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Influence of a low atmospheric pressure on the growth and lipid composition of *Chlorella vulgaris*

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ABSTRACT

Within crewed missions to Mars, microalgae could be used for the production of essential consumables. Their cultivation there could be less resource-intensive if the pressure inside bioreactors was reduced. However, whether this would decrease their growth is unclear; and how the composition of their lipidome would change (for instance, to compensate for the tendency of low pressure to increase membrane fluidity) is unknown. Here we demonstrate that the growth of *Chlorella vulgaris* is unaffected by a reduction in total pressure from 1000 hPa (ca. Earth at sea level) to 100 hPa if the partial pressure of carbon dioxide is constant. We then show that the microalga can grow vigorously, reaching above 1.5 g L^{-1} within 4 days, under cultivations conditions analogous to these foreseen on Mars: a leachate of a Martian regolith simulant mixed with synthetic urine, under an atmosphere of 100 hPa of carbon dioxide. Conversely, a decrease in pressure altered the lipidome. Some changes may have helped preserve membrane viscosity: as an example, the MGDG-to-DGDG ratio increased. However, other alterations do not clearly point in that direction: the overall ratio of unsaturated-to-saturated fatty acids, as well as the average degree of unsaturation in thylakoid membrane lipids, for instance, only decreased with pressure when carbon dioxide (rather than carbonate) was the carbon source. Regardless, these alterations are small when compared to those induced by a change in the inorganic carbon source. Our results overall suggest that relying on a low pressure would benefit the resource-efficiency of microalgal cultivation on Mars.

1. Introduction

In the 2018 Global Exploration Roadmap of ISECG, a consortium of the world's spacefaring nations, landing humans on Mars is listed as a shared driving goal [1]. This goal is also stated in documents which lay out the long-term vision of individual space agencies, such as ESA [2] and NASA [3]. It can be argued, however, that reaching the surface should not be the end objective of missions to Mars, but that a sustainable scientific exploration program should be established. This requires the development of abilities to produce and recycle on site the essential consumables such as food and oxygen. Biological systems could support this ability.

One approach could lie in using plants and microorganisms to recycle, after use, consumables imported from the Earth. An example of a project based on it is ESA's MELiSSA [4]. Another approach lies in producing consumables from the resources naturally found on site. This strategy is referred to as in situ resource utilization (ISRU) and an example of a biological concept for it lies in feeding diazotrophic, rock-leaching cyanobacteria with carbon dioxide, dinitrogen, mineral nutrients and water found in the Martian ground and atmosphere [5,6]. In addition to the oxygen they produce, their biomass could be used as a basis for downstream biotechnologies [7]. Which, between recycling and ISRU, is most relevant, largely (though not exclusively) depends on mission duration: recycling systems typically require a lower initial investment in terms of imported mass, but this mass tends to increase faster than ISRU with mission duration. In practice, the most resource-

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efficient systems may combine ISRU and recycling. Such a combination could be used for the cultivation of the microalga *Chlorella vulgaris*.

C. vulgaris is being considered for bioproduction in long-duration, crewed spaceflight due to its rapid growth, high photosynthetic efficiency, and richness in proteins, carbohydrates, and lipids [8]. It is edible [9]: products based on its biomass were notably granted Generally Recognized As Safe (GRAS) status by the USA's Food and Drug Administration, and were recognized as a traditional food by the European Union following consultation in the frame of the Novel Food Regulation (Regulation 2015/2283). It is rich in fatty acids of high nutritional value, notably those known to be essential for humans (i.e., which are required for normal physiological function but cannot be synthesized in the body): linolenic acid (ω-3) and linoleic acid (ω-6) [10].

It has been proposed that *C. vulgaris* could be cultivated with carbon dioxide sourced from the Martian atmosphere, various nutrients extracted from the Martian regolith, and urine as a source of nitrogen (as well as an additional source of elements also provided from the regolith, such as phosphorus) [11]. Here we focus on the first item on this list: the provision of carbon dioxide. *Chlorella* cultures could not be directly exposed to the Martian atmosphere: while the latter contains ca. 95% CO_2 [12], its total pressure is very low (6.1 hPa on average at altitude zero, against an average of 1013 hPa on Earth at sea level), which would be incompatible with the stability of liquid water at temperatures that support the growth of *C. vulgaris*. Martian air should thus be pressurized before being fed to a cultivation system, but this pressurization is energy-intensive and must therefore be minimized [13].

How low the atmospheric pressure under which *C. vulgaris* is cultivated could be lowered, without negatively affecting its growth or nutritional value, is unclear. If the total pressure is decreased under a constant atmospheric composition, changes in growth and biomass composition may result from changes in the partial pressure of carbon dioxide (pCO_2), the effects of which have been well-studied in microalgae—including in *Chlorella* spp. [14]. However, the effects on microorganisms of a low pressure per se, independently of the partial pressures of metabolizable gases, are poorly understood [15,16]. For *Chlorella* spp. specifically, two previous studies are worth mentioning. In the first, lowering the total pressure to 250 hPa under constant pCO_2 enhanced their growth [17]; but this positive effect was not observed in the second study (though no negative effect was observed either), where the total pressure was lowered to 565 hPa under constant pCO_2 and pO_2 [18]. More broadly, the limited evidence available in the area of hypobaric microbiology suggests that 100 hPa represents a threshold down to which the growth of most microbial species is not affected by a decrease in total pressure [15,16]. However, conclusions from most low-pressure studies can be generalized only tentatively due to confounding factors (e.g., the partial pressures of metabolizable gases are usually not kept constant) and a species-specific sensitivity to hypobaria.

The effects of hypobaria on biomass composition are known even less than those on growth dynamics. As reducing pressure tends to fluidify membranes [19], and perturbations in membrane viscosity can affect functions which are critical to cell integrity (e.g., membrane transport, intracellular signaling, and electron transport) [20], we hypothesized that the lipid makeup of *C. vulgaris* would be altered to compensate for it. Such a response would be akin to the way numerous microorganisms adapt their lipid composition to maintain a suitable membrane fluidity following changes in temperature [21] or hyperbaria [22] (a process referred to as homeoviscous adaptation). Since the lipid profile of *C. vulgaris* biomass plays a major role in its nutritional value, determining how it is affected by lowering the pressure is critical if cultivation is to be performed under hypobaric conditions.

Here we primarily sought to determine whether a tenfold reduction in pressure, down from Earth-ambient, would (i) affect the growth of *C. vulgaris* when the partial pressure of metabolizable gases is kept constant, or (ii) change the lipid composition of its biomass. For this purpose, the microalga was cultivated under normal (1000 hPa) and low (100 hPa) atmospheric pressures, either maintaining a constant partial pressure of carbon dioxide (100 hPa) or using a carbon dioxide-free atmosphere with sodium carbonate as a carbon source. As a proof-of concept of a cultivation strategy foreseen during crewed Mars missions, an additional experiment was performed where a simulant of Martian regolith, synthetic urine, and a low atmospheric pressure of carbon dioxide were used as sole nutrient and carbon sources.

2. Materials and methods

2.1. Chemicals

Analytical LC grade isopropanol, methanol, acetonitrile, acetic acid, formic acid, ammonium formate and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). Sulphuric acid (96%) and potassium chloride, both analytical grade, were purchased from Carlo Erba Reagents SAS (Val de Reuil, France). Ultrapure water was obtained from a MilliQ purification system (Merck KGaA, Darmstadt, Germany). A Splash Lipidomix standard lipid component mixture was purchased from Sigma Aldrich.

