Non-axenic microalgae cultivation in space – Challenges for the membrane µgPBR of the ISS experiment PBR@LSR

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The spaceflight experiment PBR@LSR (Photobioreactor at the Life Support Rack) shall demonstrate for the first time the technology and performance of a hybrid life support system – a combination of physico-chemical and biotechnological components – under real space conditions during an operation period of 180 days. To be launched to the International Space Station (ISS) in 2018, PBR@LSR combines the carbon dioxide (CO_2) concentrator of ESA's Life Support Rack (LSR) with an advanced microalgae photobioreactor (PBR). Accommodated in the Destiny module, LSR will concentrate CO_2 from the cabin atmosphere. A dedicated interface allows the utilization of the highly concentrated surplus CO_2 for the cultivation of the green microalgae species *Chlorella vulgaris*. Current research at the University of Stuttgart focuses on the fundamental investigation and optimization of non-axenic cultivation processes in µg capable membrane PBRs. This includes the characterization of influences of accompanying bacteria on the non-axenic microalgae culture stability within the PBR suspension loop, photosynthetic capacity as well as overall biomass composition. This paper discusses in general possible influences of emerging bacteria- or algae induced biofilm formation and cell clustering due to non-axenic processing on the long term functionality of µg adapted PBR systems, e.g. PBR@LSR.

Nomenclature

A	=	absorbance
ASL	=	algae suspension loop
CO_2	=	carbon dioxide
DLR	=	German Aerospace Center (Deutsches Zentrum für Luft- und Raumfahrt)
EC	=	experiment compartment
ECLSS	=	environmental control and life support system
EPS	=	extracellular polysaccharides
FM	=	flight model

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I/F = interface
IRS = Institute of Space Systems (Institut für Raumfahrtsysteme)
ISS = International Space Station
LiED = Liquid Exchange Device
LSR = Life Support Rack
NASA = National Aeronautics and Space Administration
O_2 = oxygen
OD = optical density
PBR = photobioreactor
PBR@LSR= Photobioreactor at the Life Support Rack
PQ = photosynthetic quotient [/]
Re = Reynolds number [/]
X = dry biomass [g/l]
µg = microgravity
µgPBR = microgravity adapted photobioreactor

I. Introduction

Space agencies plans for future human space exploration include the return to the Moon, Near Earth Objects and Mars[1-3]. These long-term, deep-space mission scenarios require environmental control and life support systems (ECLSS) with a high level of regeneration and closure in order to minimize resupply demands. Therefore, improvements in physicochemical LSS technologies are needed and the integration of biological components should be considered. Biological components offer the possibility to regenerate oxygen (O_2) from carbon dioxide (CO_2) and are additionally able to close the carbon loop by *in-situ* food production. In this scope, the use of microalgae seems highly beneficial as they are very efficient in terms of water demand, light utilization and higher growth rates compared to plants[4,5].

Newly developed technologies must be carefully analyzed and tested before applying them in spaceflight missions. Today, the International Space Station (ISS) offers the great opportunity to provide a testbed to demonstrate and characterize new technologies. Although the ISS is not a perfect analogue for deep space, it is much closer than the environment on Earth and provides invaluable operational experience[6].

The ISS experiment *Photobioreactor at the Life Support Rack* (PBR@LSR; Fig 1) shall demonstrate the synergetic combination of the biotechnological component photobioreactor (µgPBR) and the physicochemical Life Support Rack (LSR), formerly known as *Advanced Closed Loop System* (ACLS)[7-12]. Inside the PBR the microalgae *Chlorella vulgaris* is cultivated in a non-axenic manner. Fig. 2 shows the process schematic of PBR@LSR.

The PBR@LSR experiment and its development was initiated in 2014 by the German Aerospace Center (DLR) and the Institute of Space Systems (IRS) of the University of Stuttgart with Airbus Defence and Space as prime for the flight hardware. The technology demonstration experiment will be launched to ISS in November 2018 and shall demonstrate the functionality and feasibility of a hybrid LSS in a real space environment during six months operation. The experiment shall also show that the long-term cultivation of

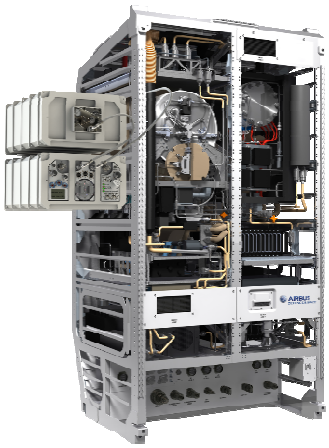

Figure 1: µgPBR (left) and LSR (right) = PBR@LSR.

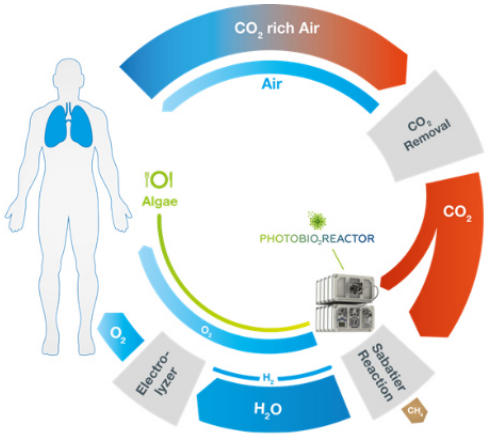

Figure 2: Process schematic of the LSR with the added PBR experiment.

C.vulgaris with a high biomass density and turn over is possible in space. Surplus CO_2 from LSR (or as a backup solution from a buffer) shall be delivered to the PBR without affecting LSR performance. Inside the PBR, the CO_2 is converted into O_2. The O_2 (together with residual air) is then delivered to cabin air. Regular liquid exchanges, performed with a special liquid exchange device, allow harvesting of algal biomass and provide fresh nutrients. Several algae samples will be taken at different time intervals and returned to ground for further analyses (the determination of µg ($<10^{-3}$ g) and radiation influence on *C.vulgaris* physiology). Several sensors allow evaluation of photosynthetic and the facility performance of the experiment.

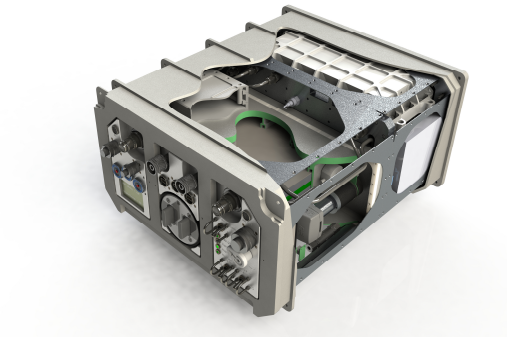

Figure 3: FM drawing of µg-adapted membrane PBR in MDL-1.

