensity.

3.4. Reversible induction of stasis

Six microalgal cultures (2 green algae and 4 cyanobacteria) were assayed for their ability to survive 6 weeks of stasis and then recover photosynthetic activity. Microalgae were placed in darkness in sealed containers and incubated at 4 or 23 °C. Microalgae were provided different carbon sources (glucose or CO_2) and various levels of nutrient availability under oxic or anoxic conditions. Microalgae did not appear to grow during stasis although all strains in nearly all conditions survived and fully recovered within 5 days. In addition, there was no apparent effect of temperature on survival or recovery, although rates of recovery did vary depending on the strain and the media conditions. Green algal strains such as *C. vulgaris* recovered more rapidly when provided glucose and oxygen ($F_{15,120} = 42$; $p < 0.001$) (Fig. 7). Cyanobacterial strains appeared to recover most rapidly in dilute, anoxic BG11 medium.

3.5. Full-length mission simulation

C. vulgaris and *D. bardawil* cultures were sealed in a black 96-well polycarbonate plate, the material that is currently the leading candidate for GraviSat fluidic discs (owing to its lower moisture permeability relative to acrylic as well as excellent biocompatibility). Both strains survived the 6-week stasis period, recovered within two days and then displayed stable photosynthetic activity for 10 months (Fig. 8). During stasis at 23 °C, culture densities and chlorophyll *a* fluorescence declined. Photosynthetic

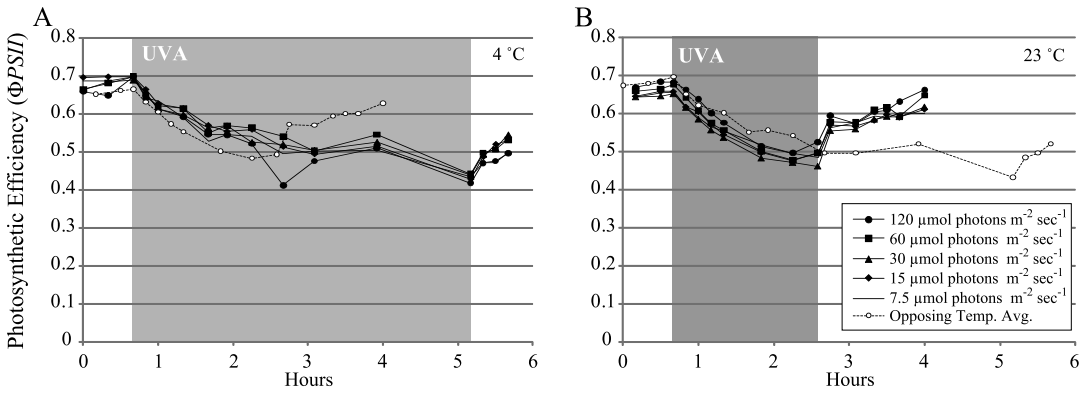

Fig. 6. Photosynthetic efficiency (*ΦPSII*) before, during (grey background), and after exposure to UVA radiation, measured using PAM fluorometry, for cultures of *Chlorella vulgaris* UTEX 29 grown in ATCC 847 medium under continuous 6.25, 12.5, 25, 50, or 100 µmol photons $m^{-2}\,s^{-1}$ visible illumination at **A**) 4 °C or **B**) 23 °C. Open circles represent the average *ΦPSII* of the five light intensities in the alternative temperature. After the first 45 min of growth, cultures were exposed to 3 $W\,m^{-2}$ UVA radiation for (**A**) 2 h at 4 °C or (**B**) 4.25 h at 23 °C.

