Ines Wagner

Clemens Posten

Department of Bioprocess
Engineering, KIT, Karlsruhe
Institute of Technology,
Karlsruhe, Germany

Research Article

Pressure reduction affects growth and morphology of *Chlamydomonas reinhardtii*

Cellular perception of pressure is a largely unknown field in microalgae research although it should be addressed for optimization of a photobioreactor design regarding typically occurring pressure cycles. Also for the purpose of using microalgae as basic modules for material cycles in controlled ecological life support systems, the absence of pressure in outer space or the low absolute pressures on other planets is an abiotic factor that needs to be considered for design of integrated microalgae-based modules. The aim of this work is to study the effects of lowered pressure and pressure changes on photosynthesis as well as morphology. Two *Chlamydomonas reinhardtii* wild-type strains were exposed to controlled pressure patterns during batch cultivations. Sudden pressure changes should test for existing threshold values for cell survival to mimic such events during space missions. Algae were grown inside a 2 L photobioreactor with an integrated vacuum pump ensuring constant pressures down to 700 mbar. Cultivation samples were analyzed for OD₇₅₀, cell dry weight, and morphology via light microscope. *Chlamydomonas reinhardtii* CC-1690 cells showed decreased growth rates, higher carbon dioxide uptake rates, and unchanged oxygen production rates at lower pressures. For sudden pressures changes in the range of 300 mbar no fatal threshold was determined. This study shows that pressure reduction affects growth, gas exchange rates, and morphology. Within the tested pressure range no fatal threshold value was reached.

Keywords: Carbon dioxide uptake rate / *Chlamydomonas reinhardtii* / Microgravity / Oxygen production rate / Photobioreactor / Pressure reduction

Received: May 6, 2016; *revised:* November 4, 2016; *accepted:* November 8, 2016

DOI: 10.1002/elsc.201600131

1 Introduction

As microalgae are interesting species for application in basic modules within controlled ecological life support systems for space [1], they have to withstand the environmental conditions present. Besides microgravity, radiation, hypoxia, and extreme temperature gradients there are low atmospheric pressures prevailing on other planets or even vacuum in space [2,3] influencing reactor design, for example, with respect to wall thickness. Also during parabolic flights, in which those systems have to be tested in, pressure reduction to almost half the atmospheric pressure on earth is acting on the hardware, software, and bio-

logical components. Even on the ground microalgae are exposed to pressure changes, local pressure peaks and reductions, within various types of photobioreactors (PBRs). These are caused either by peripheral devices such as pumps or by expansions or reduction of flow cross-sections or simply by hydrostatic pressure differences when a single cell moves along flow paths inside a reactor. The pressure aspect is also an important factor for possible locations of microalgae processes in high-altitude regions where ambient pressure decreases down to 60% of atmospheric pressure at sea level.

For the design of algae modules that shall be applied within life support systems and also for optimization of PBR geometries and hydrodynamics in general, major questions that should be taken into account are the perception of pressure and pressure changes by microalgae and the effects of this abiotic factor on photosynthetic efficiency, kinetics, physiology, and morphology of the cells.

Although much is known about the effects of other abiotic factors such as pH and temperature on the aforementioned parameters, literature on pressure perception and its effect on

Correspondence: Ines Wagner (ines.wagner@kit.edu), Institute of Engineering in Life Sciences, Department, Bioprocess Engineering, Karlsruhe Institute of Technology KIT, Fritz-Haber-Weg 2 Building 30.44, D-76131 Karlsruhe, Germany

Abbreviations: CDW, cell dry weight; CUR, carbon dioxide uptake rate; OPR, oxygen production rate; PBR, Photobioreactor; PFD, photon flux density; $\mu_{\max}$, maximum specific growth rate

microalgae and their photosynthetic performance is rare and there is no consistent conclusion. At least hints for the existence of mechanoreceptors in *C. reinhardtii* located within the cell membrane and on flagella can be found [4–6]. It is assumed that those receptors are involved in the regulation of cell volume and morphogenesis. Other mechanosensitive receptors are reported to be located within the cytoplasm and the chloroplast membrane playing a role for chloroplast organization [7].

Min et al. [8] developed a microfluidic experimental system for distortion of the cell membrane by compression and tested it on *C. reinhardtii* cells provoking deflagellation of the cells, a higher number of daughter cells and also smaller cells after cell division. Furthermore, cells under compression in that apparatus showed higher lipid productivities than under normal conditions. For green algae, other than *C. reinhardtii*, reported effects of higher pressure and pressure changes are the disassembly and uptake of flagella inside the cell (reported for *Polytomella agilis* [9]), an inhibition of photosynthesis (lower CO₂ uptake, lower O₂ production, and reduced growth) reported for different algae and diatom species [10] as well as *Scenedesmus obliquus* [11] as reaction to high hydrostatic pressures. The latter reported the reversibility of those effects on photosynthesis.

