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Bachelorarbeit

Culture optimization and characterization of three Porphyridium strains for valuable compound production

David Große
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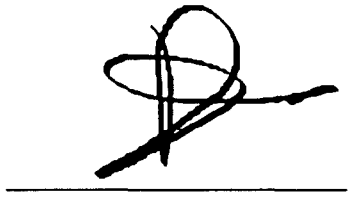

David Große

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1. **Prüfer Prof. Dr. Ingar Janzik**
2. **Prüfer Dr. Diana Reinecke-Levi**

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Abstract

The optimal conditions to maximize phycoerythrin- (PE) and exopolysaccharide (EPS) production in the three photoautotrophic red marine microalgal strains of *Porphyridium purpureum* (SAG 1380-1a & -1d) and *cruentum* (UTEX 161) were researched. Design of experiment software (DoE) was used in planning and evaluation. Dependencies were found between light intensity, nitrogen and magnesium concentrations, and the resulting PE and EPS productivities. The maximal PE productivity (4,96 µg $mL^{-1}d^{-1}$) was found in *P. purpureum* SAG 1380-1d. Contrasting, the highest EPS productivity (5,75 g L^{-1} d^{-1}) was found in *P. purpureum* SAG 1380-1a. However, optimization efforts in EPS production remained due to low dependencies under tested growth conditions. The optimal product inducing conditions for all strains were identified at ~40µmol photon m^{-2} $s^{-1}$and 9-15 mM nitrogen. The optimal magnesium concentration ranged between 5, 38 and 45 mM for UTEX 161, SAG 1380-1a and -1d, respectively. The pilot scale cultivation under greenhouse conditions could not be finished, because of temperature stress during the cultivation.

Zusammenfassung

Die optimalen Kultivierungsbedingungen für die maximale Phycoerythrin (PE) und Exopolysaccharid (EPS) Produktion durch die drei photoautotrophe rote Mikroalgen Stämme *Porphyridium purpureum* (SAG 1380-1a & -1d) und *cruentum* (UTEX 161) wurden erforscht. Eine Design of Experiment Software wurde genutzt, um die Experimente zu planen und auszuwerten. Abhängigkeiten zwischen Lichtintensitäten, Stickstoff- und Magnesium Konzentrationen mit den resultierenden PE und EPS Produktivitäten wurden gefunden. Die maximale PE Produktivität wurde bei *P. purpureum* SAG 1380-1d (4,96 µg $mL^{-1}d^{-1}$) gefunden. Hingegen wurde die maximale EPS Produktivität bei *P. purpureum* SAG 1380-1a (5,75 g L^{-1} d^{-1}) lokalisiert. Wir konnten keine Optimierung der EPS Produktion durchführen, weil niedrige Abhängigkeiten unter den getesteten Bedingungen gefunden wurden. Die besten Bedingungen liegen bei ~40 µmol photon m^{-2} s^{-1} und 9-15 mM Stickstoff Konzentration für alle Stämme. Die optimalen Magnesium Konzentrationen schwanken zwischen 5, 38 und 45 mM für UTEX 161, SAG 1380-1a und 1-d. Die Kultivierung im großen Maßstab konnte nicht abgeschlossen werden, wegen Temperaturstress während der Kultivierung.

Content

Abbreviations

μ	**Growth rate**
μ_{max}	Maximal growth rate
ASW	Artificial sea water
DoE	Design of experiment
DW	Dry weight
EPS	Exopolysaccharide
K	Relative growth constant
Mg	Magnesium
Mg25	Magnesium, 25 mM
N	Nitrogen
N5	Nitrogen, 5mM
OD	Optical density
P. c.	*Porphyridium cruentum*
P. p.	*Porphyridium purpureum*
PBP	Phycobiliproteins
PE	Phycoerythrin
PC	Phycocyanin
AP	Allophycocyanin
P_i	Productivity
RT	Room temperature
tCar	Total Carotenoid
tChl	Total Chlorophyll
tN	Total Nitrogen
tPO4-P	Total ortho-Phosphate

1. Introduction

Microalgae of the genus Porphyridium belong to the larger phylum of photoautotrophic marine red algae, the Rhodophyta. Many of them are high-value algae, due to their high content of polyunsaturated fat acids (PUFAs), phycobiliproteins (PBP), and exopolysaccharides (EPS) [1,2]. Yet, compare to the Rhodophyta-typical leafy macroalgae, Porphyridium cells lack the ability to grow in multicellular phenotypes. Instead, cells remain single cellular cultures of globular to polygonal cells ranging between 7-12 µm in diameter [3].

Thus, Porphyridium species can be grown in conventional microalgal photobioreactors and gained a high economic value in various market sectors [13]. PUFAs, EPS, PBP carotenoids, and the chlorophyll-a can be used as nutraceuticals, natural colorants or antioxidants in food, feed, and cosmetics [4]. The auto-fluorescence of PBP, phycoerythrin (PE), allophycocyanin (AC), and phycocyanin (PC), is utilized in fluorescent marker in research, and diagnostic [5]. Commercial producers can be found in Israel, USA, and China [16, 17, 18]

However, persisting challenges, problems in the up-scaling, and the down-stream processing require further research. A major problem is remains of the variable cellular composition, which causes a cost-intensive down streaming. For example, the EPS are harvested by energy intensive centrifugation or cost solvent precipitation. Furthermore, continuous biorefinery of several products include more cost intensive processes. PBP, and EPS yields can vary largely under suboptimal culture, and climate conditions. Therefore, one possible solution could be the strain optimization for given culture conditions to increase productivity while reduce production costs.