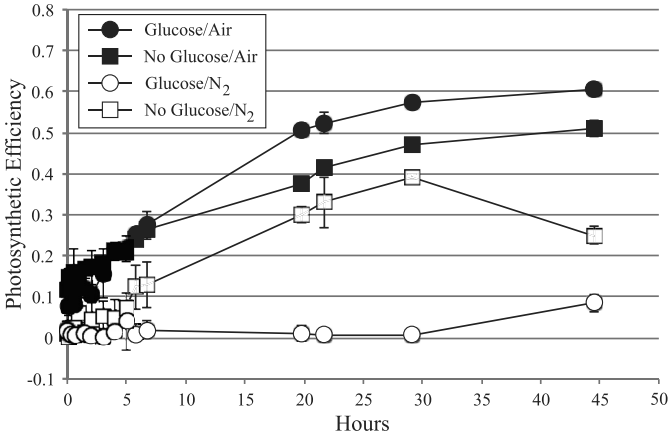

Fig. 7. Recovery of photosynthetic efficiency (*ΦPSII*) in *Chlorella vulgaris* UTEX 29 after stasis for four weeks. During stasis, cultures were kept in darkness and in oxygenated 847 proteose medium supplemented with 1 $g\,L^{-1}$ glucose (closed circles), oxygenated medium without glucose (closed squares), anoxic medium supplemented with 1 $g\,L^{-1}$ glucose (open circles), or anoxic medium without glucose (open squares). Cultures were removed from stasis by exposure to 75 µmol photons $m^{-2}\,s^{-1}$ light. Error bars represent ± one standard deviation for 3 replicates.

efficiency (*ΦPSII*), however, did not decline but remained stable during the stasis period. Upon reactivation (exposure to light), cultures were grown at 12 °C under 3 µmol photons $m^{-2}\,s^{-1}$ for 10 months. Fluorescence and culture densities increased while photosynthetic efficiency values rapidly decreased from ~0.7 to 0.55 for *C. vulgaris* and from ~0.55 to 0.25 for *D. bardawil* (Fig. 8). After this initial decline in the photosynthetic efficiency upon reactivation, values for both strains remained relatively stable or almost 10 months. For *C. vulgaris*, photosynthetic efficiency values decreased slightly with time. Additionally, biomass increased for about 105 days before reaching stationary phase. *D. bardawil* reached stationary phase ~170 days after reactivation.

4. Discussion

The analytical techniques and novel methods detailed here were developed to enable studies of photosynthesis in the space environment using microalgae on GraviSat and other similar nanosatellite, small satellite, space station or secondary payload platforms. Each technology critical to the incorporation of microalgae as a nanosatellite payload (strain selection, biocompatibility, physiological manipulation of strains, reversible induction of stasis, and the subsequent incorporation of these tests into a one mission simulation) is discussed below. Additionally, each step, including a short description, outcomes and conclusions/recommendations is summarized in Table 1.

4.1. Strain selection

GraviSat fluidic discs incorporate materials and fluidics technologies not previously used for growing microalgae in space flight experiments, necessitating laboratory experimentation to determine microalgae compatibility with the new hardware and materials. Microalgae are a polyphyletic group with a large diversity of physiologies and metabolisms. However, not all classes of mi-

