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Impact of low-dose X-ray radiation on the lipidome of *Chlorella vulgaris*

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ABSTRACT

C. vulgaris is a microalga with great potential as a source of lipids and essential fatty acids for human nutrition during extended space missions to Mars. However, the effects of Mars-like radiation on lipid composition are still poorly understood. In this study, we analyzed the effects of X-rays on the growth and lipid biosynthesis of *C. vulgaris* CCALA 269, exposing the cultures to doses of 450, 900, 1800, 3600, and 10,800 mSv, simulating approximately 354, 709, 1417, 2835, and 8504 days of exposure to Martian radiation, respectively. The results show that, although growth remained stable, doses exceeding 1800 mSv led to an increased production of specific lipid classes, suggesting an adaptive mechanism to counteract radiation stress. This adaptation was accompanied by an increase in reactive oxygen species (ROS) and changes in pigment composition, with an elevation in pheophytin-a and chlorophyll-a, and a decrease in chlorophyll-b. Our results demonstrate the ability of *C. vulgaris* to adapt to ionizing radiation, highlighting its suitability for sustainable lipid production in extraterrestrial environments, supporting human life on Mars through in situ resource utilization.

1. Introduction

Several space agencies are currently working towards a continuous human presence on the Moon [1]. Leveraging insights gained from lunar exploration, it may become feasible to send humans to Mars, thereby expanding the horizons of interplanetary exploration [1]. However, the sustainability of these ambitious space programs may be compromised by the substantial quantities of consumables needed. In the long term, a large part of these consumables should be produced on site. The capabilities to produce food on site would be essential for human settlement beyond Earth [2].

Cyanobacteria and microalgae could play a vital role in addressing these challenges, thanks to their efficient production of edible biomass and oxygen, as well as their provision of essential nutrients for human health, particularly in extreme environments like space [3–6]. These photosynthetic microorganisms have also been proposed for a range of other functions, such as metal nutrient extraction from Martian regolith and/or processing of human waste, contributing to production systems based on in situ resource utilization and recycling [3,7]. To date several studies have demonstrated the feasibility of producing biomass using resources available on Mars [4,5]. Among the microorganisms considered for this purpose, one of the most studied is the cyanobacterium *Arthrospira platensis* (Spirulina) [4,8]. Spirulina is a key component of the Micro-Ecological Life Support System Alternative (ESA's main biological life support project), owing to its efficient production of oxygen and edible biomass, as well as its provision of essential nutrients [6]. However, given the diversity and unique characteristics of microalgal and cyanobacteria species, as well as the varied technological applications for which some are more suitable than others, it is essential to integrate these systems with additional species capable of producing

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biomass with different biochemical profiles. This would enhance their versatility for utilization even in space environments. For example, among the most widely cultivated microalgae and cyanobacteria on Earth are *Chlorella*, *Spirulina*, *Dunaliella*, *Haematococcus* and *Schizochytrium* spp. [9,10]. Within these genera, species such as *Arthrospira platensis*, *Chlorella vulgaris*, and *Haematococcus pluvialis* are Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (FDA) [10]. Given their rich contents of a wide range of nutritional and high-value compounds, edible biomass derived from cyanobacteria and microalgae may represent a suitable bioresource for producing food supplements [11]. Specifically, *C. vulgaris* has garnered interest as a source of food and feed due to its high contents of essential nutrients [12,13]. For instance, it can yield up to 67 % dry matter of protein, with digestibility comparable to that of beans, oats, and wheat [13]. The lipid contents exhibit considerable variability, with determined values ranging from 5.10 % to 19.7 % of dry matter [9,13]. While the composition of these photosynthetic microorganisms has been extensively studied under Earth conditions, our understanding of the physiological changes they undergo in challenging space conditions, such as microgravity or increased radiation exposure, remains limited [14,15]. These stressors can potentially lead to significant alterations in the quality and safety of the biomass.

On Earth, the thick atmosphere and strong magnetic field act as protective shields against ionizing radiation [16,17]. Conversely, the low density of the atmosphere and the absence of a substantial magnetic field on Mars leave the surface exposed. The primary source of ionizing radiation on the Martian surface is galactic cosmic rays (GCRs) and solar energetic particles (SEPs) [18]. The composition of GCRs exhibits slight variations over time and typically comprises 85–90 % protons, 10–13 % helium nuclei, 1 % electrons, and 1 % heavier nuclei [19,20]. Skylab recorded dose rates up to 860 μGy/d, while Apollo 14 detected rates up to 1270 μGy/d [20,21]. On Mars, the Curiosity rover measured an average of 233 ± 12 μGy/d in 2012 [20,22]. During solar flares and coronal mass ejections, these values can increase by up to 50-fold.

On living cells, such radiation leads to damage to various components (including nucleic acids, proteins, and lipids), through either direct energy transfer or the indirect production of reactive oxygen species (ROS) [23]. ROS can be a singlet oxygen ($^1O_2$), hydroxyl radical (•OH), superoxide (O_2^-), or hydrogen peroxide (H_2O_2) [24], all of which are naturally generated within microalgal cells during cellular processes involving redox reactions and signalling pathways across various organelles, including mitochondria, peroxisomes, cell walls, endoplasmic reticulum, plasma membrane, and chloroplasts [25].

Microalgae's lipids are particularly susceptible to the effects of ionizing radiation due to the presence of double bonds, which can readily react with free radicals. Microalgae possess two main categories of lipids: polar and non-polar, also referred to as neutral [26]. The predominant chloroplast thylakoid membrane lipids include monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglicerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). MGDG can account for up to 50 % of polar acyl lipids, and DGDG up to 20 %. These galactolipids are crucial for the functioning of photosynthetic complexes, chloroplast structure, and photoprotection [27].

Microalgae can adjust their MGDG/DGDG ratio in response to environmental stressors, including ionizing radiation [26,27]. Since MGDGs and DGDGs are essential for maintaining the stability of the bilayer structure of photosynthetic membranes, changes in their ratio can result in deformations in the structure of the photosynthetic apparatus, leading to alterations in photosynthetic capacity. In fact, the proportion of MGDGs to DGDGs decreases under unfavourable growth conditions, such as nutrient deprivation or exposure to high light intensities [26,27]. The thylakoid membranes of microalgae also include other species, such as sulfolipid SQDGs and phosphoglycerols (PGs), which impart negative charges. Studies on *Chlamydomonas* have demonstrated their essential role in maintaining the activity of photosystem II (PSII) [28].

Triacylglycerols (TAGs) are neutral lipids that do not possess specific structural functions; instead, they are stored in the cytosol as lipid droplets, typically under stressful conditions [26]. Diacylglycerols (DAGs) serve as a precursor for the synthesis of TAGs, phospholipids, and glycolipids [26].

In addition to their physiological roles within microalgae, these lipids can present biological activities that benefit humans, particularly during space missions. For instance, MGDG and SQDG have been shown to have antitumor effects [29], and SQDG can help inhibit neoplastic and inflammatory processes and confer protection against cell death [30,31]. Furthermore, phospholipids (PL) possess antibacterial, antiviral, antitumoral, and antimicrobial properties [32] and play crucial roles in memory storage, as well as muscle control.

While dose rates on Mars are expected to be too low to affect growth significantly, the alteration of lipid composition remains a critical concern, given the susceptibility of lipids to radiation-induced damage. This study aims to elucidate the impact of ionizing radiation on the lipid composition of *Chlorella vulgaris* CCALA 269. It is important to note that the radiation flux on the surface of Mars is complex and, to the best of our knowledge, not yet available in the literature. Consequently, no facility on Earth can accurately replicate it. Therefore, this study primarily focuses on the effects of X-rays as a proxy for Mars-surface radiation, leaving the investigation of other radiation components to future research. These insights can contribute to developing strategies to preserve the nutritional quality and safety of microalgal biomass for space exploration applications, facilitating long-duration missions on Mars.