2.2. Growth experiments

2.2.1. Model microalga and routine growth conditions

The green microalga *Chlorella vulgaris* CCALA 269 was obtained from the Culture Collection of Autotrophic Organisms (CCALA), Třeboň, Czech Republic. Prior to experiments, cultures were grown inside a poly klima PK 520-LED photoincubator at 25 °C, in Bold's Basal Medium (BBM) at a pH of ca. 7.7, with a light intensity of 65–70 $\mu mol_{ph}\ m^{-2}\ s^{-1}$ (16 h/8 h day/night cycle), on a rotary shaker set at 100 rpm.

2.2.2. Cultivation at ambient and low pressure, under constant partial pressures of carbon dioxide

To test the effects of a low atmospheric pressure on *C. vulgaris*, independently of the partial pressure of carbon dioxide, the microalga was exposed to non-ambient conditions using Atmos: a photobioreactor designed for the cultivation of phototrophic microorganisms at low pressure [13,23]. It was there cultivated under 1000 hPa (circa the ambient pressure at sea level) and 100 hPa. This latter value was selected since, within the optimum temperature range of *C. vulgaris*, a lower pressure causes excessive evaporation or even cavitation, which interferes with photobioreactor operations (a constraint that applies to foreseen cultivation systems on Mars as well as to the present experimental setup).

Atmos vessels were filled with 70 mL of BBM at a pH of 7.04 ± 0.02, buffered with 20 mM HEPES and, when cultivation was to be performed under argon only, supplemented with 1 $g\ L^{-1}$ $NaHCO_3$. The medium was inoculated with precultures in late exponential phase to an optical density at 750 nm (OD_{750}) of 0.2 (0.44 $g\ L^{-1}$). The headspace air in the vessels was replaced with the experimental atmospheres: 1000 hPa of 10.02 ± 0.20 mol% N45 carbon dioxide in N50 argon (CRYSTAL mixture; Air Liquide S.A., Paris, France), argon ($\geq$ 99,999 mol%; ALPHAGAZ 1 Argon; Air Liquide S.A.), or ambient-like air (*Druckluft*; Air Liquide S.A.); or 100 hPa of either carbon dioxide (N45; Air Liquide S.A.) or argon. The light intensity was set to 50 μmol photons $m^{-2}\ s^{-1}$ per side (measured on the inner side of the vessel, where it is closest to the LED strips, at the mid-height of the culture), the temperature to 30 °C, and the stirring rate to 125 rpm. The pressure in the gas phase was automatically restored to its target value when deviations exceeded 10 hPa. The gas in the headspace was renewed (by flushing at constant pressure) 2 h after the start of the experiment and subsequently, every 12 h throughout the experiment. Six replicate vessels (in at least two separate experimental runs) were used for each tested condition. Hereafter, the conditions where carbon was provided as carbon dioxide are referred to as CO_2_AP (under 1000 hPa; AP stands for "ambient pressure") or CO_2_LP (under 100 hPa; LP stands for "low pressure"), and these where the atmosphere was composed of argon only, to Ar_AP and Ar_LP.

C. vulgaris was grown under these conditions for four days. Samples were taken every 24 h to measure the OD_{750}, and biomass concentrations were calculated from OD_{750} values using a predetermined relationship between both variables (Fig. S1). Biomass concentration values over time were then fitted to a logistic growth equation using GraphPad Prism version 10.5.0 for Windows (GraphPad Software). The significance of differences in growth dynamics was then assessed by testing (using an extra-sum-of-squares F test) whether the best-fit values of the logistic growth equation parameters differed between conditions. A P value of 0.05 was selected as the threshold for significance.

2.2.3. Cultivation under a low-pressure atmosphere of carbon dioxide, in Martian medium

To test cultivation conditions which might be provided on Mars from the local natural resources and from recycled urine, *C. vulgaris* was cultivated in Martian medium (MM; prepared as described below) buffered with 20 mM HEPES, under a carbon dioxide atmosphere at 100 hPa. This experiment was performed in Atmos, as described above.

The MM was based on the Jezero Delta Simulant (JEZ-1; Space Resource Technologies, Orlando, FL, USA), as a proxy for Martian regolith, and synthetic urine. Briefly, 50 g of JEZ-1 (<1 mm diameter grains) was mixed with 500 mL of ultrapure water. The resulting slurry was stirred for 24 h at 25 °C within a 1 L Erlenmeyer flask with a cap and then filtered with bibulous paper to yield a regolith leachate. To determine the composition of this leachate, it was filtered through a nylon filter with a pore size of 0.45 μm and acidified with 5% (v/v) suprapure-grade HNO_3 (66–67%) before running it into an Agilent 7850 inductively coupled plasma mass spectrometer (ICP–MS; Agilent Technologies, Palo Alto, CA) equipped with a collision/reaction cell operated in helium mode (to minimize polyatomic interferences). Element quantitation was carried out using an external calibration method, corrected with an internal standard solution (composed of ^6Li, GeO_2, In, Lu_2O_3, $Rh(NO_3)_3$, Sc_2O_3, and Tb_4O_7, each at 0.2 μg L^{-1}) continuously injected during the analysis. Calibration curves were constructed using five standard solutions and were considered acceptable when $R^2 \geq 0.990$. Instrument parameter values, as well as linear regression equations and correlation coefficients of the calibration curves, are given in Table S1. Synthetic human urine was produced as described by Sarigul et al. [24] and diluted with ultrapure water ten times. Finally, the regolith leachate and diluted urine were mixed (1:1 v/v) to produce the MM, whose composition is given in Table S2.

2.3. Lipidomics

2.3.1. Fatty acids methyl ester (FAME) analysis

Sample preparation followed the protocol described by Breuer et al. [25], with the modifications previously reported by Casula et al. [10]. Briefly, lipids were extracted from 25 mg of lyophilized biomass using 4 mL of a methanol/chloroform (4:5 v/v) mixture containing 50 mg/L of the internal standard tritridecanoin. They were then trans-esterified to obtain fatty acid methyl esters (FAMEs). The hexane phase containing FAMEs was transferred to glass vials for GC–MS analysis using an Agilent gas chromatograph (8860 GC system) coupled with a single quadrupole mass spectrometer (5977 MSD; Agilent Technologies).

Each sample was injected in split mode (split ratio 1:2) and separated on a fused silica capillary column (Ultra Inert 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 was used as the GC carrier gas. The oven temperature was initially held at 50 °C for 4 min before being ramped from 50 to 160 °C at 5 °C/min, ramped from 160 to 210 °C at 1.0 °C/min, and finally ramped from 210 °C to 280 °C at 5 °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). The mass fraction of the biomass represented by each individual fatty acid was determined (using the internal standard) as suggested by Breuer et al. [25].

2.3.2. Sample preparation for lipidomics analysis

Lyophilised microalgae samples (five replicates per condition) were extracted using a slight modification of the method by Folch, Lees and Stanley [26]. Briefly, 10 mg of each lyophilised biomass sample was transferred to a centrifuge tube and 10 mL bi-distilled water (previously cooled on ice) was added. Samples were then ultra-sonicated for 3 min using an ExtractorOne (GM Solution, Cagliari, Italy). Subsequently, 5000 μL of ice-cold methanol/chloroform (1/2 v/v) and 10 μL of a mixture of internal standards (Splash Lipidomix) were added. The samples were agitated every 15 min for 1 h. Afterwards, 1000 μL of an aqueous, 0.2 M potassium chloride solution was added and the samples were agitated again. The tubes were centrifuged at 24,104 rcf for 10 min, after which 1000 μL of the lipophilic layer from each was transferred into a glass vial and dried with a gentle nitrogen stream.