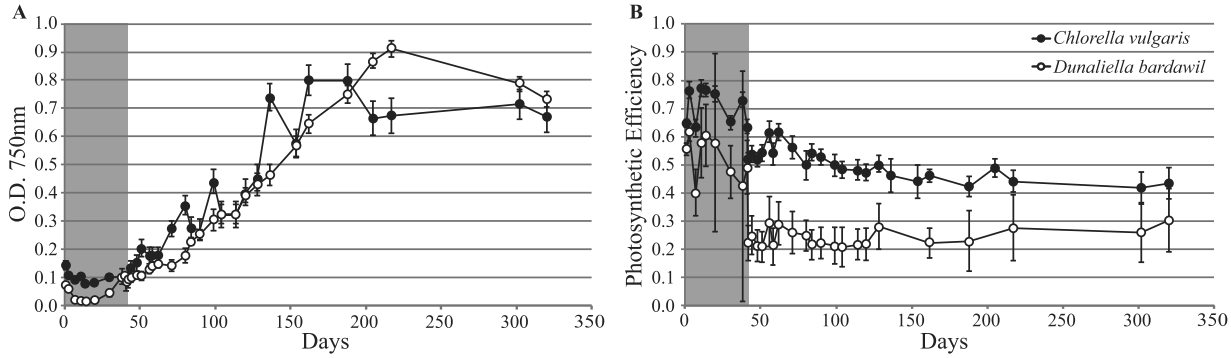

Fig. 8. Full-length (10-month) mission simulation run on microalgae strains *Chlorella vulgaris* UTEX 29 (solid circles) and *Dunaliella bardawil* 30 861 (open circles). Cultures were kept in darkness and in stasis for 45 days (shaded region of both panels) at 23 °C before exposure to light for the remainder of the run (3 µmol photons $m^{-2}\,s^{-1}$ visible light at 12 °C). **A**) Photosynthetic efficiency (*ΦPSII*) measured using PAM fluorometry. **B**) Cell density measured as OD_{750}. Error bars represent ± one standard deviation for 45 replicates.

Table 1
Goals, requirements, activities, and results for selection of photosynthetic organisms for spaceflight.

	Goal	Requirements	Possible approach(es)	Results	Conclusions/recommendations
Initial screen	• Build a collection of microalgae wherein at least one or two strains can be found to satisfy all experimental criteria	• Large diversity (CB, GA, and D) • Contain strains that are optically and spatially homogeneous in liquid culture – compatible with sensors • Contain strains with low surface adhesion – compatible with sensors and microfluidics	• N/A	• N/A	• N/A
Biocompatibility	• Determine long-term toxic/inhibitory effects of nanosatellite materials on various microalgae	• Low or no toxic materials effects for contact of organisms/media • Low or no toxicity of gases/vapors emitted from materials • Survival with minimal inhibitory effect for 2–12 months in stasis or active culture	• Use sterilization procedure developed for PharmaSat: ethylene oxide followed by bakeout to remove toxic residues	• All organisms compatible with plastics (acrylic, polycarbonate) • Some sensitivity to pressure-sensitive adhesives • All cyanobacteria & some green algae declined steadily in sealed systems regardless of material	• Ethylene oxide sterilization treatment is effective with adequate bake-out • Most microalgae are compatible with current materials
Physiological manipulations	• Identify appropriate combination of temperatures, light intensities, illumination durations, and media	• Support a minimum 2-month growing period • Support a 4–6 week stasis period • Limit gas production to minimize bubble formation	• Photoperiod manipulation • Constant or variable intensity light • Lower temperatures slow growth, increase gas solubility • Nutrient supplementation, e.g. bicarbonate	• Low L:D ratios slowed growth in all strains • CB generated bubbles even at lowest L:D ratios • Lowering constant light levels limited growth and gas production • CB made bubbles even at lowest light intensities • Bicarbonate in medium extended growth period • Bicarbonate led to greater gas production	• ~2-month growing period achievable • Photoperiod manipulation not effective to lower growth and metabolic rates • Gas removal technologies need to be developed to successfully incorporate cyanobacteria and other high gas producers
Stasis	• Identify the conditions necessary to induce stasis and to recover from stasis	• Induce physiological stasis; prevent or limit growth for 4–6 weeks at 23 °C • Reactivate cultures and rapidly return activity to prestasis levels on command	• Maintain complete darkness • Manipulate oxygen availability • Manipulate nutrient availability • Alternative carbon sources	• Growth greatly reduced in darkness • Metabolic activity reduced in darkness • CB – most rapid recovery with low nutrient, anoxic stasis conditions • GA – most rapid recovery with glucose-supplemented, oxic stasis conditions	• Microalgae can be placed into stasis for 4–6 weeks by the absence of light
Full length mission simulation	• Perform a test run (mission simulation): 4-week stasis period followed by 6 months under conditions experimental growth	• Conduct experiment in GraviSat prototype • Reversibly induce stasis at room temperature • Reactivate cells upon command • Grow cultures for 6 months without excessive gas production or loss of viability • Monitor cell growth and photosynthetic efficiency	• Utilize *C. vulgaris* and *D. bardawil* • Induce stasis at 23 °C by dark incubation • Reactivate with visible light: 3 μmole photons m^{-2} s^{-1} • Incubate cultures at 12 °C for 6 months • Measure optical density and pulse-amplitude-modulated fluorometric signal daily/weekly	• *C. vulgaris*: sustained growth for 105 days after 4 week period of stasis • *D. bardawil*: sustained growth for 170 days after 4 week period of stasis • Photosynthetic efficiencies were stable but slowly declined	• 4 weeks of stasis followed by 2–6 months of active growth is readily achievable

