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Research Article

Reactive Oxygen Species in Emulated Martian Conditions and Their Effect on the Viability of the Unicellular Alga *Scenedesmus dimorphus*

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Abstract

Formation of oxygen-based free radicals from photochemical decomposition of hydrogen peroxide (H_2O_2) on Mars may be a key factor in the potential survival of terrestrial-like organisms on the red planet. Martian conditions that generate reactive oxygen species involve the decomposition of H_2O_2 at temperatures of around 278 K under relatively high doses of C-band ultraviolet radiation (UVC). This process is further amplified by the presence of iron oxides and perchlorates. Photosynthetic organisms exhibit a number of evolutionary traits that allow them to withstand both oxidative stress and UVC radiation. Here, we examine the effect of free radicals produced by the decomposition of H_2O_2 under emulated martian conditions on the viability of *Scenedesmus dimorphus*, a unicellular alga that is resistant to UVC radiation and varying levels of perchlorate and H_2O_2, both of which are present on Mars. Identification and quantification of free radicals formed under these conditions were performed with Electron Paramagnetic Resonance spectroscopy. These results were correlated with the viability of *S. dimorphus*, and the formation of oxygen-based free radicals and survival of the alga were found to be strongly dependent on the amount of H_2O_2 available. For H_2O_2 amounts close to those present in the rarefied martian environment, the products of these catalytic reactions did not have a significant effect on the algal population growth curve. Key Words: Extremophiles—*Scenedesmus dimorphus*—Photo-Fenton reaction—Iron oxides—Perchlorate—Peroxide—UVC—EPR—Mars. Astrobiology 21, xxx–xxx.

1. Introduction

Martian soil is dominated by iron-based minerals (Bouvier *et al.*, 2018), mainly oxides. These oxides are the main source for the generation of reactive oxygen species (ROS) (Yen *et al.*, 2000). Photochemical reactions driven by ultraviolet (UV) radiation involve charge transfer from the dehydrated oxide rocks to adsorbed O_2 to form oxygen radicals (Papacosta and Corrigan, 1975; Ito *et al.*, 1985). In addition, iron and iron oxides are catalysts for the homogeneous and heterogeneous Fenton catalytic reactions, respectively, which lead to the decomposition of hydrogen peroxide (H_2O_2) into oxygen-based free radicals.

There is a highly variable amount of H_2O_2 in Marś surface (Encrenaz *et al.*, 2019), as shown in Supplementary Fig. S1A. There are two primary proposed pathways of the generation of H_2O_2: the photochemical pathway (Hunten, 1979), and through electrostatic fields during dust devils and storms (Atreya and Gu, 1994; Encrenaz *et al.*, 2012).

It is predictable that, at least in some parts of the planet, the reaction of ferrous ions (Fe^{2+}) and H_2O_2 may proceed well beyond the simple oxidation of Fe and into the production of oxygen-based free radicals, following the aforementioned heterogeneous Fenton reaction (which use iron oxides as catalysts for the decomposition of H_2O_2 into oxygen-based free radicals). By outer sphere electron transfer, the combination of Fe^{2+} and H_2O_2 produces Fe^{3+} and $[H_2O_2]$•, which then decomposes into hydroxide ion (OH^-) and the hydroxyl radical HO•. In the presence of an excess of H_2O_2, this further reacts with ferric ions (Fe^{3+}) to produce Fe^{2+} + OOH• + H^+. The unpaired electron in the outer orbit of a free radical makes it unstable, short lived, and highly reactive; their high reactivity causes the oxidation or reduction of other compounds to attain stability.

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ROS such as OH• and OOH•, and especially the $O_2^{\bullet -}$ (superoxide radical), can oxidize biomolecules in self-propagating chain reactions and, if not properly counterbalanced by cellular antioxidant mechanisms, these can lead to significant cell damage and death (Dixon and Stockwell, 2014).

Another main threat to carbon biomolecules—DNA, in particular—on the red planet is the UVC radiation (254 nm, levels near 150 kJ/m^2) (Cockell *et al.*, 2000) that penetrates the thin, ozone-lacking atmosphere of Mars, which is also missing a global protective magnetic field. Even extremophile microorganisms that are tolerant to A- and B-bands of UV radiation (UVA and UVB) would suffer deleterious effects if exposed to UVC radiation for prolonged periods of time (Ott *et al.*, 2019). UV solar light plays a key role in martian photochemistry by catalyzing the photolysis of H_2O and CO, thus generating background levels of methane (CH_4) (Bar-Nun and Dimitrov, 2006). In addition, UV radiation combined with martian iron oxides and H_2O_2 would result in heterogeneous photo-Fenton catalytic reactions, thus producing large amounts of OH•.

The variety of oxygen-based free radicals generated by these heterogeneous Fenton and photo-Fenton reactions can interact among themselves and with carbon-based biomolecules to readily recombine into different permutations, thereby propagating the oxidative aggression.

The Fenton and photo-Fenton reactions are not the only possible means for producing ROS in Mars. H_2O_2 gives rise to oxygen-based free radicals in the presence of UV radiation alone (Liao and Gurol, 1995). Also, another reason why martian soils are highly oxidizing is the presence of perchlorate salts (0.4–0.6%) (Hecht *et al.*, 2009; Ojha *et al.*, 2015), including magnesium perchlorate [$Mg(ClO_4)_2$], presumably produced as a result of atmospheric interactions between ozone and volatile chlorine compounds.

Although a metabolism based on perchlorate oxidation has been found in some terrestrial extremophiles (Myers and King, 2017), perchlorates are strong oxidizers, which are harmful *per se* to most terrestrial life-forms. In addition, decomposition of perchlorate by ionizing radiation forms highly reactive photoproducts, especially when irradiated with UVC (Georgiou *et al.*, 2017), which further enhanced the biocidal effect of perchlorate on the bacteria *Bacillus subtilis* (Wadsworth and Cockell, 2017), a common contaminant of spacecrafts.

Thus, the interaction between UVC radiation and the chemicals present in martian soil may produce an emerging variety of free radicals that are toxic for carbon-based life-forms. Previous results identified some of the oxygen-based free radicals generated from Mars precursor simulants (Lasne *et al.*, 2016; Georgiou *et al.*, 2017). Still, until now, the unequivocal identification and quantification of the oxygen-based free radicals that are produced by the UVC-radiated main precursors found on martian soil (*i.e.*, iron oxide, H_2O_2 and perchlorates) have not been determined. This information is crucial to begin to answer the question of whether any known terrestrial organism could survive in the presence of the free radical derivatives produced in martian environments.

A great diversity of carbon-based life-forms thrives in the wide range of physical and chemical conditions found on current Earth's environments. In particular, the high adaptability of some microorganisms led them to colonize even extreme environments such as the ice caps, the hypersaline Dead Sea, acid or alkaline hot springs, or places exposed to high UV radiation (reviewed by Rampelotto, 2013). Further, unicellular life, including photosynthetic cyanobacteria, emerged on Earth when its atmosphere lacked the amount of oxygen it contains today (Hong Enriquez and Do, 2012).

Since then, planets harboring conditions that are even remotely similar to early Earth, or its present extreme habitats, could still be habitable, at least by some fast-evolving microorganisms. This could be the case for Mars. On Earth, iron oxides act as electron acceptors for iron-reducing microorganisms (reviewed in Logan *et al.*, 2019).

Given the rich iron-oxide soil of Mars, diffusive redox species such as Fe ions form redox pairs that could provide free energy to sustain carbon-based life. Apropos, Mars is the second planet in the solar system, after Earth, with the highest free energy, which mostly (>98%) comes from the abiotic redox pair CO-O_2 (produced by the photolysis of CO_2 and H_2O; the remaining less than to 2% is provided by the H_2–O_2 redox pair) (Sholes *et al.*, 2019). The photolysis of CO and H_2O produces CH_4, a reduced species in an oxidant-rich environment, which hints at the possibility of redox gradients supporting life on Mars.

The free energy of Mars might support two of the most ancient metabolisms on Earth: the methanogenic oxidation of H_2 with CO_2, and the anaerobic oxidation of CO (reviewed in Martin and Thauer, 2017). The latter only requires a source of CO and water, and it might have bestowed the earliest forms of terrestrial life in submarine volcanic vents with both energy and a carbon source. Regarding the relatively high exposure to UVC radiation, Mars subsurface environments, which may also contain liquid water and/or ice at shallow depths (Malin and Edgett, 2000), seem to be more permissive for terrestrial life than the UVC-irradiated surface. Indeed, it was shown recently that water provided by deliquescence may be sufficient to reactivate the metabolism of microorganisms under conditions roughly analogous to the near-subsurface martian environment (Maus *et al.*, 2020).