For effects of lower pressure on algae cells no studies have been published yet. Moreover, characteristics of *C. reinhardtii* such as the presence of flagella, the ability for gravitaxis as well as gyrotaxis should also be taken into account. The presence and the composition of the cells flagella is important in so far as experiments indicate them being weak spots for cell wall integrity resulting in the inability of the cell to maintain a turgor pressure [12]. So far, no clear conclusion can be found in literature concerning this aspect. Furthermore, as it is assumed that mechanoreceptors also play a role for perception of gravity and orientation relating to it, this could also be important for revealing possible effects of lowered pressure.

Pressure is an important abiotic factor having influence on biological systems. For development of life support systems based on microalgae modules it is important to know which are critical, fatal thresholds or if slight deviations from earth's atmospheric pressure are beneficial for photosynthetic activity. Also concerning effects such as agglomeration and subsequent sedimentation of microalgae within ground PBRs, reasons for this behavior are highly discussed. Fast pressure changes alone or in combination with other factors might also be responsible for those phenomena leading to reduced productivity of microalgae based processes.

This work aims at studying the effects of moderate pressure reductions, also measured within parabolic flights, on growth, CO₂ uptake, and the oxygen production of the green microalga *C. reinhardtii*, wild-type CC-1690. Also effects on morphology shall be elucidated via light microscopy. Studies should show if fatal threshold values of pressure reduction can be identified and if and within which time frame adaptation mechanisms permit cell survival. Pressure borders applied for these experiments (1000–700 mbar) were a compromise between technical feasibility with the PBR used and relevant pressures for high-altitude terrestrial microalgae cultivations (600 mbar) and the proposed 54 kPa by NASA for lunar/extraterrestrial bases as a compromise between engineering and human demands [13].

2 Materials and methods

2.1 Strains and media used

Chlamydomonas reinhardtii CC-1690 wild-type mt+ (Sager 21 gr) [14, 15] was used as a model organism in this study. For preculturing and maintenance the algae were grown pure photoautotrophically on TP-NO₃ (tris phosphate nitrate) medium (per 1000: 7.5 mL KNO₃ (1 M stock), 10 mL CaCl₂ (0.034 M stock), 10 mL MgSO₄ (0.04 M), 8.33 mL phosphate solution (0.08 M K₂HPO₄ and 0.05 M KH₂PO₄ stock), 10 mL TRIS (2 M stock), 7 mL trace elements (each 1 mL) according to Kropat et al. [16], without ammonium, pH at 7.2 at low light intensities (photon flux density, PFD of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) with warm-white LED illumination.

Chlamydomonas reinhardtii wild-type wt13 [17] originating from the original Ebersold/Levine line 137c (CC-125mt+) [15, 18] was used in order to study agglomeration effects of pressure reduction in comparison to CC-1690 cells that tend to form palmelloids and secondary aggregates [19] very easily in liquid cultures. For preculturing and cultivations inside the stirred PBR the alga was grown photoautotrophically on TP-medium [19] under the same conditions as CC-1690 cells.

2.2 Model reactor for batch cultivations with reduced pressure

For batch cultivations under reduced pressure, down to absolute pressures of 700 mbar, a stirred tank 2 L model reactor (KLF 2000, Bioengineering AG, Switzerland) with a glass vessel was chosen, equipped with a customized cylindrical white LED illumination device based on SMD-LEDs (surface-mounted device). Four cultivations were performed under warm white illumination (1000, 900, 800, and 700 mbar) and three batches were conducted with cold white illumination (1000, 800, and 700 mbar) whereas the total PFD was the same in both cases. Details of the illumination device have been explained in [20]. Control of LEDs was realized via the process control system BioProCon based on LabView, National Instruments. For pressure reduction and control a vacuum pump (KnF LAB SC920) was installed inside the exhaust gas loop.

2.3 Cultivations in model reactor

Cells were cultivated photoautotrophically at PFDs of 200 $\mu\text{E m}^{-2} \text{s}^{-1}$. Cells were inoculated from precultures at approximate cell dry weight (CDW) concentrations of 0.2 g/L. Aeration was achieved by sparging 4.8% CO₂ v/v at a total aeration rate of 63 mL/min into the reactor with the intention to ensure CO₂ saturated conditions above 0.5–1% dCO₂ in the liquid also for lower pressures down to 700 mbar absolute with the corresponding solubilities according to Henry's law (Eq. 1).