CB – cyanobacteria; GA – green algae; D – diatoms; L:D – light:dark.

croalgae could nor needed to be screened. The goal of this study was to find one or two representative model organisms with a set of physiological characteristics that satisfy all experimental criteria. We focused on common and well-studied strains of green algae and cyanobacteria representative of the metabolic and physiological diversity within the microalgae. Cyanobacteria and algae differ metabolically in that algae, unlike cyanobacteria, are capable of performing oxygenic photosynthesis and respiration simultaneously (Kliphuis et al., 2011). Consequently, many algal strains are able to up or down-regulate photosynthesis and respiration activity depending on CO_2 and O_2 concentrations (Hoch et al., 1963; Foyer and Noctor, 2000). Cyanobacteria, however, are unable to conduct respiration in the light making them more susceptible to carbon limitation.

Microalgal strains were also selected with morphological traits anticipated to be compatible with microfluidic disc design and sensor technology. With microalgae, factors such as cell–cell or cell–surface adhesion can increase spatial heterogeneity even within a low-volume (~100 μL), and unmixed vessel. For example, surface-adherent cells will attach to the clear acrylic window for optical sensors or directly to the electrochemical sensors and artificially elevate local *OD*, fluorescence, or oxygen values. Spatial heterogeneity increases experimental variability and makes each individual measurement less representative of the overall sample. With this in mind, the initial pool of green algae and cyanobacteria cultures was primarily comprised of strains with a homogeneous optical cross-section (single cells or small cell clusters) and low surface adherence. Only the green alga, *Dunaliella* possessed flagella and was motile. Strains with filamentous or colonial morphologies were added to the pool for comparison, including the cyanobacteria *Calothrix, Scytonema*, and *Chroococcidiopsis*.

4.2. Biocompatibility

Biocompatibility of organisms to all nanosatellite materials is important not only to maintain microbial growth but also to remove additional, possibly confounding, materials-related effects that could complicate results. In the GraviSat hardware under development, microalgae come in direct contact with only a few materials and are then only exposed to a small area (relative to the volume of culture material). However, chemicals leached into the growth medium and/or emitted into the gas phase and dissolved into the medium – whether from these or other materials not in direct contact with the microalgae – can alter their physiology. Over a multi-month or multi-year mission, even compounds with small deleterious effects can lead to cell death, premature mission termination or, more subtly, less efficient metabolic activity manifested as decreased photosynthetic efficiency. Because GraviSat is built upon the GeneSat and PharmaSat technology, some of the biocompatibility testing was previously performed and sterilization procedures developed. However, GeneSat and PharmaSat hosted *E. coli* and *S. cerevisiae* as their respective test organisms; no biocompatibility testing was performed on microalgae in the development of these two systems. In the current study, microalgae grew in clean, sterilized PharmaSat microfluidic plates but declining health suggested that the materials may not be completely biocompatible. Individual material (pre-treated) tests, however, did not support this conclusion; no reduction in growth was observed in cultures to which each of the component materials was added. To test long-term viability in the most biocompatible containers known to us, cultures were placed in commercially available 96-well cell-culture plates, which were then sealed with a gas permeable (Breathe-Easy®) membrane. Again, cultures (primarily cyanobacteria) exhibited sustained growth for only 1 to 2 weeks, showed declining fitness over that time and reached stationary phase at relatively low cell densities. Since the possibility of material toxicity was extremely low in the 96-well plates and with the Breathe-Easy® membrane, we hypothesized that declining fitness was most likely the result of resource limitation and not due to a lack of biocompatibility. Subsequent experiments indicated cultures were carbon limited.