2. Materials and methods

2.1. Microalgal cultivation and preparation for experimental inoculation

The green microalga *Chlorella vulgaris* CCALA 269 was obtained from the Culture Collection of Autotrophic Organisms (CCALA), Třeboň, Czech Republic. *C. vulgaris* was cultivated in modified Bold's Basal medium (BBM, Table S1) at 25 °C with a light intensity of 40 μmol_{ph} m^{-2} s^{-1} (12 h/12 h day/night cycle).

Before the experiment started, cultures designated for inoculation underwent cultivation on a rotary shaker set at 60 μmol_{ph} m^{-2} s^{-1}. The inoculum was prepared using cultures in the exponential growth phase.

The experiment was conducted in vented cap flasks filled to 50 mL, in quadruplicate for each radiation dose. The inoculum was prepared by washing a preculture twice in ddH_2O and resuspending the pellet in fresh BBM medium, to an optical density (OD) at 750 nm of 0.35. Following irradiation, the flasks were incubated for 9 days at 25 °C, with a light intensity of 70 μmol_{ph} m^{-2} s^{-1}. Shaking was maintained at 100 rpm. Concurrently, control cultures of *C. vulgaris* were prepared and subjected to an identical treatment, save for irradiation. Growth kinetics was assessed by monitoring OD at 750 nm.

2.2. Experimental setup and X-ray irradiation conditions

The samples underwent X-ray irradiation (as a proxy for Mars's ionizing irradiation) using a linear accelerator (LINAC SIEMENS ONCOR) situated at the *S.C. Radioterapia Oncologica* of the ARNAS (*Azienda di Rilievo Nazionale ed Alta Specializzazione*) G. Brotzu in Cagliari (Sardinia, Italy). Bremsstrahlung photons were generated via a 6 MV electron beam impinging on a tungsten target. The flasks containing the samples were positioned on a water-equivalent slab 5 cm thick, itself placed on a bed (Fig. S1). To mitigate the build-up effect and ensure uniform irradiation across all samples, an additional slab of the same thickness was placed atop the flasks (Fig. S1). Each irradiation session employed two beams (top and bottom), facing each other, to achieve a consistent dose distribution throughout the entire volume. The dose rate remained constant, at 2.50 Sv/min, during irradiation. Five distinct doses were administered: 450, 900, 1800, 3600, and 10,800

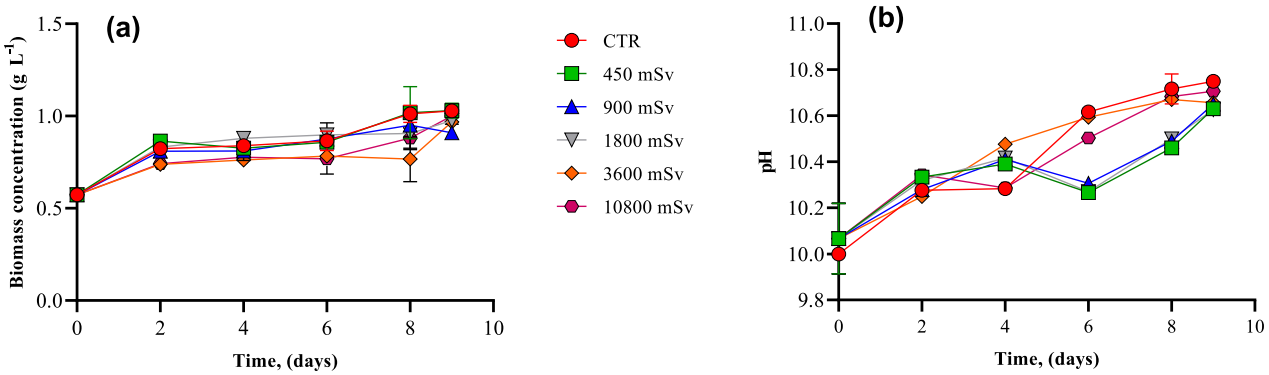

Fig. 1. Growth dynamics (a) and pH (b) time evolution of *Chlorella vulgaris* after initial exposure to 450, 900, 1800, 3600 or 10,800 mSv of X-ray.

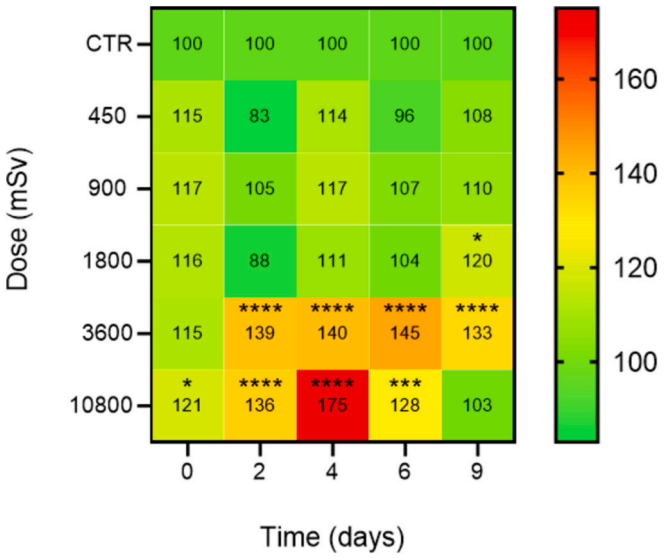

Fig. 2. Heatmap of intracellular ROS contents of *Chlorella vulgaris* exposed to X-ray doses of 450, 900, 1800, 3600 and 10,800 mSv. The values are expressed as % of control (mean ± SD). The mean differences have been tested by using ANOVA and Dunnett's correction for multiple comparisons ($n = 12$).

mSv.

2.3. Equivalent time of exposure on Mars

To compute the number of days on the Martian surface in which *C. vulgaris* would receive radiation doses corresponding to those administered in this study, the equivalent dose measured by the Mars Science Laboratory – Radiation Assessment Detector (MSL-RAD) on Mars was considered: 600 μSv/d [22].The exposure time on Mars can be obtained by dividing that dose by the daily equivalent dose determined by MSL-RAD. These times are 750, 1500, 3000, 6000, 18,000 days.

2.4. Biomass characterization

Nine days after irradiation, cultures were transferred to Falcon tubes, centrifuged, and washed three times with ddH_2O. Tubes were centrifuged and supernatants removed, and the pellets frozen at −80 °C. They were then freeze-dried for two days with a LIO-5PDGT freeze-dryer (5 Pa). Dried samples were then pulverized with mortar and pestle.2.4.1 Effect of low doses of X-ray irradiation on ROS production.

ROS formation was measured on the day of irradiation, and again after 2, 4, 6 and 9 days, by using the 2′,7′-dichlorodihydrofluoresceindiacetate (DCFH-DA) assay (DCFH-DA). DCFH-DA is hydrolyzed by cellular esterases to form the non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) after penetrating the cell of the test organisms, and DCFH is immediately transformed to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS [33].

The stock solution of DCFH-DA was prepared in DMSO at a concentration of 25 mM and stored at −20 °C. 100 μL of microalgae suspension of determined OD were transferred into a 96- well microplate and incubated with 25 μM of DCFH-DA solution for 30 min at 37 °C, in the dark. The fluorescence of DCF was measured with a microplate reader (Perkin Elmer Victor X5 2030 Multilabel HTS Fluorescence) at room temperature, with excitation and emission filters at 485 and 530 nm, respectively. Fluorescence was related to OD and results were expressed as % of the positive controls [34].

2.4.1. Fatty acids methyl ester (FAME) analysis

Sample preparation followed the protocol described by [35], but with modifications. 10 mg of lyophilized biomass were weighted within a glass tube and suspended in 4 mL of a methanol/chloroform (4:5 *v*/v) solution containing the internal standard tritridecanoin at a concentration of 50 mg/L. Subsequently, the solution was vortexed eight times. 8 mL of chloroform, and 2.5 mL of double-distilled H_2O containing 50 mM of 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and 1 M NaCl, were added. Samples underwent ultrasonication thrice for 3 min using ExtractorOne (GM Solution, Cagliari, Italy) and were subsequently centrifuged for 10 min at 177 rcf at 5 °C. The chloroform phase was then dried under a gentle nitrogen stream.