In this bachelor thesis, the (i) effect and (ii) optimization of three independent variables for the EPS- and PBP-productivity in the three microalgal strains, *Porphridium pupureum* SAG1380-1a and -1d, and *Porphyridium cruentum* UTEX 161 were studied. All three strains are known for their commercial EPS and PBP production [12, 17].

DoE software was used for planning and evaluation of the experiments. The selection and range of the independent variables (A) light intensity (B), nitrogen, and (C) magnesium concentration was based on literature review. Light intensity and quality are the most important growth factors for photosynthetic microalgae. Thus, the synthesis of phycobiliproteins, and chlorophylls in Porphyridium are highly dependent on the available light [6]. According to the literature, Porphyridium prefers lower light intensities (A) of 300-

500 µmol photon m^{-2} s^{-1} [6, 21]. Nitrogen (B), and magnesium (C) are essential ions in the photosynthetic pigments and other cell compounds [12]. The highest rate of photosynthesis and product formation are expected under elevated nitrogen and magnesium concentrations. Optimal concentrations are indicated at 5-15 mM nitrogen [7, 14], and 5-45 mM magnesium [8].

Following lab-scale optimization, the Porphyridium strains with the highest PBP- and EPS productivity will be tested at pilot scale under the local climatic conditions in North Rhine Westphalia.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Tab. 1. Chemicals compositions of the standard growth medium artificial seawater (ASW)

Chemical	g L^{-1}	medium
1. NaCl	27 g	27 g
2. $MgSO_4 + 7\ H_2O$	6,6 g	6,6 g
3. $MgCl_2 + 6\ H_2O$	5,6 g	5,6 g
4. $CaCl_2 + 2\ H_2O$	1,5 g	1,5 g
5. KNO_3	1,0 g	1,0 g
6. KH_2PO_4	0,07 g	0,07 g
7. $NaHCO_3$	0,04 g	0,04 g
8. Chelated Iron stock solution		1 mL
$FeCl_3 + 4\ H_2O$	240 g	
0,05 M EDTA	14,6 g	
9. 1 M TRIS HCL, pH 7,6	121 g	20 mL
10. Trace metal stock solution		1 mL
$ZnCl_2$	40 mg	
H_3BO_3	600 mg	
$CoCl_2 + 6\ H_2O$	15 mg	
$CuCl_2 + 2\ H_2O$	40 mg	
$MgCl_2 + 4\ H_2O$	400 mg	
$(NH_4)_6\ Mo_7O_{24} + 4\ H_2O$	370 mg	

Tab. 2. Other used chemicals

≥99,5%Dimethylsulfoxid (DMSO)	20 mM Acetate buffer
30% (v/v) Methanol	96% Ethanol
0,9 M KOH solution	3,7% HCl

2.1.2 Devices

Tab. 3. Devices used in cultivation and analysis

Device	Name and Type	Producer
Centrifuge	RC 6+ Plus	Sorvall®
Spectrophotometer	D-5000	Hach-Lange®
Balance	MS Semi-Micro Balances	Mettler Toledo®
Ultrasonic device	75186	Vibra cell®
Heat block	Rotilabo-Block-Heater H250	Roth®
Incubator	Minitron Floor	Infors®
Orbital shaker	DOS-20L	ELMI®

2.2 Growth parameters & analytical methods

2.2.1 Culture conditions

The microalgal strains *Porphyridium purpureum* (SAG 1380-1a and -1d) were ordered from the Algal Culture of the University of Goettingen, Germany. The strain *Porphyridium cruentum* (UTEX 161) was ordered from the University of Texas, USA. All three strains were cultivated in standard artificial seawater (ASW) medium of Jones (1963) [8], see 2.1.1. The cultures were established in Erlenmeyer flasks of 50 to 2000 mL and shacked at 170 rpm, 20°C and light intensity and cycle of 30-60 µmol photon m^{-2} s^{-1} and 12:12 h, respectively, Fig. 1A. Cultures were kept in batch mode and diluted at most 1:5, once a week. During experiments, cultures were tested in modified ASW-medium at a light-path of 1 cm under the light intensities of 40 to 500 µmol photon m^{-2} s^{-1}, Tab. 4. The nitrogen and magnesium concentrations were adjusted between 5-15 and 5-45 mM, respectively, Tab. 4. Cultures for biomass production were kept in 2000 mL Erlenmeyer flasks at 170 rpm, RT and diurnal 300-600 µmol photon m^{-2} s^{-1}, Fig. 1A. Cultures for pilot scale were kept in sharp bags of 5 L, which are aerated with air, 20-30°C and diurnal 100-400 µmol photon m^{-2} s^{-1}, see Fig. 1B.

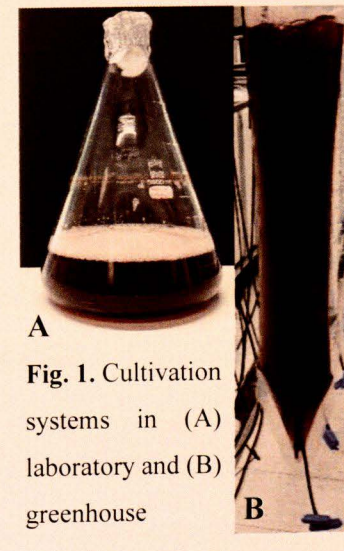


Fig. 1. Cultivation systems in (A) laboratory and (B) greenhouse

2.2.2 Optical density

Optical density (OD) was measured in a spectrophotometer (D5000, Hach-Lange®). 1 mL culture was sampled and measured at 750 nm. Samples were diluted if the density exceeded $OD_{750} = \geq 0{,}4$ [9]. The growth rate (1) and doubling time (2) was calculated as noted below:

$$(1) \qquad \mu\,[d^{-1}] = \frac{\ln(OD_2) - \ln(OD_1)}{t_2 - t_1}$$

$$(2) \qquad g\,[d] = \frac{\ln 2}{\mu}$$

2.2.3 Dry weight

Dry weight (DW) was determined by filtering 5 mL [V] of culture through pre-weight [m_1], and rinsed disk-filter (GF/C ™, Whatman®). Filters were rinsed with 5 mL deionized water, dried at 100°C for 1h, and left to cool in an exicator, overnight. Filter with retained cells were weight again [m_2] (MS Semi-Micro Balances, Mettler Toledo®) and the dried biomass weight calculated according to [9]:

$$DW\,[g\,L^{-1}] = \frac{m_2\,[g] - m_1\,[g]}{V\,[L]}$$

2.2.4 Total Chlorophylls

The total chlorophyll content (tChl) was determined according to Boussiba *et al.* (1999) [11]. 1-5 mL culture were sampled, centrifuged at 3500 rpm for 5 min, and the supernatant was discarded. The cell pellet was extracted in 5 mL DMSO at 70°C for 5 min and centrifuged again. The absorption of the supernatant was measured at 665 nm (D5000, Hach-Lange®). The chlorophyll content was calculated as noted below [10]:

$$1\,OD_{665} = 11{,}49\,\mu g\,mL^{-1}$$

2.2.5 Total Carotenoids

The total Carotenoid content (tCar) was determined according to Boussiba *et al.* (1999) [11]. 1-5 mL culture were sampled, centrifuged at 3500 rpm for 5 minutes, and the supernatant was discarded. The pellet was treated with 5 mL of 0,9 M KOH in 30% (v/v) methanol for 5 min, centrifuged, and the supernatant discarded again. 50 µl of acetic acid and 5 mL of DSMO were added, and extracted at 70°C for 5 min. After centrifugation, the adsorption of the supernatant was measured at 490 nm (D5000, Hach-Lange®). The carotenoid content was calculated as noted below [10]:

$$1\ OD_{490} = 5{,}59\ \mu g\ mL^{-1}$$

2.2.6 Phycobiliprotein

The phycobiliprotein (PBP) content were determined according to Kathiresan *et al.* (2006) [12]. 10 mL culture were centrifugalized at 3500 rpm for 5 min. The supernatant was discarded, and the cell pellet was washed twice with 10 mL acetate buffer with followed centrifugation for 5 min and dried overnight. 15 mL of acetate buffer was added. The cells were disrupted with an ultrasonic device for 5 min at an amplitude of 100% and a pulse of 5:1. The sample was filled up with acetate buffer to the final volume of 50 mL. After the centrifugation at 3500 rpm for 5 minutes, the absorption of the supernatant was measured at 280, 565, 620, and 650 nm (D5000, Hach-Lange®). The calculation of the individual pigment phycocyanin (PC), allophycocyanin (AC), and phycoerythrin (PE) can be seen below [11]:

$$PC\ [mg\ mL^{-1}] = \frac{OD_{620} - 0{,}7 \times OD_{650}}{7{,}38}$$

$$AC\ [mg\ mL^{-1}] = \frac{OD_{650} - 0{,}19 \times OD_{620}}{5{,}56}$$

$$PE\ [mg\ mL^{-1}] = \frac{OD_{565} - 2{,}8 \times [PC] - 1{,}34 \times [AP]}{12{,}7}$$

2.2.7 Exopolysaccharide

The exopolysaccharide (EPS) yield was determined gravimetrically according to an intern work instruction [10]. An unloaded tube was weighed [m_1], filled with 50 mL of culture, and centrifuged at 3500 rpm for 5 minutes. The pellet was discarded, and the volume of the supernatant measured [V]. 150 mL of pure ethanol were subsequently added, and blended, until the EPS precipitated. The suspension was centrifuged at 3500 rpm for 5 minutes, the supernatant discarded, and the pellet dried at 40°C overnight. After drying, the tube was weighed again [m_2]. The calculation can be seen below:

$$EPS\ [g\ L^{-1}] = \frac{m_2\ [g] - m_1\ [g]}{V_{supernatant}\ [L]}$$

2.2.8 Calculation productivity

The productivity of biomass, -tChl, -tCar, -PBP and -EPS were calculated as noted below:

$$P_i(X) = \frac{x_1 - x_0}{t_1 - t_0}$$

x_0 = content at the start
x_1 = content at the end
t_0 = time at the start
t_1 = time at the end

2.3 Design of Experiment (DoE)

2.3.1 Selection and range of independent variables

The range of the variables (A) light intensities, (B) nitrogen and (C) magnesium concentrations were chosen according to literature. The light intensity was between 40-500 µmol photon m^{-2} s^{-1} [6, 21], nitrogen concentration between 5-15 mM [7, 14], and magnesium concentration between 5-45 mM [8, 12]. The experimental planning and analysis were supported by DoE Software, Design Expert 13®. The calculated number of experiments ($3^3 = 27$ experiments) was reduced by removal of redundant combinations. Table 4 shows the chosen experimental combinations by DoE.