2.3.3. Complex lipid analysis

A lipidomic analysis was performed with the previously described UHPLC-QTOF/MS method [8]. Briefly, the dried lipophilic phase was dissolved in a methanol/chloroform mixture and diluted before injection. Complex lipids were analyzed using a UHPLC-QTOF/MS system (Agilent 1290 Infinity II LC) in both positive and negative ionization modes, using a Kinetex EVO C18 column. Chromatographic separation and MS parameters were as detailed by Casula et al. [10]. Chromatographic areas were obtained by acquiring samples in ESI full scan mode and normalized using PE 15:0–18:1(d7) (added to a concentration of 10 μg/mL during extraction) as an internal standard. The abundance data presented below therefore corresponds to peak area ratios (i.e., the peak area of each analyte divided by the peak area of the internal standard).

2.3.4. Identification of lipid classes and pigments

Lipid identification was performed using iterative MS/MS experiments as previously described [10], using collision energies of 20 and 40 eV. Lipid classes were annotated using Lipid Annotator (version 1.0, Agilent MassHunter workstation) and confirmed by comparison with an online mass database. Specific diagnostic fragments were used for the identification of complex lipid species, following the criteria outlined previously [10]. Chlorophylls and their derivatives were quantified according to Hyvärinen et al. [27] and Milenkovic et al. [28].

2.3.5. Statistical analysis of lipidomics data

A multivariate statistical analysis (MVA) was performed with the SIMCA-P+ program (Version 14.1, Umetrics, Sartorius, Germany). An unsupervised Principal Component Analysis (PCA) was used for dataset overview, and the results were represented in two dimensions as score (related to observations) and loading (related to variables) scatter plots. *t*-tests (corrected where applicable for multiple comparisons using the Bonferroni-Dunn method) were performed using GraphPad Prism version 10.5.0 for Windows. Mean differences between groups were considered significant when P values (or, where applicable, corrected P values) were below 0.05.

3. Results and discussion

3.1. Growth of Chlorella vulgaris following a tenfold decrease in pressure

To assess whether a low atmospheric pressure affects the growth of *C. vulgaris*, the microalga was cultivated in Atmos under 1000 hPa and 100 hPa and its optical density measured over time. Two independent approaches were followed to exclude the effects of the partial pressures of metabolizable gases. In the first, *C. vulgaris* was grown under argon, a gas whose presence was shown not to affect the growth of *Chlorella* spp.

even at high concentrations [17,29]; there, the carbon source was sodium bicarbonate. In the second, the partial pressure of carbon dioxide in the inlet was increased tenfold when the total pressure was decreased tenfold. It should however be noted that, in the vessels, water vapour accounted for a larger fraction of the total pressure at 100 hPa than at 1000 hPa (as its saturation vapour pressure hardly depends on total pressure). Given that the total pressure is roughly the sum of the partial pressures (Dalton's law), this resulted in a lower partial pressure of carbon dioxide. This effect was deemed negligible since carbon dioxide was non-limiting and non-toxic in both cases, and since the medium was buffered.

The results are shown in Fig. 1. Growth was higher under carbon dioxide (reaching close to 3 g L^{-1} within four days) than under argon only (where it remained below 1 g L^{-1}). This is presumed to stem primarily from the difference in carbon source: the use of a large, and renewed, amount of carbon dioxide is known to support higher productivities of *C. vulgaris* than an initial supply of bicarbonate (e.g., [30,31]). The effect could not be compensated for by increasing this supply: higher concentrations of bicarbonate could have increased growth only slightly at most (e.g., [32,33]).

More importantly, under neither type of atmosphere did a reduction in the total pressure lead to a change in growth rates or in culture density at the plateau. This is at odds with the observation by others, already mentioned above, that lowering the total pressure from ambient to ca. 250 hPa (under a constant pCO_2 of 50 hPa) had a positive impact on the growth rates of *Chlorella sorokiniana* [17]. It may be that the positive effect reported there came from an increase in diffusion coefficients with decreasing pressure, and that in our setup carbon dioxide diffusion was not limiting (and was taken out of the equation in the argon-only comparison). Whether or not that is the case, our results show that a reduction in pressure over that range does not negatively affect the growth of *C. vulgaris*. They are consistent with these of a recent study where lowering the pressure of an argon-only atmosphere from 1 bar to 80 hPa did not reduce the growth rates of the cyanobacterium *Anabaena* sp. PCC 7938 [13], and with previous observations that shifting pressure in the 100–1000 hPa range seems not to largely affect the growth of most bacterial species when the partial pressures of metabolizable gases are not limiting [15]. It therefore seems that a gas mixture directly drawn from the Martian atmosphere would only need to be pressurized to 100 hPa or less, which, compared to a system operated under an Earth-like pressure, could significantly reduce the energy expenditure associated with microalgal cultivation [13].

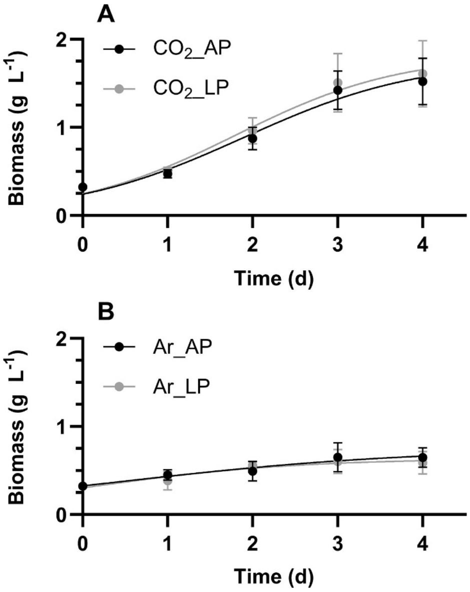

Fig. 1. Growth of *C. vulgaris* under different atmospheric pressures and compositions. These comparisons were performed to test whether a tenfold reduction in atmospheric pressure affects growth when the partial pressures of metabolizable gases are constant. A: Biomass concentration over time in Bold's Basal Medium (BBM) under an atmospheric pressure of either 1000 hPa (ambient pressure, AP) or 100 hPa (low pressure, LP). The partial pressure of CO_2 (pCO_2) was in either case of circa 100 hPa (10% CO_2 in Ar_AP; 100% CO_2_LP). B: Biomass concentration over time in BBM under atmospheres with the same pressure values (1000 or 100 hPa) but with CO_2-free argon.

3.2. Growth in Martian medium under a low-pressure atmosphere of carbon dioxide

As a proof of concept, *C. vulgaris* was grown under conditions (referred to as "Mars cultivation" below) analogous to those previously proposed for the cultivation on Mars of photosynthetic microorganisms [34], but under a low pressure. In short, it was grown in a leachate of a Martian regolith simulant mixed with synthetic urine ("Martian medium") under an atmosphere of 100 hPa of carbon dioxide, which provides a pCO_2 comparable to that of Martian air pressurized tenfold. It should be noted that these conditions only account for the composition of the liquid medium and the artificial atmosphere under which the microalgae are foreseen to be cultivated, and not for other factors which are specific to Mars. Most critical among these may be the partial gravity or the higher levels of ionizing radiation. However, while these may significantly affect cultivation systems, no large effect is expected on growth at least in the short term (e.g., [8,10]).