The main research focuses of PBR@LSR are: the verification of the hybrid system approach, the stability of the gas conversion (CO_2 from LSR into O_2 to cabin air), the production of algal biomass and operational handling in a non-axenic cultivation system. For the first time, these flight data will reveal the long-term performance, the system stability and reliability as well as the biological stability of a high density and high turn over algae culture (embedded in a synergistically integrated biotechnological LSS component) under space conditions. Besides the introduction of the general µgPBR system design and the used microalgae strain *C.vulgaris* SAG 211-12, in the following sections a first long term non-axenic cultivation approach within a µg-capable PBR system is presented. Entirely conceivable problems due to successive biofilm layering, based on experimental data and observations, are discussed.

II. General µgPBR System Design

The µg-adapted membrane PBR FM is shown in Fig. 3. The PBR and periphery parts are customized to the standard mid-deck locker (MDL) size for an Express Rack on the ISS. Visible components are the front panel for crew interaction, the experiment compartment (EC, green, in the middle part), peristaltic pump and super absorber unit. A second MDL (MDL-2, not shown) conatins a backup CO_2 bottle, for experiment periods with limited excess to the LSR. The schematic setup of PBR@LSR is shown in Fig. 4. The majority of the components are located within the gas and water tight EC. The EC contains a total volume of ~10 l and provides access to the algae suspension loop (ASL) and the defined gas atmosphere. The functional group ASL is composed of two µg-adapted photobio-reactor flow chambers (µgPBR; Fig. 5) which are individually covered with a fluorethylenepropylene (FEP) gas exchange membrane, tubing and connectors, sensors to monitor pH and biomass density (via absorbance measurement at $\lambda = 660$ nm) and access ports for feeding and harvesting via a separate liquid exchange device (LiED). V_{total} of the ASL is ~650 ml filled with algae suspension. Lighting is realized by a dichromatic red/blue LED panel placed outside the ASL[11]. Algae are lighted throught the gas exchange membrane

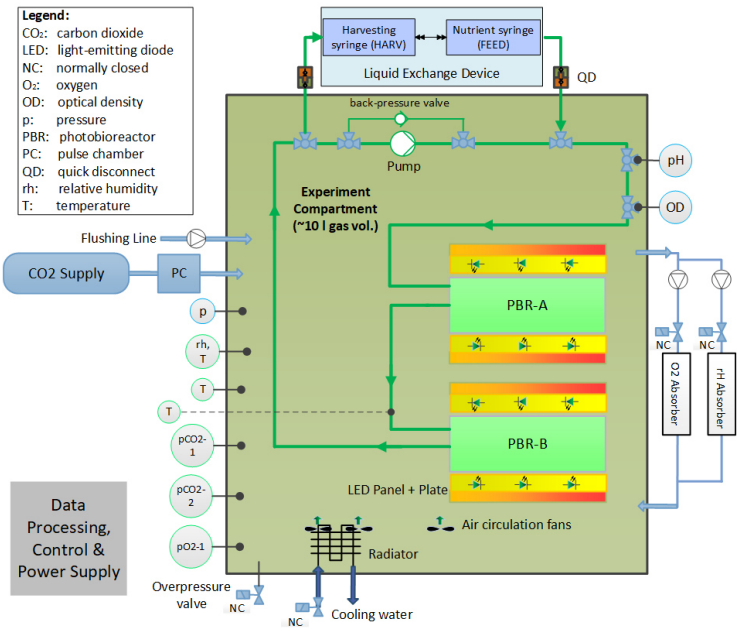

Figure 4: FM Setup of PBR@LSR.

(transmission >98%). Gases were pulsed into the EC by a pulse chamber and homogeneously distributed within the EC by air circulation fans. Gas evolution through the experiment is monitored by several gas sensors: pCO_2 (COZIR Wide Range 20%, Cozir®, Gas Sensing Solutions, UK, sensing principle: nondispersive infrared detector), pO_2 (FIGARO SK25F, Figaro®, Osaka, Japan, principle: sensing galvanic cell), pressure (p), relative humidity (rh) and temperature (T). O_2 and rh within the EC are actively regulated by absorbers, T is controlled by a cold plate connected to the ISS cooling water loop (16-23 °C). The connection between the µgPBR and LSR (former name ACLS) will be realized by a specific interface (I/F), which allows the transfer of excess CO_2 to external experiments. Nominally, the µgPBR will be supplied with CO_2 from LSR. LSR is planned to be launched with HTV-7 and will be accommodated in the US lab "Destiny" in November 2018[16].

III. *Chlorella vulgaris* – A small cell with great potential

Microalgae are uni- or multi-cellular, aquatic, eukaryotic microoganisms. For photoautotrophic growth, they conduct photosynthesis given by the top-level formula (1) of:

$$6\ CO_2 + 12\ H_2O + \Delta H_{hv} \rightarrow C_6H_{12}O_6 + 6\ H_2O + 6\ O_2 \qquad (1)$$

(where $\Delta H_{hv} = 2870$ kJ mol^{-1} glucose)

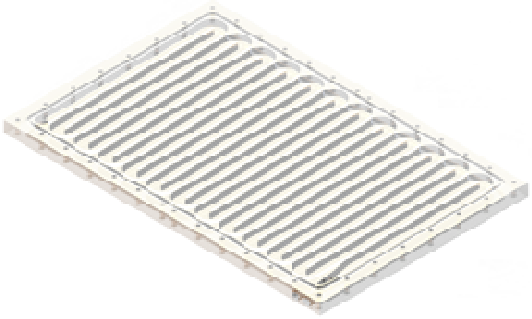

Figure 5: FM drawing of meandric µg-adapted PBR chamber.

which is a key ability for production of O_2 and edible biomass (glucose, $C_6H_{12}O_6$) from CO_2 and water (H_2O) in a ECLSS of a space station by using light energy. The auspices of long-term space missions make it important to investigate the influence of the space environment including microgravity (µg) and cosmic radiation on microalgal metabolism as well as the efficient cultivation of microalgae in the space environment in a µg-adapted photobioreactor (µgPBR) system. Compared with higher plants, microalgae have a higher harvest index (H_i >95 %) and a five times higher biomass productivity, a higher light utilization (> 10%) and lower water demand[5,13]. Photoautotrophic cultivation of microalgae is a promising key factor and reasonable technological step from a state-of-the-art physico-chemically based LSS to a hybrid LSS due to mass and energy savings and the *in-situ* biosynthesis of complex and high molecular biomolecules[14-17].