4.3. Growth limitation in sealed fluidics cards

In a sealed vessel, like the GraviSat fluidic disc or a 96-well plate covered with a gas-permeable membrane, the diffusion of gases between the atmosphere and the culture medium may affect a number of important photophysiological processes. Algae and cyanobacteria rely on gas exchange with the atmosphere to provide CO_2 and remove excess O_2 generated through photosynthesis. The carbon requirement for green algae and cyanobacteria is extremely high compared to other nutrients (124:16:1 C:N:P) (Quigg et al., 2003). Without CO_2 as an electron acceptor, photosynthetic efficiency decreases; at least for obligate phototrophs, they cannot produce biomass and thus cannot grow. Carbon limitation was primarily evident in sealed cultures of cyanobacteria and was temporarily alleviated by the addition of sodium bicarbonate to the medium. In addition to CO_2 limitation, microalgae can be negatively affected by oxygen produced as a result of photosynthesis. Oxygen concentrations can reach 300% air saturation levels in the water in which algae grow, even in their natural environment. At low CO_2 or high O_2 levels, oxygen out-competes CO_2 for RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase, the enzyme that catalyzes the reduction of CO_2) catalysis thereby inhibiting carbon fixation (Keys, 1986). At such high concentrations algae can also experience oxygen toxicity (Shelp and Canvin, 1980; Fridovich, 1998). High concentrations of O_2 also lead to the generation of reactive oxygen species that then damage cellular components and inhibit photosynthesis (Asada and Takahashi, 1987; Apel and Kirt, 2004). Microalgae experiencing negative effects from high concentrations of oxygen is a distinct possibility in sealed growth chambers like the GraviSat fluidic disc.

4.4. Physiological manipulations to limit growth rate

GraviSat was designed to support microalgal cultures for at least 6 months. However, under optimal growth conditions, algae and cyanobacteria displayed declining photosynthetic activity after 1–2 weeks in a PharmaSat card or a sealed 96-well plate. If GraviSat design iterations that incorporate the use of media changes (up to one or two complete volume replacements) to replenish nutrients and lower cell concentrations are taken into account, microalgal cultures would still need to remain viable and active for 2–3 months. Growth periods of 2 months or more could only be achieved by decreasing the growth rate and metabolic activity of the microalgae. This was achieved by decreasing temperature more than 10 °C below optimal growth temperature (~23 °C) to slow all metabolic processes, and lowering the average light intensity to 3 μmol photons $m^{-2} s^{-1}$ to also slow photosynthetic activity. A light intensity of 3 μmol photons $m^{-2} s^{-1}$, although low, is ~10% and ~5% the optimal light intensity for growth in *C. vulgaris* and *D. bardawil*, respectively. Furthermore, the microalgae were photosynthetically active at this low light intensity which should therefore support meaningful results on any future mission.

Although effective in lengthening the growth period, lowering metabolic activity and limiting energy generation can lead to greater sensitivity to certain environmental stresses (Roos and Vincent, 1998). By lowering temperature and light intensity the amount necessary to prolong the growing period to 2 months, the microalgae could become highly sensitive to other stresses, such as solar/cosmic ionizing radiation in the space environment. Elevated stress sensitivity could complicate results, diminish photosynthetic

efficiencies, or, at worst, lead to cell death and the loss of the culture. As we could not replicate cosmic radiation, we used UVA to induce stress. In this study, the negative effects of UVA on photosynthetic efficiency were similar under all conditions tested (4 and 23 °C, and 7–120 μmol photons m^{-2} s^{-1}), which suggests that the ability of *C. vulgaris* and *D. bardawil* to deal with stress is most likely not limited in the low-temperature, low-light environment planned for the GraviSat nanosatellite.