Although archaea and bacteria are the pioneering colonizers of all possible environments on Earth, some unicellular algae were the first photosynthetic eukaryotes (Sánchez-Baracaldo *et al.*, 2017), and, as such, they have a number of evolutionary traits that may prove very advantageous for their survival in Mars. In the first place, unicellular photosynthetic microorganisms have fast evolved to fight and control the ROS produced by the photo-oxidation that accompanies their photosynthetic metabolism (Niyogi, 2017; Foyer, 2018). In particular, many species of algae, including microalgae of the genus *Scenedesmus* (*Chlorophyceae*), have proven to tolerate a variety of highly oxidative compounds such as perchlorates (Brinkmann and Senger, 1978) and H_2O_2 (Mallick, 2002), two strong oxidizers present on Mars. Second, unicellular algae developed resistance to strong UVC radiation (Kovácik *et al.*, 2010). And third, as eukaryotes, their metabolism is much more efficient than bacteria and archaea, and the production of energy based on several mitochondria outpaces that of prokaryotes.

This aerobic respiration process only occurs in the presence of oxygen, which is extremely low, yet variable, on Mars. Although many species of algae are tolerant to low levels of dissolved oxygen in water (Haas *et al.*, 2014), they do need humid environments and water, which is lacking in its liquid state in most of Marś surface environments. However, there are near-surface flows of brines in some equatorial sites (Ojha *et al.*, 2015), and at subglacial martian

polar regions (Lauro *et al.*, 2020). Liquid brines are formed because regolith salts, especially perchlorates, can lower the freezing point temperature and the saturation water vapor pressure (Martínez and Renno, 2013). In addition, this water may have also originated from the melting of subsurface ice and the discharge of shallow aquifers (Grimm *et al.*, 2014; Ojha *et al.*, 2015). The presence of H_2O_2 and other ROS in this underground water cannot be discarded since the most accepted hypothesis is that H_2O_2 diffuses in martian soils from the atmosphere (Zent, 1998), and then it may well be part of the martian hydrologic cycle.

In this study, we first identified and quantified the oxygen-based free radicals produced in some ''emulated martian conditions.'' These are defined as an iron oxide substrate immersed in an aqueous solution that contains the appropriate concentration of the main oxidizing elements [H_2O_2 and $Mg(ClO_4)_2$] commonly found in martian environments and is irradiated by UVC light. With this aim, we used Electron Paramagnetic Resonance (EPR) spectroscopy to analyze the free radical species produced in liquid media with these components of martian soil subjected to UVC radiation.

To determine whether a photosynthetic unicellular microorganism with a fast-evolving antioxidant metabolism and terraforming capabilities could survive in the adverse photochemical conditions found in non-oxygenic Mars, we assessed the viability of extremophile green microalga *Scenedesmus dimorphus* after exposure to the oxygen-based free radicals produced in the aforementioned emulated martian conditions in the presence and absence of oxygen dissolved in the aqueous media. The results obtained by EPR were then correlated with the survival rate of the alga for each of the tested conditions.

To further explore whether *S. dimorphus* has the necessary mechanisms to cope with the oxidative stress present in martian environments, we used a fluorescent method, to determine the production of intracellular ROS in response to exposure to oxygen-based free radicals expected on Mars. This combination of methodological approaches allowed us to establish a correlation between the viability of this unicellular green alga and the amount of oxygen-based free radicals produced in UVC-irradiated emulated martian conditions.

2. Materials and Methods

2.1. Characterization of the chemicals used to emulate the martian soil

We used a commercial micrometric iron oxide compound (Fe^{3+}: 99.999% of purity) that presents a small amount of the magnetic ferrite γ-Fe^{3+} phase (maghemite, γ-Fe_2O_3) together with the expected dominant weak-ferromagnetic corundum α-Fe^{3+} phase (hematite, α-Fe_2O_3). Although the purity regarding the ionic Fe (Fe^{3+}) is quite high, the presence of two distinct ionic coordination states for the iron should induce different catalytic activity, since this activity is strongly dependent on the electronic orbital configuration and population for the 3d transition metals (Wang *et al.*, 2019).

The presence of small amounts of magnetic ferrite phase in commercial ferric oxides is well known, and it depends on the supplier and grain size (Ahmadzadeh *et al.*, 2017). Therefore, from X-ray diffraction (XRD) analysis shown in Supplementary Fig. S2, we estimated the concentration of corundum and ferrite phase in our sample to be about 95% and 5%, respectively. In the scope of this work, it is important to correlate this phase ratio with the iron oxide composition estimated for Mars. In fact, different studies on martian soil composition indicate the presence of magnetic and no-magnetic iron-oxide phases (Hviid *et al.*, 1997; Gunnlaugsson *et al.*, 2002), with predominance of the latter. In this way, our commercial iron compound with the mentioned mixture of phases could be a good choice to understand the role played by photo-Fenton reactions in emulated martian conditions.

Supplementary Figure S2 also shows the XRD pattern of the prepared $Mg(ClO_4)_2$ used in the experiments, showing only the expected $Mg(ClO_4)_2 \cdot 6H_2O$ phase. Scanning electron microscopy (SEM) images (Supplementary Fig. S3) show a granular morphology with grain size in the micrometric range. Energy-Dispersive X-ray Spectroscopy (EDS) analysis shows the expected Fe and O % for the phases previously identified [Supplementary Table S1), and the presence of other metallic ions was not detected. The EDS analysis of $Mg(ClO_4)_2 \cdot 6H_2O$] also presents no-contamination with other metallic ions.

2.2. Free radicals measured by EPR

The EPR measurements were performed with an ELEXSYS II-E500 spectrometer (Bruker) with an X-band resonant cavity (9.4 GHz) at the temperature of 278 K. The spectra were acquired with an attenuation of 10 dB (20 mW microwave power) and 1 Oe of amplitude of the modulation field. Problems with the signal saturation were corrected and taken into account in the data processing. The solutions for the EPR experiments were prepared by dispersing in Bold media: 120 µg of iron oxide, 0.6 wt % of Mg Perchlorate, and 50 µL of 5,5-dimethyl-1-pyrroline N-oxide/dimethyl sulfoxide (DMPO/DMSO) solution (0.33 g/mL). The concentrations of H_2O_2 used were 10 m*M*, 100 µ*M*, 1 µ*M*, and 0 (without H_2O_2). For this, a 30 vol % solution of H_2O_2 was used. The starting point of the reaction was assumed at the moment H_2O_2 was added to the solution, and the spectra were collected 10 min after. To quantify the amounts of the free radicals, the EPR spectrum of each solution in a quartz tube was recorded simultaneously with a pattern attached to the quartz tube—an MgO pattern crystal doped with a known concentration of Mn^{2+}. The collected spectra were fitted with the software SPIN from Bruker by using the same procedure for all measurements. To identify each species, we used the fitted values of hyperfine constant in the Spin Trap database of the National Institute of Environmental Health Sciences-NIEHS (https://tools.niehs.nih.gov/stdb/index.cfm).

The concentration of each identified free radical was obtained by comparing the area of the EPR-fitted spectrum of the respective species with the area of the MgO/Mn^{2+} pattern component, as reported elsewhere (Chang *et al.*, 1978; Tobia *et al.*, 2014).

2.3. Algal strain and culture conditions

Axenic cultures of the alga *S. dimorphus* (UTEX 417) were maintained in Bold's Basal Medium (BBM) (Andersen, 2005) in Erlenmeyer flasks in a climate-controlled chamber at 21°C. The illumination was provided by a combination of grow lux and 18 W cool white fluorescent light at 80 µmol photons/($m^2 \cdot s$) and a 14 h photoperiod. Cultures and growth

were monitored routinely by microscope observations and cell counting in a Neubauer chamber.

2.4. Experimental design, culture conditions, treatments, and statistical analysis

The martian treatment on *S. dimorphus* cells was carried out *in batch* conditions in wells of 12-well plates. Iron oxide was present in excess as a solid substrate in the form of cylindrical tablets of 1.5 cm diameter and 1 mm thickness. They were placed in the wells, whereas the $Mg(ClO_4)_2$ and H_2O_2 were in 1 mL solution of Bold media. The solution in the well forms a layer about two times the thickness of the iron oxide pellet (1 mm). We used $Mg(ClO_4)_2$ in the martian concentration: 0.6 wt % (Hecht *et al.*, 2009) and H_2O_2 were present at different concentrations, including that reported for Mars of 1 μ*M* (30 ppbv) (Encrenaz *et al.*, 2019). Cells of *S. dimorphus* at a density of 6×10^6 cells/mL were added to the reaction mix, which was then exposed to a collimated UVC radiation from the commercial lamp for 5 min, unless otherwise indicated.