The controlled cultivation in a PBR requires a complex infrastructure consisting of illumination, nutrients supply, gas exchange, thermal control, media/solution control, harvesting and stowage/processing[18]. In addition to the technical realization of a sufficient cultivation environment, the choice of the microalgae species and the development of an individualized cultivation process are crucial to provide sufficient growth rates at high biomass concentrations through long cultivation periods. Besides optimized and reproducible growth dynamics, the following factors have to be taken into account for a sustainable process assessment: Cell morphology, phyiology and impacts on biomass composition, cell-cell interaction, photosynthetic yield (CO_2, O_2, evolution or ΦPSII), regeneration potential and genetic stability under space conditions.

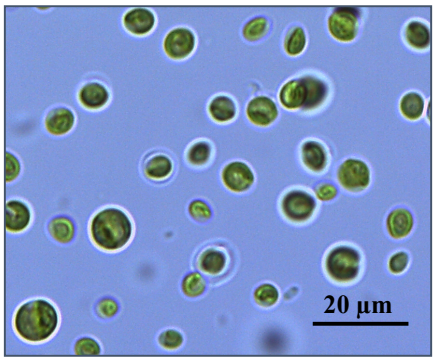

Figure 6: *Chlorella vulgaris* SAG211-12 at Insitute of Space Systems, Stuttgart.

Unicellular green algae meet the requirements for application in a LSS in space[19,20]. Since 2010, several algae species have been investigated at the IRS Stuttgart for usability in space applications, *Chlorella vulgaris* particularly showed very good results due to its versatility. The eukaryotic green algae *Chlorella vulgaris* (*Chlorophyta*) is an immotile single cell organism of spherical shape with a diameter of 2-15 µm[21-23], see also Fig. 6. Depending on culture growth status and culture condition *C. vulgaris* can form small cell aggregate structures[24]. *C.vulgaris* shows a wide temperature and pH tolerance[25-27] and grows within a wide range of CO_2 concentrations[28]. By choosing a selective lighting strategy[29] or by variation of medium composition[30,31], the growth behavior and proliferation of *C. vulgaris* can be actively controlled. Due to high resistance of the algae to bacterial cross contamination the cultivation process can be performed in a non-axenic manner[32]. This is an important factor for a robust cultivation

process serviced by the space crew. Biomass from *C. vulgaris* is also a nutritive food source containing 10-18 % carbohydrates, 40-58 % proteins and 14-25 % fats with addition of several vitamins, minerals and mono/polyunsaturated fatty acids, i.a. ω3 and ω6 fatty acids[33]. Due to theoretical and experimental studies[34,35] and space flight experience[8], *C.vulgaris* (SAG 211-12) emerges to be a promising candidate for a long-term cultivation experiment (> 180 d) on the ISS.

IV. Long term cultivation of *C.vulgaris* in µg-adapted membrane raceway PBR

The long-term cultivation in µgPBRs was conducted with the wild type microalgae strain *C. vulgaris* (SAG 211-12) obtained from the Department of Experimental Phycology and Culture Collection of Algae (EPSAG), University of Goettingen, Germany. For inoculation only cells at the end of the exponential growth phase of the preculture with a living cell count LCC = 98-100%[36] and mobile single (non-plasmolytic) morphology were taken. No clustering was observed. To make storage conditions representing conditions of the later cultivation scenario on ISS, the cells were dark adapted before inoculation to maximize photosynthetic activity[37]. Light energy was provided by red/blue (R/B) LED panels, which meet the requirements for photoautotrophic growth of *C. vulgaris*. *C. vulgaris* absorbs light energy primarily through chlorophyll a/b in the red (660-680 nm) and blue (435-475 nm) spectral range[10].

Table 1: Cultivation parameters during the longterm cultivation phase of PBR@LSR.

Parameter	Setting
Vloop	~650 ml
T	27 °C (±2)
pH	7 (±2)
CO_2 in EC	7-10 vol.-%
O_2 in EC	10-25 vol.-%
rh in EC	50-80 vol.-%
PPFD average	100-400 µmol/(m²s)
[NH_4^+-N]	100-700 mg/l
[PO_4^{3-}]	50-300 mg/l

The culture was non-axenic but free of major contaminants and from other algae strains, resulting in *C.vulgaris* being the dominant species within the closed loop system. The cultivation was conducted in fed-batch mode according to the parameters in Tab. 1. Mobile algal cells were perfused through two serial flat bed, meandering raceway reactors of the closed ASL by a peristaltic pump, see Fig 5. Before inoculation, the EC was initially flushed with N_2. A defined CO_2 atmosphere was established. Background nutrient solution was a diluted seawater nitrogen medium (DSN). In the current study, dry-biomass concentration (X) was determined by measurement of optical density (OD) at 750 nm and 680 nm using a *Hach* spectrophotometer (DR 2800). OD and X were correlated and caculated according to Eq. 2, see also[12].

$$X = (0.2312 \times OD_{680}) + (0.2886 \times OD_{750})/2 \quad [g/l] \tag{2}$$

For determination of nutrient uptake ([NH_4^+-N] and [PO_4^{3-}]) the culture supernatant (SN) was collected. SN samples were prepared and medial ion conentrations were measured according to manufacturer protocols (*Hach* GmbH, Berlin, Germany). Total cell counts (TCC), living cell counts (LCC) and bacterial stainings were performed according to protocols presented in[12].

Nominal long term operation in µgPBR

The long term cultivation was conducted for 186 d. In the first phase, day 0 to day 40 (D0-D40), a molar distribution of R/B photons of 50% each was set as baseline. A few hours after inoculation (X= 1,46 g/L) a decrease of biomass within the PBR loop down to X_{mobil} = 0,03 g/L could be observed (see Fig. 7; D0). The fast apparent loss of biomass can be explained by adhesion of the algal cells within the flow channels resulting in biofilm creation through the whole surface of the chamber and gas exchange membrane. A possible explanation for this behavior could be the adaption of the cells to the new cultivation conditions and environment. Sedimentation processes enhanced local accumulation of the algae cells. This could be confirmed by observations in former experiments. Nevertheless a minor fraction of mobile cells could grow within the liquid mobile phase with a mean growth rate of 0.007 g/L/d (D2-40), see also cells at D0 and D34. Kim *et al.* described the influence of different light wavelengths on the growth behaviour of *C. vulgaris*. Although the related signaling pathways are not fully understood yet, it is shown in experiments with monochromatic lighting that blue photons enhance pure cell growth up to a critical size, necessary for efficient cell proliferation. On the other hand, red photons induce an enhanced proliferation of cells[29].

To increase the proportion of mobile cells within the suspension loop without disturbing single cell growth, the lighting regime was changed to a molar distribution of R/B = (62.5 % / 37.5 %) resulting in a stepwise release of cell clusters from the PBR chambers and proliferation of single cells within the PBR loop, see also cells at D82, D161 and D178. Mean growth rates of 0.009 g/l/d (D41-80), 0.014 g/l/d (D81-120) and 0.044 g/l/d (D121-180) could be proven. Caused by cell clustering, the heterogeneous cell distribution within the mobile phase led to the scattering of biomass data.