Equation 1: Henry's law for gas solubility.

$$c_i = \frac{p_i}{H_i}$$

Figure 1. Batch cultivation at 700 mbar pressure with dissolved gas concentrations. Overview of process data from a batch cultivation at 700 mbar pressure. The stepwise pressure reduction from atmospheric conditions to the set point after inoculation is displayed by the pressure sensor signal.

Where c_i is the concentration of dissolved gas i in the liquid [mol/L], p_i is the partial pressure of the gas i in the gas phase [atm], and H_i is the Henry coefficient of the component i [L · atm/mol], which is dependent on pressure, temperature, and salinity of the liquid phase. According to Dalton's law (Eq. 2), the total pressure of a gas mixture is the sum of the partial pressures of the gases.

Equation 2: Dalton's law of partial pressures.

$$p = \sum p_i$$

For small pressures differences the influence of the overall pressure on the Henry coefficient can be neglected and for this reason the calculation of gas solubilities for pressures down to 700 mbar was performed with Henry coefficients for atmospheric pressure of 1013 mbar.

Flow rates were controlled via two mass flow controllers (MKS Instruments) that were calibrated for each absolute pressure applied so that the volumetric gas composition was the same for all pressures applied. Temperature was kept constant at 25°C; pH was controlled at 7.2 by dosing 2 M HCl and 2M NaOH via the integrated controllers of the type IFM, Bioengineering AG. Mixing and dispersion of gas bubbles was realized by two six-blade Rushton stirrers at 250 rpm. Probes for pH, pO_2 , and pCO_2 were used to measure dissolved gas concentrations in the liquid phase and pH. Additionally, a pressure sensor and pressure gauge were integrated in the exhaust gas loop. The exhaust gas was analyzed batchwise (every 15 min intervals) for carbon dioxide and oxygen concentration via a Maihak Multor 610 system. Around 12 h after inoculation, before cells started to grow exponentially, pressure was reduced stepwise to the respective absolute pressure desired. Batch cultivations at 1 atm, 900, 800, and 700 mbar were conducted where pressure, once lowered, was kept constant for the rest of the cultivation. Figure 1 exemplarily shows online data of a batch cultivation at 700 mbar pressure. The signal of the pressure sensor shows how pressure was reduced stepwise after inoculation and how concentrations of dissolved gases develop. As the medium was not saturated yet with the gas mixtures there is an increase in pCO_2 in the beginning. Con-

cerning CO_2 limitation it is shown that even during phases where carbon dioxide uptake rate (CUR) was maximum pCO_2 values are higher than the critical pCO_2 of 0.5–1% in the liquid medium whereas optimum growth rates are considered to correlate with CO_2 partial pressures above 0.5%. This critical concentration can be extracted from CO_2 kinetics [21]. Therefore throughout all the experiments of this study CO_2 limitation conditions can be excluded even though gas solubility decreased with lowered pressure. Furthermore, pressure was changed stepwise during the exponential phase of one cultivation to determine a fatal threshold value and below that value, adaptation of cells.

2.4 CDW determination

In order to determine CDW, concentration samples were taken during the linear growth phase and CDW was calculated by the gravimetric method. For dry weight measurements, a total volume of 90 mL of algal suspension was centrifuged in 30 mL aliquots for 30 min at 4500 rpm and 4°C (Rotina 420R, Hettich Lab Technology, Germany) in predried and weighted stainless steel centrifugation tubes. The supernatant was discarded and the pellet was resuspended in 30 mL deionized water and the centrifugation step was repeated. Pellets were washed twice to remove salt residues and the pellet was dried for 48 h at 80°C. The tubes were cooled down in a desiccator and weighed again. Every sample was measured in triplicate.

A correlation factor was calculated between CDW concentration and the respective OD. This correlation factor was used to convert daily measured OD of the samples to CDW concentration. OD was measured offline at a wavelength of 750 nm using a UV-Vis-spectrophotometer (Lambda 35, Perkin Elmer, USA).

2.5 Cell morphology via light microscopic analysis

Morphology and cell diameter was determined regularly, especially before and after decrease of pressure by means of a light microscope (Zeiss AXIO Scop. A1) and the corresponding

software (Infinity Analyze, Lumenera Corporation). Samples were analyzed within 5 min after a pressure step was exerted whereas at least a number of 20 cells were monitored for cell diameters.