The commercial UVC lamp has an emission peak close to 260 nm and power of 5 W collimated on a controlled area, which is equivalent to a power of 350 W/m^2 on the wells irradiated for 5 min.

Similarly, control experiments to evaluate normal cell growth and treatments varying the H_2O_2 concentration or UVC exposition time were carried out as specified in the figure legends. Experiments in anoxia were performed by degassing the Bold's media by boiling and then gassing it with a stream of nitrogen during the cooling of the media to remove the oxygen. After each treatment, 200 μL of the reaction mix was diluted in 2 mL of fresh Bold's media, and the tubes were incubated at room temperature (21°C ± 2°C) by illumination with a 18 W cool white fluorescent light at 80 μmol photons/(m$^2 \cdot$s) and a photoperiod of 14 h. Cell recovery and growth were evaluated by cell counting in the Neubauer chamber at different time points.

Each experimental condition and replicates were normalized by initial cells counting (time 0 h), and data are expressed relative to the growth level of the control condition. For most of the experiments and conditions, viability was reported at 168 h, which is a point of the growth curve at which *S. dimorphus* is on the exponential phase. All results are expressed as mean values ± standard deviation. Statistical analysis was performed with OriginLab Corporation. Significant differences were evaluated by one-way analysis of variance with a Tukey multiple-comparisons test and a significance level of $p < 0.05$.

2.5. Determination of ROS generation by dichloro-dihydro-fluorescein diacetate

Aliquots of cell cultures exposed to the different conditions were harvested at 0 and 19 h by centrifugation at 3500 rpm at room temperature for 10 min. The oxidation-sensitive fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used to stain the cells and evaluate intracellular ROS levels. Pellets were suspended again in 1 mL of BBM and incubated with 3 μ*M* DCFH-DA for 10 min in the dark. Afterward, cells were recovered by centrifugation (3500 rpm at room temperature 10 min), and the excess of dye was removed with the supernatant. Cell pellets were suspended again in 1 mL of phosphate-EDTA buffer (0.5 M phosphate: 0.1 M EDTA) and disrupted by using ultrasound for 3 min on ice (sonicator Omni Sonic Ruptor 400 Ultrasonic Homogenizer), and samples were centrifuged at 15,000 × *g* for 10 min at 4°C.

The supernatant (cells extracts) samples were then analyzed by spectrofluorometry. An aliquot was taken for protein analysis. Data were recorded as relative fluorescence units with 480 nm as the excitation wavelength by using a spectrofluorometer (Horiba_Fluoromax-4). The emission spectra between 500 and 600 nm were obtained for all experiments. Fluorescence was normalized and expressed relative to the protein content of each sample. Total protein content was determined by the Bradford method. In addition, images of DCFH-DA-stained cultures were obtained by using a fluorescence microscope (Nikon Eclipse E800); the excitation and emission wavelengths were 480 and 530 nm, respectively.

3. Results

3.1. Identification and quantification of free radicals produced in emulated martian conditions

The EPR is a suitable technique for the identification and quantification of individual free radical species (Eaton *et al.*, 2010; Davies, 2016). To facilitate detection of highly unstable and short-live free radicals by EPR spectroscopy, the spin trapping technique was used. Spin traps are diamagnetic molecules that react covalently with the free radicals that form more stable spin adducted species. This paramagnetic molecule has a characteristic EPR spectrum that allows the unambiguous identification and quantification of the adducted free radical. This is because the resonance lines originate from the hyperfine interaction between the unpaired electrons of the free radical and the nearby nuclei with non-zero nuclear spin.

As the chemical structure and spatial configuration of each adducted species is different, the strength of the hyperfine interaction also varies for each case. For this work, we used the DMPO spin trap. This method has already been applied to the study of peroxidase-like activity of iron oxide compounds in the decomposition rate of H_2O_2 (Chen *et al.*, 2012; Wang *et al.*, 2013; Moreno Maldonado *et al.*, 2019; Raineri *et al.*, 2019).

Following this procedure, we performed a comparative study of the free radical formation as a function of the reagents' concentrations to identify the free radicals produced in the emulated martian conditions. The studied media included (1) commercial micrometric iron oxide containing a mixture of α-Fe_2O_3 and γ-Fe_2O_3; (2) $Mg(ClO_4)_2$ at the concentration reported of Mars (0.6%) (Hecht *et al.*, 2009); and (3) H_2O_2 at different concentrations (from 0 to 10 m*M*), including that reported for the martian environment (Encrenaz *et al.*, 2019). Phases present in the iron oxide and $Mg(ClO_4)_2$ were analyzed by XRD—results presented in Supplementary Fig. S2—whereas compositional analysis in terms of metallic ions was determined by Energy-Dispersive X-ray Spectroscopy (EDS) combined with SEM, as shown in Supplementary Fig. S3. According to these analyzes, iron oxide was mainly in the hematite phase (α-Fe_2O_3, ∼95%), and a small fraction in the ferrite phase (∼5%) was probably maghemite (γ-Fe_2O_3), since the used material was 99.999% Fe^{3+}. The synthesized perchlorate gave the expected unique phase $Mg(ClO_4).6H_2O$. In both cases, no cations other than Fe and Mg were observed in the EDS analyses.

In addition, to reproduce the series of chemical reactions that lead to the production of free radicals that occur when unfiltered UVC rays reach these components on the surface of Mars, we exposed the reaction solution described earlier to UVC radiation for 5 min. The source of radiation was generated by a commercial UVC lamp with emission peak close to 260 nm and power of 5 W collimated on a controlled area, which is equivalent to a power of 350 W/m^2 on the wells irradiated for 5 min.

Typical EPR spectra measured for H_2O_2 at the highest concentration of 10 m*M* with and without exposure to UVC are presented in Fig. 1A and B, respectively. As shown by the fitting of these data, the signal of each free radical adducted species can be separated, according to the catalogued hyperfine parameters (see Section 2). The free radicals identified in the reactions are the OH• (hydroxyl), OOH• (hydroperoxyl), CH_3• (methyl), oxidized DMPO (DMPOX), N•, and Cl-based reactive species. The production of hydroxyl radical was dominant in both the presence and absence of UVC radiation; although its concentration showed a great increment when the reaction mix was exposed to UVC. This behavior was not observed for the other radicals.

The second most abundant species were either OOH• or CH_3•, depending on the conditions. The CH_3• radical is associated with a secondary reaction of the OH• radical with the DMSO used to dissolve the DMPO. The Cl-based reactive species and the DMPOX were observed at lower concentrations, as well as the radical that interacts only with the N in the DMPO molecule. We noted that this adduct was also present in the original DMPO compound.

To discriminate the contribution of each component, that is, iron oxide, $Mg(ClO_4)_2$, and UVC, we measured the concentration of free radicals produced under different combinations of these agents in a solution containing 10 m*M* of H_2O_2 as shown in Fig. 1C. This figure shows the predominant generation of the OH• radical in two conditions: iron and iron plus perchlorate, both UVC-radiated. This result indicates that the hydroxyl species plays a main role as a source of oxidative

