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Ionizing radiation impacts photochemical quantum yield and oxygen evolution activity of Photosystem II in photosynthetic microorganisms

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Abstract

Purpose: Long-term space exploration requires biological life support systems capable of coping with the deleterious space environment. The use of oxygenic photosynthetic microorganisms represents an intriguing topic in this context, mainly from the point of view of food and O_2 production. The aim of the present study was to assess the effects of space ionizing radiation exposure on the photosynthetic activity of various microorganisms.

Materials and methods: Ground-based irradiation experiments were performed using fast neutrons and gamma rays on microorganisms maintained at various light conditions. A stratospheric balloon and a European Space Agency (ESA) flight facility were used to deliver organisms to space at the altitude of 38 and 300 km, respectively. During the balloon flight, the fluorescence activity of the organisms was real-time monitored by means of a special biosensor.

Results: The quantum yield of Photosystem II (PSII), measured directly in flight, varied among the microorganisms depending on the light conditions. Darkness and irradiation of cells at 120 and 180 μmol m^{-2} s^{-1} enhanced the radiation-induced inhibition of photosynthetic activity, while exposure to weaker light irradiance of 20 and 70 μmol m^{-2} s^{-1} protected the cells against damage. Cell permanence in space reduced the photosynthetic growth while the oxygen evolution capacity of the cells after the flight was enhanced.

Conclusions: A potential role of PSII in capturing and utilizing ionizing radiation energy is postulated.

Keywords: *Space ionizing radiation, Photosystem II activity, photochemistry, oxygen evolution, survival, microorganisms*

Abbreviations: D1 and D2, main proteins of Photosystem II core; F_0, basic fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence; F_v/F_m, maximum potential quantum yield of PSII in dark-adapted cells; PAR, photosynthetically active radiation in the range 400-750 nm; PSII, Photosystem II; $Pheo^-$, reduced pheophytin.

Introduction

The experimental activities carried out at the Russian Mir space station and by the USA and European Space agencies demonstrated that space environment is stressful for living organisms, the main problems being the presence of space radiation, the absence of gravity, low atmospheric pressure, extreme temperatures, etc. For long-duration flights such as exploratory missions to the moon and Mars, biological life support systems will be needed, especially for food, nutraceutics and oxygen production (Sonnenfeld & Miller 1993, Ohnishi et al. 2002, Biolo et al. 2003, Dicello 2003, Yamashita et al. 2006). Like on Earth, the biomass production will be based on photosynthesis. Oxygenic photosynthetic organisms are unique in the biosphere, since they can use light energy to split water and evolve oxygen by means of PSII in a process that produces storable energy-rich products from atmospheric carbon dioxide (Barber 2006). Therefore, with respect to future space colonization, research on the response of the photosynthetic PSII apparatus to space conditions is of great interest.

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Space ionizing radiation is characterized by fluxes of complex radiation of variable energy and intensity that are very difficult to measure. It may be classified according to its origin as: galactic cosmic radiation, with energies between 1 and 10^3 GeV, comprising protons, alpha particles and high-ionizing high energy particles (HZE); solar energetic radiation, from particles emitted during solar storms, mainly composed of protons with energies that can reach about 1 GeV; geomagnetically-trapped particle radiation, comprising electrons with energies up to 7 MeV, protons with energies up to several hundreds of MeV, and low energy heavy ions. HZE and neutrons represent the components with the highest relative biological effectiveness. They are particularly dangerous since their interaction with the shielding material of spacecraft leads to the formation of secondary radiation such as gamma rays and neutrons with various energies that have high biological effectiveness (Mitaroff & Silari 2002, Miroshnichenko 2003). HZE constitutes only 1% of the total space ionizing radiation while neutrons constitute 20%.

The interaction of ionizing radiation with biological material can cause damage to DNA and proteins modifying their structural and biochemical properties. Damage could occur by direct deposition of energy or indirectly through the generation of reactive oxygen species derived from water breakdown (i.e., free electrons, hydrogen free radicals, or hydroxyl radicals). Genetic damage can also arise from DNA repair or errors in replication of damaged DNA, or chromosome aberration leading to cell death and carcinogenesis (Vladimirov et al. 1992, Benkhaled et al. 2006).

Several studies report that the absence of gravity could affect some physiological parameters of higher plants (Clément 2005). Microgravity is able to modify the ultrastructure of storage reserves in mature dry seeds and the quality of seeds produced by space-grown *Brassica rapa* L. plants without affecting reproduction (Musgrave et al. 2000, Kuang et al. 2000, 2005). In space, the absence of gravity particularly affects the development of the root apparatus of higher plants; evidence of root zone hypoxia in *Brassica rapa* L. grown in microgravity has been biochemically and cytochemically demonstrated and correlated to microgravity-induced changes in fluid and gas distribution (Stout et al. 2001). Higher plants can partially tolerate the reduced atmospheric pressure (or hypobaria) that, without a proper increase in CO_2 partial pressure, is responsible of a reduced plant growth (Corey et al. 1997, Massimo & André 1999). Richards and colleagues analysed the consequences of low pressure environments on arabidopsis plants and indicated the pressure values observed for normal plant growth (Richards et al. 2006).