2.6 Starch analysis

A sulfur anthrone method was used to analyze the starch content of *C. reinhardtii* cells during the linear growth phase in triplicates for each batch cultivation in the respective pressure environment. With deionized water the cell pellets were resuspended and diluted to a CDW of 0.2 to 0.5 g/L to achieve a volume of 400 μ L. Eight hundred microliters of anthrone reagent was added, which was composed of 1 g/L anthrone in 96% sulphuric acid. The mixture was gently mixed and the sample was put on ice for 5 min. The sample was then placed in a thermomixer for 16 min at 95°C and 300 rpm. Afterwards the tubes were cooled down on ice for another 5 min followed by centrifugation (Mikro 220R, Hettich Lab Technology) for 10 min, at 4°C and 10 000 rpm. The supernatant was then used for the absorption measurement at 625 nm against a blank consisting of 400 μ L deionized water and 800 μ L anthrone reagent. A calibration curve for different starch concentrations and the corresponding A625 was established by using solutions of 0.01 to 0.15 g/L starch concentration. Specific starch contents were calculated with the respective CDW concentrations at the respective times.

3 Results and discussion

Experiments with *C. reinhardtii* CC-1690 and wt13 were conducted in batch mode by starting with atmospheric pressure and lowering absolute pressure to a particular value kept constant for the rest of the cultivation. During one experiment pressure changes were applied during the exponential growth phase to test for cells adaptation and a possible fatal threshold.

3.1 Pressure reduction affects growth rates and CUR values

Figure 2 shows the results of cultivations with CC-1690 cells in batch mode under different constant pressures, lower than atmospheric pressure, at the same PFD of 200 μ E m⁻² s⁻¹ regarding maximum specific growth rate $\mu_{\max}$, maximum volumetric and specific oxygen production rate (OPR), maximum volumetric and specific CUR, and specific starch concentrations. The experiments were conducted with two different LED panels, four of them with warm white light, three of them with cold white light.

Figure 2A shows that the $\mu_{\max}$ in control accounts for 0.060 h⁻¹ (warm white) and 0.050 h⁻¹ (cold white) and lowers for decreasing absolute pressure for both lighting conditions (900 mbar: $\mu_{\max}$ = 0.051 h⁻¹ warm white light; 800 mbar: $\mu_{\max}$ = 0.045 h⁻¹, 0.035 h⁻¹ cold white) with a minimum at 700 mbar at 0.035 h⁻¹ (warm white, ww), 0.033 h⁻¹ (cold white, cw). For maximum values of volumetric OPRs (compare to Fig. 2B), the assumption that lower pressures lead to a change in volumetric

OPRs, cannot be verified by the experiments within the limits of measurement accuracy for absolute pressures down to 700 mbar for both white lights. All volumetric OPR_{max} values lie within the range between 0.71 and 0.95 mmol O₂ L⁻¹ h⁻¹ (warm white), respectively 1.15 and 1.56 mmol O₂ L⁻¹ h⁻¹ for cold white light (1000 mbar: 0.71 mmol O₂ L⁻¹ h⁻¹ (ww), 1.23 mmol O₂ L⁻¹ h⁻¹ (cw); 900 mbar: 0.90 mmol O₂ L⁻¹ h⁻¹ (ww); 800 mbar: 0.95 mmol O₂ L⁻¹ h⁻¹ (ww), 1.56 mmol O₂ L⁻¹ h⁻¹ (cw); 700 mbar: 0.77 mmol O₂ L⁻¹ h⁻¹ (ww), 1.15 mmol O₂ L⁻¹ h⁻¹ (cw)). Specific OPR values slightly increase with decreasing pressure for warm white light, which is not shown for cold white light. Values account for: 1.15 mmol O₂ g⁻¹ h⁻¹ (ww), 1.89 mmol O₂ g⁻¹ h⁻¹ (cw) (1000 mbar), 1.58 mmol O₂ g⁻¹ h⁻¹ (ww, 900 mbar), 1.81 mmol O₂ g⁻¹ h⁻¹ (ww), 2.12 mmol O₂ g⁻¹ h⁻¹ (cw) (800 mbar), and 1.97 mmol O₂ g⁻¹ h⁻¹ (ww), 1.51 (cw) mmol O₂ g⁻¹ h⁻¹ (cw) (700 mbar). Further experiments should verify these results for oxygen production of *C. reinhardtii*.