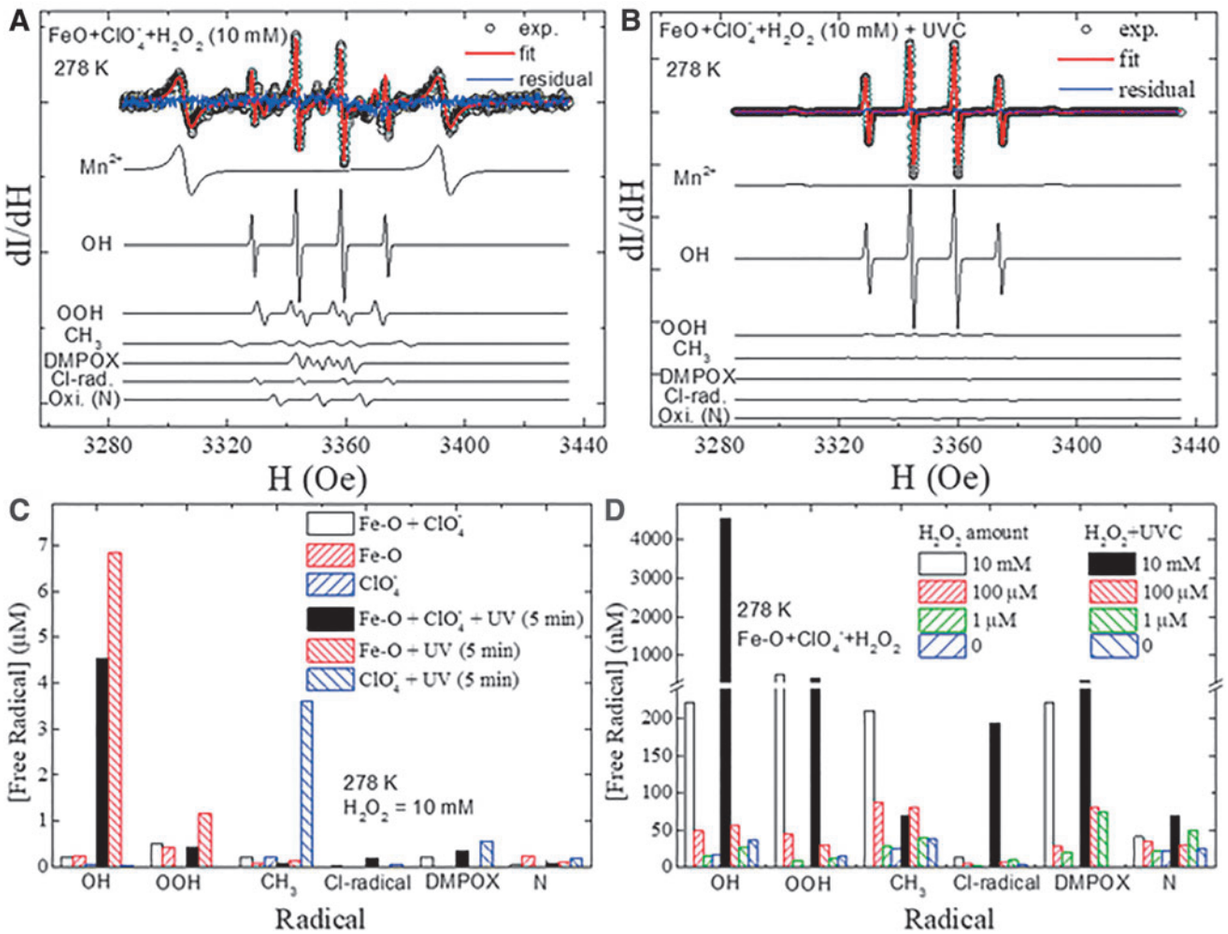

FIG. 1. EPR analysis of free radical production in emulated Mars conditions: **(A, B)** gives a representative EPR spectrum of a solution containing iron oxide (Fe-O), $Mg(ClO_4)_2$, and 10 m*M* H_2O_2 at 278 K, with and without exposure to UVC, respectively, as well as the EPR resonance lines of Mn^{2+} diluted in an MgO crystal used as standard. The figure includes the simulated spectrum of each radical after the fitting procedure. **(C)** Free radical concentrations produced in different systems containing 10 m*M* of H_2O_2 and **(D)** effect of UVC exposition on free radical production in the presence of Fe-O and ClO_4^- with different amounts of H_2O_2. EPR, Electron Paramagnetic Resonance; H_2O_2, hydrogen peroxide; $Mg(ClO_4)_2$, magnesium perchlorate; UV, ultraviolet. Color images are available online.

stress in these emulated martian conditions. Note that these results correspond to a media containing 10 m*M* of H_2O_2, which is four orders of magnitude higher than the levels of H_2O_2 detected on Mars (Encrenaz *et al.*, 2019).

The dependence of the concentration of free radicals on the amount of H_2O_2 in the media that contained the described components was examined next (Fig. 1D). A strong decrease was observed in the free radical's generation with decreasing H_2O_2 concentrations.

We examined next the effect that the different species and the amount of free radicals identified by EPR produced in the earlier specified emulated martian conditions would have on the survival of the microalga *S. dimorphus.* As mentioned in the introduction, photosynthetic cells have evolved over billions of years such that the development of antioxidant mechanisms to protect from photo-oxidation has occurred as a result of their photosynthetic metabolism. Also, primitive phototrophs first developed in anoxic conditions and later evolved to thrive in oxygen-rich environments. Although there likely is no extremophile on Earth that may fit all the required criteria for survival on Mars (anoxia and a rarefied atmosphere, radiation, low temperatures, atmospheric pressure, lack of water, presence of toxic chemicals), we chose *Scenedesmus* sp. as our test subject given that this microalga has proven to tolerate UVC radiation and certain levels of perchlorate and H_2O_2. We designed an experimental protocol to answer the question of whether this eukaryote unicellular alga could survive the types and levels of ROS generated under emulated martian conditions.

Regarding the putative oxidative stress posed by the martian environment on the biology of the alga *S. dimorphus*, two key parameters should be taken into account: the concentration of OH• free radical generated when all the components are present (H_2O_2, iron oxide, perchlorate, and UVC) and its dependence on the concentration of H_2O_2. According to the presented results, it is expected that the oxidative stress and the toxicity would be proportional to the amount of H_2O_2 as well.

3.2. Survival of the microalga S. dimorphus exposed to emulated martian conditions

To determine whether the free radicals produced under emulated martian conditions have an effect on the survival rate of the microalga *S. dimorphus*, cells growing at an exponential rate were UVC-irradiated in the presence of different combinations of the main chemicals detected in martian soil: iron oxide (Hviid *et al.*, 1997; Gunnlaugsson *et al.*, 2002), $Mg(ClO_4)_2$ (Hecht *et al.*, 2009), and H_2O_2 (Encrenaz *et al.*, 2019). Iron oxide was present in excess as a solid substrate (Wadsworth and Cockell, 2017), whereas the $Mg(ClO_4)_2$ was in solution in the martian concentration: 0.6 wt % (Hecht *et al.*, 2009). H_2O_2 was also in solution at different concentrations, including that reported for Mars of 1 μ*M* (30 ppbv) (Encrenaz *et al.*, 2019). Cells of *S. dimorphus* in the concentration of $6–10 \times 10^6$ cells/mL were added to the reaction mix and exposed to a collimated UVC radiation from the commercial lamp (3.5 kW/m or ~1000 W/well plate) for 5 min, unless otherwise indicated.

We used this time window for the reaction because the kinetics of production and the half-life of oxygen-based free radicals are quite fast (Dickinson and Chang, 2011), and this time was enough to produce detectable levels of free radicals in the batch conditions used in the experiment (Fig. 1). It is also expected that the reaction time of the oxygen-based free radicals with the algal cell components is also very fast, and it occurs concomitantly with their production.

We determined the effect that controlled exposure to the ROS, generated by irradiating martian soil components with UVC, had on the survival rate of *S. dimorphus*. For this, after the 5 min treatment with UVC, the reaction mix that included the cells was diluted 1:20 in culture media (Bold; inoculum density = 3×10^5 cells/mL) and incubated at room temperature for 2 weeks to monitor growth rate.

The effect of the treatment on the viability of *S. dimorphus* was quantified by counting cells at 168 hrs of culture after the treatment and calculating the ratio of surviving cells over the starting concentration ($n = n_{168}/n_0$). This parameter gives the number of cell divisions that took place during culture time, and it depends on the number of cells that survived the treatment. The control received no treatment and included only cells of *S. dimorphus* in bold media at the same density. The results are expressed as the number of divisions relative to the divisions undergone by the control cells in each experiment. We ran a control experiment to test the effect of exposure to 5 min of UVC radiation alone.

As expected, based on previous studies (Kovácik *et al.*, 2010), exposition to UVC radiation alone during the experimental time frame had no negative effects on the growth of *S. dimorphus*. On the contrary, sole irradiation with this wavelength had an enhancing effect on the growth of this microalga compared with the control (Fig. 2A). Nevertheless, a comprehensive analysis of the effect of UVC radiation on the survival rate of *S. dimorphus* at different times was beyond the scope of this study and was examined in the work of Kovácik *et al.* (2010). In the subsequent experiments, we tested the effect of free radicals produced by different combinations and proportions of the main martian soil components and enhanced exposure to UVC radiation.

Given that perchlorates (identified as P) present in emulated martian conditions enhance the biocidal effects of UV light on bacteria (Wadsworth and Cockell, 2017), we assessed the effect of UVC-irradiated perchlorate on the survival rate of *S. dimorphus*. We used a concentration of 0.6 wt % of $Mg(ClO_4)_2$, as this is the highest concentration of perchlorate anions found in Mars (Hecht *et al.*, 2009). As shown in Fig. 2A, this treatment (P UVC) had a significant negative effect on growth rate compared with UVC-irradiated cells, though not in comparison to the non-irradiated control. A similar effect was observed when iron oxide (identified as Fe in the sample names) was present in the reaction mix (Fe P UVC) (Fig. 2A).