Fatty acid methyl esters (FAMEs) were obtained by trans-esterifying the dried lipids with 3 mL of methanol containing 5 % (*v*/v) sulfuric acid, followed by incubation for 3 h at 70 °C. After this, 3 mL of MilliQ water and 3 mL of n-hexane were added, and the samples were agitated for 15 min before being centrifuged for 10 min at 177 rcf at 5 °C. The hexane phase containing FAMEs was transferred to glass vials for GC–MS analysis using a Thermo Fisher Trace 1300 gas chromatograph coupled with a triple quadrupole mass spectrometer (TSQ 9000).

Each sample was injected in split mode (split ratio 1:20) and separated on a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness: 0.25 μm, Agilent Technologies Inc., Santa Clara, CA). The front inlet temperature was set at 250 °C, and helium gas was used as the GC carrier gas. The oven temperature was initially held at 50 °C for 1 min before being ramped from 50 to 175 °C at 10 °C/min, held at 175 °C for 10 min, ramped from 175 to 210 °C at 5.0 °C/min, held at 210 °C for 10 min, ramped from 210 °C to 230 °C at 5.0 °C/min, held at 230 °C for 9.5 min, and finally ramped from 230 °C to 300 °C at 10 °C/min.

The mass spectrometry transfer line and ion source temperatures were set at 250 and 300 °C, respectively. Ions were generated at 70 eV with electron ionization and recorded at 1.6 scans/s over the mass range *m/z* 50 to 550. Peak identification was performed by comparing peak retention times with these of the Supelco 37 Component FAME Mix (Sigma Aldrich). Data are presented as a percentage of dry weight (%

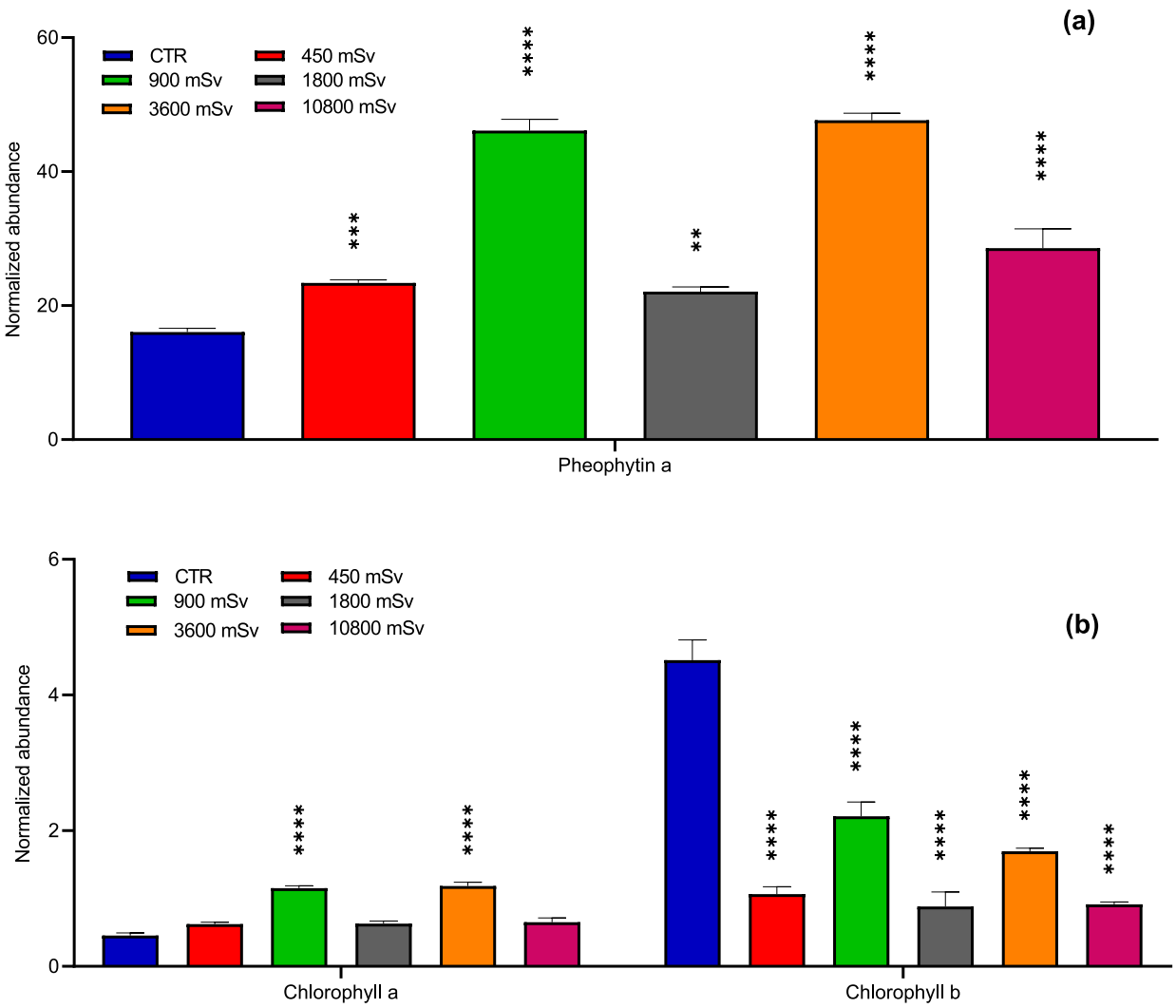

Fig. 3. Column plot of pigment contents in *Chlorella vulgaris* exposed to X-ray doses of 450, 900, 1800, 3600 and 10,800 mSv. (a) Pheophytin-a and (b) Chlorophyll-a and b. The mean differences have been tested by using one-way and ANOVA and Dunnett's correction for multiple comparisons ($n = 4$).

dwt, mean ± standard deviation).

2.4.2. Complex lipid and pigment analysis

Intact complex lipids and pigments were extracted from the samples obtained nine days after irradiation, using a modified Folch method [36]. In brief, 10 mg of dried biomass were weighted into a glass tube, and 10 mL of a mixture of methanol/chloroform (2:1 *v*/v, 10 mL), along with 1 mL of 0.2 M KCl, were added. The samples were ultrasonicated for 3 min and repeated thrice. After that, they were vortexing every 15 min over a 1-h period. Subsequently, the samples were centrifuged for 10 min at 12,298 rcf, and the chloroform phase was dried under a gentle nitrogen stream.

The dried phase was dissolved in a mixture of methanol/chloroform (1:1 *v*/v, 20 μL) and diluted with a mixture of 2-propanol/acetonitrile/water (2:1:1 v/v/v, 380 μL) containing the internal standard Cer(d18:1/25:0). Complex lipids were analyzed using a UHPLC-QTOF/MS coupled with an Agilent 1290 Infinity II LC system, with injections of 5 μL and 8 μL in the positive and negative ionization modes, respectively. Chromatographic separation was achieved using a Kinetex 5 μm EVO C18 100 A, 150 mm × 2.1 μm column (Agilent Technologies, Palo Alto, CA), maintained at 50 °C and with a flow rate of 0.2 mL/min.

For the positive ionization mode, the mobile phase comprised (A) a 10 mM ammonium formate solution in 60 % milli-Q water and 40 % acetonitrile, and (B) a 10 mM ammonium formate solution in isopropanol and acetonitrile mixture (9:1 *v*/v). The gradient in positive ionization mode consisted of an initial 60 % A, followed by a linear decrease to 50 % A over 2 min, then to 1 % over 5 min, maintaining this percentage for 1.9 min before returning to the initial conditions in 1 min. The mobile phase in negative ionization mode differed only in the use of 10 mM ammonium acetate instead of ammonium formate.