For maximum volumetric CUR values (Fig. 2C), a dependency on absolute pressure was detected for both lighting colors: values increase from atmospheric pressure with a volumetric CUR_{max} of 1.04 mmol CO₂ L⁻¹ h⁻¹ (ww), 0.86 mmol CO₂ L⁻¹ h⁻¹ (cw) to 3.6 mmol CO₂ L⁻¹ h⁻¹ (ww), and 3.82 mmol CO₂ L⁻¹ h⁻¹ (cw) for 700 mbar (900 mbar: 1.73 mmol CO₂ L⁻¹ h⁻¹ (ww), 800 mbar: 2.29 mmol CO₂ L⁻¹ h⁻¹ (cw)). The same tendency is shown for the specific CUR values for both light colors: they increase from 0.92 mmol CO₂ g⁻¹ h⁻¹ (ww), 0.98 mmol CO₂ g⁻¹ h⁻¹ (cw) at atmospheric pressure up to 5.14 mmol CO₂ g⁻¹ h⁻¹ (ww), and 4.19 mmol CO₂ g⁻¹ h⁻¹ (cw) for 700 mbar (900 mbar: spec. CUR_{max} = 3.04 mmol CO₂ g⁻¹ h⁻¹ (ww), 800 mbar: 2.16 mmol CO₂ g⁻¹ h⁻¹ (cw)). Figure 2D shows specific starch concentrations for the tested pressures. Whereas results under warm white illumination show a minimum at 800 mbar, results under cold white light are in the same range: for 1000 mbar c_{starch} = 0.46 $\pm$ 0.01 g g⁻¹ (ww), 0.45 $\pm$ 0.01 g g⁻¹ (cw), 900 mbar: 0.34 $\pm$ 0.01 g g⁻¹ (ww), 800 mbar: 0.20 $\pm$ 0.09 g g⁻¹ (ww), 0.46 $\pm$ 0.01 (cw) g g⁻¹ and 700 mbar: 0.31 $\pm$ 0.01 g g⁻¹ (ww), 0.47 $\pm$ 0.02 g g⁻¹ (cw).

Results indicate that at least for CO₂, there seems to be an influence of pressure on gas exchange rates. As experiments were conducted under CO₂ saturating conditions, also for the lowest pressure applied, a decreased specific growth rate provoked by CO₂ limitation as a result from lower gas solubilities at lower pressure at the same volumetric inlet gas composition can therefore be excluded. As CUR and OPR values were calculated based on exhaust gas analysis at atmospheric pressure and not on CO₂ and O₂ sensor data, there is no influence of reduced gas solubility for lower pressures on these calculated values. Concerning dissolved CO₂ concentrations in the liquid there is a reduction of dCO₂ at lower pressure as a result of reduced solubility (compare to Fig. 1). However, the inlet CO₂ gas concentration of 4.8% v/v was chosen high enough to ensure a sufficient CO₂ concentration in the liquid (above the critical 0.5 to 1% dCO₂) even at lowest tested pressures of 700 mbar. Above this concentration, according to CO₂ kinetics, optimal growth rates are enabled.

Further experiments should verify these preliminary results and should reveal reasons why OPRs seem to be unaffected also in regard to the application of microalgae based modules in life support systems for space application. As analysis of specific starch content of the cells yielded similar results for those

Figure 2. Influence of lowered pressure on growth and photosynthesis of *C. reinhardtii* CC-1690 cells during batch cultures. (A) Dependency of μ_{max} on pressure. (B) Influence of pressure reduction on maximal volumetric (darker columns) and specific (brighter columns) OPR and (C) on maximal volumetric (black columns) and specific (grey columns) CUR. (D) Specific starch concentrations in dependence on lowered pressure during batch cultivations. Experiments were conducted in batch mode at a PFD of $200 \mu\text{E m}^{-2} \text{s}^{-1}$, and 4.8% v/v CO_2 , at $T = 25^\circ\text{C}$, using TP- NO_3 medium. Two different light sources were used: warm white and cold white LED light.

different absolute pressures tested (compare to Fig. 2D), future analysis should also focus on possible enhanced lipid production of this alga under different pressures.

3.2 Cells survive sudden pressure changes up to 300 mbar

In order to test for fatal threshold values of sudden pressure changes, nine pressure steps at 1 h intervals were performed during the exponential phase of a batch culture. Figure 3A shows the overview of this batch cultivation with CC-1690 cells where online OD signals, pressure, and the natural logarithm of these online OD values (as $\ln(\text{OD})$) as well as pCO_2 and pO_2 signals (both in %) are plotted versus process time. By regarding $\ln(\text{OD})$ values, μ_{max} of exponential growth phase can be determined as the slope of the linear curve and so possible changes of μ within this exponential phase with maximum growth rates should be detected in response to these sudden pressure drops. Figure 3B shows an enlarged section of part A where the phases of sudden pressure changes are more easily visible, numbers refer to microscopic pictures in Fig. 3C. For dissolved gases the stepwise reduced pressure and therefore reduced solubilities are clearly

seen from the signals, whereas the steps are more easily detected for dissolved O_2 , as the optic sensor has a shorter response time than the pCO_2 sensor.