3.3. Effect on the survival of S. dimorphus of free radicals produced in emulated martian conditions with different concentrations of H_2O_2

H_2O_2 is an oxidant agent that is capable of inducing oxidative stress by itself in living cells, including algae (Rezayian *et al.*, 2019). A great variability in the distribution and abundance of H_2O_2 on Mars has been recorded as shown in Supplementary Fig. S1A, with great inter annual variations as well (Encrenaz *et al.*, 2019). Recent maps, which were derived from data recorded in the 1232–1242 cm^{-1} range (8.1 μm) by the Texas Echelon Cross Echelle Spectrograph

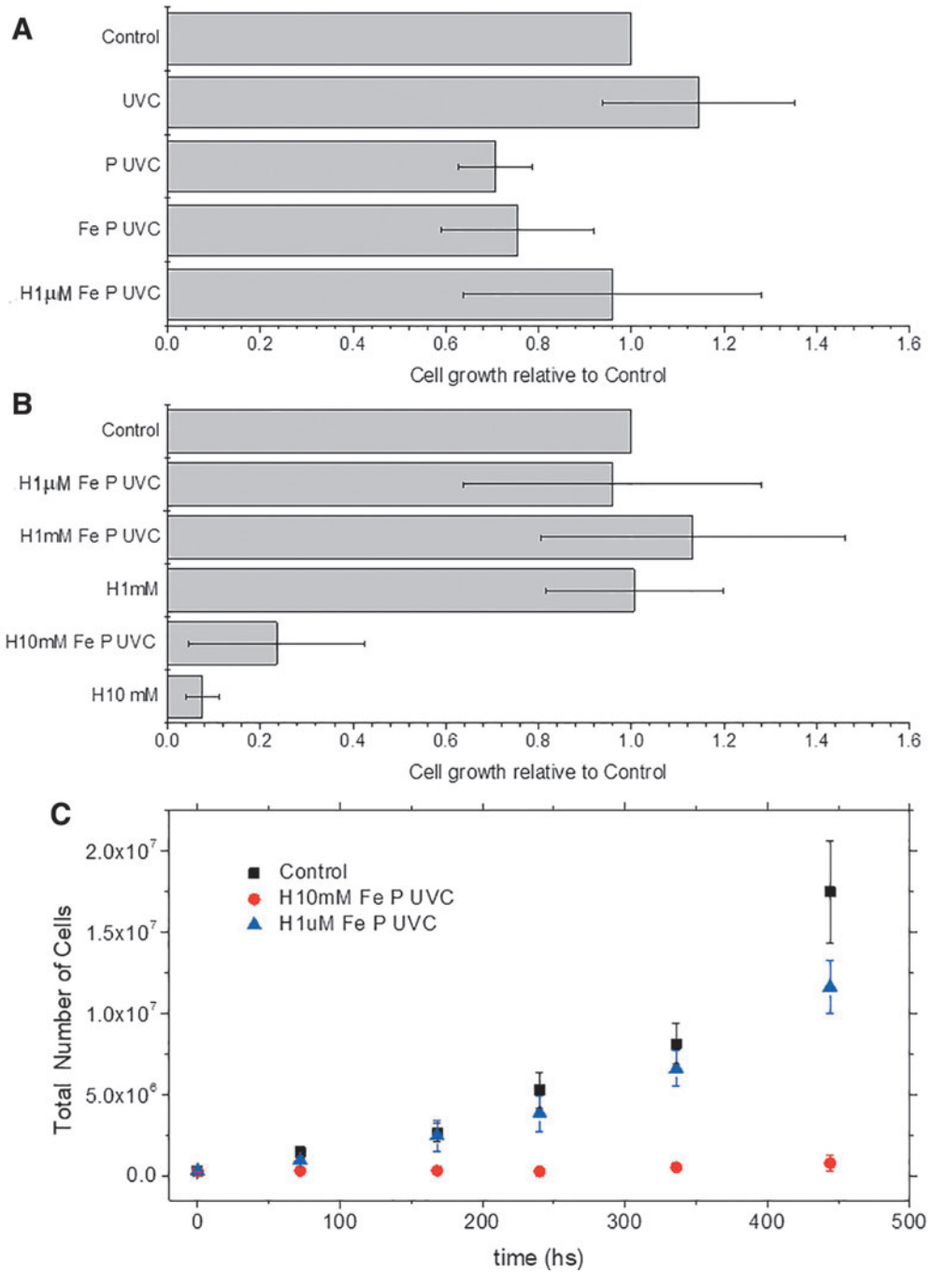

FIG. 2. Cell survival in emulated martian conditions. The bar graphs in **(A, B)** show *Scenedesmus dimorphus* cells' survival—relative to the control treatment—at 168 h after the exposition to different elements that emulate martian environments. **(A)** Gives the results for different combination of elements in the average concentrations found in Mars: UVC radiation, iron oxide (Fe), $Mg(ClO_4)_2$ (P; 0.6 wt %) and H_2O_2 (H; 1 μ*M*). **(B)** Gives the results for the combination of Fe P UVC, in addition to different concentrations of H_2O_2, compared with the effect of these concentrations of H_2O_2 alone. **(C)** Gives the number of algal cells after progressing times in culture from the time of exposure to the combination of Fe P UVC and H_2O_2 in the concentrations of 1 μ*M* and 10 m*M*. Color images are available online.

(TEXES) mounted at the 3 m Infrared Telescope Facility (IRTF) at the Mauna Kea Observatories, gave an average value of 1 μ*M* (30 ppbv) (Encrenaz *et al.*, 2019). The addition of this concentration of H_2O_2 (identified as H in the sample names) to the mix of iron oxide and perchlorate followed by UVC radiation (H 1 μ*M* Fe P UVC) did not have a statistically significant ($p < 0.05$) effect on the viability of *S. dimorphus* compared with the other UVC-irradiated combinations or with the non UVC-irradiated control (Fig. 2A).

Given the high variability of H_2O_2 levels in the soil of Mars as shown in Supplementary Fig. S1A (Encrenaz *et al.*, 2019), we tested the effect of H_2O_2 concentrations that were up to four orders of magnitude higher than the average value. The UVC-exposed treatments conducted with up

to 1 m*M* H_2O_2 in addition to iron oxide and perchlorate (H 1 m*M* Fe P UVC) (Fig. 2B) did not have an effect on *S. dimorphus* viability compared with the non-irradiated control. Only when 10 m*M* H_2O_2 was included in the reaction mix (H 10 m*M* Fe P UVC) (Fig. 2B) was a drastic reduction in cell growth observed, which was statistically significant compared with the control conditions and with the martian conditions (H 1 μ*M* Fe P UVC, Fig. 2B).

To evaluate whether the decrease in viability might be associated with the oxidative toxicity posed by this high concentration of H_2O_2 alone, we carried out a control performed with only 10 m*M* H_2O_2 in the reaction mix. This treatment (H 10 m*M*) (Fig. 2B) had the same negative effect on the growth of *S. dimorphus*, which indicates that the sole presence of 10 m*M* H_2O_2 in the reaction mix is sufficient to produce a cytostatic effect in the cells' growth.

To rule out that the presence of diluted H_2O_2 during the culture time was not harmful to the cells, we performed a control with no treatment and with 0.5 m*M* H_2O_2 in the culture media, which corresponds to a 1:20 dilution of the 10 m*M* H_2O_2 in the reaction mix, during the 168 h of the duration of the culture. This condition did not have a statistically significant effect ($p < 0.05$) on cell growth compared with the control (Supplementary Fig. S4). The concentration of 10 m*M* H_2O_2 was also used by Wadsworth and Cockell (2017) to demonstrate that the bacteria *B. subtilis* is sensitive to the photo-Fenton-generated free radicals produced in emulated martian conditions. Supplementary Figure S4 gives the survival rate of the microalga *S. dimorphus* exposed to different combination of martian soil components. These data further confirm that H_2O_2 is the key factor that determines the toxicity of the environment; however, it was only relevant at the highest concentration used in this study (10 m*M*), which is four orders of magnitude higher than the average value found on Mars (Encrenaz *et al,* 2019).

As seen in Supplementary Figure S4, the effect of other combinations of UVC, iron oxide, perchlorate, and H_2O_2 at concentrations of 1 m*M* and lower was only a moderate reduction in the survivor rate of the microalga. Interestingly, the presence of UVC increased the survival rate when combined with 10 m*M* of H_2O_2; compare, for example, sample H10mM Fe P UVC 300 s with sample H10mM Fe P in Supplementary Fig. S4. Further, the survival rate remains the same and/or increases with increasing UVC exposure time (see samples H10mM Fe P UVC 300 s, H10mM Fe P UVC 60 s, and H10mM Fe P UVC 30 s in Supplementary Fig. S4). These results could be explained by the fact that the decomposition of peroxide is faster in the presence of UVC. Indeed, we measured the kinetics of decomposition of 10 m*M* H_2O_2 by Fourier Transform Infrared Spectroscopy in the presence and absence of UVC radiation (Supplementary Fig. S5). We also found that the peak that corresponds to H_2O_2 completely disappears only after 300 s, whereas the same peak is no longer observed after 100 s in the presence of UVC. These results suggest that the free hydroxyl radicals produced by the decomposition of H_2O_2 by UVC are not as toxic to this alga as is H_2O_2 alone.