Studies in unicellular organisms suggest that space flights can cause changes in growth rate, mobility, developmental cycle and morphological and ultrastructural characteristics (Wang et al. 2004, Lehto et al. 2006). The specific target of the damaging effect on the fine structure of the photosynthetic apparatus was determined in pea, brassica and arabidopsis plants grown in space or during clinorotation. An overall decrease of photosynthetic activities was observed. Moreover, modifications of the chloroplast ultrastructural features were observed mainly in the Photosystem I (PSI) and, to a lesser extent in the PSII apparatus (Jiao et al. 2004).

A major problem for microorganisms in space seems to be related to the presence of ionizing radiation. Despite the numerous studies carried out on plants and microorganisms under space conditions, very little is known about the effects of space ionizing radiation on PSII at molecular level. PSII is the supramolecular pigment-protein complex that catalyses the light-induced transfer of electrons from water to plastoquinone with the oxidation of water molecules, generating the Earth's entire atmospheric oxygen. PSII consists of more than 25 polypeptides, making up the oxygen-evolving complex (OEC), a light-harvesting chlorophyll protein complex (LHCII) and a reaction center core composed of two proteins, D1 and D2, which are involved in the primary charge separation. LHCII captures light energy and transfers it to the reaction center chlorophyll (P680). This excitation leads to a primary charge separation and the formation of chlorophyll $P680^+$ with reduced pheophytin ($Pheo^-$). The radical pair is stabilized when $Pheo^-$ transfers its electron to Q_A, the primary quinone acceptor bound to the D2 protein, and successively to the secondary quinone acceptor Q_B. The electron is then transferred via the cytochrome b6/f complex and plastocyanin to PSI for NADP reduction and the synthesis of organic compounds (Giardi & Pace 2005, Barber 2006). For the space experiments presented here, we selected *Chlorella sorokiniana*, *Chlorella zofingiensis*, *Chlamydomonas reinhardtii*, *Arthrospira platensis*, *Chlorococcum sp.*, *and Monodus subterraneus*, since they represent significant stages in the evolutionary scale of photosynthesis. Another important reason supporting our choice was the potential application of such organisms as a source of nutraceuticals in space missions; the nutritional value of these microorganisms relies in their high content of protein, polyunsaturated fatty acids, vitamins and carotenoids (Torzillo et al. 2003, Walker et al. 2005).

Ground experiments using irradiation sources are limited by the fact that it is usually possible to obtain

only single, monoenergetic radiation, often produced in a narrow unidirectional single beam. On the contrary in space an organism is exposed to a shower of radiation with a wide spectrum of atomic mass and energies between 1 MeV and 10^3 GeV (Miroshnichenko 2003). Therefore, we conducted experiments in real-time space conditions in a stratospheric balloon flight, at 38 km altitude, and in a Soyuz flight at about 300 km altitude. A fluorescence-based sensor was utilized to monitor in flight the photosynthetic electron transfer activity of PSII (Angeli et al. 2001, Esposito et al. 2002, 2006).

Materials and methods

Chemicals, strains and culture conditions

All reagents were purchased from Sigma Chemical Co., St Louis, MO, USA. Wild-type *Chlorella sorokiniana*, *Chlorella zofingiensis*, *Chlorococcum sp.*, *Chlamydomonas reinhardtii* (Chlorophyta), *Arthrospira platensis* (Cyanobacteria), and *Monodus subterraneus* (Eustigmatophyta) cells were obtained from the culture collection at the Institute for Ecosystem Study-National Research Council (CNR) of Florence (Tuscany, Italy). Microalgae and cyanobacteria cultures were grown in liquid Tris/acetate/phosphate (TAP) medium, pH 7.0 (Gorman & Levine 1965) and Blue Green 11 (BG11) liquid medium, respectively (Rippka et al. 1979), at 25°C and 50 μmol m^{-2} s^{-1} photon irradiance. Cells were harvested during the exponential growth phase by centrifugation and resuspended in the same medium at a chlorophyll (Chl) concentration of 50 μg ml^{-1}. Cell suspensions were used for the determination of oxygen evolution or layered on agar-medium in plastic devices for the flight and irradiation experiments. Microorganisms were subsequently exposed to light intensities ranging from 0–180 μmol m^{-2} s^{-1} according to the specific experimental requirements.

Immunoblot analyses and densitometry

For immunoblot analyses, thylakoid membranes were isolated from cell cultures at the exponential growth phase, after gamma radiation exposure. Cell cultures were harvested by centrifugation at 3,000 *g* for 3 min at 4°C. Pellets were diluted with sonication buffer containing 100 mM tris(hydroxymethyl) aminomethane-HCl (Tris-HCl) (pH 6.8), 10 mM NaCl, 1 mM *p*-aminobenzamidine-2HCl, 1 mM 6-aminocaproic acid, 10 mM ethylene diamine tetra-acetic acid (EDTA), and 100 μM phenylmethanesulphonylfluoride (PMSF). Cells were disrupted by sonication for 2 min in a Branson Sonifier Cell Disruptor 200 (Branson, Ultrasonics Corp., Danbury, CT, USA) operated in the pulsed mode with a 50% duty cycle and an output power setting of 5. Unbroken cells and other large cell fragments were removed by centrifugation at 3,000 *g* for 3 min at 4°C. Chlorophyll concentration was measured upon pigment extraction in 80% acetone after removal of cell debris by centrifugation, and by measuring the absorbance of the solutions at 652 nm with a Perkin Elmer Lambda BIO spectrophotometer (Perkin Elmer, Monza, Lombardy, Italy) (Geiken et al. 1998).