A growth curve is shown in Fig. 2. The same figure also shows, as a control, a growth curve of *C. vulgaris* in a standard growth medium, BBM, under ambient-like air ("Earth control" below; note that growth in this setup does not reflect that in a system where the air would be renewed continuously). Using the Martian medium rather than BBM did not reduce growth significantly (cf. "Mars" in Fig. 2 vs. "CO_2_LP" in Fig. 1A), consistently with previous results [11]. While it may be that other factors (e.g., light) were limiting, and that in otherwise optimized conditions a difference would be observed between BBM and Martian medium, a more telling comparison is that between Mars cultivation and Earth control conditions: the productivity of *C. vulgaris* can be remarkably high in cultivation conditions that combine ISRU and waste recycling under a low-pressure, CO_2-rich atmosphere. However, decreasing the total pressure from Earth-ambient levels can impact microalgae in ways which are not reflected in the growth dynamics. Pigment contents, for instance, were affected (Fig. S1); and changes to the lipidome are presented below.

3.3. Effects of a tenfold reduction in atmospheric pressure on the fatty acid profile of the biomass

Increases in atmospheric pressure tend to compact biological structures [35]; membranes are particularly sensitive to it and conversely,

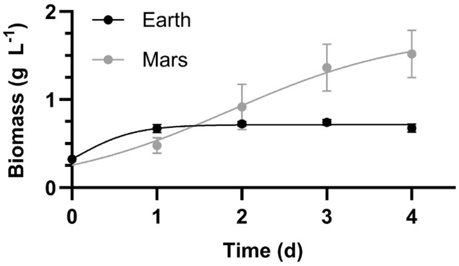

Fig. 2. Biomass concentration over time in BBM supplemented with carbonates and under 1000 hPa of ambient-like air (Earth), or in Martian medium (leachate of a Mars-like regolith mixed with diluted synthetic urine) and under 100 hPa of carbon dioxide (Mars). The associated experiment represents a proof-of-concept for cultivation conditions foreseen during crewed Mars missions.

reducing pressure tends to fluidify them [19]. We therefore tested whether the lipid composition of *C. vulgaris* changes as a response, as was shown to occur in various organisms facing an increase in temperature (which also promotes an increase in membrane fluidity) [36–38].

The best understood and most frequently reported mechanisms of homeoviscous adaptation lie in the modification of acyl chains (increases in the degree of saturation and in length promoting an increase in viscosity). The abundance of FAMEs from the biomass decreased with a decrease in pressure, both when carbon was provided as CO_2 (163.6 ± 9.8 mg per g of dry weight [gdw] for CO_2_AP vs. 118.4 ± 4.0 mg gdw^{-1} for CO_2_LP; unpaired, two-tailed *t*-test, $p < 0.05$) and when the atmosphere was composed of argon only (25.8 ± 0.9 vs. 31.0 ± 0.3 mg gdw^{-1}, $p < 0.05$). For further analysis, the abundances of individual FAMEs were normalized to their combined abundance. Results evidence an influence of pressure (Fig. 3A, B), although the way in which the abundance of specific fatty acids was affected depends on how carbon was provided. In the presence of atmospheric carbon dioxide, reducing the pressure from 1000 hPa (CO_2_EP) to 100 hPa (CO_2_LP) led to an increase in the abundance of palmitic acid (C16:0), stearic acid (18:0) and *cis*-octadecadienoic acid (C18:2), as well as a decrease in the abundance of oleic acid (C18:1) and linolenic acid (C18:3). Among these changes, the most drastic was the decrease in oleic acid, whose concentration is known to play a key role in the homeoviscous adaptation of a wide range of organisms (e.g., [39–41]). As an example, this fatty acid was highly overproduced in a psychrotolerant, piezophilic, deep-sea bacterium when pressure was increased, and an oleic acid-auxotrophic acid mutant of this strain was shown to be both low-temperature sensitive and high-pressure sensitive in the absence of an exogenous supply of this fatty acid [42]. Under an argon-only atmosphere, however, a pressure reduction (from Ar_EP to Ar_LP) led to an increase in the abundance of oleic and linoleic acids and a decrease in that of stearic acid.

For both atmospheric compositions, pressure affected the abundance of monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and the unsaturated-to-saturated fatty acid ratio (Fig. 3C–F). When atmospheric carbon dioxide was the carbon source, these values decreased when pressure was lowered, which would be consistent with an adaptive response: double bonds in fatty acid chains create kinks that prevent tight lipid packing, thereby increasing membrane fluidity [37,43–51]; a decrease in saturation levels therefore tends to compact membranes, which may help preserve rigidity when pressure is reduced. This decrease in fatty acid unsaturation mirrors the increase observed in piezophilic or piezotolorant microorganisms as a response to increasing pressure (e.g., [52,53]). Under an argon-only atmosphere, the opposite trend was observed. The underlying reason is unclear; however, the degree of unsaturation of fatty acids under 1000 hPa was already very low and, in spite of its (slight) increase at low pressure, it remains below that observed in the presence of carbon dioxide.

3.4. Effects of a tenfold reduction in atmospheric pressure on the lipidome

How a low pressure affects the lipid composition of *C. vulgaris* was

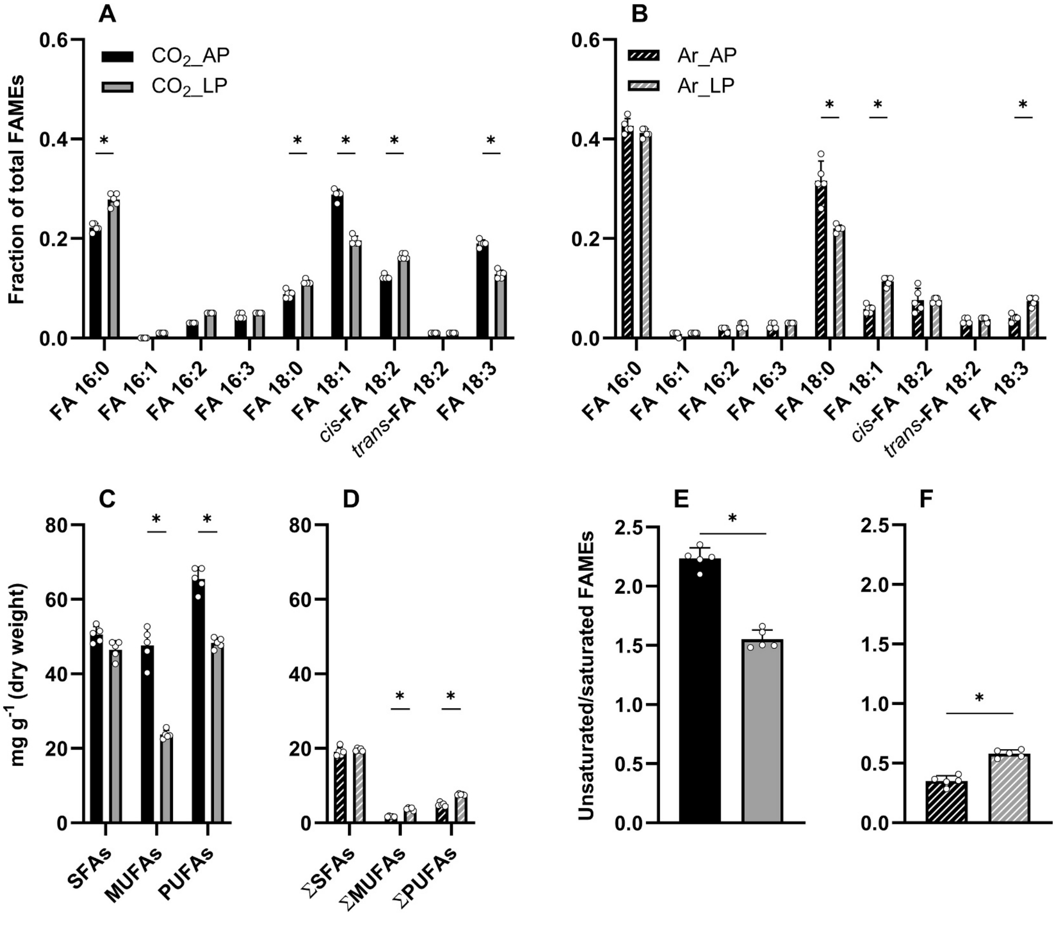

Fig. 3. Characterization of the total fatty acids (as FAMEs) of *Chlorella vulgaris* grown under either 1000 (AP) or 100 hPa (LP) of either 100 hPa of carbon dioxide and argon as a balance gas (CO_2), or argon only (Ar). A, B: Fraction of the total FAMEs represented by each fatty acid. C, D: Abundance of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). E, F: Ratio of unsaturated-to-saturated fatty acids. The significance of mean differences was tested with *t*-tests, corrected where applicable for multiple comparisons using the Bonferroni-Dunn method. An asterisk indicates a *P* value (adjusted where applicable) below 0.05. Numbers in the designation of the fatty acids (e.g., "16:0" in "FA 16:0" represent the number of carbon atoms and of double bonds.