Four media were prepared for experiments 1-4 and 12-15, and five media were prepared for the experiments 5-11, Tab. 4. An example for calculation of the required KNO_3 can be found below:

Tab. 4. Values and combinations of independent variables

Run	A: Light [µmol photon m^{-2} s^{-1}]	B: N [mM]	C: Mg [mM]
1	40	5	25
2	40	10	5
3	40	10	45
4	40	15	25
5	270	5	5
6	270	5	45
7	270	10	25
8	270	10	25
9	270	10	25
10	270	15	5
11	270	15	45
12	500	5	25
13	500	10	5
14	500	10	45
15	500	15	25

$$0{,}015 \text{ mol } L^{-1} \times M\,(N)\,[g\ mol^{-1}] = 0{,}015 \text{ mol } L^{-1} \times 14{,}01 \text{ g } mol^{-1} = 0{,}21 \text{ g } L^{-1}$$

$$c\,(KNO_3)\,[g\ L^{-1}] \times \frac{0{,}21 \text{ g } L^{-1}}{c\,(N/KNO_3)\,[g\ L^{-1}]} = 1 \text{ g } L^{-1} \times \frac{0{,}21 \text{ g } L^{-1}}{0{,}14 \text{ g } L^{-1}} = 1{,}5 \text{ g } L^{-1}$$

The same applied to the calculation of $MgSO_4 + 7H_2O$ admixture.

Experiments were conducted in biological triplicates and batch-cultures of 7 days. The dependent variables (OD, DW, PBP, EPS, tChl, tCar, tPO4-P, tN) were measured at the start and end of each experiment. These data were used for identifying correlations and optima of variables by the DoE Software, see Section 3.3 to 3.6. Furthermore, the OD was measured throughout to characterize the growth behavior, Fig. 2.

3. Results

As described in chapter 2.3.1, the experimental data were analyzed via DoE software to describe the correlations (R^2) between the independent variables (A, B, C) and dependent variables such as biomass, and PBP and EPS productivity.

3.1 Growth rate & biomass productivity

Optical densities were measured to monitor growth and calculate growth rate (μ). The highest growth rate (0,09 d^{-1}) and doubling time was found in the *P. p.* SAG 1380-1a under 270 μmol photon m^{-2} s^{-1}, at N10 and Mg25, Fig. 2A and Tab. 5. All strains showed a lag-phase in the first two days, Fig. 2A-C. Then the growth rates recovered from day 2 to 8, Fig. 2A-C. A slight decrease in growth was found in *P. p.* SAG 1380-1a from day 6 to 8, Fig. 2A. The highest biomass productivity was found in strain UTEX 161 (0,63 g L^{-1} d^{-1}), Tab 5.

The uptake of tN, and tPO_4-P from the medium were monitored and used to calculate the specific uptake rate for each strain under the tested growth conditions. The highest uptake rate for tPo_4-P was found in *P. p.* SAG 1380-1a under 270 μmol photon m^{-2} s^{-1}, at N10 and Mg25, Tab. 5. The highest N-uptake rate was found for the same conditions in *P. p.* SAG-1380-1d, Tab. 5.