The MS source was operated with the following parameters: gas temperature, 200 °C; gas flow (nitrogen), 10 L/min; nebulizer gas (nitrogen), 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage, 3500 V for positive and 3000 V for negative; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V; octapole RF, 7550 V; mass range, 50–1700 *m/z*; collision energy, 20 eV in positive and 25 eV in negative mode; mass precursor per cycle = 3; threshold for MS/MS, 5000 counts. Chromatographic areas were obtained by acquiring samples in ESI full scan mode and were normalized using Cer (d18:1/25:0) as an internal standard. Consequently, results are expressed as a ratio to an internal standard and are referred to as normalized abundance.

2.4.3. Identification of complex lipid classes and pigments

To identify lipids class, iterative MS/MS experiments of quality controls (QCs) and SPLASH LIPIDOMIX standard mixture (Sigma Aldrich) were carried out, at two different collision energies (CEs, 20 and 40 eV) to improve mass fragmentation. This method consists in

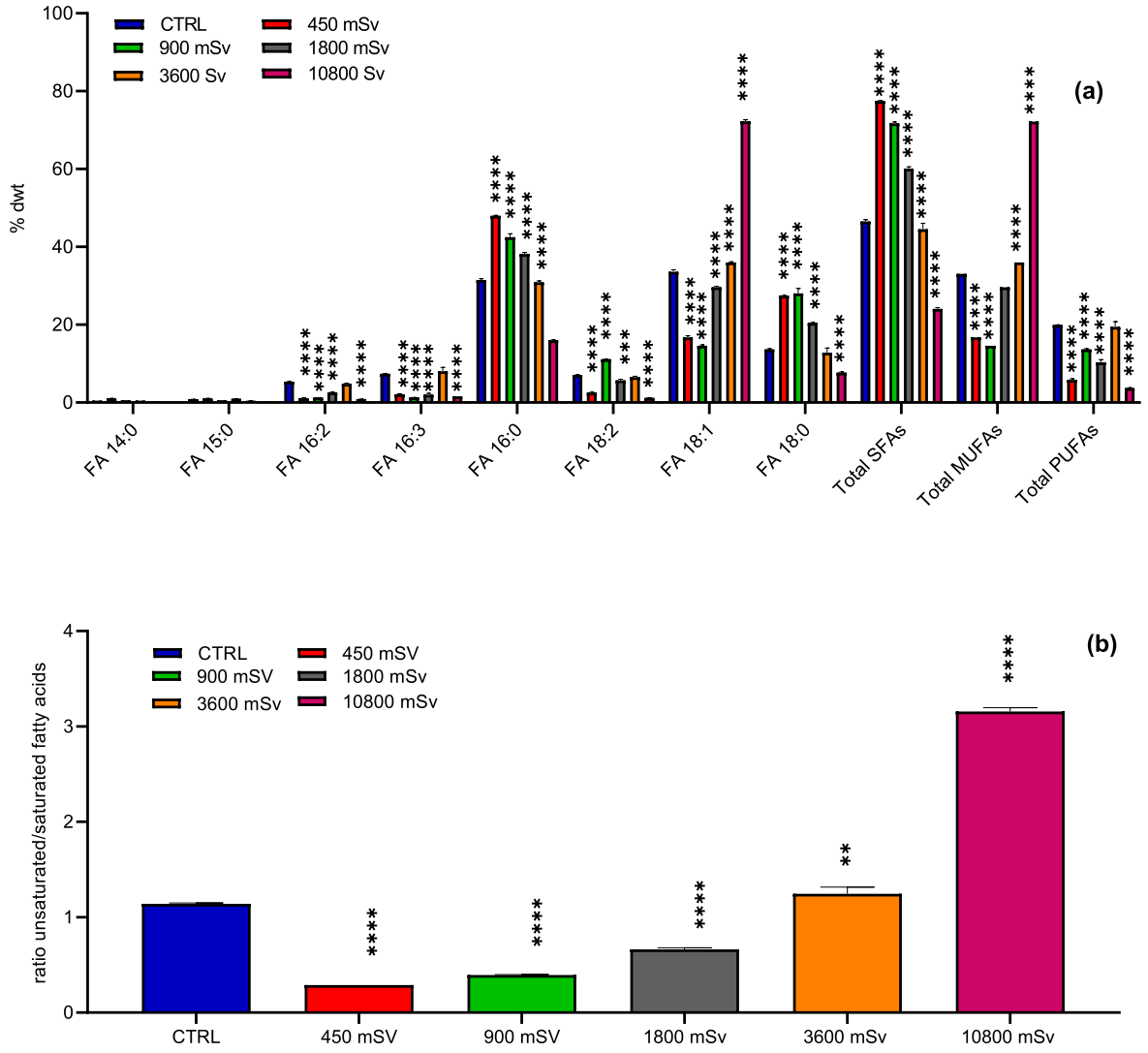

Fig. 4. Column plot of (a) fatty acids (as a FAMEs) content of *Chlorella vulgaris* and (b) unsaturated/saturated fatty acid ratio (U/S, % dwt). Mean differences have been tested by using ANOVA and Dunnett's correction for multiple comparisons ($n = 3$).

injecting the same sample multiple times, while precursors previously selected for MS/MS fragmentation are excluded on a rolling basis. Four different iterative analyses were performed for maximizing the maximum number of lipid species with a mass error tolerance of 20 ppm and a retention exclusion tolerance of 0.2 min. Lipids were identified in the MS/MS spectra using the putative mass annotation provided by Lipid annotator (version 1.0, Agilent MassHunter workstation), comparing with an online mass database [37] and analyzing the diagnostic fragment for each lipid class [38–41]. MGDG and DGDG were identified as $[M + NH_4]^+$ by using the product ion resulting from the combined neutral loss of NH_3 and galactosyl unit (−197 Da) and for DGDG by using the neutral loss of NH_3 and digalactosyl unit (−359 Da). Others product ions for MGDG and DGDG resulted from the loss of fatty acyl groups as acylium ions plus 74 Da $[RCO + 74]^+$. Phosphatidylcholine (PC) was detected in positive ionization mode and annotated as $[M + H]^+$, while the fatty acyl composition was determined in negative using the $[M + CHO_2]^-$ adduct. Diacylglycerol (DAG) and triacylglycerol (TAG) were detected in positive ionization mode and annotated as $[M + NH_4]^+$. Fatty acyl chains were determined using the three typical DG^+ (or the two MG^+ in the case of DG) ions corresponding the loss of FA, after which the FA composition of TAG molecule was deduced using the mass difference. Sulfoquinovosyldiacylglicerol (SQDG) was detected in negative ionization mode and identified as $[M-H]^-$. The diagnostic product ion at *m/z* 225.0 due to the loss of the polar head was used to confirm SQDG species. The fatty acyl composition was determined using the product ions resulting from the neutral loss of fatty acyl group as free carboxylic acid (RCOOH). Phosphatidylinositol (PI) was detected as $[M-H]^-$ and confirmed using the product ions at *m/z* 241 (derived from the cyclic anion of inositol phosphate) and at *m/z* 223 (loss of water from *m/z* 241). Phosphoglicerol (PG) was determined as $[M-H]^-$ and using the product ions at *m/z* 153 (corresponding to the cyclic phosphate anion) and at *m/z* 391 (resulting from the loss of fatty acyl group at *sn-2* with the polar headgroup glycerol). Chlorophyll *a* and *b*, and pheophytin *a*, were quantified according to Hyvärinen et al. [42] and Milenkovic et al. [43]. Due to their molecular structure, chlorophylls and their derivatives can be worked on with either ESI^+ or ESI^-. Here molecular peaks as $[M + H]^+$ were observed, but the most abundant ion was derived from the loss of phytil chains (as phytadiene). This may appear at *m/z* values corresponding to $[M + H\text{-}C_{20}H_{38}]^+$ and $[M + H\text{-}CH_3COOC_{20}H_{39}]^+$.