3.4. Effect of emulated martian conditions on the growth of S. dimorphus at prolonged times in culture

The effect that free radicals produced under emulated martian conditions on *S. dimorphus* at longer times in culture is presented in Fig. 2C, which shows the growth curves, expressed as total number of cells versus time, as obtained for the control condition (squares). Figure 2C also shows two UVC-irradiated treatments that included the iron oxide substrate, the presence of 0.6 wt % of $Mg(ClO_4)_2$, and either the average martian concentration of H_2O_2 (1 μ*M*, triangles) or 10 m*M* H_2O_2 (circles). At longer times in culture (more than 250 h), there was a more marked effect on the growth of the cells exposed to martian conditions (triangles) than occurred at shorter times. The cells exposed to the treatment that included 10 m*M* H_2O_2 did not recover, not even at longer times. Interestingly, the evaluation of cell mortality by safranin staining at 250 h did not reveal the presence of cell deaths with damaged membranes (data not shown), indicating that even this high concentration of H_2O_2 could be merely cytostatic rather than biocidal for this unicellular alga.

3.5. Effect of free radicals produced under emulated anoxic martian conditions on S. dimorphus survival

Given that the atmosphere of Mars is deprived of oxygen, we carried out experiments to investigate whether ROS produced under emulated martian conditions would have the same effect on the survival of *S. dimorphus* if they were produced in the presence or absence of oxygen. The reaction mix was oxygen-depleted by warming up the culture media during sterilization and by cooling it down in a nitrogen gas atmosphere, as described in Section 2. Experiments were done *in batch*, under the same conditions used in the previous assays for the concentration and presentation of martian soil components (iron oxide, $Mg(ClO_4)_2$, and H_2O_2) and the exposure to UVC. A control experiment, by triplicate, in the presence of oxygen was included for comparison. Figure 3 shows viability at 168 h for the different treatments. Carrying out the reaction under anaerobiosis did not have an effect on any of the treatments. Consistent with previous results, a significant reduction on cell viability was observed when 10 m*M* of H_2O_2 was included in the reaction mix.

3.6. Production of endogenous free radicals in the microalga S. dimorphus exposed to emulated martian conditions

The ROS generated by UVC-irradiated martian soil components would react with the biomolecules of *S. dimorphus* cells, first inducing lipid peroxidation and, subsequently, oxidative damage inside the cell, spreading the formation of ROS. To evaluate the formation and accumulation of intracellular ROS, we stained the cells with DCFH-DA. This compound is hydrophobic and, hence, plasma membrane-permeable (Eruslanov and Kusmartsev, 2010). Once inside the cell, it is cleaved by intracellular esterases at the two ester bonds, becoming cell membrane-impermeable as H_2DCF and thus trapped inside, where it accumulates. Its subsequent oxidation by ROS originates from the fluorescent product DCF. This method is widely used to monitor levels of intracellular ROS (Eruslanov and Kusmartsev, 2010).

Experiments were done for the most relevant conditions evaluated in the previous sections. We first evaluated the formation, and eventual localization, of intracellular ROS by examining the stained cells under a fluorescence microscope. Figure 4A shows representative images of the different treatments as follows: Control; H 1 μ*M* Fe P UVC (Mars

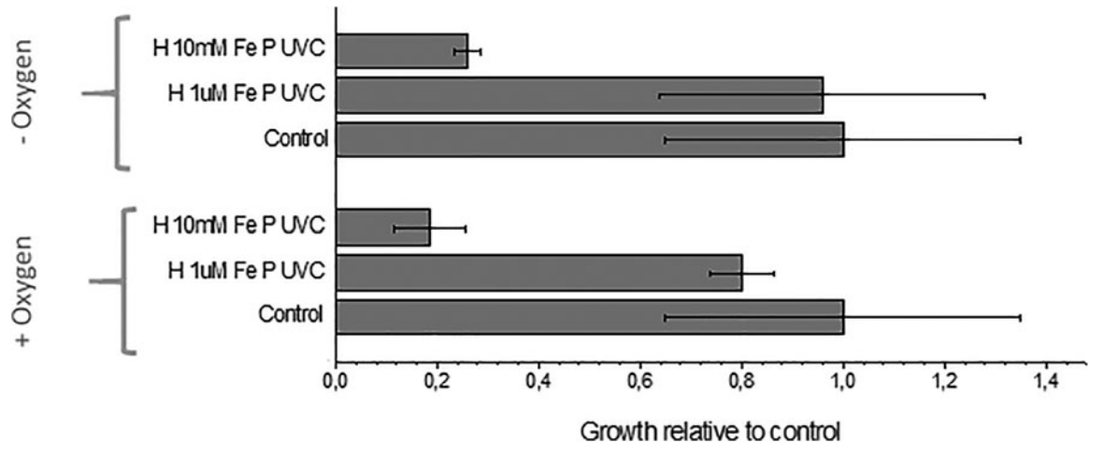

FIG. 3. Cells survival in emulated Mars conditions, including anoxia. The bar graph shows *Scenedesmus dimorphus* cells' survival—relative to the control treatment—at 168 h post-treatment in the presence (+ Oxygen) and absence (– Oxygen) of oxygen. Bars represent the average values of triplicates for each condition. The labels correspond to Fe: ferrite (iron oxide); H: hydrogen peroxide (1 μ*M* and 10 m*M*); P: perchlorate (0.6 wt %); UVC: ultraviolet C light (5 min).

conditions); and H 10 m*M* Fe P UVC (Mars conditions but with 10 m*M* of H_2O_2), at time 0 h. The images show that the fluorescent signal is intracellular with no background, and in some cells, the dye strongly stains the chloroplast (presumably the pyrenoid), as indicated by white arrows in Fig. 4A. This staining pattern is more evident in the Control, whereas in the martian treatments, the staining is more homogeneously distributed in the cytoplasm.

To quantify the levels of intracellular ROS, cell pellets from the same experimental conditions were extracted by cell sonication in phosphate-EDTA buffer, and DCF levels were evaluated by spectrofluorometric analysis. To perform this measurement, the samples were excited with 480 nm and the emission spectra were evaluated between 500 and 600 nm, showing a peak at 520 nm. Figure 4B shows the results obtained at 0 h and at 19 h of incubation after treatments. We observed similar basal levels right after treatments, at time 0 h; whereas the signal reflecting intracellular ROS levels increased with increasing H_2O_2 concentrations when the measurement was done at 19 h post-treatment. This result indicates that levels of extracellular ROS production during the reaction can translate into intracellular ROS formation, and that these intracellular levels positively correlate with increasing H_2O_2 concentrations, as expected based on the viability assays (Fig. 2B).

4. Discussion

In the present work, we emulated martian chemical conditions to study the photo-Fenton reaction that takes place when the main components of martian soil are combined under the effect of UVC radiation. The reaction products are oxygen-based free radicals that are generally known to have deleterious effects on carbon-based living organisms, but the exact types and proportions of the radical species generated in martian conditions are not known. Their effect on the terrestrial microorganisms abler to withstand UVC radiation and oxidative stress – i.e., unicellular green algae – is unknown as well.

In this context, the main achievements of this work are the identification and quantification of the free radical species generated during the reaction in a martian emulated environment and the evaluation of their effect on the microalga *S. dimorphus*. EPR spectroscopy is a precise and sensitive technique widely used to study the generation of free radicals by the spin trapping technique. In particular, the EPR spectra of the DMPO spin trap molecule combined with ROS are very well known, and each spectrum is like a fingerprint of the free radical specie adducted. Therefore, the nature of the free radicals that are generated in each reaction can be identified; further, given that the EPR adsorption intensity is proportional to the number of paramagnetic species (Abraham and Bleaney, 1970), the free radicals can also be quantified through a direct comparison of the EPR intensity with a standard sample that has a known concentration of magnetic ions.

In this way, the different free radicals that were generated by UVC-irradiation of different combinations of martian soil components could be analyzed. From these experiments, several free radical species were identified: OH•, OOH•, CH_3•, Cl-radical, DMPOX, and N•. In all the conditions tested, the predominant species was always OH•. Although the mentioned radical species are produced in the presence of iron oxide and $Mg(ClO_4)_2$, a remarkable increase of the ROS is produced when H_2O_2 is added to the reaction mix, due to the decomposition of H_2O_2 catalyzed by the heterogeneous Fenton reaction. In addition, the photo-Fenton reaction takes place when the reaction components are exposed to UVC radiation. Although the measurements of the kinetics formation of free radicals by EPR were performed in liquid media, the results obtained gave us key information about the species of free radicals that can be found in the drier conditions of Mars.