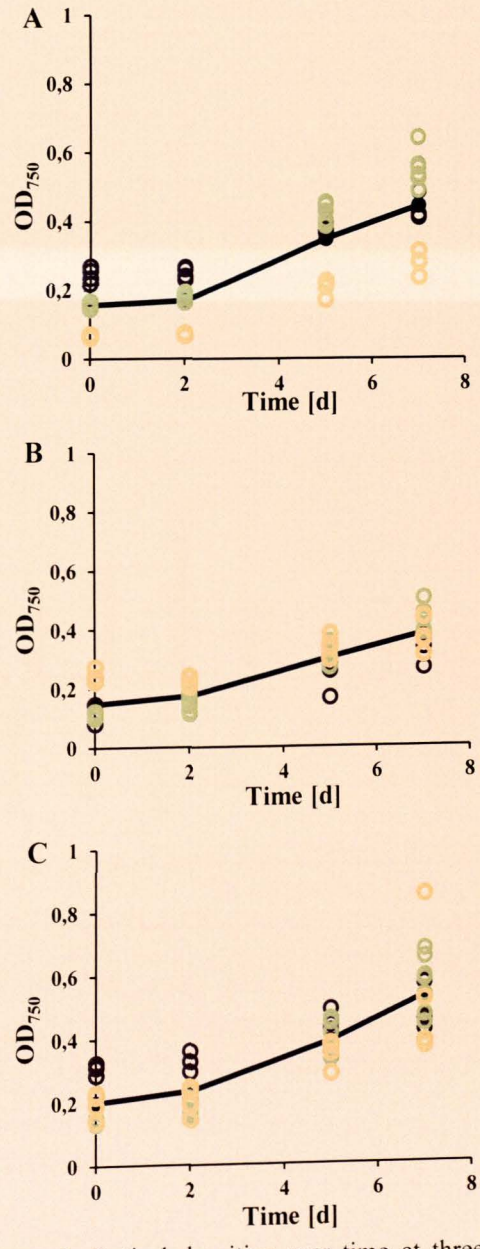


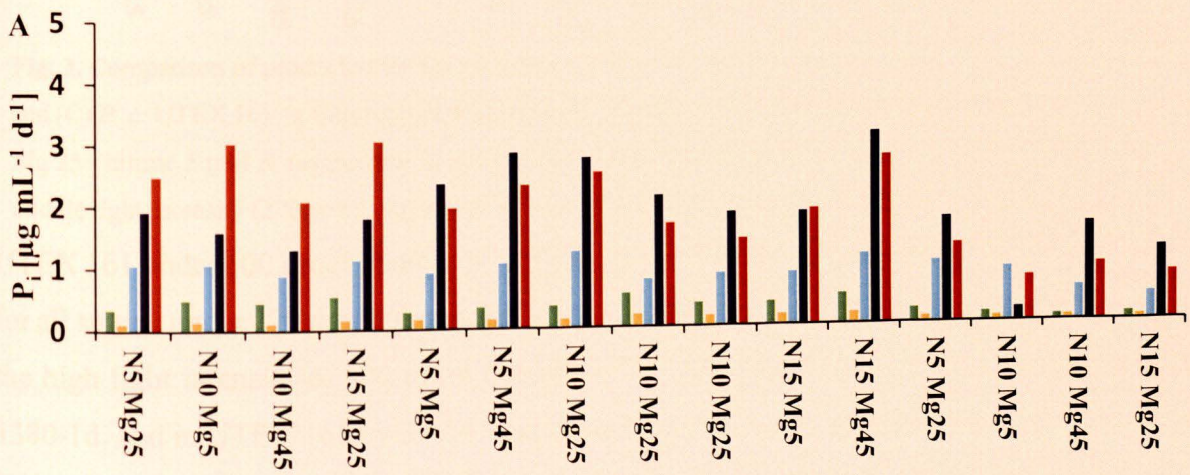
Fig. 2. Optical densities over time at three light intensities in (A) *P. p.* SAG 1380-1a, (B) *P. p.* SAG 1380-1d, and (C) *P. c.* UTEX 161. Light intensity: ◦, 40 μmol photon m^{-2} s^{-1}, ◦, 270 μmol photon m^{-2} s^{-1}, ◦, 500 μmol photon m^{-2} s^{-1}, —●— average optical density

Tab. 5. Results of three Porphyridium strains concerning of growth parameters and nutrients remove under 270 µmol photon m^{-2} s^{-1}, at N10 and Mg25.

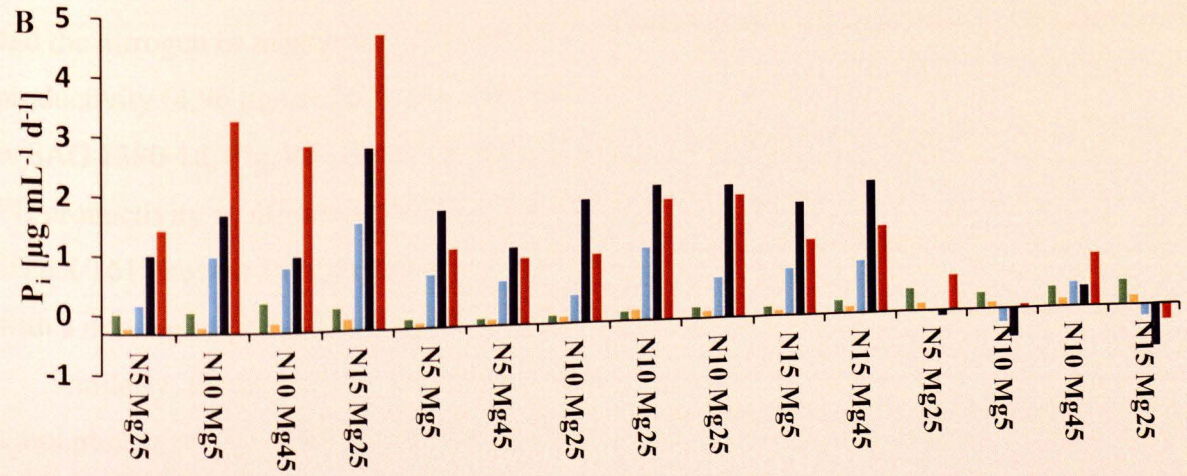
	SAG 1380-1a	SAG 1380-1d	UTEX 161
P_i Biomass [mg mL^{-1} d^{-1}]	0,60	0,62	**0,63**
µ[d^{-1}]	**0,09**	0,07	0,08
Double time [d]	**7,6**	9,6	8,6
Remove tPo_4-P [mg L^{-1} d^{-1}]	**0,47**	0,41	0,45
Remove tN [mg L^{-1} d^{-1}]	0,28	**1,43**	0,57

3.2 Pigment content

Pigment contents were measured and compared for experimental combinations. In all strains were pigment productivity and light intensity inversely correlated, Fig 3A-C. The highest chlorophyll productivity (tChl = 0,54 µg $mL^{-1}d^{-1}$) was found in SAG 1380-1a under 40



µmol photon m^{-2} s^{-1}, N15 and Mg25, Fig. 3A. The highest carotenoid productivity (tCar = 0,18 µg $mL^{-1}d^{-1}$) was identified in strain SAG 1380-1a under 270 µmol photon m^{-2} s^{-1} N15 and Mg 45, Fig. 3A. The tChl P_i decreased in all strains under the high light intensity of 500 µmol photon m^{-2} s^{-1} by 77-90%, and tCar P_i by 52-64%, Fig. 3A-C. However, the highest



phycoerythrin (PE = 4,96 µg $mL^{-1}d^{-1}$) and phycocyanin (PC =1,80 µg $mL^{-1}d^{-1}$) productivity was yielded in the strain *P. p.* SAG 1380-1d under the low light intensity of 40 µmol photon m^{-2} s^{-1}, and N15 and Mg25, Fig. 3B. The highest allophycocyanin (AC = 3,48 µg $mL^{-1}d^{-1}$) productivity was identified in UTEX 161 under 270 µmol photon m^{-2} s^{-1}, N10 and Mg25, Fig. 3C. The PE productivities decreased in all strains by 75-100% and negative productivities in