2.5. Statistical analysis

Univariate analyses were performed with the GraphPad Prism software (version 8.3.0, Dotmatics, Boston, Massachusetts). Mean differences between groups were tested for statistical significance using ANOVA with Dunnett's correction for multiple comparisons. The

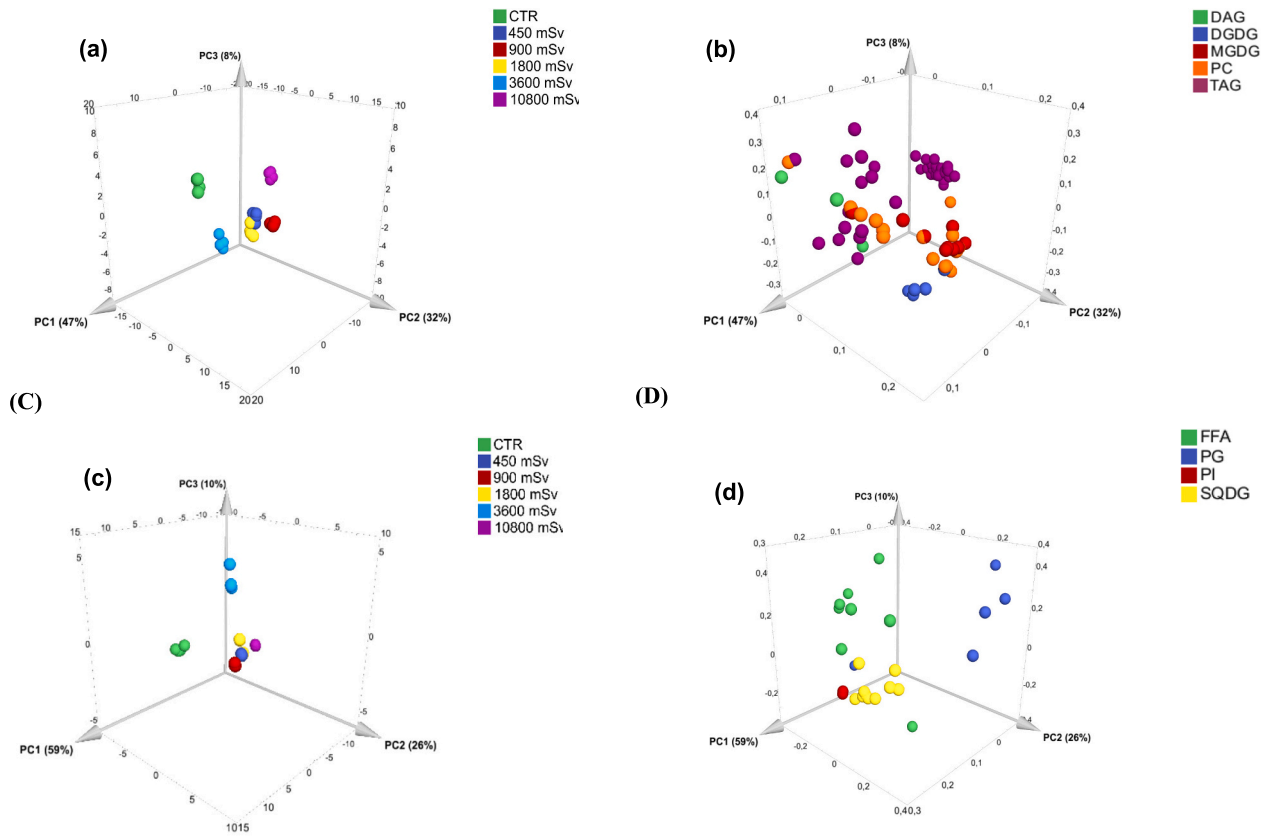

Fig. 5. *Chlorella vulgaris* PCA. 3D-Score plots from positive and negative LC-QTOF/MS data of lipids (a) and (c) and their respective 3D-loading plots (b) and (d). R^2X and Q^2 were 0.86, 0.81, and 0.95, 0.90 respectively for positive and negative data ($pc = 3$, $n = 4$). DAG, diacylglycerols, DGDG, digalactosyldiacylglicerol, MGDG, monogalactosyldiacylglycerol, PC, phosphatidylcholines, TAG, triacylglycerol, FFA, Free fatty acid, PG, phosphoglycerol, PI, phosphatidylinositol, SQDG, sulfoquinovosyldiacylglycerol.

significance levels based on the *p*-values are indicated by asterisks (*). No asterisk corresponds to a *p*-value >0.05, * to 0.005 < *p*-value <0.05, ** to 0.0005 < *p*-value <0.005, *** to 0.0005 < *p*-value <0.0001 and **** to $p < 0.0001$.

Unsupervised Principal Component Analyses (PCA) were performed for dataset overview. Results are shown in two dimensions as score (related to observations) and loading (related to variables) scatter plots. This multivariate analysis was performed with the SIMCA-P+ software (Version 14.1, Umetrics, Sartorius, Germany). The quality of the models and the optimum number of principal components were evaluated based on the cumulative parameters R^2X (goodness of fit) and their analogues in cross validation Q^2 (goodness of prediction).

3. Results and discussion

3.1. Effect of low dose irradiation on growth kinetics

C. vulgaris cells were subjected to low-energy X-ray irradiation generated from the bremsstrahlung of electrons in an electron beam accelerator. The irradiated cells received 450, 900, 1800, 3600, or 10,800 mSv. While Mars's radiation flux is complex, and dose is not the only parameter relevant to biology, no facility on Earth can reproduce it accurately. X-rays are, therefore, a valuable proxy.

Growth dynamics were monitored over a period of 9 days. As illustrated in Fig. 1, exposure did not exert discernible effects on microorganism growth or pH levels.

The observed stability in the microalgae growth and pH levels throughout the growth period shows the resilience of *C. vulgaris* to the applied radiation. Abo-State et al. [44] reported analogous findings while, Pradhan et al. [45] detected no differences in the growth of *Chlorella* sp. (BUACC06) exposed to 10 Gy of γ radiation. It has also been shown that low-dose of radiation may even stimulate plant and photosynthetic microorganism [46–48], for example accelerating cell division [49], or stimulating auxin-responsive genes [50]. It is nonetheless plausible that cells experienced slight damage, but they swiftly repaired it [51]. Given that ionizing radiation possesses the ability to deeply penetrate materials, unlike ultraviolet radiation which can be effectively shielded by thin layers, the observed resilience of *C. vulgaris* to the applied radiation doses is of paramount importance. This resilience is critical for the potential application of this microalga in sustainable food production systems for Martian environments, where effective radiation shielding may be challenging to achieve.

3.2. Effect of low doses of X-ray irradiation on intracellular ROS production

Reactive oxygen species (ROS) can be produced by biological and chemical redox reactions. A wide range of factors can cause them to accumulate [25,52]. Although they may play a role in cell signalling in microalgae, their accumulation can cause oxidative stress, which is detrimental to lipids, proteins and nucleic acids [25,53]. To assess the oxidative stress and potential lipid damage induced by our irradiation protocol, intracellular ROS levels were quantified bi-daily and at the conclusion of the growth period. The results are shown in Fig. 2.