We examined the effect of these radicals on the survival of the photosynthetic unicellular eukaryote alga *S. dimorphus.* The viability analysis of *S. dimorphus* in this oxidative environment, generated by Fenton and photo-Fenton reactions from martian soil components and UVC radiation, was conducted by an *in batch* experimental design, which has been previously used to explore similar questions (Wadsworth and Cockell, 2017). We exposed the alga to conditions that

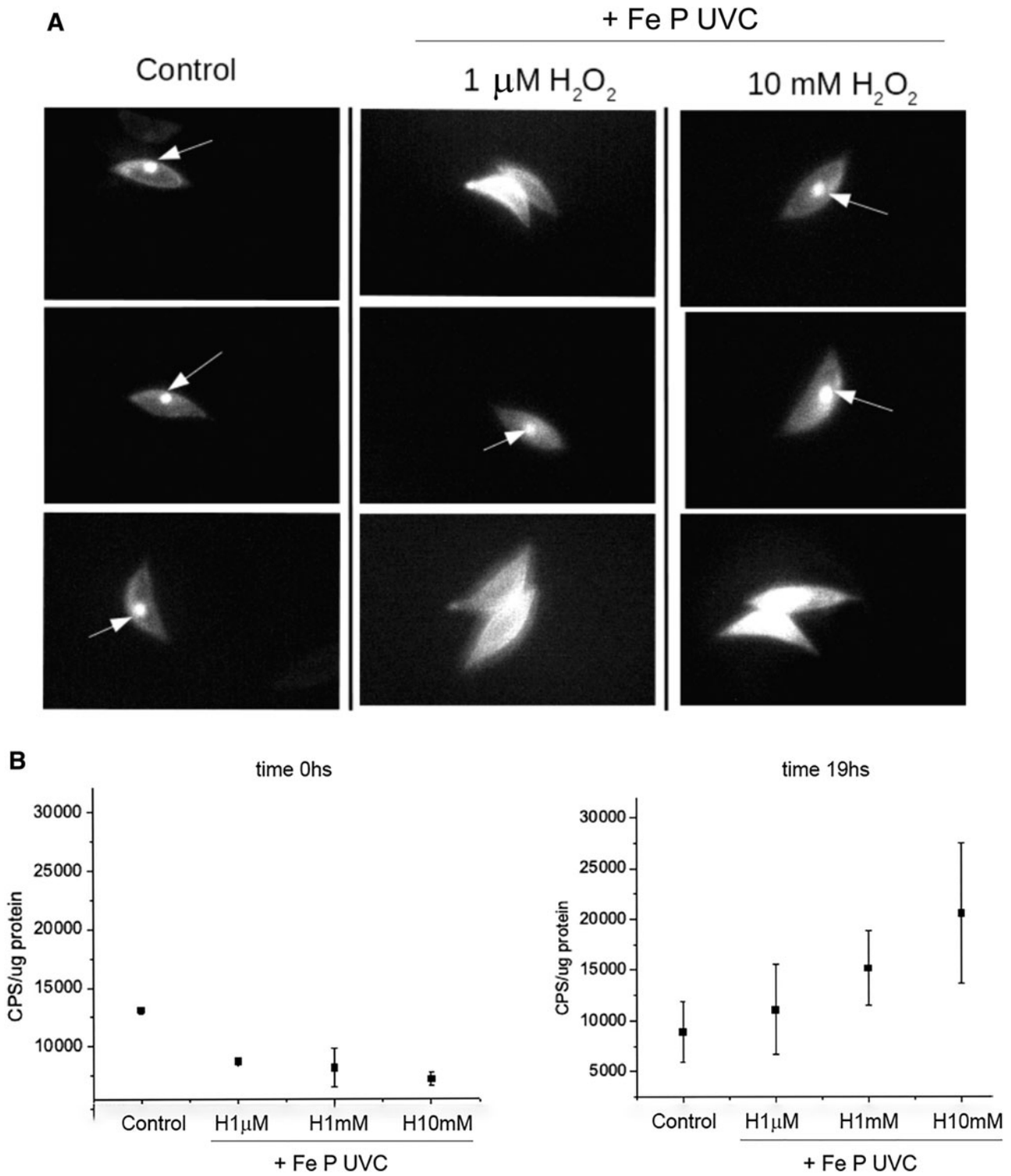

FIG. 4. Free radicals' production in emulated Mars conditions. **(A)** The pictures show cells stained with the fluorescent probe DCFH-DA at time point 0 h after the treatment for the control (no treatment), the Mars treatment (H1 μ*M* Fe P UVC), and the treatment that included 10 m*M* H_2O_2 (H10mM Fe P UVC). Pictures were taken with a 100× objective, and they are at a magnification of 1000×. **(B)** Fluorometric analysis of the cell pellets extracted at times 0 and 19 h post-treatment for the Control, H1 μ*M* Fe P UVC (Mars condition), and two other concentrations of H_2O_2 (H1mM Fe P UVC and H10mM Fe P UVC). Fe, iron oxide; H, hydrogen peroxide; P, Perchlorate (0.6 wt %); UVC, 5 min. The plotted values are average values of triplicates for each treatment. DCFH-DA, dichloro-dihydro-fluorescein diacetate.

emulate the martian environment (UVC exposure, iron oxides, perchlorate, and H_2O_2), and we varied the reaction conditions and the composition of the reaction mix to examine the contribution of the different components.

Our experiments show that different combinations of the martian elements with UVC did not have an effect on the cellś survival even at 168 h post-treatment (Supplementary Fig. S4). In particular, the highest concentration of H_2O_2 present on Mars, either alone or in combination with iron oxide and perchlorates under UVC radiation, did not pose a threat to the survival of the microalga (Fig. 2B). This is not surprising since algae, including *Scenedesmus* sp., are tolerant to various concentrations of H_2O_2 (Mallick, 2002). The same applies to other potent oxidant reagents such as perchlorates, since species from *Scenedesmus* sp. have been shown to be more resistant to perchlorates than prokaryote organisms such as the cyanobacteria *Microcystis aeruginosa* (Brinkmann and Senger, 1978) and methanogenic archaea (Maus *et al.*, 2020).

The sensitivity of bacteria, in general to perchlorates and UVC, may also explain the discrepancy between our results and those from the work of Wadsworth and Cockell (2017). In that study, the authors showed that potassium perchlorate significantly increases the bactericidal effect of UVC on *B. subtilis* (Wadsworth and Cockell, 2017).

In our case, the microalga *S. dimorphus* was resistant to the effect of UVC, in both the presence and absence of perchlorates. The particular tolerance of photosynthetic algal cells to elements with high oxidative potential is most likely due to their adapted capacity to overcome oxidative stress. All these facts are in agreement with our observations, and we can conclude that the ROS produced from the combination of these highly oxidant martian components exposed for 5 min to UVC radiation have a non-statistically significant effect on the exponential growth of the alga *S. dimorphus* (Fig. 2C).

From the EPR analysis during the Fenton and photo-Fenton experiments, we learned that the production of free radicals was dependent on the concentration of the most variable martian component, H_2O_2. The H_2O_2 values recorded on Mars range between 45 ppbv on the north side and <10 ppbv on the south side, as shown in recent maps at high spatial resolution (Supplementary Fig. S1). These values are three orders of magnitude lower than the maximum concentration of H_2O_2 (1 m*M*), which did not have a significant effect on the growth of *S. dimorphus* at 168 h post-treatment (Fig. 2B).

To examine further the effect of higher concentrations of H_2O_2 on cell viability, we tested the effect of 10 m*M* H_2O_2, a concentration that gave the highest production of ROS, 4000 n*M*, when combined with the other martian components [iron oxide, $Mg(ClO_4)_2$, and UVC radiation] (Fig. 1). We found that this dose of H_2O_2 was deleterious to the survival of *S. dimorphus*, regardless of the presence of the other martian components in the reaction mix (Fig. 2B and Supplementary Fig. S4). It is worth noting once again that this concentration of H_2O_2 is more than three orders of magnitude larger than the highest H_2O_2 concentration recorded on Mars (Supplementary Fig. S1), and that all the conditions that included H_2O_2 at the martian value had no significant effect on the viability of the *S. dimorphus* in comparison to the control conditions.