Samples containing equal amounts of chlorophyll (5 μg $lane^{-1}$ and lower quantities) were separated on denaturing discontinuous 12–17% polyacrylamide gel as previously described (Geiken et al. 1998). After electrophoresis, gels were stained with Coomassie brilliant blue, destained and photographed. Alternatively, resolved proteins were electrotransferred onto a nitrocellulose filter using the Trans-Blot Transfer Cell (Bio-Rad, Milan, Lombardy, Italy) for 1 h at 300 mA at 4°C. Filters were blocked with high salt 1 × TBST (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.05% Tween 20), washed with low salt 1 × TBST, and incubated with specific polyclonal antibodies against the OEC, comprising the extrinsic proteins of 33, 23, and 16 kDa and against the D1 N-terminus, D2, CP43 and CP47 core proteins, kindly provided by Dr R. Barbato (University of Alessandria, Piedmont, Italy). All antibodies were diluted 1:2,000 in high salt 1 × TBST. For signal detection, secondary antibodies conjugated to alkaline phosphatase (Promega, Milan, Lombardy, Italy) were used at a concentration of 1:5,000 (in 1 × TBST) and the reaction was visualized using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega). Proteins were quantified on filters by scanning densitometry using a Shimadzu CS 930 densitometer (Shimadzu, Tokyo, Japan). The amount of proteins was analysed in several dilutions to determine the linear zone of the corresponding standard curve.

PSII fluorescence measurements

The PSII fluorescence measurements were carried out with an automatic multi-fluorimeter interfaced with the cells (''BioLumi'', Biosensor S.r.l., Rome, Lazio, Italy). The bio-device provides a simultaneous determination in various samples of the main chlorophyll fluorescence parameters, such as basic fluorescence F_0, maximum fluorescence F_m, variable fluorescence F_v, and as a result, the F_v/F_m ratio, and the area over the fluorescence curve (Papageorgiou & Govindjee 2005). The basic fluorescence F_0 is calculated using an algorithm that determines the line of best fit for the initial data points recorded at

the onset of illumination. This line is extrapolated to time zero in order to determine F_0. The multi-fluorimeter also recorded the external temperature and the light intensity by the BPW34 sensor, purchased from RS (Milan, Lombardy, Italy).

Oxygen evolution measurements

Oxygen evolution was measured at 25°C by exposing cells to saturating irradiance in a Clark-type oxygen electrode connected to a YSI Biological Oxygen Monitor (model 5300, Yellow Springs, Ohio, USA). To ensure that oxygen evolution was not limited by the carbon source available to the cells, 100 μL of 0.5 M $NaHCO_3$, pH 7.4 was added to a 2.5 ml aliquot of the culture prior to the oxygen evolution measurements (Melis et al. 1999). Measurements were performed with the O_2 electrode, beginning with the registration of dark respiration in the cell suspension and followed by measurement of the light-saturated rate of O_2 evolution. The rate of each process was recorded for about 5 minutes. To compare the relative photon yield of photosynthesis between the different samples, about the same Chl concentration (15 mg l^{-1}) was loaded in the oxygen electrode chamber.

Statistics

All statistical tests were performed using analysis of variance (ANOVA). The statistical significance of differences was evaluated by *p*-level. *P*-values have been calculated comparing protein levels, fluorescence values and oxygen concentration in irradiated samples with respect to the control.

Stratospheric balloon and space flight

Chlorella sorokiniana, *Chlorella zofingiensis*, *Chlorococcum sp.*, and *Monodus subterraneus* cultures were layered as described above and integrated in a bio-device carried to an average altitude of 38 km by a 200 m high balloon. The balloon, inflated with helium, was launched from the Milo base (Trapany, Sicily, Italy) of the Italian Space Agency. The bio-device was recovered some hours later in Spain, about 1400 km from the launch site. The external box containing the microorganisms and the bio-fluorimeter had a 1 cm-thick aluminium-polycarbonate wall with a steel frame (described by Angeli et al. 2001), able to withstand temperature changes from -60°C to $+70$°C. The experiments were conducted at internal constant pressure of 0.8 atm. The box was fixed to the frame of the balloon by six 4 mm-thick electrically isolated supports, in order to keep its lower surface always in the shade. It could also be insufflated with air to maintain a controlled temperature. To avoid excess lighting of the micro-organisms, the external solar light was filtered through a black cover reducing the external illumination by 90%.

For the space flight, *Chlamydomonas reinhardtii* cultures were layered on agar-TAP medium (see above) enclosed in a sealed device. In this medium, the algae can potentially grow photoautotrophically as well as chemioheterotrophically in the presence of acetate as a carbon source. Many replicates of the same culture were inserted into the experimental flight module. The final payload was mounted inside the ESA facility called Biopan (www.esa.int). Biopan is an unmanned and recoverable capsule successfully used to deliver scientific experiments to near orbit since 1992. The experimental module was located in the Biopan bottom part and the temperature was maintained at 15°C. Biopan was fixed at the external wall of a Foton spacecraft, which was launched to 250–300 km altitude. Once in orbit Biopan was opened and algae were directly exposed to space conditions. Algae were protected from vacuum, heat and cold as well as from a too intense visible and ultraviolet (UV) radiation, but they were exposed to all other kinds of radiation without any metal cover protection. Visible (VIS) and UV radiation protection was obtained using a combination of special glass and quartz filters (provided by Kayser-It, Livorno, Italy) that provided attenuation of the external solar light between 95–99%.