further investigated through an untargeted lipidomic analysis. A total of 128 putative lipids were annotated (Table S3). A principal component analysis (PCA) of positively ionized lipid species (Fig. S2, top) revealed a clear separation along principal component 1 (PC1) of samples exposed to 100 hPa of carbon dioxide from those exposed to ambient-like air or argon, suggesting that the source and abundance of carbon was the main discriminating factor in lipid profile variation. This is consistent with observations by others that the nature and abundance of inorganic carbon can influence the lipid makeup of microalgae (e.g., [30,31,54]). Among samples exposed to 100 hPa of carbon dioxide, a separation according to atmospheric pressure was observed along PC2. A PCA performed on negatively ionized lipid species (Fig. S2, bottom) showed a less pronounced distinction, but again the main discriminating factor appeared to be the carbon source. Consistently, the loading plot (Fig. S5) suggests that some lipids correlate with specific compositions of atmosphere or liquid medium (e.g., digalactosyldiacylglycerols with “Mars conditions”) rather than with atmospheric pressure. Others (e.g., triglycerides and free fatty acids) are evenly distributed.

3.4.1. *Plasma membrane lipids*

The total abundance of the quantified lipids which are extraplastidic, and therefore predominantly located in the plasma membrane (phosphatidylcholines [PCs], phosphatidylinositols [PIs] and diacylglyceryl-*N*-trimethylhomoserines [DGTSs], hereafter collectively referred to as plasma membrane lipids) increased significantly with a decrease in pressure when carbon was provided as carbon dioxide (peak area ratio [PAR]: 8.39 ± 3.58 for CO_2_AP vs. 16.40 ± 4.26 for CO_2_LP; unpaired, two-tailed *t*-test, $p < 0.05$). Under an argon-only atmosphere, the difference is not significant (PAR: 15.03 ± 3.56 for Ar_AP vs. 18.65 ± 3.87 for Ar_LP; $p > 0.05$). It is important to note, however, that due to technical limitations (in particular, low biomass availability), not all plasma membrane lipids could be quantified; sterols, for instance, were not, and these lipids may play an important role in the preservation of membrane fluidity [55].

The fractional abundance (relative to total plasma membrane lipids) of PCs, PIs and DGTs is given in Fig. 4A, B (values for each lipid within these classes, e.g., PC 32:0, are given in Fig. S5). The numbers following the abbreviated lipid name (e.g., “32:0” in PC “32:0”) represent the total

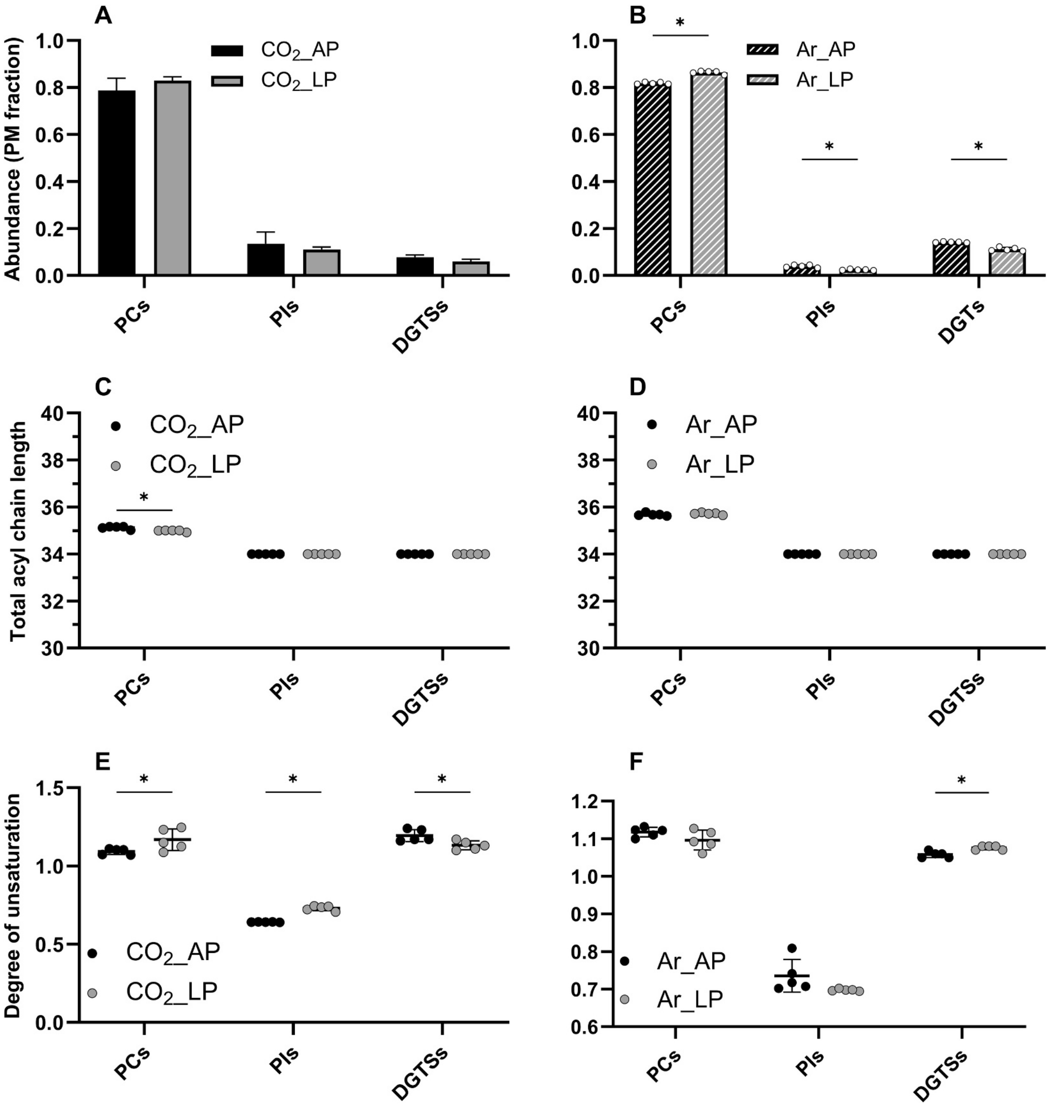

Fig. 4. Characterization of the main classes of plasma membrane lipids in *Chlorella vulgaris* grown under either 1000 (AP) or 100 hPa (LP) of either 100 hPa of carbon dioxide and argon as a balance gas (CO_2), or argon only (Ar). A, B: Abundance (relative to the total abundance of quantified plasma membrane lipids) of phosphatidylcholines (PCs), phosphatidylinositols (PIs) and diacylglyceryl-*N*-trimethylhomoserines (DGTSs). C, D: Average total acyl chain length (sum of both chains) of plasma membrane lipids. E, F: Average degree of unsaturation (per acyl chain) of plasma membrane lipids. The significance of mean differences was tested with *t*-tests corrected for multiple comparisons using the Bonferroni-Dunn method. An asterisk indicates an adjusted *P* value below 0.05.