The lowest doses (450 and 900 mSv) caused no significant increase in ROS, suggesting that the microalga's antioxidant mechanisms [25,54,55] are sufficient to neutralize any ROS that may have been produced. These mechanisms involve the production of different

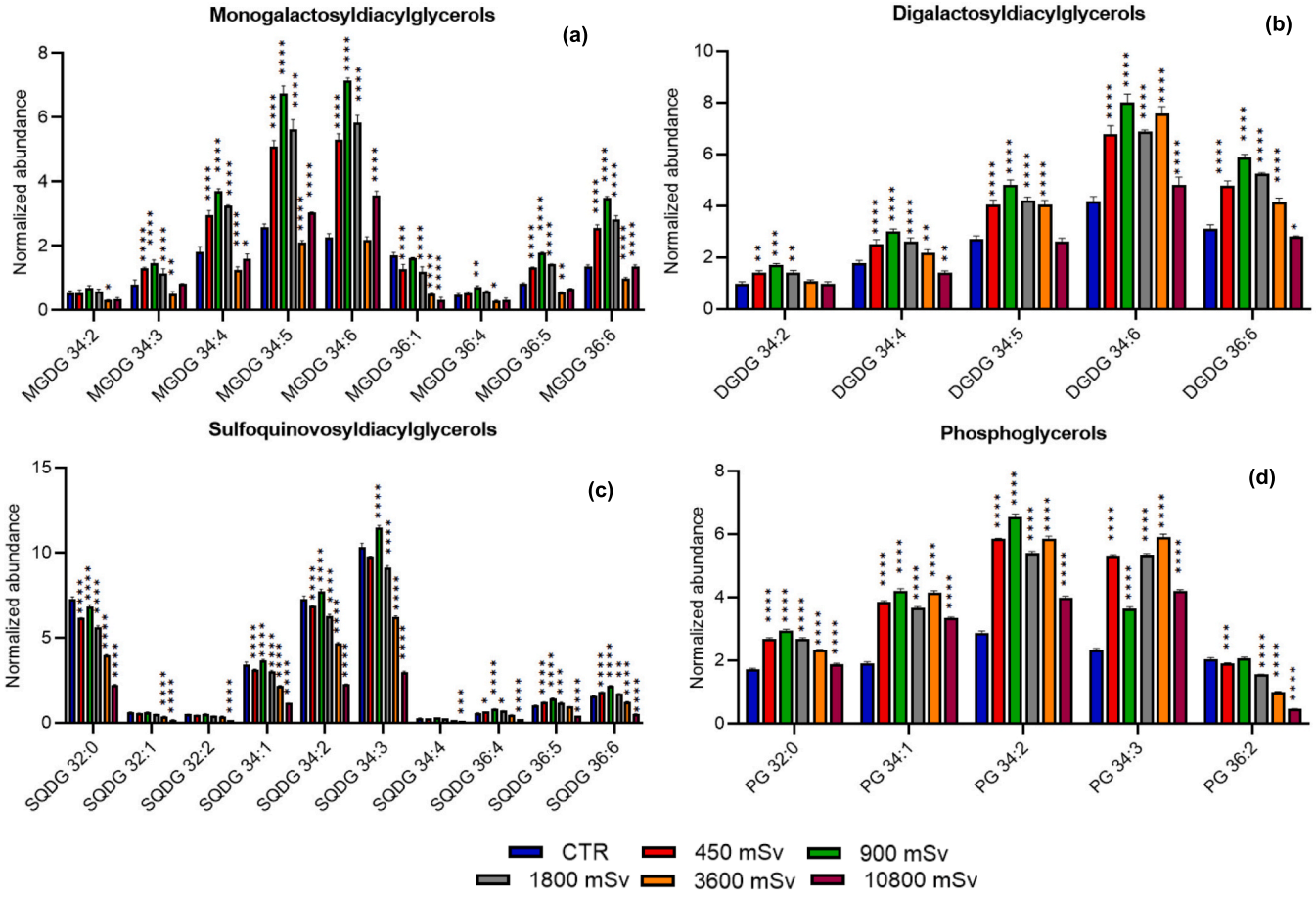

Fig. 6. Column plot of thylakoid membrane lipids after irradiation at 450, 900, 1800, 3600 and 10,800 mSv. (a) MGDG, (b) DGDG, (c) SQDG and (d) PGs. Mean differences were tested by using ANOVA and Dunnett's correction for multiple comparisons ($n = 4$).

metabolites such as carotenoids, fatty acids, sterols and enzymes [25,56]. Conversely, higher doses (1800, 3600 and 10,800 mSv) led to the intracellular accumulation of ROS. After the highest of these doses (10,800 mSv), ROS levels came back to normal between days 6 and 9.

This may be due to a stronger activation of antioxidant defence mechanisms. It should be noted that the radiation was here applied over a short time span, while on Mars they would be received over multiple months or years, during which cells would be active and able to inactivate ROS. ROS accumulation is therefore unlikely to be caused by Mars's ionizing radiation levels in themselves.

3.3. Chlorophyll and its derivatives

Chlorophyll *a* (Chl *a*) is a pigment that plays a fundamental role in light absorption and conversion processes within photosynthetic organisms [57]. Pheophytin *a* (Pheo *a*), structurally akin to Chl *a* but lacking the central magnesium ion in its porphyrin ring, acts as the primary electron acceptor of PSII and operates between P680 and Q [43,58,59]. Chlorophyll *b* (Chl *b*) is an accessory pigment which in the photosynthetic process [57]. To further investigate the molecular effects of X-rays radiation, the levels of Chl *a*, Chl *b*, and Pheo *a* were determined using an LC-QTOF/MS system (Table S2). As depicted in Fig. 3, alterations in the pigment content of *C. vulgaris* were observed following irradiation. The abundance of Pheo *a* was significantly increased by all tested radiation doses, though increasing the dose did not necessarily increase abundance. A similar pattern was observed for Chlorophyll a, although the differences with the control were significant only for the doses of 900 and 3600 mSv. Conversely, a decrease in Chl *b* contents was observed after all tested doses. Marcu et al. [60] reported a decrease in the Chl *a* content of *Lactuca sativa* after irradiation with γ radiation. Toghyani et al. [61] irradiated *C. vulgaris* UTEX 265 at 300, 600 and 1200 Gy of γ radiation and reported a decrease in Chl *a* and *b* contents,. Pradhan et al. [45] reported a decrease in Chl *a* and *b* contents of *Chlorella* sp. (BUACC06) at doses lower than 75 Gy, and an increase at 75 Gy. Singh et al. [62] have examined the responses of *C. sorokiniana* to different doses of UV (0.25, 0.5, 1 and 2 J/cm^2) and γ radiation (0.5, 1, 2 and 4 kGy) and observed a dose-dependent decrease in growth and chlorophyll contents. The discrepancies between these different studies point out a need to further investigate the complex relationships between radiation and pigment contents.

3.4. Fatty acid methyl esters (FAMEs)

The fatty acid composition of *C. vulgaris* is depicted in Fig. 4. Total saturated fatty acids (SFAs), palmitic acid (FA 16:0) and stearic acid (FA 18:0) increased following the lower doses of X-ray irradiation (450, 900, and 1800 mSv) but decreased after higher doses (3600 and 10,800 mSv). Conversely, total monounsaturated fatty acids (MUFAs) and oleic acid (FA 18:1) initially decreased with radiation dose but then increased; MUFAs reached twice the contents of the controls after 10,800 mSv. Total polyunsaturated fatty acids (PUFAs), hexadecadienoic acid (FA 16:2), hexadecatrienoic acid (FA 16:3) and linoleic acid (FA 18:2) exhibited a dose-independent response, with in most cases a reduction after irradiation. Overall, the unsaturated-to-saturated fatty acid ratio (U/S ratio) decreased after the lowest irradiation dose, but from there increased with dose. Kumar et al. [63] reported analogous findings in *C. sorokiniana* subjected to UV irradiation: monounsaturated fatty acids increased while polyunsaturated fatty acids decreased.