The satellite, with a 7.8 km/s speed, orbited the Earth in 90 min. During this time it was exposed to the sun for 55 min and in darkness for 35 min. Because of the spacecraft self-rotation, Biopan experienced a permanent change between light and dark during 55 min. The Biopan was opened for a total of 351 h; at the same time the Foton spacecraft orbited the Earth 234 times. One day before returning to Earth, Biopan was closed and after nearly 250 orbits and about 379 h in flight the capsule landed in an uninhabited area in Kazakhstan. After landing, the experimental module was opened in sterile conditions and the solid agar containing algae was transferred to a 500 ml flask filled with 200 ml TAP medium. The flasks were gently shaken overnight at 50 μmol m^{-2} s^{-1} on a rotator to completely resuspend the cells. From each flask 1 ml culture was removed and plated onto a TAP solid medium to estimate the number of surviving cells. Further sampling was carried out to determine the oxygen evolution and the growth rate during a four-day period.

Space radiation environment and radiation source facilities

For the period of the balloon flight, an evaluation of the radiation environment was performed in order to

Table I. Details of the stratospheric and space flight experiments. Time is given as Coordinated Universal Time (UTC) or days.

Mission	Altitude (km)	Duration	Radiation dose rate
Stratospheric balloon	~38	From 2 July 2001 (05:47 UTC) To 3 July 2001 (02:32 UTC)	0.8 ± 0.3 μSv/hour
Foton-M2 Space	250–300	~15.6 days (from 31 May to 16 June 2005)	8.9 ± 0.9 μGy/hour (average value)*

*The dosimeter used in the Foton flight calculates the absorbed dose in Gray. At the present time, for this type of instrument, no method is available to convert the absorbed dose into dose equivalent.

determine the relative contribution to the measured dose of the various types of ionizing radiation present in the stratosphere. Because of the difficulty in measuring different radiation components at the same time over a wide energy range, both experimental detection techniques and a Monte Carlo simulation were used. An accurate neutron dose assessment was performed considering the high biological effectiveness of neutrons due to their quality factors (Miroshnichenko 2003). Integral neutron dose equivalent rates in the range of 100 keV–20 MeV were directly detected by BD 100 R neutron bubble dosimeters. The neutron spectrum in the 10 keV–20 MeV range was analysed by a passive detector system based on the BDS spectrometer and by an unfolding technique according to Zanini et al. (2001, 2005).

The Monte Carlo method was applied in order to evaluate the total absorbed dose, using the GEANT3 code (GEometry ANd Tracking), that simulates the passage of various particles through the matter in the 10 keV–10 TeV energy range (Zanini et al. 2005). Protons (87%) were considered as a source of primary particles, with the following distribution:

$$\Phi_{primary}(E_p, X) = E_p^{-\gamma} \exp\left[-\frac{X(1-\eta)^{(\gamma-1)}}{\lambda_p}\right]$$

where $\Phi_{primary}$ = energy distribution of the primary particles (protons) impinging on the top of the atmosphere; $E_p > 1$ GeV = kinetic energy of primary particles; $\gamma = 2.7$, spectral index; $\eta = 0.5$, coefficient of elasticity; $\lambda_p = 90$ g/cm^2, absorption mean free path (for protons); and X = atmospheric depth (g/cm^2).

A flux of protons with the mentioned energy distribution (10^{-4}-10^3 GeV) interacted at the top of the atmosphere with a flux of 0.3 protons cm^{-2} s^{-1} sr^{-1}. The atmosphere was simulated with 15 layers of decreasing density; the GEANT3 code made it possible to obtain the spectra of various secondary particles produced by proton interaction with oxygen (21%), nitrogen (78%), hydrogen (0.3%) and argon (0.75%) nuclei. In particular, the neutron energy spectra at the balloon flight altitude were evaluated using the conversion factors from the ambient dose equivalent (H*) to fluency taken from ICRP74 (International Commission on Radiological Protection, cf. reference ICRP, 1996), where the corresponding spectra in terms of H* as a function of neutron energy were obtained (Zanini et al. 2001, 2005).

During the 15.6 days of Foton space flight, the cell cultures, covered by a 2 mm glass top, received a dose of ~3.4 mGy, as extrapolated from the measurements of the active dosimeter R3D-B2 located in the Biopan facility (Dachev et al. 2005). Information about the balloon and space flights concerning altitude, duration and ionizing radiation doses experienced by the organisms is summarized in Table I.

During the space flight, the solar and absolute radiation intensities received by the samples in Biopan was 54.8 SCh (1 SCh = 4932 kJ/m^2 × h) as communicated by ESA. According to the position of the device and the measured excitation light peaks during the rotation around the Earth, the minimum and maximum light intensities reaching the samples are presented in the Results section.

Various sources of radiation were used for experiments on the ground. A source facility is installed at one of the secondary beam lines of the Super Proton Synchrotron in CERN (*Conseil Européen pour la Recherche Nucléaire*). A positive hadron beam (35% protons, 61% pions, 4% kaons) of energy 120 GeV is secured in a copper target which can be installed in two different positions inside an irradiation cave. The neutron spectrum has a second pronounced maximum at about 70 MeV which resembles the high-energy component of the radiation field created by cosmic rays at plane flight altitude (Mitaroff & Silari 2002). The intensity of the primary beam is monitored by an air-filled precision ionization chamber (PIC) at atmospheric pressure. A PIC-count corresponds to 2.2×10^4 particles (error ± 10%) impinging on the target. The energy distribution of the various secondary particles at the various exposure locations were obtained by means of Monte Carlo simulations performed using the Fluka Code. Another radiation source facility was the Dosimetry Division of the Joint Research Centre (JRC) at Ispra, Varese, Lombardy, Italy, where gamma radiation was obtained using a ^{60}Co source.