In a biological context, the ROS are formed as a by-product of the metabolism of oxygen. In photosynthetic organisms, ROS (O_2•, OH•, and H_2O_2) are continuously formed by leakage of electrons onto molecular oxygen from the electron transport activities of chloroplasts, mitochondria, and the plasma membrane. The production of H_2O_2 by photo-reduction of oxygen during photosynthesis in chloroplasts is unescapable (Fridovich, 1995; Niyogi, 2015). For these reasons, algae thrive in environments with low oxygen and simultaneously develop mechanisms that allow them to colonize more oxygenated environments.

As part of this adaptation to oxidative stress, algae evolved to minimize free radical damage by triggering antioxidant defenses, both enzymatic (*e.g.*, superoxide dismutase, catalase, ascorbate peroxidase) and non-enzymatic (*e.g.*, ascorbate, glutathione, tocopherols, carotenoids) (Niyogi, 2015). Usually, these cellular antioxidants counteract normal concentrations of ROS, including H_2O_2. However, very high levels of free radicals may lead to severe oxidative stress and production of more free radicals than the cell's defense mechanisms can possibly control, leading to cell death.

Our EPR results indicate that there is a correlation between the amount of free radicals produced and the amount of H_2O_2 (Fig. 1), as well as between the amount of H_2O_2 and the growth rate of *S. dimorphus* (Fig. 2B). This correlation is clearly evident in Fig. 5, where *S. dimorphus* growth, expressed as the number of cell divisions, is plotted as a function of the total ROS obtained for each concentration of H_2O_2 tested. Besides varying amounts of H_2O_2, all the treatments also included the martian concentrations of iron oxide and $Mg(ClO_4)_2$, and they were irradiated with UVC.

As shown in Fig. 5, there is, indeed, a direct correlation between the concentration of ROS produced by the catalyzed decomposition of H_2O_2 and the viability of the microalga *S. dimorphus* exposed to these treatments. This dependence is strongly supported by the experiments in

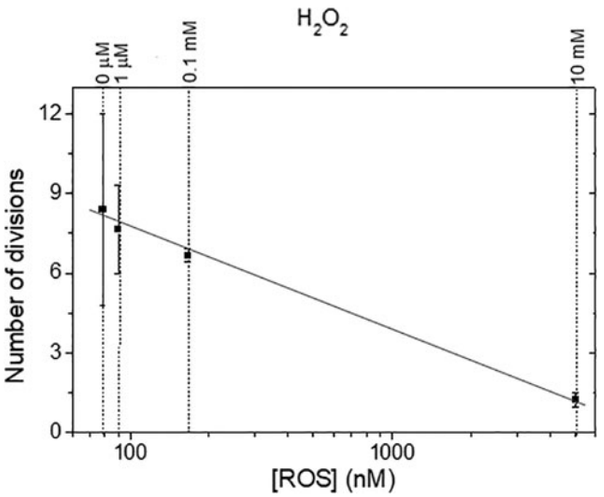

FIG. 5. Growth rate of *Scenedesmus dimorphus* after exposure to martian conditions with different concentrations of H_2O_2 concentration versus the concentration of total ROS produced in each case. The growth rate, expressed as number of cell divisions, was plotted as function of the total ROS concentration generated in the UVC-exposed treatments that included iron oxide, $Mg(ClO_4)_2$, and the different concentrations of H_2O_2 shown in the upper axis, ranging from 1 µ*M* (Mars condition) to 10 m*M*. ROS, reactive oxygen species.

which we demonstrated that extracellular ROS induce intracellular ROS production in an H_2O_2 concentration-dependent manner (Fig. 4).

The fact that the relationship between the cell's growth and the logarithm of the production of ROS follows an Arrhenius-like behavior, as observed in Fig. 5, may indicate that there is a threshold of ROS levels above which the cell defenses are unable to fend off the oxidative stress. This threshold is probably the result of a competition between the oxidation promoted by ROS and the antioxidant reactions that comprise the cellular response.

From our results, the dose of 10 m*M* H_2O_2 is clearly above that threshold. This amount of H_2O_2 generated enough extracellular ROS to trigger the accumulation of high levels of intracellular ROS still observable at 19 h post-treatment. The cellular damage produced by these levels of ROS would explain the observed reduction in cell viability. However, more studies are required to confirm this linear correlation and determine exactly which is the level of oxidative stress that the antioxidant defenses of these algal cells can manage. Nevertheless, our results indicate that this threshold of ROS that leads to a lethal oxidative stress is way above the concentration of free radicals that are produced under the emulated martian conditions examined here, which was shown not to be a threat to the survival of the microalga *S. dimorphus*.

We observed that, in the time frame tested, UVC radiation alone had a slight positive effect on cell proliferation (Fig. 2A). The fact that species of the genus *Scenedesmus* sp. tolerate UVC radiation has been previously reported (Kovácik *et al.*, 2010). Also, it has been shown that exposure to UV light stimulates antioxidant enzymes in response to ROS production in plants and algae (Prasad *et al.*, 2005). Presumably then, the UVC activation of the antioxidant response of the alga may explain not only the tolerance to UVC and possibly even the enhanced growth, but more importantly, it may also prime the cells to withstand the oxidative aggression posed by ROS. More experiments are needed to test this hypothesis. Nevertheless, Mars is being constantly irradiated by UVC and galactic cosmic rays; hence, still open is the question with regard to the possibility of survival of *S. dimorphus* to prolonged UVC exposure and ongoing generation of ROS on a rather anoxic, dry, and cold planet.

In fact, atmospheric conditions, such as temperature and pressure, can also play a fundamental role in how the microalga could survive the martian oxidative stress. On Mars, the observed temperature range varies between 170 K (−103.1°C) and 300 K (26.85°C), as shown in Supplementary Fig. S1C. In spite of the seasonal variations, there are regions on Mars that register temperatures well above 273.15 K (0°C) all year around (see red areas in the northern hemisphere of Supplementary Fig. S1C). The Fenton and photo-Fenton catalytic reactions are more effective, and thereby, more ROS are generated, at higher temperatures. Therefore, for this study, we carried out the *in vivo* experiments at 21°C, and EPR measurements at 21°C and 5°C, which is close to the highest temperature value recorded on Mars. In this way, we were able to test the effect of ROS from emulated martian conditions at temperatures where production of ROS would be maximal.

Even in this worst-case scenario, the amount of ROS generated by Fenton and photo-Fenton reactions from emulated martian components tested here had very low or no effect on the viability of *S. dimorphus* cells. Based on the capacity of *S. dimorphus* to survive the oxidative stress in these simulated martian conditions, future studies could include a combination of mutagenesis and classical conditioning to produce a variant of *S. dimorphus* that further adapts to actual martian conditions.

5. Conclusion

We identified and quantified ROS radicals in an emulated martian chemical environment that included iron oxide, perchlorate, and hydrogen peroxides under UVC radiation. Mainly, the OH radical was dominant, with the production also of OOH and other radicals. The amount of radicals was directly proportional to that of OH present, as well as to UV exposure in a photo Fenton-type chemical reaction. We determined the effects of these radicals produced under these conditions on the viability of a eukaryotic unicellular alga, *S. dimorphus*, which has the ability to survive oxidative stress and a moderate dose of UVC. We found the remarkable result that this microalga is fully capable of fending off these emulated martian levels of oxidant aggression. We observed that a determinant factor in both the production of ROS and the survival of *S. dimorphus* was the concentration of H_2O_2. However, it took a concentration of H_2O_2 that was four orders of magnitude higher (10 m*M*) than the average concentration of H_2O_2 (1 μ*M*) on Mars to finally inhibit the growth of *S. dimorphus.*

We conclude that this amount of H_2O_2 is above the threshold of antioxidant defense mechanisms that this alga possesses, which, on the other hand, is enough to bestow on it the protection necessary to fight off the ROS produced in emulated martian conditions. Although these are encouraging results, it is a preliminary step. Whether this oxygen-producing microalga has the potential to evolve naturally or artificially and survive under actual martian conditions—perhaps to play a role in future terraforming processes—remains to be seen.

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Author Disclosure Statement

The authors declare no conflict of interest.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Table S1

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Abbreviations Used

BBM = Bold's Basal Medium
CH_4 = methane
DCFH-DA = dichloro-dihydro-fluorescein diacetate
DMPO = 5,5-dimethyl-1-pyrroline N-oxide
DMPOX = oxidized DMPO
DMSO = dimethyl sulfoxide
EDS = Energy-Dispersive X-ray Spectroscopy
EPR = Electron Paramagnetic Resonance
H_2O_2 = hydrogen peroxide
$Mg(ClO_4)_2$ = magnesium perchlorate
ROS = reactive oxygen species
SEM = scanning electron microscopy
UV = ultraviolet
XRD = X-ray diffraction