number of carbon atoms and the total number of double bonds in the fatty acyl chains. Overall, a decrease in pressure slightly changed the relative abundance of several lipids in the plasma membrane, though changes differed under different atmospheric compositions. When carbon was provided as carbon dioxide, the abundances of PCs 34:2, 34:5, 34:6 and 36:5 increased while these of PC 36:2 and DGTS 36:3 decreased. Under argon only, changes were minor: the abundances of PI 34:2 and DGTS 36:2 decreased (Fig. S5). More coarsely, the abundance of PCs increased, while those of PIs and DGTSs decreased, in samples under argon only (samples under carbon dioxide followed the same trend but the differences are not significant; Fig. 4A, B). How these modifications affect membrane fluidity is unclear: the impact of changes in the abundance of extraplasmidic polar lipid classes is far less understood than that of changes in the acyl chains.

When carbon was provided as carbon dioxide, the average total length of acyl chains (the sum of carbon atoms in both chains) across all lipid classes in plasma membranes did not change significantly with a decrease in pressure (34.88 ± 0.10 for CO_2_AP vs. 34.82 ± 0.03 for CO_2_LP; unpaired, two-tailed *t*-test, $p > 0.05$), though the average length of PC acyl chains decreased (Fig. 4C). The average degree of unsaturation (reported as the average number of double bound per acyl chain) increased in PCs and PIs but decreased in DGTSs (Fig. 4D), resulting in an overall significant ($p < 0.05$) but very small increase (1.04 ± 0.02 for CO_2_AP vs. 1.12 ± 0.06 for CO_2_LP). Results differ under argon only: the average total acyl chain length increased significantly overall ($p < 0.05$), though only slightly (35.38 ± 0.06 for Ar_AP vs. 35.48 ± 0.05 for Ar_LP) and without a significant difference within any given lipid class (Fig. 4D), and the overall degree of unsaturation was unaffected (1.09 ± 0.01 for Ar_AP vs. 1.09 ± 0.02 for Ar_LP; $p > 0.05$) in spite of a very minor increase in DGTS unsaturation (Fig. 4F). Overall, changes in total acyl chain length and degree of unsaturation were minor and, taken alone, do not indicate any significant homeoviscous adaptation of the plasma membrane.

3.4.2. Thylakoid membrane lipids

The lipid composition of thylakoid membranes is highly conserved among plants, algae, and cyanobacteria, and mainly features four lipid classes (monogalactosyldiacylglycerols [MGDGs], digalactosyldiacylglycerols [DGDGs], phosphatidylglycerols [PGs] and sulfoquinovosyldiacylglycerols [SQDGs]) which provide a matrix for the photosynthetic complexes, stabilize them, and in other ways are necessary to photosynthesis [56]. Our decrease in pressure affected the relative abundance of several lipids within these classes when carbon dioxide was provided (Fig. S6). As with plasma membrane lipids, the total abundance of thylakoid membrane lipids increased significantly under carbon dioxide (PAR: 15.46 ± 2.38 for CO_2_AP vs. 43.17 ± 14.13 for CO_2_LP; unpaired, two-tailed t-test, $p < 0.05$) but did not change significantly under argon only (PAR: 13.87 ± 4.20 for Ar_AP vs. 13.42 ± 2.30 for Ar_LP; $p > 0.05$). Under the latter conditions, MGDG 34:4 was the only one whose relative abundance decreased significantly (Fig. S6).

Under both atmospheres, the fraction of thylakoid membrane lipids represented by DGDGs increased with a decrease in pressure. This change is noteworthy: an increase in the abundance of DGDG has been shown to favour thermotolerance (higher temperatures, as lower pressures, tend to fluidify membranes) in photosynthetic organisms [57–59]. Besides, changes in the MGDG-to-DGDG ratio are also known to help maintain the properties of thylakoid membranes [60], and its decrease has often been suggested to be associated with a higher tolerance to temperature-induced stress. This has been proposed with regard to both high (e.g., [59,61,62]) and low temperatures (e.g., [63,64]) but the prime driving mechanisms may differ. As MGDGs are cone-shaped and DGDGs more cylindrical, a low ratio tends to promote membrane viscosity and therefore to serve homeoviscosity when temperature increases (or, presumably, when pressure decreases). However, this geometry also implies that MGDGs promotes non-bilayer structures while DGDGs promote lamellar ones, and a decrease in the MGDG-to-DGDG ratio can therefore support the maintenance of the thylakoid membrane's structural and functional integrity under various stress conditions, including cold stress (e.g., [6,63]). Here we expected a decrease in this ratio at low pressure, and this occurred under argon (1.15 ± 0.42 vs. 0.52 ± 0.04; $p < 0.05$). Under carbon dioxide, however, the fractional abundance of MGDGs increased (Fig. 5A), and so did the MGDG-to-DGDG ratio (0.24 ± 0.01 vs. 0.30 ± 0.04; $p < 0.05$). In absolute numbers, however, the increase in DGDG exceeded that in MGDG, and one can presume that the increase in the fractional abundance of DGDG within the thylakoid membrane weighed more heavily on membrane fluidity than the small decrease of its abundance relative to MGDG.

In the fractional abundance of SQDGs and PGs, we observed a decrease with pressure under carbon dioxide but no change under argon only (Fig. 5A, B). In the case of PGs, the shift under a carbon dioxide atmosphere mirrored the increase observed in piezophilic bacteria following an increase in pressure (e.g., [53,66,67]) and in a wide range of organisms following a decrease in temperature (e.g., [63,68]). It has been presumed that this increase was due to the rather large head group of PGs, which disrupts membrane packing and thereby favors fluidity [66,69]. While less has been reported on the fluctuations of SQDGs, their biophysical properties are close to those of PGs [70] and a decrease in their levels has been found to be associated with heat adaptation in wheat [71] and in a marine red alga [72] suggesting a role in homeoviscous adaptation analogous to that of PGs.

When carbon was provided as carbon dioxide, the average total acyl chain length of PGs decreased slightly with a decrease in pressure, and that of SQDGs increased slightly, while those of MGDGs and DGDGs remained constant (Fig. 5C). PGs represent a small fraction of the total thylakoid membrane, and the overall average total acyl chain length of thylakoid membrane lipids increased, though to a very small extent (33.32 ± 0.08 for CO_2_AP vs. 33.63 ± 0.08 for CO_2_LP; $p < 0.05$). The average degree of unsaturation of MDGGs, DGDGs and SQDGs increased, and so did the average degree of unsaturation of thylakoid membrane lipids (0.97 ± 0.08 for CO_2_AP vs. 1.31 ± 0.05 for CO_2_LP; $p < 0.05$). Under a pure argon atmosphere, a decrease in pressure did not lead to a change in the average total acyl chain length (Fig. 5D) or average degree of unsaturation (Fig. 5F) in any lipid class. Consistently, across all thylakoid membrane lipids, neither the average total acyl chain length (33.64 ± 0.04 for Ar_AP vs. 33.69 ± 0.07 for Ar_LP; $p > 0.05$) nor the average degree of unsaturation (1.17 ± 0.08 for Ar_AP vs. 1.19 ± 0.04 for Ar_LP; $p > 0.05$) changed significantly.