4.5. Physiological manipulations to limit gas production

Microalgal cultures produce copious amounts of oxygen as a byproduct of photosynthetic activity. As stated previously, high concentration of O_2 can induce oxygen toxicity in the algal culture and also lead to the formation of bubbles. Gas production and bubble formation is a singular problem for microfluidically based biological growth systems for space microgravity experiments, as these systems cannot be designed to use gravitation to direct bubbles "up" and away from sensors or optical measurement paths (Ricco et al., 2007; Nicholson et al., 2011). In fact, gas bubbles can also be problematic for terrestrial microfluidic systems, where they can interfere with fluid movement and organism growth, as well as various types of measurement, particularly if they form in features so small that surface tension forces overcome bubble buoyancy (Zheng et al., 2010). If they were to form in the GraviSat fluidic disc, gas bubbles could interfere with its optical (PAM fluorometry) measurements and electrochemical sensors in addition to creating spatial inhomogeneity in dissolved gas and nutrient concentrations.

Oxygen production, like growth rate, can be reduced by lowering the temperature or light intensity. However, in a sealed chamber, even low rates of metabolic or photosynthetic activity will result in O_2 accumulation and bubble formation. Therefore, net oxygen production, not just the rate of O_2 production, must be reduced to prevent bubble formation. Net oxygen production can be lowered by decreasing the photosynthetic rate (oxygen production) relative to the respiration rate (oxygen consumption). In green algae, the two metabolic processes are separated within the cell (chloroplasts and mitochondria) and, under illumination, occur simultaneously. Therefore, reducing the light intensity will lower photosynthetic activity relative to respiratory activity and most likely result in lower net O_2 production. Additionally, the rates of respiration, photosynthesis and photorespiration (the consumption of O_2 through RuBisCO) can be affected by temperature and the O_2/CO_2 ratio (Davison, 1991). Respiration and photorespiration are typically stimulated under high O_2/CO_2 ratios and photosynthesis under low ratios. These characteristics inherent to green algae may explain their superior performance compared to other groups tested in this study. The green algal strains performed well under continuous, low intensity light (<5 μmol photons m^{-2} s^{-1}) displaying long (~2 month) growth periods with little or no gas production.

Unlike green algae, however, cyanobacteria (obligate phototrophs) contain their photosynthetic and respiratory systems in the same compartment and even share components (Scherer, 1990). Even though some cyanobacteria can perform the two processes simultaneously, photosynthetic rates are most likely much greater than respiratory rates except under darkness and extremely low light intensities (Scherer et al., 1988). To stimulate O_2 consumption (respiration) a light-dark cycle is needed to oscillate between the two processes. In the current study, light cycle manipulation lengthened the growth period of many cyanobacterial strains and limited their gas production. However, cyanobacteria appeared to require extremely small L:D ratios to prevent bubble formation. Consequently, cyanobacterial cultures would spend almost the entirety of an experiment respiring in darkness, which contradicts one of the main purposes of using cyanobacteria: to study photosynthesis. Additionally, irregular phenomena with short- or long-term effects (e.g., the peak of a solar particle event) could occur within one period of darkness, one period of illumination, or during a transition period. For GraviSat, exposing cultures to continuous light is therefore preferable to a L:D cycle. Cyanobacteria were thus removed from consideration for incorporation into long-term experiments aboard the GraviSat platform because of their high oxygen production characteristics and their inability to perform photosynthesis and respiration simultaneously. Although their high oxygen production capability may make cyanobacteria important components of future renewable life support systems, any such systems will have to be engineered with appropriate gas control/removal mechanisms. For the GraviSat studies, cyanobacterial strains continued to be studied in subsequent assays, but primary focus was placed on green algal strains particularly that of *C. vulgaris* and *D. bardawil*.