Nevertheless, a mean biomass concentration of 4.6 g/l in the mobile phase could be reached at the end of the experiment and gives evidence for fundamental growth in a high biomass concentration range in a µgPBR for the first time. Until D78 liquid exchanges (LEs) were performed in periods D4-46 to reduce biomass concentration within the loop and provide the culture with fresh macronutrients. A cultivation period of 14 days between two LEs was chosen for further cultivation runs.

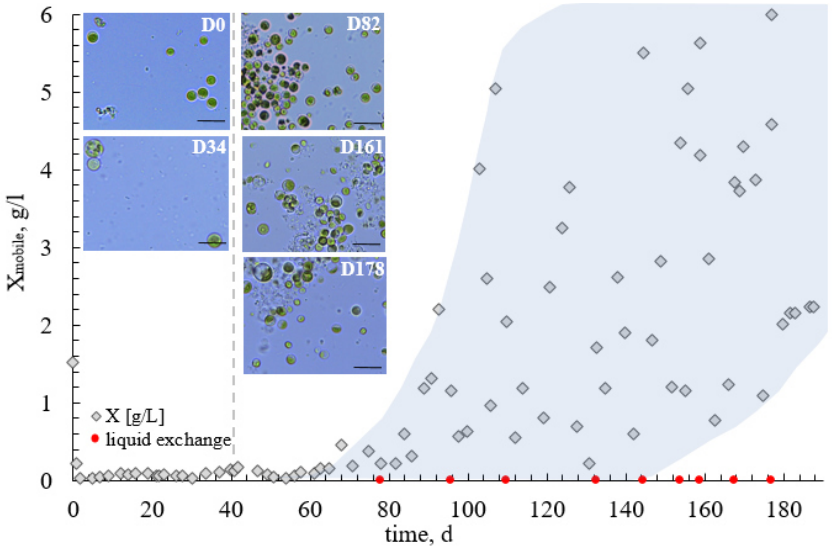

Fig. 7: First long-term cultivation (186 d) of *C. vulgaris* in µgPBR. The grey area demonstrates the tendency of algal growth behavior after process adaption. Biomass determinations rely on $OD_{680,750}$ measurements and correlation only on cells within the mobile phase, N = 1. X, biomass conc.

V. Assesment of biofilms in non-axenic long term cultures

A stable long term functionality of algae driven PBR systems depends in a large part on the equal distribution of free microalgal cells within the ASL, in particular the raceway flow channels of the PBR chambers. Especially a homogeneous availability of inorganic macronutrients and the "dispersion" of light energy influx within the algal suspension are basic requirements for a controlled and stable photoautotrophic process. Hence, uncontrolled immobilization of cells and extracellular biomolecules could affect the capacity of a culture to produce oxygen and to integrate environmental CO_2 into algal biomass (represented by photosynthetic quotient, PQ) and the suspension properties for potential biomass harvesting.

With an increasing duration of the total cultivation approach (includes no intermediate purification of the cultivation environment), the probability for biofilm layering due to direct adhesion of cells, biological deposits, e.g. extracellular polysaccharides (EPS) or cellular debris increases dramatically. Furthermore, a resulting increase of cell clustering due to interconnection with mobile bulk EPS or the excretion of soluble EPS could have a vast impact on viscosity and flow dynamics of the liquid phase within the ASL. In non-axenic micoralgae cultures, biofilms based on both algal and bacterial EPS and cell debris, are plausible. According to the microbiome composition and the resulting interactions within the "ecosystem PBR", these EPS based biofilms (EPS proportion often >90% of total biofilm[38]) could strongly vary in complexity and characteristics. The following environmental parameters were observed to affect EPS production and characteristics of algal biofilms:

Light intensity and temperature

The intensity and composition of light influx affect biomass composition, due to its influence on the carbohydrate metabolism. Is has been shown that high light intensities enhanced the proportion of bulk & soluble EPS[39]. For the current study the light influx to biomass ratio was chosen to be 40-150 µmol/m²*s per g biomass to prevent light inhibition of mobile cells resulting in a minimal tendency for light induced EPS synthesis. The parameter temperature could also synergistically affect EPS synthesis as it affects light inhibition processes[40].

Availability of carbon, nitrogen and phosphorous

A high CO_2 concentration could prevent EPS accumulation due to the carbon concentrating mechanism (CCM) by which microalgae concentrate medial inorganic carbon under low carbon conditions[39]. For example, for *C. kessleri* is has been demonstrated that a reduction of the medial CO_2 level resulted in an enhanced EPS synthesis[41]. In the current study the CO_2 level was regulated between 7-9 vol.% to ensure a stable photosynthetic process, but also to avoid C-depletions within the algal cells.

The impact of medial nitrogen and phosphorous levels on the EPS biogenesis are controversially discussed in literature. Nevertheless, at N-starving conditions it was proven for *C.vulgaris* to accumulate carbohydrates[42]. The most frequently observed effect of P-starvation is the switch from protein synthesis to carbohydrate and lipid accumulation[43]. This could potentially result in an enhanced excretion of carbohydrate polymers resulting in the forming of a basal EPS.

Stress response - bacteria and mechanical forces

EPS can be formed in respose to stress caused by a bunch of biotic (e.g. bacteria[44]) and abiotic factors[45-47]. Generally, EPS-induced multicellularity of microalgae could appear as a defense mechanism against cross cultivated bacteria or predators (own data, not shown).

The current ASL contains ~650 ml of culture volume and is driven by a peristaltic pump (*Watson Marlow® 114 series*). Although, comparing other pump types (e.g. gear pump, membrane pump, peristaltic pump) the peristaltic pump generally reduces mechanical forces to a minimum[11,48], the occurring periodic shear stress due to pressure swings or vibrations could induce certain cellular responses resulting in the enhanced creation of algal or bacterial EPS. The impact of the mechanical stress on the algae cells depends on the algal cell size, cell wall composition, algal regeneration capacity, the total number and the frequency of passes through the pump head and the rotating velocity of the pumphead[49].