The details of the radiation source facilities, energies and doses utilized for ground tests are reported in Table II.

Results and discussion

PAR influence on the cell response to neutron and gamma radiation

With a view to future space colonization it is important to improve our knowledge of the defence responses elicited by cosmic radiation in oxygenic microorganisms and determine the growth conditions that maximize the oxygen evolution capacity of space-grown microalgae cultures (Lehto et al. 2006). However, understanding the response of biological organisms to space stress is complicated by the multifaceted nature of space ionizing radiation (Miroshnichenko 2003).

In this study we analyzed the behavior of green algae and cyanobacteria in response to ionizing radiation in both ground experiments and space flights.

A variety of ionization radiation sources were used to examine the effects of radiation on the photochemical activities of PSII in ground experiments. *Chlamydomonas reinhardtii* cells were exposed to a total dose of 4.8 mSv fast neutrons at different PAR as indicated in Figure 1. Cells irradiated in the dark showed a 40% reduction in F_v/F_m ratio. In contrast, the Fv/Fm ratio actually increased by 18% in cells simultaneously exposed to neutron radiation and to 70 μmol m^{-2} s^{-1} light intensity (Figure 1). Light intensity of 120 μmol m^{-2} s^{-1} and higher were photoinhibitory, inducing a 20% decrease in Fv/Fm values compared to the non-irradiated controls. In the latter, the F_v/F_m values remained high over time at all various light conditions (not shown). In irradiated cells, oxygen evolution increased with the increase in PAR from 20–120 μmol m^{-2} s^{-1} and then declined.

Table II. Kinds of ionizing radiation and facilities used in ground experiments.

Ionizing radiation quality	Ground-based facility	Radiation source	Energy (MeV)	Equivalent dose rate	Equivalent total dose
Neutrons	CERN (Switzerland)	CERF, Super Proton Synchrotron	0–800	0.23 mSv/h	4.8 mSv
Gamma rays	JRC Ispra (Italy)	^{60}Co		1.54 Sv/h	3.1 Sv
			2.5	4.2 Sv/h	8.4 Sv
				4.2 Sv/h	> 10 Sv

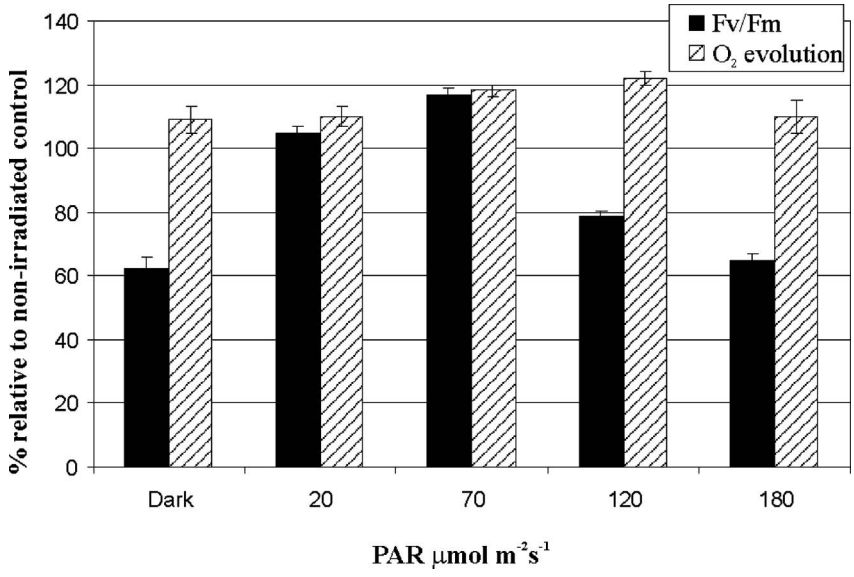

Figure 1. Changes in the F_v/F_m ratio and in the oxygen evolution of *Chlamydomonas reinhardtii* after exposure to fast neutrons under various light intensities. Exposure to fast neutrons was carried out at CERN: energy 0–800 MeV, dose rate 0.23 mSv h^{-1} for a total dose 4.8 mSv. The initial fluorescence value was F_v/F_m 0.753 $\pm$ 0.003. The percentage of Fv/Fm modification is relative to a non-irradiated control kept under the same light conditions. The values were obtained as the difference between the photosynthetic efficiency of irradiated and non-irradiated samples as a percentage of the control. Oxygen evolution was measured using a Clark electrode after irradiation of 15 mg l^{-1} Chl in liquid culture under saturated light conditions. The experiments were repeated five times. Standard deviation (SD) is reported in the graph. *P*-values $\leq$0.05 were calculated as reported in *Materials and methods*.

Concerning cells exposed to γ-rays and treated with doses of 3.1 Sv, no modification of the photochemical quantum yield was observed (data not shown). At a dose of 8.4 Sv, which is much higher than that experienced in short-term space flights, the photochemical quantum yield decreased only slightly in all microorganisms, with minor differences among the species (Table III). The Fv/Fm values decreased slightly, but reproducibly, in the following order: *Arthrospira platensis* (Cyanobacteria), *Chlamydomonas reinhardtii* (Chlorophyta), *Monodus subterraneus* (Eustigmatophyta), *Chlorococcum sp*., *Chlorella sorokiniana* and *Chlorella zofingiensis* (Chlorophyta). In contrast, the oxygen evolving capacity increased in all the irradiated strains and in particular in Chlorella (Table III).