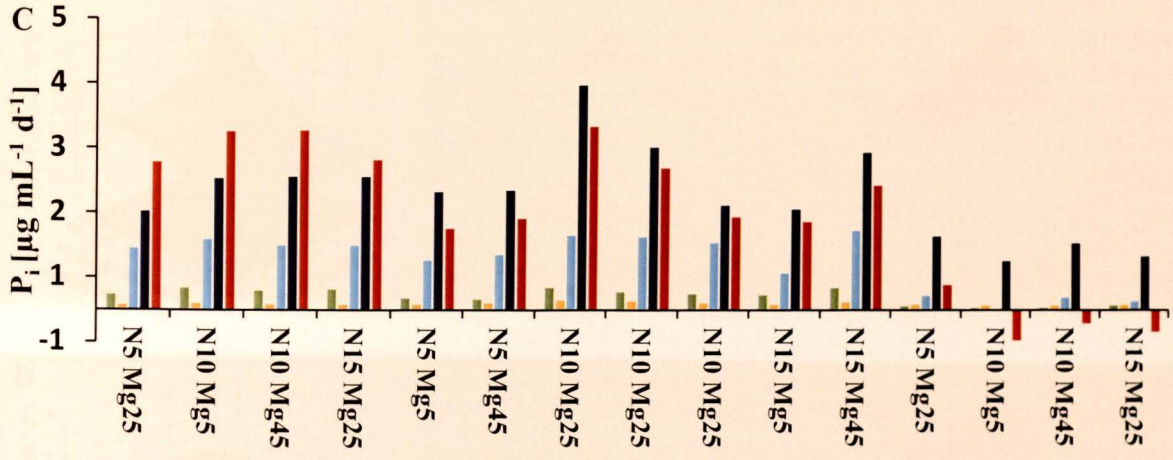


Fig. 3. Comparison of productivities for pigments synthesis in (A) *P. p.* SAG1380-1a, (B) *P. p.* Sag 1380-1d and (C) *P. c.* UTEX 161. ● Chlorophyll ● Carotenoid ● Phycocyanin ● Allophycocyanin ● Phycoerythrin; N5 Mg 25= nitrate 5 mM & magnesium 25 mM; exp. 1-4 low light intensity (40 µmol photon m^{-2} s^{-1}), exp. 5-11 middle light intensity (270 µmol photon m^{-2} s^{-1}), exp. 12-15 high light intensity (500 µmol photon m^{-2} s^{-1}).

UTEX 161 under 500 µmol photon m^{-2} s^{-1}, Fig. 3A-C. We found increases of AC productivities for all strains under 270 µmol photon m^{-2} s^{-1} by 48-58%, but the AC P_i decreased 73-93% under the high light intensity of 500 µmol photon m^{-2} s^{-1}, Fig. 3A-C. The PC P_i decreased in SAG 1380-1d, and in UTEX 161 by 39-74 % and negative productivities under 500 µmol photon m^{-2} s^{-1}, Fig. 3B & 3C. In contrast, the PC productivities remained relative constant in SAG 1380-1a, Fig. 3A. These results illustrate again, that light intensity is the most important variable in pigment production.

3.3 Phycoerythrin productivity

3D surface contour plots illustrate the dependency of PE-productivity of the light intensity, and the nitrogen or magnesium concentration in Porphyridium strains, Fig. 4. The highest PE-productivity (4,96 µg $mL^{-1}d^{-1}$) was identified at high nitrogen level but low light intensity in *P. p.* SAG 1380-1d, Fig. 4B. Both *P. p.* strains, SAG 1380-1a and -1d, showed a sharp maxima in PE productivity at 40 µmol photon m^{-2} s^{-1}, and 15 mM N, Fig. 4A & 4B. Contrasting, *P. c.* UTEX 161 showed an high maxima in PE productivity over a wide nitrogen range (5-15mM) with a maximum (PE = 3,88 µg $mL^{-1}d^{-1}$) at 15 mM N, Fig. 4C.

Similarly, the highest phycoerythrin productivity (PE = 3,23 µg $mL^{-1}d^{-1}$) was found at 40 µmol photon m^{-2} s^{-1} (R^2=0,96), Fig. 4F. The strain *P. c.* UTEX 161 showed a broad maximum

in PE productivity over a wide range of Mg concentrations (5-45 mM) with a maximum (PE = 3,23 µg $mL^{-1}d^{-1}$) at 5 mM Mg, Fig. 4F. In strong contrast, the strains *P. p.* SAG 1380-1a and 1-d showed moderate increase in PE productivity for all tested light intensities, and Mg concentrations, Fig. 4D & 4F. Light is most decisive variable for the PE productivity.