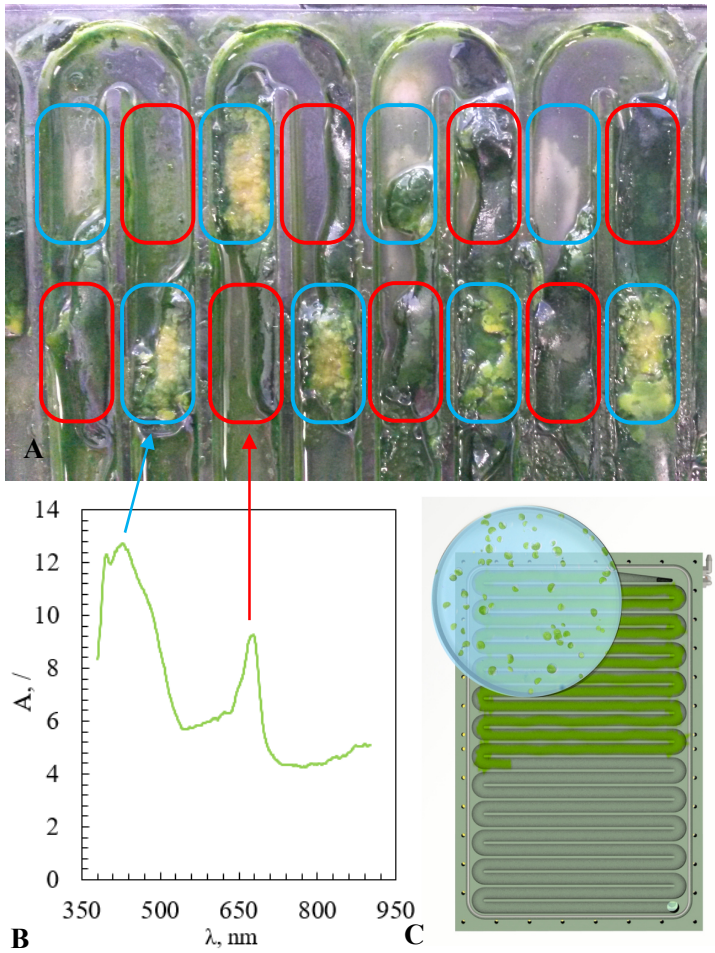

Figure 8: Raceway PBR-chamber after cultivation of 186 d. A, heterogenic algal biofilm in raceway PBR chamber. The blue and red borders indicate the positions of the blue and red LED spots; B, rel. i*n-vivo* absorbance of *C.vulgaris* SAG 211-12; C, *C.vulgaris* in raceway chamber (Airbus DS).

A. Biofilm in µgPBR chamber

Both µgPBR chambers represent the major growth compartment of the ASL. Inside the meandric flow channels the light influx, main mixture of the nutrients and cells as well as the gas exchange are realized. A stable long term operation requires a homogeneous suspension circulation without major deposits which could disturb the flow behaviour. During the running cultivation process only cell material and biopolymeric structures (e.g. EPS) of the mobile liquid phase could be sampled and analyzed. Free single cells and cell clusters composed of algae cells, bacteria and bulk EPS in sizes between 150-9000 µm² could be identified. The collection and analysis of

immobilized algal or bacterial cells, multicellular adhesion layers or clusters were performed after completion of the experiment (186 d) and manual opening of the reactor chambers, see also Fig. 8. Previously, residual algae suspension has been collected separately.

Fig. 8 shows algal biofilm within the open PBR chamber. According to the defined position of red and blue LEDs on the LED-panel, areas of photo-bleached algae could be observed only under blue light spots. This pattern could be explained by the relatively high energy intake of short-wave radiation. Carefully removed top cell layers showed a bleaching impact down to a layer depth of at least ~1 mm. Cells under red spots were apparently not stressed. According to this observation ~17 % of the immobilized algal material was calculated to be influenced or even damaged. Excess light exposure could lead to a degradation or destruction of algal light-harvesting pigments[38], e.g. Chlorophyll a and b (Chl a/b) of *C.vulgaris*.

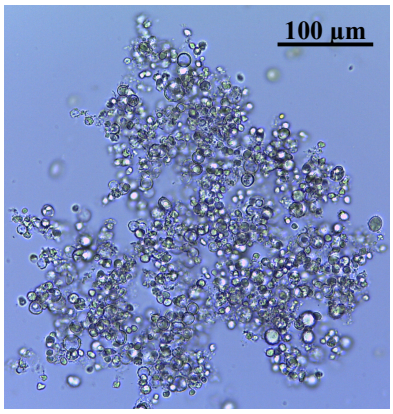

Figure 9: Cluster of photo-bleeched but viable *C.vulgaris* cells, crosslinked by bulk EPS.

Vitality assays of isolated photobleached cells (Fig. 8 A) showed a LCC >80%. The residual ~20% biomass could be assumed as dead cell material due to photoinhibition and photooxidation as a result of immobilization. The viable bleached *C.vulgaris* cells (Fig. 9) are not further able to grow photoautotrophically, but compensate energy demands by changing their metabolism into respiration (via oxygenase activity of RuBisCO)[38]. Using organic carbon sources, e.g. free floating or EPS-bound cellular debris, these cells survive as oxygen consumers/CO_2 producers. Due to further EPS-driven crosslinking, a propagation of the consumers is a realistic problem for the long term efficiency of the O_2 producing and carboxylating system, measurable by a decreasing algal photosynthetic activity quantified by the photosynthetic quotient, PQ.

The PQ could be used as an adequate online indicator for influences of environmental process parameters on microalgal physiology[50], in the current case the increased gradual biofilm layering within the PBR chamber. For the current experiment, light deficiency impacts due to a potential high mobile biomass concentration can be excluded (Fig. 7). PQ values were calculated according to Eq. 3:

$$PQ = O_2 \text{ production}/CO_2 \text{ consumption} * M(CO_2)/M(O_2)\ [/] \quad (3)$$

(where M = molar mass, $M(CO_2)$ = 44,0095 g/mol and $M(O_2)$ = 31,9988 g/mol)

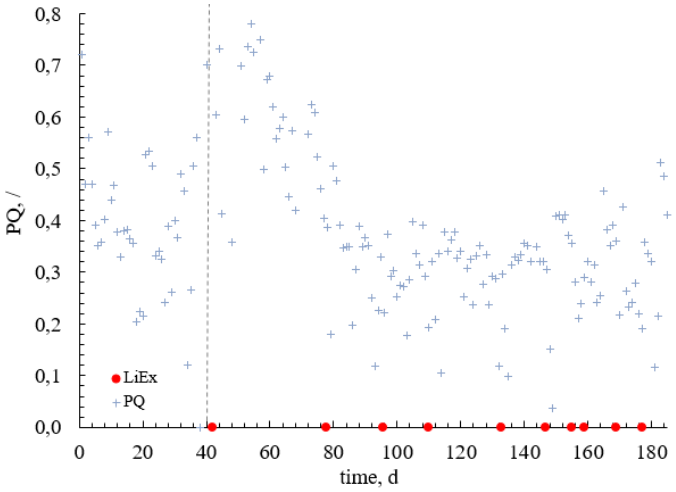

Figure 10: Photosynthetic activity of *C.vulgaris* SAG211-12 during long term cultivation. PQ values are averaged over 24 h. Dashed line, change of lighting regime.