In order to understand the effects of radiation on the PSII apparatus at the biochemical level, *Arthrospira platensis*, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* cells were exposed to high doses ($>$ 10 Sv) of gamma radiation for 12 h and the corresponding isolated thylakoids were analysed with polyclonal antibodies against the major PSII proteins. The results show that the exposition to such doses causes a 40% reduction of the 33, 23 and 16 KDa OEC extrinsic protein accumulation, compared to non-irradiated controls, in all the analysed microorganisms. On the contrary, the accumulation levels of the D1 and D2 protein were slightly affected, while the content of the internal antennae chlorophyll proteins 43 and 47 (CP43 and CP47) appeared to remain constant, as measured by densitometric analyses reported in Table IV. These results are consistent with the observations on *Brassica rapa* plants flown aboard the space shuttle Columbia in 1997, where the levels of OEC proteins in the cotyledons were found to decrease by 32% with only slight reductions of D1, D2 and LHCII proteins (Jiao et al. 2004).

Survival and viability of microorganisms in response to stratospheric and space flights

In order to unravel the response of the photosynthetic apparatus to real space conditions, microorganisms were delivered to the stratosphere at a maximum altitude of 38 km by a balloon flight. The algal species that in simulation studies proved to be relatively more tolerant to ionizing radiation were sent to space in the balloon flight under various experimental conditions: in the light, in the dark and in the dark partially shielded from space radiation. The fluorescence activity was monitored by the special automatic biodevice described in *Materials and methods*.

During the balloon flight the solar activity was at very low levels and relevant X-ray flares were not observed. Figure 2 shows that the Eustigmatophyta *Monodus subterraneus* maintained the highest photochemical efficiency after the period in the stratosphere, while *Chlorella sorokiniana* showed the highest inhibition. Dark conditions in flight particularly affected the activity of *Chlorella sorokiniana,* but in a less efficient way in shielded samples (Figure 2).

The passive physical dosimeters inside the box detected a dose equivalent rate of 0.8 ± 0.3 μSv hour^{-1} (Table I). The main radiation component seemed to be protons (51%), while neutrons were about 35%, gamma ray was less than 13% and HZE less than 1%, distribution in accordance with data by Spurný (2001).

During the Foton-M2 space mission, *Chlamydomonas reinhardtii* cells were sent to 250–300 km altitude in a special container, where it experienced solar radiation of different intensities using glass attenuation filters (see *Materials and methods*). The light experienced by the cells was variable depending on the rotation of the spacecraft, but it was always in the physiological range (2–180 micromoles photon m^{-2} s^{-1}). A total ionizing radiation dose of $\sim$ 3.4

Table III. Changes in the F_v/F_m ratio and in the oxygen evolution in microorganisms during exposure to gamma radiation. The values were obtained as the difference between the photosynthetic efficiency of irradiated and non-irradiated samples as a percentage of the control. Oxygen evolution was measured by a Clark electrode at saturating light intensity. Exposure to gamma ray at JRC-ISPRA (Exposure time 20 min; total dose 8.4 Sv) with illumination at 70 μmol m^{-2}s^{-1}. Data (mean values $\pm$ SD, $n = 5$) are expressed as percentages relative to non-irradiated controls. *P*-values $\leq$0.05 were calculated as reported in *Materials and methods*.

			% F_v/F_m	% 0_2
			relative to non-irradiated control	
Organisms	Average of cell size	Control Fv/Fm		
Arthrospira platensis	10 μm	0.759 ± 0.003	96 ± 2.4	101 ± 2
Chlamydomonas reinhardtii	10 μm	0.750 ± 0.003	96 ± 2.1	115 ± 2
Monodus subterraneus	9.0 μm	0.760 ± 0.001	95 ± 2.0	102 ± 2
Chlorococcum sp.	4.0 μm	0.781 ± 0.004	94 ± 2.2	107 ± 5
Chlorella zofingiensis	3.7 μm	0.749 ± 0.002	93 ± 1.9	122 ± 7
Chlorella sorokiniana	3.7 μm	0.777 ± 0.003	90 ± 2.1	115 ± 6

Table IV. PSII protein accumulation in green algae and cyanobacteria following exposure to high doses of gamma radiation. Cultures of the different microorganisms at a exponential growth phase were exposed to a ^{60}Co source for 12 h, receiving a total dose >10 Sv. Thylakoid membranes were isolated from harvested cell cultures and their membrane proteins fractionated by SDS-PAGE (12–17% gradients; Geiken et al. 1998). Gels were loaded on an equal chlorophylls basis (5 μg lane^{-1}) and the proteins were detected by BCIP and NBT colorimetric assay. Proteins were quantified on filters by scanning densitometriy and the amount of proteins was analysed in several dilutions to determine the linear zone of the corresponding standard curve. D1 and D2: heterodimeric protein of the PSII reaction centre; 33, 23 and 16 KDa: OEC extrinsic proteins of the PSII; CP43 and CP47: internal antennae proteins of the PSII reaction centre. Data (mean values $\pm$ SD, $n=5$) are expressed as percentage relative to non-irradiated controls. *P*-values ≤ 0.05 were calculated as reported in *Materials and methods*.