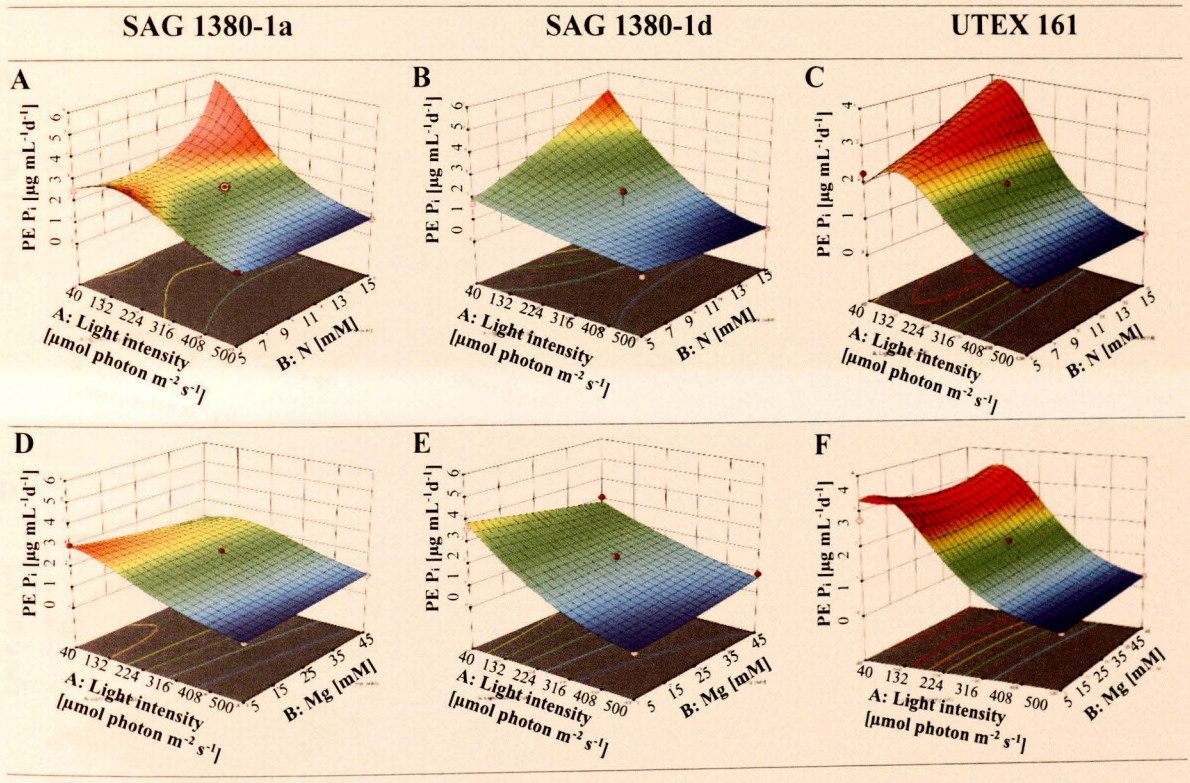


Fig. 4. 3D surface contour plots illustrating the dependency of phycoerythrin productivity of the light intensity, and nitrogen (A, B, C) or magnesium (D, E, F) concentration. Porphyridium strains left, SAG 1380ß-1a; center, SAG 1380-1d; right UTEX 161. Axis labeling: X1, light intensity [µmol photon m^{-2} s^{-1}]; X2, nitrogen concentration [mM]; X3, magnesium concentration [mM]; Y, PE P_i [µg $mL^{-1}d^{-1}$]

3.4 EPS productivity

3D surface contour plots illustrate the dependency of EPS productivity of the light intensity, and the nitrogen or magnesium concentration in three Porphyridium strains, Fig. 5. R^2 vary between 0,64-0,75 and the mathematical model were modified by addition of a summand (K = 2 g $L^{-1}d^{-1}$), because of negative productivities. The highest EPS P_i (1,53 g $L^{-1}d^{-1}$) was identified in *P. p.* SAG 1380-1d under the low light intensity of 40 µmol photon m^{-2} s^{-1} and 5 mM N, Fig. 5B. The strain *P. p.* SAG 1380-1a and -1d showed sharp maxima in EPS productivity at 40 µmol photon m^{-2} s^{-1}, and 5 mM N, Fig. 5A & B. Contrasting, the productivity in UTEX 161 showed a sharp maximum over a wide nitrogen-range, Fig. 5C.

The highest EPS P_i (1,76 µg $mL^{-1}d^{-1}$) was identified in SAG1380-1a at 40 µmol photon m^{-2} s^{-1}, and 45 mM Mg, Fig. 5D-F. The strains *P. p.* SAG 1380-1a and SAG1380-1d showed a sharp maximum in EPS P_i, Fig. 5D. In strong contrast, the strain *P. c.* UTEX 161 showed an increase in EPS P_i over wide magnesium range, Fig. 5F.