4.6. Reversible induction of stasis

Nanosatellites are relatively inexpensive platforms for conducting biological experiments in space, in part because they usually reach orbit as secondary payloads and/or rocket ballast. As a secondary payload, however, nanosatellites have low priority with respect to launch preparation and schedule. They typically are integrated with a space vehicle well in advance – some 4–8 weeks – of the scheduled launch date and must remain unpowered and without monitoring or measurement until orbital deployment. Test subjects inside the nanosatellite are exposed to ambient and variable temperatures (usually with protection from freezing and excessive heat) and go without light, which can be a challenge for phototropic organisms. Therefore, it is often desirable or even necessary to place study organisms into some form of stasis where metabolism is reduced or inhibited until deployment in space and initiation of the experimental measurement phase of the mission, at which time a reliable means of exiting stasis is required.

Various methods have been employed to initiate and maintain metabolic stasis on previous nanosatellite missions. On PharmaSat, *S. cerevisiae* cells were integrated and launched in a state of stasis in nutrient-free aqueous buffer, a condition they endured for about seven weeks. The cells had very low metabolic activity, and were thus in stasis; they were reactivated in orbit by introducing nutrient-containing growth medium. GeneSat-1 also launched its study organisms, two strains of *E. coli* in nutrient-free liquid culture. The *E. coli* were reactivated in orbit by flushing the cultures with nutrient-replete medium. For the O/OREOS-SESLO nanosatellite payload, *Bacillus subtilis* were loaded, integrated, and launched into space as dried spores (Nicholson et al., 2011). They were returned to the vegetative state in multiple "batches" after two weeks, three months, and six months in Earth orbit by the addition of aqueous growth medium, which displaced the air in the microwells through an integral hydrophobic membrane; the last culture to be activated had been in stasis as spores for nearly one year.

Microalgae, like other microbes, are evolved to enter and exit a type of physiological stasis on a regular basis (Agrawal, 2012), "waiting out" stressful events by lowering metabolic activity. Some strains produce specialized cells like spores, while others make modifications to their vegetative cells. For microalgae, particularly cyanobacteria, stasis can be induced by simply turning off the lights. In the absence of light, some microalgae will respire if oxygen is present and ferment if it is absent. Physiological and metabolic responses to darkness are strain specific.

In the current study, stasis was induced for the microalgae by placing them in darkness for 4 to 6 weeks. All strains survived and rapidly recovered upon illumination. For cyanobacteria, the appar-

ent benefit of anoxic conditions observed during stasis may have been due to limited oxidation of their cellular systems during prolonged metabolic inactivity. Green algae, however, most likely need to respire during prolonged periods of darkness to maintain physiological health. These results suggest microalgae can be loaded into a GraviSat disc as a liquid culture and safely placed into stasis by incubating cultures in darkness with little or no modifications to the medium or the nanosatellite. Additionally, microalgal insensitivity to temperatures between 4 and 23 °C while in darkness (stasis) will ensure that the cultures remain healthy during the period between loading the cultures onto the disc and the deployment of the satellite in space, during which time GraviSat is expected to be unpowered and temperature regulated only within rather broad limits (e.g., 4–37 °C).

4.7. Full-length mission simulation

Laboratory test runs are important to identify potential late-mission (4–6 month) experimental problems and reduce overall mission risk. We performed a one-year mission simulation (6 months longer than planned) on the two best-performing microalgal strains – *C. vulgaris* and *D. bardawil*.