As can be seen in Fig. 3A and B no influence of algal growth (μ_{max}) can be detected. Even after the last pressure change no reduction of exponential growth rate is visible from online process data. Cells survived those sudden pressure changes (highest step with 300 mbar) and no fatal threshold was reached during this experiment indicating that cells can tolerate pressure changes within the tested magnitudes.

3.3 Lower pressures and pressure changes affect cell morphology

Figure 3C shows that a pressure difference of 50 mbar and increasing step sizes result in cell swelling and also an increase in cell diameter. Under atmospheric pressure (1) cells mostly show diameters around $10 \mu\text{m}$, whereas for the highest pressure difference exerted on the cells (6), diameters up to $18 \mu\text{m}$ can be seen. Furthermore, the number of daughter cells is increased with increasing pressure steps: whereas under atmospheric conditions two or four daughter cells per cell division prevail, there

Figure 3. Influence of sudden pressure changes during exponential growth phase of batch cultures on cell morphology and survival of *C. reinhardtii* CC-1690 cells. (A) Overview of process data from batch culture with pressure changes starting at atmospheric pressure. (B) Section of diagram in panel A showing numbered pressure steps correlating to microscopic pictures in part C (1), ($\Delta p = 0$ mbar) from 30 mbar step size down to 970 mbar (2), ($\Delta p = 30$ mbar), increasing negative step size each time by 10 mbar (3), ($\Delta p = 50$ mbar), down to 670 mbar absolute pressure (4), ($\Delta p = 80$ mbar) followed by positive step sizes of 100 and 200 mbar (5), ($\Delta p = 200$ mbar), ending with a negative step size of 300 mbar from 1000 mbar down to 700 mbar (6), ($\Delta p = 300$ mbar). (C) Light microscopic pictures (bright field, 400× magnification) taken immediately after the stepwise changes of pressure starting with a microscopic picture taken at atmospheric pressure; each picture is equipped with scales indicating randomly chosen cell diameters (measured from top apical side with flagellas to basal side of the cell); green boxes on the right upper corner show the applied Δp for each step respectively. (D) Light microscopic picture (bright field, 400× magnification) taken at 700 mbar after pressure changes were finished. Cells were more prone to breaking while handling and samples showed cell debris.

Figure 4. Influence of lowered pressure on formation of palmelloids and aggregates of *C. reinhardtii* CC-1690 and *C. reinhardtii* wt13 cells during batch cultures at 700 mbar pressure. (A) Microscopic pictures (100× bright field) of *C. reinhardtii* CC-1690 cells in preculture at atmospheric pressure (1), in reactor at atmospheric pressure before stepwise pressure drop (2), directly after stepwise pressure reduction from 1000 to 700 mbar (3), cells at the end of the batch cultivation at 700 mbar (4). (B) Microscopic pictures (100× bright field) of *C. reinhardtii* wt13 cells in preculture at atmospheric pressure (5), in reactor at atmospheric pressure before stepwise pressure reduction (6), directly after stepwise pressure drop from 1000 to 700 mbar (7), cells at the end of the batch cultivation at 700 mbar (8).

are mostly eight daughter cells observed for pressure differences higher than 80 mbar (4). This effect is detected after cells were exposed to a step of 80 mbar which means that lower and earlier pressure steps were able to trigger this reaction. However, as microscopic analysis only involved a small number of cells, future studies should verify these findings by particle size analysis together with microscopic image analysis software.

For *C. reinhardtii* cells, it is known that cells divide into, either two, four, or eight daughter cells [19] where for optimal conditions also 16 daughter cells are reported [22]. In general this is known as multiple fission [23], and higher light intensities and optimal temperatures, as the main key natural components, are assumed to lead to a higher number of resulting cells after division. Thereby light conditions seem to be the most important factor influencing multiple fission that is seen as an adaptation mechanism to light cycles occurring in nature. Signals relating cell-cycle progression and photosynthesis are assumed to exist. Moreover, cell size seems to be an important factor deciding over the division number [23].

Relating to the results of our study, a higher number of daughter cells after division was also described by Min et al. [8] for *C. reinhardtii* cells after distortion of their cell membrane. This is remarkable as a higher number of resulting cells generally is originating from environmental conditions enabling higher growth rates where multiple fission is an evolutionary advantage over binary fission [23].

After the sudden pressure changes exerted on the cells were finished, pressure was kept constant at 700 mbar. Further samples were taken several hours after and cells showed a higher sensitivity during handling compared to cells cultivated from the beginning under such lowered absolute pressures, resulting in breaking of the cell wall and cell death where cell debris was seen with the help of light microscopic analysis (compare to Fig. 3D). This was a rather qualitative observation and should be further studied in future experiments by quantitative means, e. g. shear stress tests. Concerning aggregate formation as response to sudden pressure changes, there are no aggregates detected for any of the Δp tested on CC-1690 cells.