Fig. 10 shows the photosynthetic activity of the *C.vulgaris* culture during the long term cultivation experiment. Due to culture adaption a decrease was observed until D 40. After changing the lighting regime, the higher absolute energy influx of red wavelength could increase the mass related PQ to a maximum (PQ ~0,8). An physiological adaption to the new set up was observed, resulting in a $PQ_{average}$ = 0,351 after D 80. In accordance with the stepwise release of cells into the mobile phase, the lighting distribution has been improved. This results in an increased mean PQ at the late experiment phase (D150-D186).

B. Biofilm on FEP gas exchange membrane

The FEP membrane shall provide a constant exchange of CO_2 and O_2 between the PBR chambers and the EC (Fig. 12 A). As shown for the PBR chambers, a pattern of photobleached and non-bleached cells could be observed on the membrane side after long term operation (Fig. 11). The hydrophilic membrane side was oriented to the aqueous medium and thereby provided potential anchor points for a cell binding directly by surface proteins

(adhesion) or in-directly by carbohydrate chains of the EPS. This allows a successive accumulation of algal cells or their immobilized proliferation within the EPS matrix directly on the membrane. A plane EPS covering throughtout the entire membrane could be verified, independent of the membrane orientation to the upper or lower side of the PBR chamber, see also Fig. 12 B. This gives evidence for the biofilm exposure to occur mainly due to adhesion events. Sedimentation effects only enhance the layering. Similar covering could also be observed after shorter cultivation periods (data not shown). The careful removing of several algal cell layers also showed that on the basal EPS layer the majority of rod-shaped bacteria are localized. This suggests that the EPS was bacteria-induced. Predominantly gram-negative bacteria were identified (Gram staining, Fig. 12 C). According to this, the accumulation of algal cell multilayers occurred by crosslinking with the EPS. To receive a deeper understanding of the algae-bacterial physiological interactions and their influence on the cultivation process, a detailed characterization of the bacterial microbiome by next generation sequencing (NGS) is currently in progress. Despite an obvious membrane covering, the constant CO_2 consumption and O_2 production through the entire cultivation gives first evidence for the long-term functionality of the gas transfer in the non-axenic bioprocess, see also accumulated gas data in[51]. The assessment of the relative gas transfer efficiency will be given after a process optimization towards a homogeneous distributed cell culture is realized. At least, this is necessary for a constant reliable controlling of the photosynthetic process and biomass density by cyclic harvesting (LiEx).

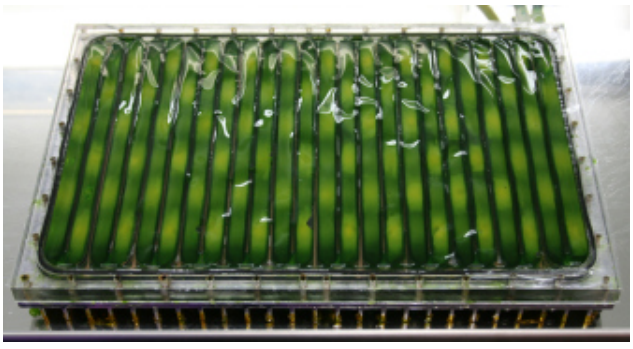

Fig 11: FEP membrane on raceway PBR chamber after 186 d: "Pattern of (non)-bleached cells".

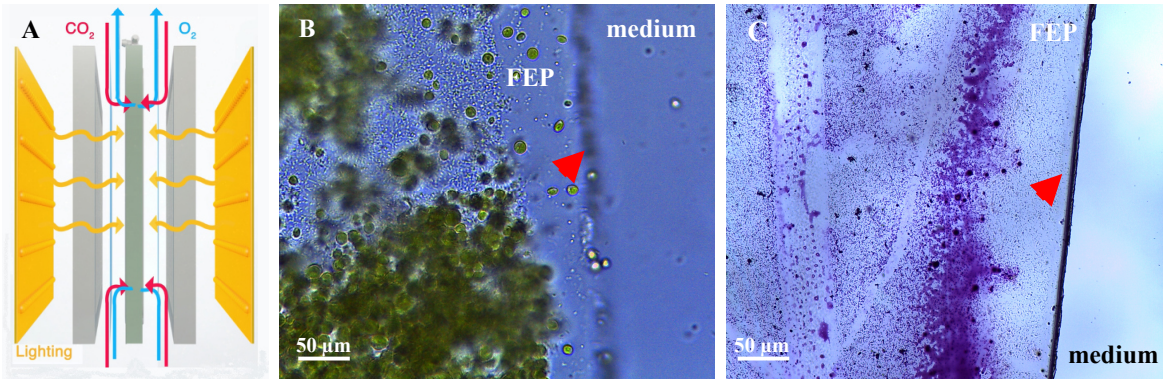

Figure 12: Layered biofilm on gas transfer membrane. A, O_2/CO_2 transfer principle through FEP membrane; B, layering of bacterial/algal biofilm; C, Gram staining of bacterial cell layer. The arrowheads indicate the edge of the membrane.

C. Biofilm in biomass sensor

The absorbance, *A* is a measure for the opacity of a liquid, and can be correlated with the biomass concentration (in g/l), allowing the *in-situ* measurement of algal culture growth. Commercial biomass density sensors are available, but they are too heavy or big for this space application. Therefore, an biomass-sensor has been developed at the IRS, specifically for the PBR@LSR experiment.

Operation principle and chamber assembly

The biomass sensor measures the amount of light "lost" in a specific length of algae suspension. Therefore, the sensor is equipped with two LEDs with a specific wavelength (λ = 660 nm) that work independently. Each LED sends an initial flux I_o through the suspension. A sensor next to the LEDs measures this flux density. On the other side of the algae solution (with a predefined specific liquid depth, d ≈ 4 mm), another light sensor measures the remaining photon flux density (Fig. 13). Following the fundamental principles of the law of Lamber-Beer, the absorbance *A* can be calculated according to Eq. 4:

$$A_1 = \log (I_0/I_1) \text{ for LED-1 and } A_2 = \log (I_0/I_2) \text{ for LED-2} \quad [/] \qquad (4)$$

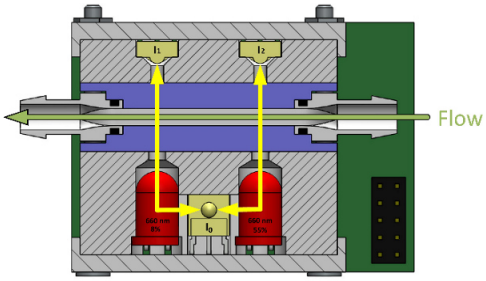

Figure 13. Working principle optical density sensor.