From the perspective of homeoviscous adaptation, the results presented so far on the lipidome paint a rather nuanced picture. Some changes suggest a response that would avoid an increase in membrane fluidity at lower pressure. These are, for instance, the decrease—when the atmosphere contained carbon dioxide—in the overall ratio of unsaturated-to-saturated fatty acids, in the average degree of unsaturation in thylakoid membrane lipids, and in the abundance of PGs; and the increase, regardless of atmosphere composition, in the fractional abundance of DGDGs in the thylakoid membrane. However, others of the observed changes would tend to fluidify membranes (e.g., the increase in an unsaturated-to-saturated fatty acid ratio under an argon-only atmosphere). Such seemingly contradictory responses to factors affecting membrane viscosity have been reported by others. Across strong temperature gradients within hydrothermal systems, for instance, higher temperatures tended to lengthen the fatty acid chains of bacterial lipids (which decreases fluidity) while unsaturation levels rose (which tends to increase fluidity) [73], and when a bacterium from deep subseafloor sediments was exposed to 25 MPa rather than ambient pressure, its membrane underwent changes that would tend to foster fluidity (an increase in PG esters and mono-unsaturated fatty acids) but also a lengthening of fatty acid chains [53]. A particularly relevant, additional example comes from an experiment where *Bacillus subtilis* was grown under 50 hPa: the proportion of saturated fatty acids increased, but so did that of anteiso-fatty acids (which tend to increase fluidity); and a

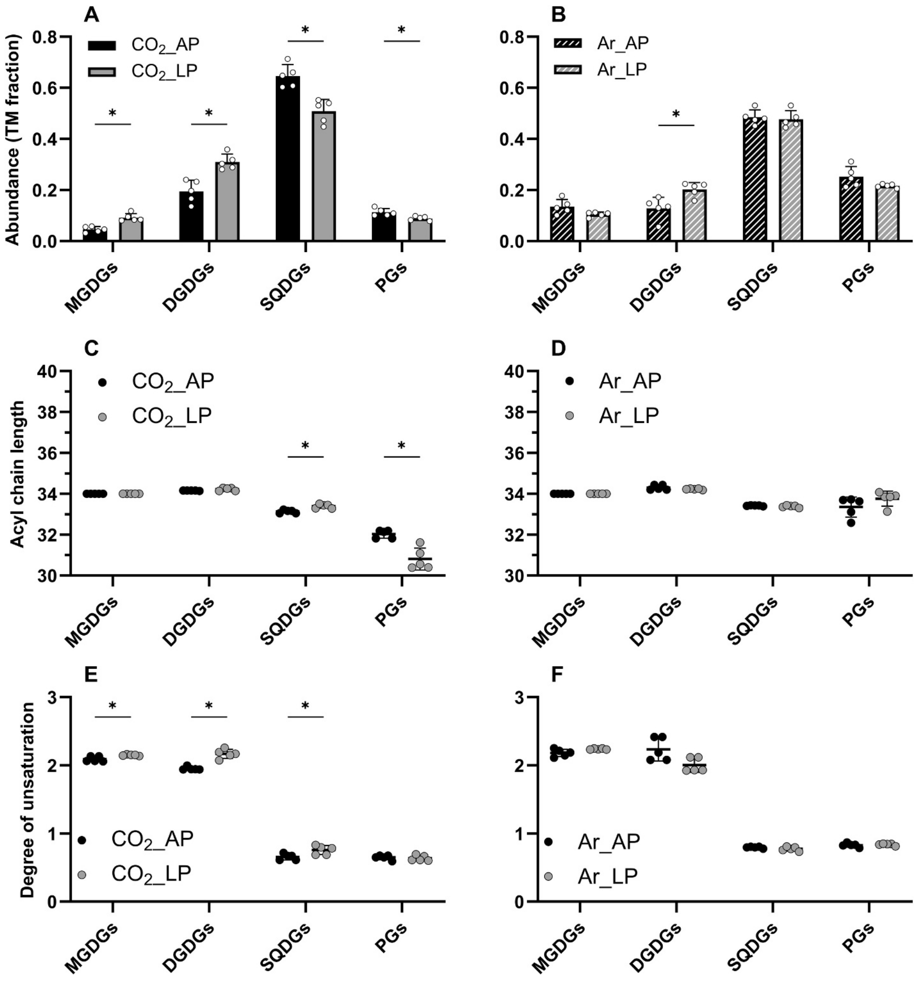

Fig. 5. Characterization of the main classes of thylakoid membrane lipids in *Chlorella vulgaris* grown under either 1000 (AP) or 100 hPa (LP) of either 100 hPa of carbon dioxide and argon as a balance gas (CO_2), or argon only (Ar). A, B: Abundance (relative to the total abundance of quantified thylakoid membrane lipids) of monogalactosyldiacylglycerols (MGDGs), digalactosyldiacylglycerols (DGDGs), sulfoquinovosyldiacylglycerols (SQDGs) and phosphatidylglycerols (PGs). C, D: Average total acyl chain length (sum of both chains) of thylakoid membrane lipids. E, F: Average degree of unsaturation (per acyl chain) of plasma membrane lipids. The significance of mean differences was tested with *t*-tests corrected for multiple comparisons using the Bonferroni-Dunn method. An asterisk indicates an adjusted *P* value below 0.05.

strain evolved under 50 hPa overexpressed genes involved in the des-desKR system (which fluidifies membranes when temperatures go down) [74]. These discrepancies may in some instances be attributed to a maladaptive response [74], especially at low pressure: the highest pressure at the surface of the Earth, at the top of Mount Everest, is above 300 hPa, and there is therefore little selective pressure towards higher fitness under hypobaria. It may however be rather explained by (i) the numerous changes in membrane composition that occur simultaneously, which renders tentative any assessment of membrane fluidity through lipid composition alone; (ii) by the complex effects that a given change can have (it has, for instance, been suggested that while one or two unsaturations lower the membrane's phase transition temperature by creating kinks that disturb membrane packing, additional unsaturations caused a decrease in the chain rotation freedom that may counterbalance this effect [67]; and (iii) by the functional roles played by lipids beyond their direct influence on fluidity.

3.4.3. Neutral lipids and free fatty acids

Unlike polar lipids, neutral lipids (diglycerides [DGs] and triglycerides [TGs]) and free fatty acids (FFAs) are not membrane-associated. Their relevance to hypobaric adaptation was therefore assumed to be low and indeed, their abundance was little affected by a decrease in pressure (Fig. 6). The overall abundance of FFAs did not change, neither when carbon dioxide was supplied (PAR: 761.2 ± 28.9 for CO_2_AP vs. 824.3 ± 91.6 for CO_2_LP; unpaired, two-tailed t-test, $p > 0.05$), nor under argon only (PAR: 812.4 ± 75.16 for Ar_AP vs. 864.1 ± 84.97 for Ar_LP; $p > 0.05$), though the relative abundances of some individual FFAs varied slightly (Fig. 6A, B). These minor differences consist in the decreased abundance of unsaturated fatty acids or in the increased abundance of a saturated one. The abundance of DGs varied slightly when the pressure decreased (it decreased under carbon dioxide and increased under argon only), but the variation is small and overall, the abundance of DGs and TGs hardly changed (Fig. 6C, D). Small changes in non-membrane lipids may therefore occur as part of a response to low pressure. Consistently, and why little research on high-pressure adaptation has focused on changes in intracellular, free lipids, one study evidenced an increase in the unsaturation of wax esters (a storage lipid) in a piezotolerant bacterium, isolated from deep seawater bacterium, when grown under 35 MPa rather than ambient pressure [75] (and a similar change was observed in a psychrophilic bacterium upon temperature decrease [76]).