To test whether lower constant pressures are responsible for triggering aggregation formation of *C. reinhardtii* cells, batch cultivations of two wild-type strains of this alga, CC-1690 and wt13, were conducted under a constant lowered pressure of 700 mbar. The second wild-type strain, wt13, was chosen, as for CC-1690 cells it is known that cells tend to build so called palmelloids [19] as well as secondary aggregates, triggered by various abiotic (*T*, pH) and biotic (bacterial contamination) factors where dependencies and causes are not yet fully revealed and understood. For wt13 cells no tendency for palmelloids formation and strong aggregate formation is described or has been detected in earlier works. In Fig. 4A, microscopic pictures of CC-1690 cells are shown: (i) in preculture at atmospheric p before inoculation; (ii) directly after inoculation, inside the PBR at atmospheric pressure; (iii) after the stepwise pressure reduction from 1000 to 700 mbar; (iv) at the end of the cultivation at 700 mbar. In Fig. 4B the same phases during a wt13 cultivation under 700 mbar were analyzed by microscopy denoted with numbers five to eight. For the CC-1690 cells, it can already be seen in the preculture that larger aggregates were prevailing, which seem to increase in diameter after inoculation, still under normal pressure. With stepwise pressure reduction these seem to suspend whereas at the end of the cultivation at 700 mbar smaller aggregates of dividing cells prevail, smaller than those present in the beginning. In contrast to that, wt13 cells show neither palmelloids nor secondary aggregates for any of the phases monitored, from preculturing, over pressure reduction to the end of the cultivation at 700 mbar pressure. This shows that at least for both of the strains of *C. reinhardtii* tested in this study, constant lower pressure does not trigger aggregate formation.

4 Concluding remarks

This study shows that a lowered pressure, down to 700 mbar absolute pressure, decreases $\mu_{\max}$ of *C. reinhardtii* CC-1690 cells, whereas volumetric and specific CUR increases; volumetric and specific OPR values stay at the same level indicating no influence of lowered pressure on light reaction of photosynthesis,

and to a small degree the impairment of growth. Neither an increase of OPR nor a reduction of it was caused by these sublethal stresses of lowered pressure. A decreased $\mu_{\max}$ for cultivation of cells at lowered pressure might be seen as a stress reaction to the elongation of the cell membrane and wall in accordance to literature [10, 11]. Open questions remain for starch production, as in this study no change of specific starch content could be detected for cells grown at lower pressures compared to the control at atmospheric pressure. Future experiments should also focus on a possible changed production of lipids as reported for cells after distortion of their membrane [8] and also discover long-term effects of lowered pressure during continuous cultivations.

Furthermore, it can be concluded that at least for both of these wild-type strains of *C. reinhardtii* tested, lowered pressure, or sudden pressure drops or changes in the range of 300 mbar are not causing aggregate formation of cells, which was presumed before. No lethal threshold of sudden pressure changes was reached with the conducted experiments, although cells are more susceptible to shear and mechanical stress after exposure to a sudden pressure change of 300 mbar.

For a possible application of microalgae within life support systems it is important to note that photosynthetic OPR seems not to be affected down to pressures of 700 mbar; moreover, no cell death was provoked by sudden pressure changes in the range of 300 mbar.

However, deeper knowledge on how pressure and pressure changes are perceived by microalgae and which effects on photosynthetic activity, physiology, and morphology are resulting is necessary to employ them for space application and also further optimize PBRs on the ground.

Practical application

This study deals with the effects of pressure reduction and sudden pressure changes on growth and morphology of the green alga *Chlamydomonas reinhardtii*. Information about effects of this important abiotic factor is essential for the design of life support systems with integrated microalgae modules for remote application such as outer space or other planets. Also for designing bioreactors for application on ground where certain pressure cycles resulting from pumps or just different orientations in the hydrostatic pressure field can occur, this knowledge is crucial. Additionally, high-altitude near-tropical regions are regarded as potential sites for microalgae-based processes where pressure or more precise a lower pressure is a critical factor possibly having an influence on productivity; for regions at 4000 m altitude pressure decreases down to almost 600 mbar.

This work is part of the ModuLES project (grant number 50 WB 1263) and is funded by DLR (Deutsches Zentrum für Luft- und Raumfahrt), German Aerospace Center.

The authors would like to thank the student Manuel Merkel for his valuable experimental support.

The authors have declared no conflict of interest.