The observed high, steady levels of photosynthetic efficiency during the 6 week period of stasis suggests that the microalgal cells remained viable and healthy. Although optical density and chlorophyll fluorescence declined during the stasis period, these results are most likely indicative of cellular adaptation to darkness and a deactivation of photosystems rather than a result of cellular damage. Additionally, both *D. bardawil* and *C. vulgaris* recovered rapidly upon reactivation and displayed stable growth and activity. The observed rapid drop in photosynthetic efficiency upon reactivation is a natural response to an increase in light intensity. In the absence of light, $\Phi PSII$ represents photosynthetic capacity, as there is no light to drive photosynthesis. PSII reaction centers are "open" in that they can pass electrons to downstream carriers resulting in elevated $\Phi PSII$ values. Photon absorption induces PSII reaction center closure as donor side carriers become reduced and unable to accept electrons. This process, in combination with other regulatory mechanisms, lowers PSII efficiency even though photosynthetic activity is increased (Schreiber et al., 1994). After this initial decline, $\Phi PSII$ remained relatively stable, as light conditions did not change, but did show a slight decline overtime (Fig. 8).

The full-length mission simulation suggests that experimental runs of 2 to 6 months are possible without the addition of nutrients or removal of wastes. The incorporation of one or two flushes with fresh medium could easily extend mission length beyond a year allowing the study of low-level radiation effects and possibly the evolution of microbial cultures in space.

5. Conclusions

The importance of microalgae to support future human space exploration cannot be underestimated. Their ability to produce oxygen, sequester CO_2 and, in so doing, generate food cannot be reproduced through any physicochemical mechanism used today. GraviSat was designed to measure the short- and long-term effects of microgravity and radiation on microalgal physiology and photosynthesis by growing microalgae for ~6 months or longer in a spinning microfluidic disc that simulates a desired gravitation, including Earth gravity to provide an *in-situ* control experiment, aboard a 3U nanosatellite. Microalgae are largely biocompatible with typical nanosatellite biopayload materials and design, and can be maintained in good health in low-volume growth chambers for multiple months under the proper conditions. Further, microalgae can be reversibly placed in stasis by simply placing them in darkness. This is very useful for delaying the start of an experiment to comply with launch and other mission constraints typical for low-cost secondary payloads. In a hypothetical mission, GraviSat could be launched into a high or low Earth orbit. Once in orbit, LEDs would illuminate the microalgal samples and bring them out of stasis. The ground control would be performed concurrently using an identical unit. After the microalgae recover from stasis, one disc would begin spinning and then incrementally increase the rate of rotation to generate a series of microgravity conditions. The duration at each microgravity level would be sufficiently long to allow the microalgae to acclimate to the new microgravity conditions (~12 h). Photosynthetic activity would be monitored in real time (every minute) throughout the mission but, possibly most importantly, during transition periods between microgravity levels. This treatment regime could be performed multiple times during a 6-month mission. This would be particularly useful if GraviSat passed through different radiation environments.

The ability to perform meaningful measurements in multiple 400 µL wells, demonstrated here, facilitates replication of treatments and the ability to conduct statistically robust studies on metabolic and genetic processes in microalgae. However, microalgae also present unique challenges to nanosatellite hardware development. Significant obstacles to successfully incorporating microalgae into a microfluidics-based nanosatellite include CO_2 limitation and excessive oxygen production. In a sealed chamber, microalgae rapidly become carbon limited, preventing growth and potentially limiting experiment duration to less than two weeks. When CO_2 is available (e.g., supplemented with bicarbonate additions to the media), oxygen production can result in bubble formation that can subsequently interfere with sensor function. Oxygen production and CO_2 limitation can be managed by incubating cultures at lower-than-optimal-growth temperatures and with intermittent and/or low-light intensities (2–10 µmol photons m^{-2} s^{-1}).

Single-cell or small colonial green algal strains are optimal for incorporation in small payloads like that of GraviSat. Their ability to perform and regulate respiration and photosynthesis simultaneously, unlike cyanobacteria, greatly decreases CO_2 limitation and limits bubble formation. Cyanobacteria will, however, likely be important components of any bioregenerative life support system in the future, warranting the development of new technology to permit gas exchange and to facilitate the study cyanobacteria and other microalgae in the space environment.

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