Strain	PSII proteins	% protein relative to non irradiated control
Arthrospira platensis	D1	85 ± 11
	D2	90 ± 7
	OEC (33-23-16)	64 ± 4
	CP43 + CP47	98 ± 5
Chamydomonas reinhardtii	D1	82 ± 12
	D2	87 ± 7
	OEC (33-23-16)	62 ± 6
	CP43 + CP47	95 ± 4
Chlorella sorokiniana	D1	81 ± 13
	D2	83 ± 8
	OEC (33-23-16)	59 ± 6
	CP43 + CP47	94 ± 4

mGy was measured during the flight. After the flight, all algae retained the green and damp phenotype and also the medium consistency and colour were not affected. Following the re-suspension procedures, the algae were used to estimate the cell surviving capability, the growth rate and the oxygen evolution capacity (Table V).

The cells plated on TAP solid medium formed colonies after 14 days. Surviving cells were found with a percentage of 0.001% and 0.01% in algae exposed to 1% VIS-UV radiation (range 2–10 μmol m^{-2} s^{-1}) and 5% VIS radiation (range 30–80 μmol m^{-2} s^{-1}), respectively; on the contrary no survivors were observed with 10% VIS radiation (range 90–180 μmol m^{-2} s^{-1}). The two survival cultures were further analysed comparing their growth rate in liquid media to those obtained in the on ground control culture. The comparison showed that an increase of light intensity decreases the algae percentage growth rate to about 80%. Interestingly, although the growth rate was lowered, the survival culture improved oxygen production up to 22%, exceeding the rate found in on ground controls (Table V).

The first observation is that, in space, the effect of ionizing radiation is enhanced compared to that observed in on ground facilities with a single beam of radiation; in fact in the stratospheric flight, PSII activity was affected even at the low doses of 20 μSv (see Figure 2). The sensitivity of PSII to ionizing radiation seems to be reasonable since it works as an

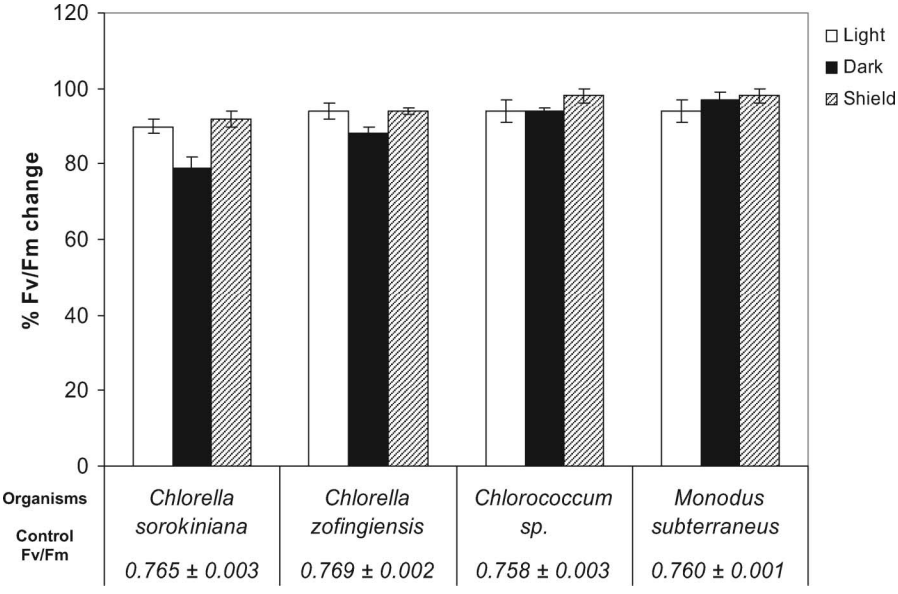

Figure 2. Changes in the F_v/F_m ratio in microalgal samples after a 10-hour balloon flight in the stratosphere at 38 km altitude. The cells received external solar light filtered through a black cover reducing the illumination by 90%. During the flight, a light intensity range of 15–40 μmol m^{-2}s^{-1} was measured. Average temperatures were 25° ± 5°C and a total dose of 20 μSv was determined. Shield: samples were partially shielded from radiation by a 5 mm thick cadmium cover layer. Double samples were analysed in triplicate measurements. Standard deviations (SD) are reported in the graph.

Table V. Survival and oxygen evolution activity of *Chlamydomonas reinhardtii* after a space flight. After 1 week re-suspension, oxygen evolution was measured by a Clark electrode at saturating light intensity in post-flight re-cultured cells at 15 mg l^{-1} Chl concentration. Growth rate was followed over four days and reported as percentage compared to on-ground control cells. The measurements were repeated three times. The SD is indicated.

Range of light intensity (μmol $m^{-2}s^{-1}$) Maximum and minimum peak experienced during flight	% Post-flight survivors Values relative to initial layered cells	% Oxygen evolution Relative to on ground control	% Growth rate
2–10	0.001	103 ± 3	82 ± 4
30–80	0.01	122 ± 6	80 ± 4
90–180	nr	–	–

electron transport chain extremely sensitive to the presence of free radicals (Jansen et al. 1999, Booij-James et al. 2000).

During the stratospheric flight, the main disturbance to the PSII apparatus complex seemed to be caused by ionizing radiation, since the effect on its activity was similar to that registered in ground experiments.