5 References

- [1] Gitelson, I. L., Terskov, I. A., Kovrov, B. G., Sidko, F. Y. et al., Life support system with autonomous control employing plant photosynthesis. *Acta Astronaut.* 1976, 3, 633–650.
- [2] Wang, G. H., Li, G. B., Li, D. H., Liu, Y. D. et al., Real-time studies on microalgae under microgravity. *Acta Astronaut.* 2004, 55, 131–137.
- [3] Rea, G., Esposito, D., Damasso, M., Serafini A. et al., Ionizing radiation impacts photochemical quantum yield and oxygen evolution activity of photosystem II in photosynthetic microorganisms. *Int. J. Radiat. Biol.* 2008, 84, 867–877.
- [4] Yoshimura, K., A novel type of mechanoreception by the flagella of *Chlamydomonas*. *J. Exp. Biol.* 1996, 199, 295–302.
- [5] Fujiu, K., Nakayama, Y., Iida, H., Sokabe, M., Yoshimura, K., Mechanoreception in motile flagella of *Chlamydomonas*. *Nat. Cell Biol.* 2011, 13, 630–632.
- [6] Yoshimura, K., Mechanosensitive channels in the cell body of *Chlamydomonas*. *J. Membr. Biol.* 1998, 166, 149–155.
- [7] Nakayama, Y., Fujiu, K., Sokabe, M., Yoshimura, K., Molecular and electrophysiological characterization of a mechanosensitive channel expressed in the chloroplasts of *Chlamydomonas*. *Proc. Natl. Acad. Sci. U. S. A.* 2007, 104, 5883–5888.
- [8] Min, S. K., Yoon, G. H., Joo, J. H., Sim, S. J. et al., Mechanosensitive physiology of *Chlamydomonas reinhardtii* under direct membrane distortion. *Sci. Rep.*, 4, 4675; DOI: 10.1038/srep04675 (2014).
- [9] Brown, D. L., Rogers, K. A., Hydrostatic pressure-induced internalization of flagellar axonemes, disassembly, and reutilization during flagellar regeneration in *Polytomella*. *Exp. Cell Res.* 1978, 117, 313–324.
- [10] Pope, D. H., Berger, L. R., Algal photosynthesis at increased hydrostatic pressure and constant pO₂. *Arch. Microbiol.* 1973, 89, 321–325.
- [11] Schreiber, U., Vidaver W., Hydrostatic pressure: A reversible inhibitor of primary photosynthetic processes. *Z. Naturforsch. C.* 1973, 28, 704–709.
- [12] Bisson, M. A., Kirst, G. O., Osmotic acclimation and turgor pressure regulation in algae. *Naturwissenschaften* 1995, 82, 461–471.
- [13] NASA, Advanced Life Support Program. *Bounding the Design Space for Space-Craft Internal Atmosphere Pressure and Composition*. Office of Biological and Physical Research, NASA Headquarters, Houston, TX 2004.
- [14] Sager, R., Inheritance in the green alga *Chlamydomonas reinhardtii*. *Genetics* 1955, 40, 476–489.
- [15] Pröschold, T., Harris, E. H., Coleman, A. W., Portrait of a species: *Chlamydomonas reinhardtii*. *Genetics* 2005, 170, 1601–1610.
- [16] Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent P. et al., A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J.* 2011, 66, 770–780.
- [17] Schönfeld, C., Wobbe, L., Borgstädt, R., Kienast, A. et al., The nucleus-encoded protein MOC1 is essential for mitochondrial light acclimation in *Chlamydomonas reinhardtii*. *J. of Biological Chemistry* 2004, 279, 50366–50374.

- [18] Harris, E. H., *Chlamydomonas* as a model organism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 2001, 52, 363–406.
- [19] Harris, E. H., Stern, D. B., Witman, G. (Eds.), *The Chlamydomonas Sourcebook*, 2nd ed., Academic Press, Amsterdam, Boston 2009.
- [20] Jacobi, A., Steinweg, C., Rosello-Sastre, R., Posten C., Advanced photobioreactor LED illumination system: Scale-down approach to study microalga growth kinetics. *Eng. Life Sci.* 2012, 12, 621–630.
- [21] Schirmer, M., Posten C., Chapter three—Modeling of microalgae bioprocesses. in: *Advances in Chemical Engineering* 48, Elsevier 2016, pp. 151–184.
- [22] Vítová, M., Bišová, K., Umyšová, D., Hlavová, M. et al., *Chlamydomonas reinhardtii*: Duration of its cell cycle and phases at growth rates affected by light intensity. *Planta* 2011, 233, 75–86.
- [23] Bisova, K., Zachleder V., Cell-cycle regulation in green algae dividing by multiple fission. *J. Exp. Bot.* 2014, 65, 2585–2602.