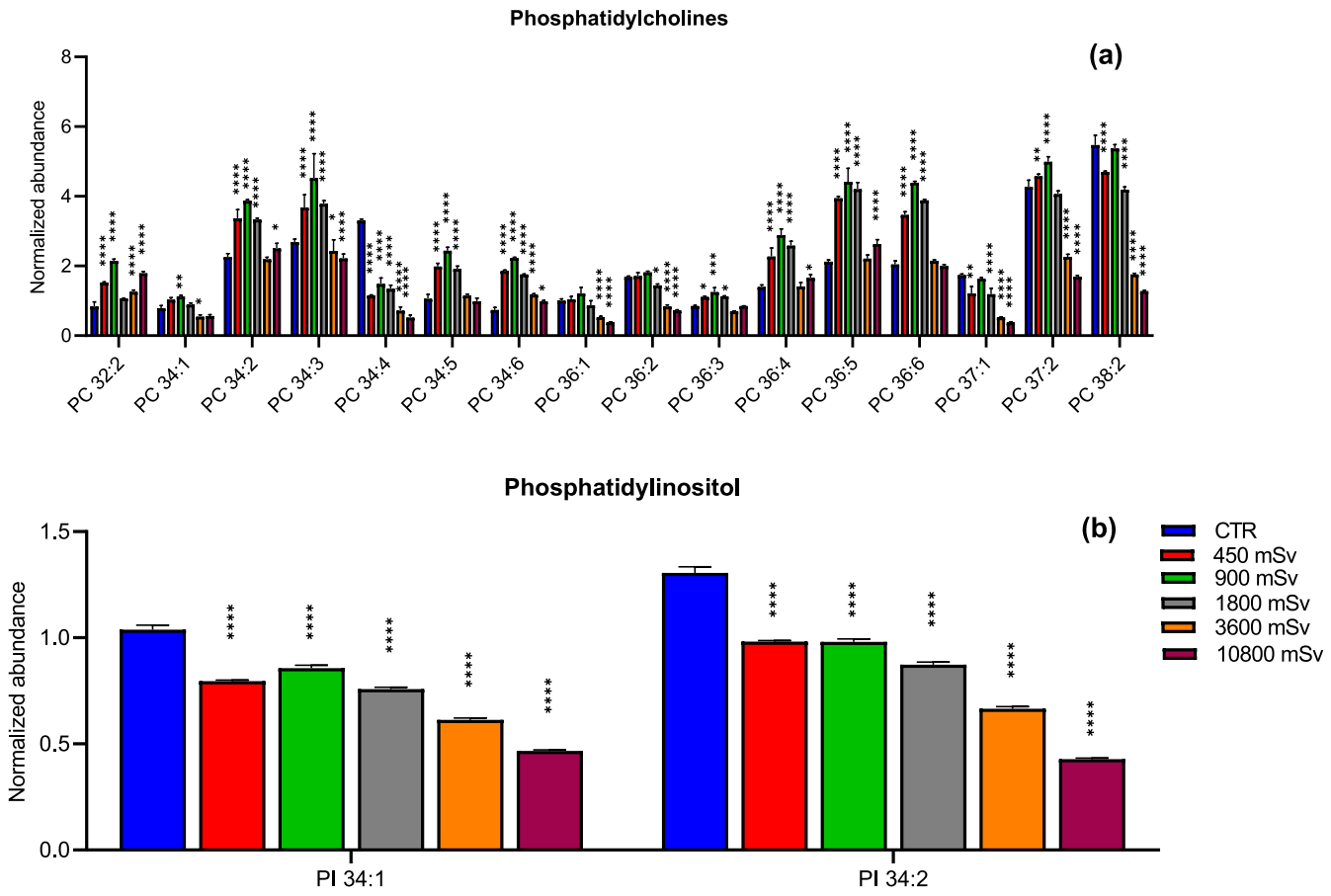

Fig. 7. Column plot of PC and PI after irradiation at 450, 900, 1800, 3600 and 10,800 mSv. (a) PCs and (b) PIs. Mean differences were tested by using ANOVA and Dunnett's correction for multiple comparisons (n = 4).

X-rays possess enough energy to ionize water molecules, thereby generating free radicals. These radicals can interact with the double bonds of polyunsaturated fatty acids and include membrane damage through lipid peroxidation [64,65]. This is consistent with the decrease in PUFAs we observed, as well as the increase with dose of the U/S ratio. Another cause may be linked to variations in ATP contents induced by X-ray irradiation. PUFA synthesis necessitates substantial ATP levels; however, under radiation-induced stress, ATP may be mobilized for cellular protection against oxidative stress. A depletion in ATP may therefore have contributed to a decrease in PUFA synthesis [66,67].

3.5. Lipidomic profile

To investigate the potential impact of X-ray irradiation on the lipid profile of *C. vulgaris*, we employed an LC-QTOF/MS untargeted approach. A total of 104 complex lipids were annotated and distributed across 9 classes (Fig. S2, S3 and Table S3): SQDG (10), MGDG (9), DGDG (5), PG (5), PI (2), FFA (9), PC (16), DAG (3) and TAG (45). This composition aligns with previous reports [68]. The detailed high-resolution mass spectrometry characteristics are provided as supplementary materials.

A 3D-PCA analysis was conducted using SIMCA-P^{+}. The results, depicted in Fig. 5, revealed significant differences among the irradiated samples. While the controls (CTR), 3600, and 10,800 mSv samples each formed their own cluster, the 450, 900, and 1800 mSv samples clustered together (Fig. 5a). A similar pattern (although with a less marked segregation of the 10,800 mSv sample) was observed with data obtained in negative ionization mode (Fig. 5c). These groupings underline distinct alterations to the lipid profile of *C. vulgaris*. This hints at a nuanced response of the microalgae to varying radiation levels, potentially linked to cellular stress responses or physiological adaptations. Triacylglycerols (TAGs) were distributed among the 10,800 mSv, 3600 mSv, and CTR groups, while DGDGs clustered near the 3600 mSv group and MGDG were predominantly located among the 450, 900 Sv, and 1800 mSv samples (Fig. 5b). PC, on the other hand, were distributed among CTR, 450, 900 mSv, and 1800 mSv samples (Fig. 5b). Free fatty acids were predominantly in CTR, whereas PG were more prominently in 10,800 mSv samples. SQDG and PI were distributed between CTR and irradiated samples (Fig. 5d).

As it is influenced by alterations in factors including radiation levels, temperature and soil composition, the lipid profile of *C. vulgaris* could be used to help assess environmental stress on Mars. Furthermore, insights into how radiation affects the lipid metabolism of *C. vulgaris* could inform bioproduction strategies for generating food, oxygen, and other essential resources in space.

3.5.1. Thylakoid membrane lipids and phospholipids

The thylakoid membrane lipids MGDG, DGDG, SQDG and PG were largely affected by X-ray irradiation (Fig. 6). For MGDG with insaturations ranging from 2 to 4, irradiation at doses between 450 and 1800 mSv stimulated synthesis, while doses of 3600 and 10,800 mSv had an inhibitory effect. The same effect was observed for DGDG 34:2, 34:4 and 34:5. This may be caused by a hormetic effect at low doses. Although it was demonstrated that low doses of radiation may cause stimulation in plant and photosynthetic microorganism [46–48], there is little knowledge regarding the effects of low dose of ionizing radiation on lipids production [48]. Jeong et al. [48] reported that low-dose radiation significantly increases the lipid contents of marine microalgae. Döhler and Biermann [69] observed that UV-B exposure affected the biosynthesis of thylakoid membrane lipids (MGDG, DGDG, SQDG, and PG) in