In the experiment in space, *Chlamydomonas reinhardtii* was only protected by 2 mm thick glass filters during exposure to the space environment. The new discovery is that photosynthetically active cells can, in principal, survive under such extreme conditions. Once again, it was observed that the effect of space stress on algae survival varies depending on the light conditions to which they were exposed during the flight. Surprisingly, despite the low survival of the organisms, the flight experience caused a stimulation of PSII oxygen evolution of the cells, as also registered in ground experiments with neutrons and gamma rays (Figure 1 and Table IV). This contradictory phenomenon has been already observed in other organisms e.g., *Thalassiosira weissglogii* and *Monodus subterraneus* under stress (Torzillo et al. 2003, and Giardi et al. unpublished results). In these species it was found that a decrease in the population of functional PSII reaction centres of up to 50% could be compensated by means of an increase in the rate of electron transport rate through the resting functional PSII reaction centres. The increased oxygen evolution activity in cells exposed in space may be the result of modification of thylakoid stacking due the presence of new charged ions and to the formation of hydroxyl radicals, triggered by the ionizing radiation. It is known that oxygen evolution can originate from hydroxyl radicals that in normal conditions are produced by mitochondria respiration (Pospísil et al. 2004).

It was interesting to observe that the consequence of space stress and gamma exposure on the microorganisms seems to be inversely correlated to cell dimensions. *Monodus subterraneus* was significantly less affected than *Chlorella sorokiniana* and *Chlorella zofingiensis*. Indeed, among the organisms tested, *Chlorella* (Chlorophyta), which showed the highest sensitivity, is the smallest in size (average cell diameter: 3.7 μm). On the other hand, *Monodus subterraneus* (Eustigmatophyta), which showed the least inhibition, has the largest diameter (more than 9 μm) (Figure 2). Similar results were obtained following gamma ray exposure which, in addition to *Monodus subterraneus*, indicates that the largest microorganisms *Chlamydomonas reinhardtii* and *Arthrospira platensis* are the less sensitive microorganisms (Table III). It seems that large cell cross-sections, with their content of lipids, antioxidants and enzymes, can partially shield internal structures. However, other possible factors could account for the higher sensitivity observed in green algae as opposed to cyanobacteria such as, for instance, the content of photoprotective pigments. In a screening of microalgal species exposed to UV-B, damage was correlated to cell dimensions, in accordance with our findings on ionizing radiation damage; the lower the cell diameter, the higher the damage recorded (Xiong et al. 1996).

The OEC of PSII seems to be particularly affected by ionizing radiation resulting in a stimulation at the lowest doses of mSv and in a reduction of the OEC proteins at the highest doses over 10 Sv. Interestingly PAR plays a very important synergistic role in the response of PSII to ionizing radiation: both in the dark and under relatively intense illumination, the damaging effect of space radiation increased, while the effect of ionizing radiation was negligible under weak light (Figure 1). Perhaps this synergistic effect is due to a phenomenon similar to photoinhibition; both ionizing radiation and high light intensities are known to cause the formation of free radicals that, in addition, could damage the photosynthetic apparatus. A similar synergism has been previously observed by Jansen and colleagues (1996) in the aquatic higher plant *Spirodela*, where a simultaneous exposure to PAR and ultraviolet-B radiation determined an increase of the D1-D2 heterodimer protein degradation (Jansen et al. 1996). This behaviour suggests that the light-dependent D1 protein turn-over may play a role in the PSII resistance to ionizing radiation stress. This assumption is in accordance with previous observations that D1 protein is a main target of high light radiation damage in PSII and is supported by the fact that recovery of D1 content in the dark is known to be precluded (Mattoo et al. 1999).

The synergetic effect of ionizing radiation and light on the photosynthetic apparatus must be considered in relation to the survival of photosynthetic organisms in space since bio-regenerative life-support systems can be severely disturbed. All the analysed microorganisms – *Chlorella sorokiniana*, *Chlorella zofingiensis*, *Chlamydomonas reinhardtii*, *Arthrospira platensis*, *Chlorococcum sp.*, and *Monodus subterraneus* – on account of their high nutritional value and human health-promoting activity might be suitable as bio-regenerative life supporting systems in space (Torzillo et al. 2003, Walker et al. 2005). In particular, in our experiments *Arthrospira platensis* and *Chlamydomonas reinhardtii* seem to maintain the highest photosynthetic efficiency in flight experiments. Moreover, several data in the literature address the potential of the multicellular, filamentous cyanobacterium *Arthrospira platensis* as a life supporting system since it shows tolerance to heat, high light intensity and alkaline environment (Lehto et al. 2006).

In addition, radioresistant strains of *Chlorella* and *Chlamydomonas* have been previously isolated and characterized, revealing the presence of all the basic DNA repair pathways following radiation exposure. The connection between the radioresistance and the adaptive repair processes is linked to other cellular functions like the regulation of the cell cycle, which is strictly correlated to developmental processes, mandatory for every bioregenerative supporting system in space (Boreham & Mitchel 1993, Chankova et al. 2005, Vlbek et al. 2008).

The ability to adjust in the presence of ionizing radiation existed in the early stages of the Earth's history, prior to the establishment of photosynthesis. The creation of an oxygenic atmosphere, with its ozone layer, protected Earth against space radiation and led to the creation of microorganisms incapable of adapting to space conditions. In accordance with this knowledge, our experiments have demonstrated that ionizing radiation can modify the photochemical properties of PSII and the oxygen-evolving apparatus activity. These observations raise intriguing questions about a potential role of PSII in ionizing radiation energy capture and utilization.

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