The measured absorbance values were correlated to manually premeasured OD_{660} (Hach, DR2800). The biomass sensor will be integrated into the FM-ASL as well as the refurbished on-ground reference µg-capable PBR-ASL for the parallel *in-situ* measurement of cell density during the flight experiment.

The sensor consist of a measurement chamber (Fig. 14), which is divided in two parts, the frame (3D printed, Accura® ABS Black) and 2 translucid covers at each side (polycarbonate). An O-ring ensures the tightness of the chamber. This chamber will be integrated into the algae-loop. The LEDs and the sensors are mounted at both sides of this chamber, in LED/Sensors housing, specifically designed for this sensor. The LEDs and sensors are controlled using a microcontroller (Arduino©).

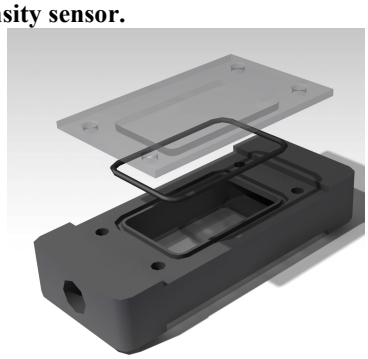

Figure 14: Measuring chamber of the biomass sensor (flow cell).

Biofilm formation testing

The most critical aspect to be considered in a long-term use of the sensor, is the accumulation of EPS and cells on the transparent surfaces of the measurement chamber, which might have an influence on the sensor signal over time. A biomass-sensor prototype (flow cell version) has been used for over 50 days integrated in the ASL under current operation conditions. This should allow the synthesis of an adequate biofilm layer. After finishing the experiment, basal DSN and algae suspension (OD_{660} = 10.49) have been pumped through the flow chamber and have been measured, before and after cleaning the sensor. The *A* difference for the medium was found to be marginal ($\Delta A_{660} = 0.02$), whereas the tested suspension showed a $\Delta A_{660} = 1$. This equals a relative sensor error of 9.87 % after 50 d of cultivation. This could be explained by the higher sensitivity of the biomass-sensor to variations of Chl a/b (*in-vivo* absorbance maxima in the red spectral range for Chl a = 670-680 nm and Chl b = 650 nm[52]), than to other cellular structures like carbohydrates, e.g. cellulose (A= 260 nm[53]). In both cases the value before cleaning was higher due to biofilm formation. In conclusion, a successive biofilm layering has to be considered, which could result in a sensor signal drift.

VI. Conceivable countermeasures

For long term non-axenic cultivation operations in µgPBRs a successive synthesis of bacterial and algal biofilm within the ASL has to be considered. Especially deposits on interaction surfaces with the environment (EC or LED), like the flow channels of the PBR chambers, the gas exchange membrane and the measurement chamber of the biomass sensor are in the focus of the current work. Therefore, a reduction of biofilms to a minimum is a major goal in the context of bioprocess control and upcoming optimizations for the technical realization, not only of the current experiment, but for the future development of µg capable (membrane) PBR system technologies.

To reduce bacteria or algae induced biofilms within the running cultivation process and to reach a higher level of mobile cells, several strategies or treatments are conceivable. In Tab. 2 µg-relevant strategies and their advantages/disadvantages are presented. In principle, several strategies could be combined to use synergetic treatment effects. All suggested treatments are based on a non-axenic wild type *C.vulgaris* culture. Cultures, based on genetically modified microorganisms, were not considered.

Tab. 2: Conceivable coutermeasures against biofilm synthesis in membrane µgPBRs before and during PBR operation.

Principle	Method	Effect	+	-
physical	Constriction of flow channel width (PBR chamber design)	Increased flow velocity, enhanced *Reynolds number,* but laminar flow	Enhanced mixing, cluster breaking in PBR chamber	Enhanced mechanical stress due to turbulent flow, no effect on existing biofilm
	Flow breaker	Local change of flow dynamics, local turbulences	Enhaced mixing, cluster breaking in PBR chamber, potential for flashing light effect	Enhanced mechanical stress due to turbulent flow, no effect on existing biofilm
	Flow particles	Mechanical removing of cell / biofilm	Abrasion of already consisting deposits & biofilm, cluster breaking in PBR loop	Potential cell immobilization on partilces, artificial adhesion nuclei, risk of clogging
	PBR chamber surface treatment	Smoothing of surfaces	Reduced anchor sides for adhesive molecules	Possibly cost and work intensive
	Pulsed electric field	Selective electroporation	Selective *in-situ* bacteria inactivation/lysis, non-invasive	Possible uncontrolled fusion of algal cell organelle membranes, changes of photosynthesis apparatus or cellular damage[54]
	Low energy electron beam	Partial disinfection	Selective *in-situ* bacteria inactivation/lysis, non-invasive	Currently limited knowledge of free electrons influencing algal cells metabolism, viability or productivity
	Cross flow filtration	Separation and removal of bacteria	Selective *in-situ* bacteria removal, combinable with continuous cultivation and DSP (harvesting),	Necessity of multi level filtration systems bypass loop
	Selective lighting	Enhanced proliferation, reduced clustering	Prevention of anoxygenic photosynthesis, *in-si*tu algae cell remobilization, reduction of current bacteria number, non-invasive	Highly specific for individual algae species or even strain
biological	Enzymatic treatment	Digestion of EPS components	Utilization of indigestible carbohydrates (cellulose) into glucose or EPS proteins in free aminoacids	Invasive, short enzyme half life, expensive, post treatment purification necessary
	Antibiotic treatment	Reduction of bacteria number	Cyclic sharp decrease of total bacteria number, potential avoidance of bacterial endotoxins	Invasive, potential of bacteria to generate resistances, unexpected negative impacts on algal metabolism (e.g. chloroplasts)[55]; no inhibition of spore germination; no safe usage as food source
	Axenic cultivation (selective bioprocessing)	Algae strain as singular microorganism, no microbiome	Higher algal growth rates, higher photosynthetic yield, pure algae biomass	High cultivation effort, not feasible for long term application, potentially only restricted biomass composition (e.g. B_{12} synthesis)[32]
chemical	Surface hydrophobing	Reduced adhesion spots on ASL surfaces	Reduced planar biofilm spreading, reduced starting points for release of bulk EPS/cell clusters	Putative biochemical incompatibilities
	Selective media design, e.g. N-source, pH	Shift of selective pressure within the µgPBR	Promoted algal growth, reduced bacteria number, reduced bacterial EPS	Highly specific for individual algae species or even strain
	Immobilization, e.g. by alginate drops	No free cells within the ASL	Immobilized but mobile algae cells, cyclic cleaning and full recovery of algal cells possible	No quantitative algal biomass harvesting, possible accumulation of bacteria within the ASL possible, risk of clogging

In the following, the possibility for an improvement of cultivation processes is illustrated and discussed by the example of the hardware component µgPBR chamber. In dependence of the manufacturing process quality, like milling of the flow channels from a massive polycarbonate plate, this results in the formation of individual groove profile. The defined surface roughness of the cultivation chambers could serve as niches for algal cells (2-15 µm, depending on maturation status), but in particular for the smaller bacterial cells (mean dm ~ 1 µm). The niches support cell deposition due to microscopic wake space and could form the basis for the synthesis of cross-linking EPS.