4. Conclusions

Decreasing the total atmospheric pressure tenfold, without changing the partial pressure of any metabolizable gas, did not affect the growth

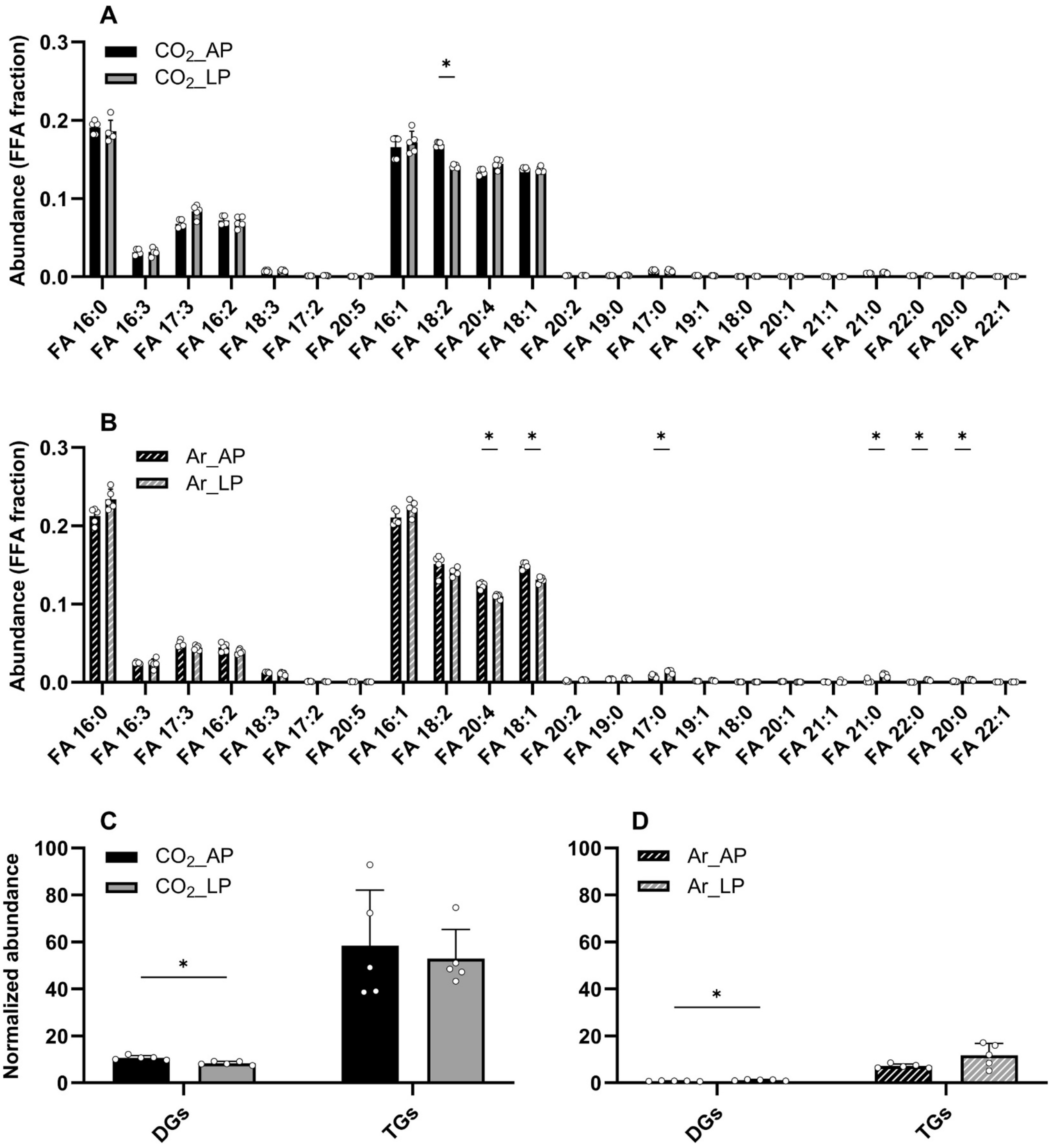

Fig. 6. Characterization of the reserve lipids of *Chlorella vulgaris* grown under either 1000 (AP) or 100 hPa (LP) of either 100 hPa of carbon dioxide and argon as a balance gas (CO_2), or argon only (Ar). A, B: Abundance (relative to their combined abundance) of the detected free fatty acids (FA). C, D: Abundance of diglycerides (DGs) and triglycerides (TGs). The significance of mean differences was tested with *t*-tests corrected for multiple comparisons using the Bonferroni-Dunn method. An asterisk indicates an adjusted *P* value below 0.05.

of *C. vulgaris*. It therefore appears that pressurizing Martian air, in an enclosed cultivation chamber, to approximately ten times its ambient pressure (rather than circa a hundred times, to reach an Earth-like pressure, which would be much more energy intensive) would be suitable as a cultivation atmosphere for this microalga during long-duration, crewed missions. Assuming water vapour is close to saturating, its partial pressure is approximately 50 hPa [77], leaving approximately 50 hPa of pCO_2, which is around the optimum for *C. vulgaris* [78]. Most of the other required nutrients could be sourced from the regolith and astronaut urine be used as a main nitrogen source [11]. As a proof-of-concept, the microalga was cultivated under 100 hPa of an atmosphere consisting of carbon dioxide and water vapour from medium evaporation, in a leachate of a simulant of Martian regolith containing diluted synthetic urine and no additional nutrients. Under these conditions, simulating a system combining ISRU with the recycling of metabolic waste, *C. vulgaris* grew vigorously.

The lipidome of *C. vulgaris* was modified by a decrease in pressure. Taken as a whole, the observed changes suggest an adaptation that preserves membrane viscosity. All do not, however, point in that direction, and a definitive conclusion in that regard could only be tentative. To shed further light on that matter, future studies may include the analysis of a more comprehensive set of lipids (e.g., including sterols) and include more direct assessments of membrane fluidity based, for instance, on fluorescence anisotropy. Either way, the changes are rather subtle: the nature of the carbon source had a larger effect on the lipidome than the change in pressure. No strong impact on practical applications, such as the production of dietary supplements or of biofuels, should therefore be expected. Here, parameters outside atmospheric conditions (e.g., pH, temperature, salinity and light intensity) were kept constant. It may be some of these parameters' effects interact with these of a low pressure. The combined fluidizing tendencies of a higher temperature and of a low pressure may for instance affect membranes to an extent that exceeds the capacity of the microalga for homeoviscous adaptation; or, the destabilizing effects on membranes of chaotropic salts (such as perchlorates, which are present in Martian regolith but not in the regolith simulant used here) may affect cells in a way which is synergistic with a low pressure. It would therefore seem worthwhile to test the effects of a low pressure under a range of values for other key cultivation parameters.

On a fundamental level, much is left to be discovered on the molecular effects of hypobaria on microorganisms. For the purposes of Mars exploration, however, our results suggest that relying on a low pressure could increase the cost-efficiency of microalgal cultivation.

CRediT authorship contribution statement

Mattia Casula: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Paola Scano:** Formal analysis. **Giacomo Fais:** Writing – review & editing, Supervision, Conceptualization. **Pierluigi Caboni:** Writing – review & editing, Supervision, Conceptualization. **Marcus Elvert:** Writing – review & editing. **Alessandro Concas:** Writing – review & editing, Supervision, Conceptualization. **Giacomo Cao:** Writing – review & editing, Supervision, Conceptualization. **Cyprien Verseux:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, 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.2026.104649.

Data availability

Data will be made available on request.

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