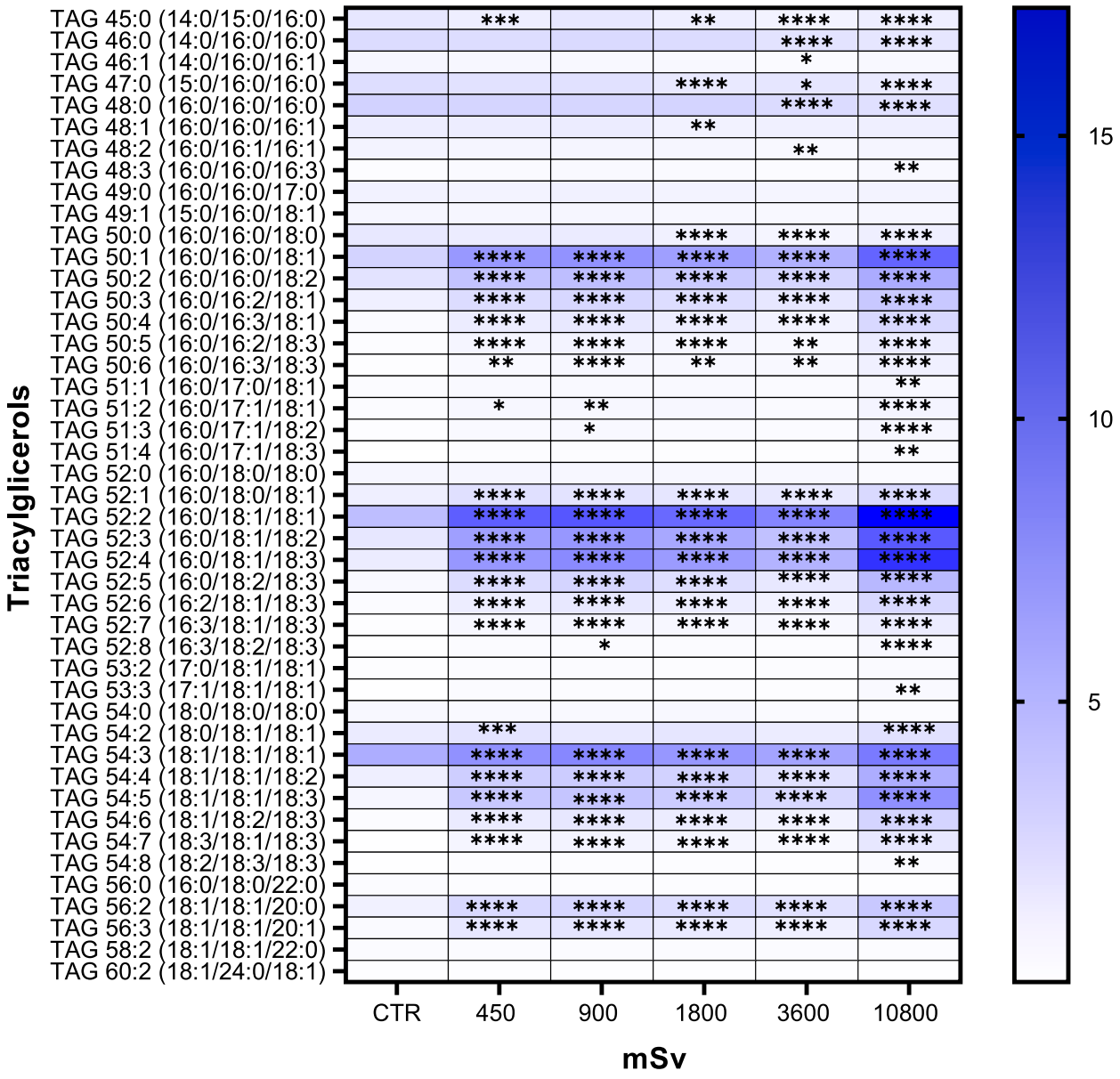

Fig. 8. Heat map of TAGs produced by *Chlorella vulgaris* after irradiation with different X-rays doses (450, 900, 1800, 3600 and 10,800 mSv). Mean differences (obtain by using normalized abundance) were tested by using ANOVA and Dunnett's correction for multiple comparisons ($n = 4$).

Ditylum brightwellii, likely due to reduced substrate and ATP availability, which inhibited enzyme activity or de novo lipid synthesis. Similarly, the observed decrease in certain polar lipids after 10,800 mSv could be attributed to a similar mechanism.

SQDGs 32:0, 32:1 and 32:2 decreased with increasing doses of X-rays. Other SQDGs underwent a reduction at all doses except 900 mSv, while SQDG 36:4, 36:5, and 36:6 experienced greater inhibition at higher doses. Previous studies demonstrated that SQDGs may act as scavengers of free radicals in plants and be peroxidized preferentially to other lipids that compose cell membranes. This may be explained by the high affinity that SQDG hydroperoxide has for ascorbic acid peroxidases, which maintain low levels of hydroperoxides in plant cells [70]. PG biosynthesis was stimulated overall; only PG 36:2 contents were reduced. Changes in SQDG and PG levels are known to be a potential adaptive response to changes in irradiance levels [71]. In addition, it is known that a loss of SQDG can be compensate by an increase in PG [27].

PCs exhibited an increase following irradiation with 450, 900 and 1800 mSv (Fig. 7), possibly as part of a stress response. However, at the higher doses of 3600 and 10,800 mSv, PC levels decreased and PI levels decreased with increasing radiation dose, suggesting that even low doses adversely affect the biosynthesis or stability of PI. Given the critical role of PI in lipid signalling and signal transduction, such a reduction may significantly affect cellular functions.

3.5.2. Neutral lipids

The heatmap in Fig. 8 indicates that TAGs with 45–49 carbon atoms were not notably affected by radiation, while larger TAGs, particularly those containing oleic acid, showed an increase. This may be related to ROS accumulation, which can influence lipid production in *C. vulgaris* and promote a shift from carbohydrate to lipid storage in *C. protothecoides* [72,73]. In *Lobosphaera incisa*, the accumulation of oleic acid can reduce ROS levels and improve lipid productivity [74]. In this context, radiation might have stimulated the de novo formation of fatty acids like oleic acid, which were then used in the production of these larger TAGs.

4. Conclusions

Microalgae, such as *Chlorella vulgaris*, could be valuable for producing metabolites, essential fatty acids, and complex lipids during long-term space missions. However, ionizing radiation on Mars could affect its growth and composition. This study evaluated the effect of low doses of X-rays on *C. vulgaris* and showed that even an exposure equivalent to 8504 days on Mars did not significantly compromise its growth. Doses of 450 and 900 mSv did not significantly increase the ROS content in the cells. Conversely, doses of 1800, 3600, and 10,800 mSv led to an increase, but the values returned to normal by the end of the growth period. Irradiation caused an increase in chlorophyll *a* and pheophytin a, but a decrease in chlorophyll *b*. As these changes in pigment levels was not associated with any increase in growth, however, they did not seem to strongly affect photosynthetic functions, which will be crucial in extraterrestrial environments if biomass and oxygen are to be produced. Lipid profiles also changed: saturated and polyunsaturated fatty acids increased at low doses and decreased at higher doses. Monounsaturated fatty acid contents followed an opposite trend. Overall, these results indicate an influence of radiation on lipid profiles, which has important implications for biomass and lipid production outside the protection of Earth's magnetosphere. Some of the radiation effects could be beneficial: an example is the increase in triacylglycerol levels containing oleic acid observed here after irradiation. Other effects, however, could be deleterious. Although X-ray radiation is a valuable proxy for the radiation flux that reaches the Martian surface, the latter is a complex mixture of electromagnetic radiation and particules that cover a range of masses, energies, and charges, and thus at a given dose damage organisms in different ways. Besides, the doses used here would be received over a much longer timeframe on Mars, allowing cells to repair damage along the way. Further investigations, with other doses, dose rates and radiation types, are therefore warranted to better understand the effects of irradiation on microalgal physiology and cell composition.

CRediT authorship contribution statement

Mattia Casula: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giacomo Fais:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Debora Dessì:** Formal analysis, Data curation. **Cristina Manis:** Formal analysis. **Alessandra Bernardini:** Writing – review & editing. **Cyprien Verseux:** Writing – review & editing, Writing – original draft, Validation. **Viviana Fanti:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Pierluigi Caboni:** Writing – review & editing, Validation, Supervision, Methodology. **Giacomo Cao:** Project administration, Investigation, Funding acquisition. **Alessandro Concas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103783.

Data availability

Data will be made available on request.

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