In the case of the standard manufactured PBR chambers, the mean groove depth was ~ 9 µm (Fig. 15 A). A typical algae cell in the current µgPBR process (size ~ 6 µm) could easily inhere and start crosslinking by EPS or direct cell-cell adhesion (Fig. 15 C). After optimization of the manufacturing process, realized by a special fine milling, the mean groove depth could be reduced to ~ 2,8 µm (Fig. 15 B). This went hand in hand with a smoothing of the groove profile (Fig. 15D). Thus, this counteracts the fundamental adhesion of algae cells, resulting in a reduction of the chance for the creation of a planar layering by algae.

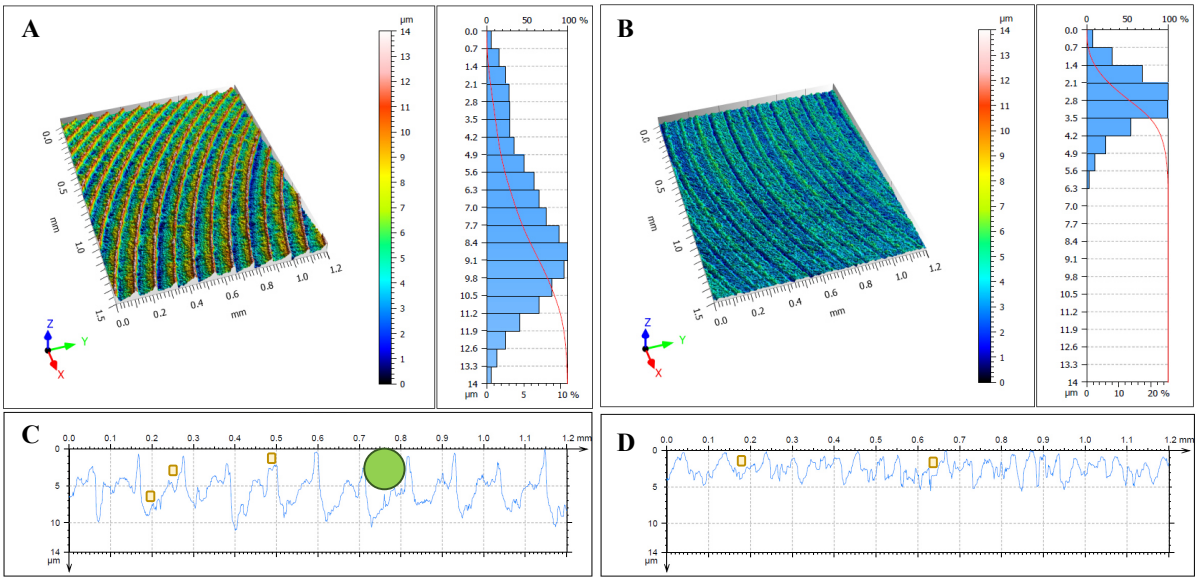

Figure 15: Interferogram of milled surface (µgPBR chamber section) in dependence of manufacturing process. Surface section and maximal groove depth before (A), after (B) and surface profile of µgPBR chambers before (C) and after (D) manufacturing optimization. Green circle, schematic algal cell (~ 6 µm); small beige square, schematic bacterial cell (~ 1 µm). Data was collected in cooperation with the Insitute for Machine Components (IMA), University of Stuttgart.

The sole optimization of the surface roughness resulted in a slight increase of cell number within the mobile liquid phase of the ASL (data not shown). An accompanying adaption of the bioprocess relized by optimized cultivation conditions currently represents the most promising option for the (re-)mobilization of algae cells of a non-axenic culture in a meandric membrane PBR system. According to significant changes of metabolic responses of different microalgae strains to process parameters like lighting strategy, harvest/feed design, temperature scenario[56], an individual characterization of the mutual influence of the microalgae physiology and the cultivation environment is crucial for an optimized, sustainable and reliable operation of the µgPBR system. That is especially the case for long term cultivation processes.

VII. Conclusion and Outlook

To be launched to the International Space Station (ISS) in 2018, PBR@LSR follows the hybrid LSS approach by combining a microalgae PBR and the CO_2 concentrator of ESA's LSR. The PBR@LSR experiment and its development was initiated in 2014 by the German Aerospace Center (DLR) Space Administration (grant) and the

Institute of Space Systems (IRS) of the University of Stuttgart with Airbus Defence and Space as prime contractor for the flight hardware. The two µg-adapted PBR, the LiED and the syringes are built by IRS.

This paper presented the final configuration of PBR@LSR with a focus on process important components of the algae suspension loop (ASL), like the cultivation chamber, the gas exchange membrane and the biomass sensor. The interaction of this components with the non-axenic culture could result in a successive creation of biofilm layers, which could strongly influence the long-term functionality, photosynthetic efficiency and finally the operational handling of a µgPBR. Depending on the individual cultural microbiome, biofilms could vary in structure and characteristics. Regarding this, the biofilm deposits of the presented long-term approach were investigated. Appropriate countermeasures for reduction of biofilm deposits within the given PBR set up were presented and discussed. With the current long term experiment of 186 days basically successful on-ground long-term cultivation in a protoflight breadboard setup of PBR@LSR could be proven. This was verified by the overall biomass growth within the ASL and the net O_2 production.

Future work at the Institute of Space Systems, Stuttgart (IRS) will include the optimization of the cultivation process within the given PBR set up, pursuing the goal to increase mobility of the total algal cell biomass as well as the photosynthetic capacity. Therefore, a comprehensive characterization of the used *C.vulgaris* strain SAG 211-12 will be performed in the context of long-term cultivation and individual processing in µg-capable meandric membrane PBR systems.

The flight data of PBR@LSR will, for the first time, reveal the long-term performance, the system stability and reliability as well as the biological stability of a synergetically integrated biotechnological LSS component in a real space environment. Microalgae samples taken during the experiment will be returned to earth and analyzed. The sequencing of isolated genetic material could be highly beneficial for the evaluation of putative alterations of photosynthesis associated *C.vulgaris* genes, which could have a significant impact on algal photosynthetic performance for permanent application as a biotechnological component in hybrid LSS.

Acknowledgments

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