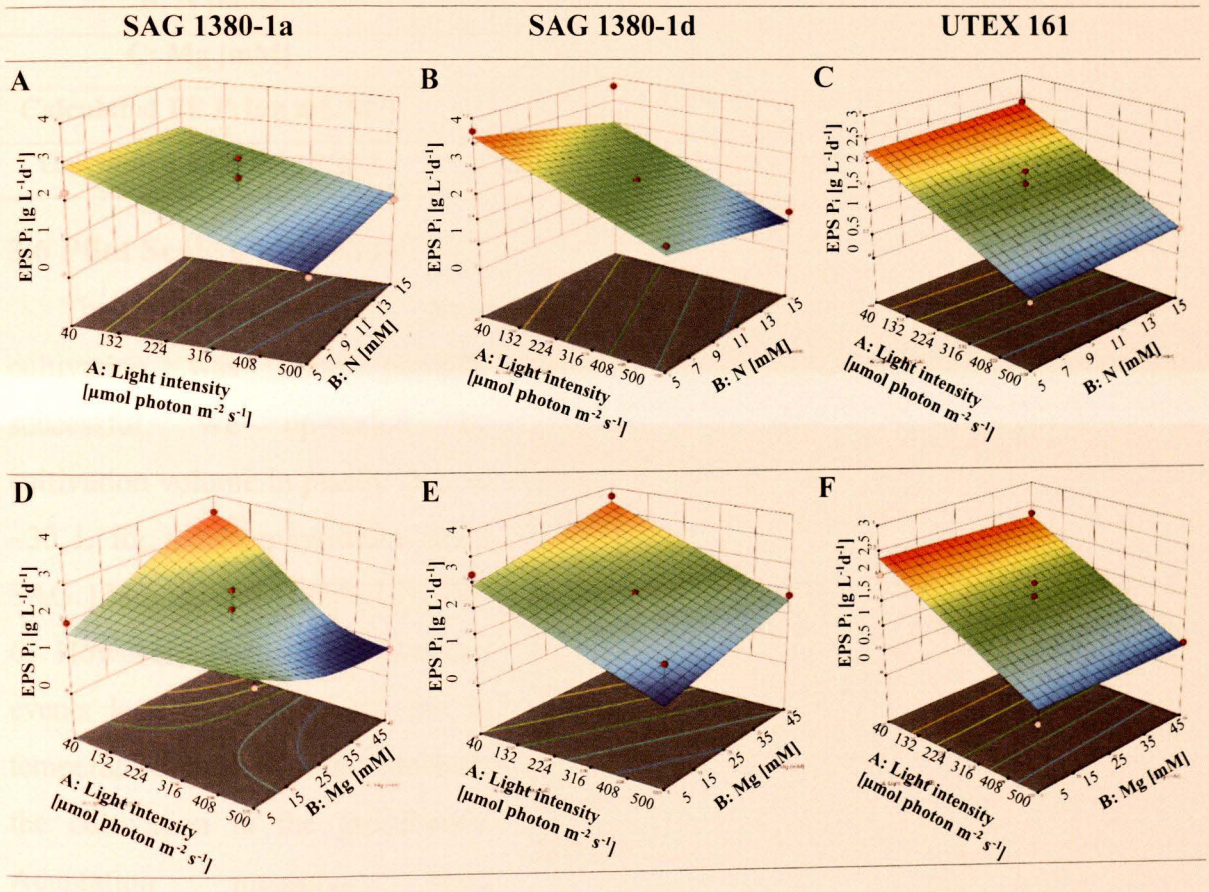


Fig. 5. 3D surface contour plots illustrating the dependency of EPS productivity of the light intensity, and nitrogen (A, B, C) or magnesium (D, E, F) concentration. Porphyridium strains left, SAG 1380ß-1a; center, SAG 1380-1d; right UTEX 161. Axis labeling: X1, light intensity [µmol photon m^{-2} s^{-1}]; X2, nitrogen concentration [mM]; X3, magnesium concentration [mM]; Y, EPS P_i [µg $L^{-1}d^{-1}$]

3.5 Optimization

The optimization of three independent variables for maximization of PE and EPS P_i were calculated with the software Design Expert 13, see Tab. 6. The optimum would be 40-41 µmol photon m^{-2} s^{-1}, 9-15 mM nitrogen, and for all strains. The magnesium concentration is rather low for UTEX 161 compared to SAG 1380-1a and -1d. The optimization showed that a high production of PE will be expect in *P. c.* UTEX 161, and EPS production in *P. p.* 1380-1a, see Tab. 6.

Tab. 6. Calculated optimization for maximization of PE and EPS productivity.

	SAG 1380-1a	SAG 1380-1d	UTEX 161
A: Light intensity [µmol photon m^{-2} s^{-1}]	40,01	40,74	40,01
B: N [mM]	13,92	8,98	15
C: Mg [mM]	38,29	44,99	5,01
Calculated PE P_i [µg $mL^{-1}d^{-1}$]	3,54	3,06	**4,54**
Calculated EPS P_i [g $l^{-1}d^{-1}$]	**5,75**	3,15	2,26

3.6 Pilot Scale Cultivation

The planned pilot scale cultivation was done partially successful. We up-scaled the cultivation volume in plastic bags to ~30 L for the Porphyridium strain SAG 1380-1d, and UTEX 161, Fig. 6. However, sudden hot weather events lead to a sharp increase in temperature (~50 °C) and hindered the cultivation in the greenhouse. Adaptation measures were implemented, but the planned comparative experiments could not be finalized, due to the lack of time.

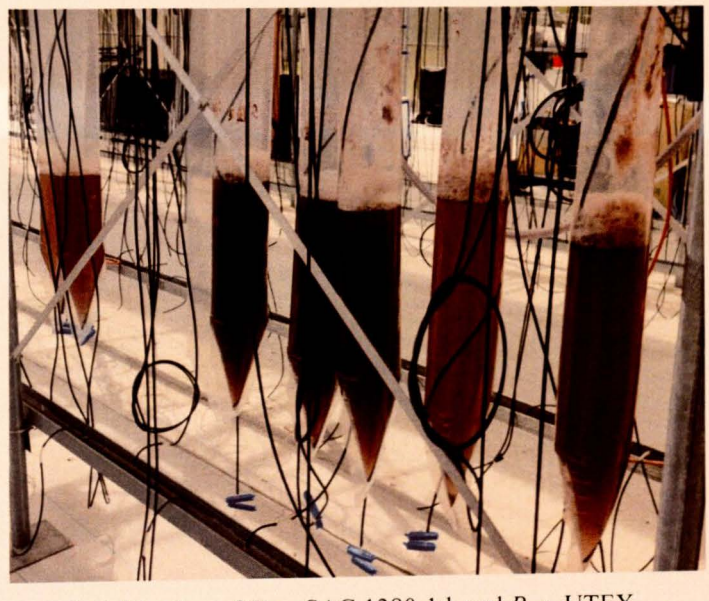


Fig. 6. Cultivation of *P. p.* SAG 1380-1d, and *P. c.* UTEX 161 in plastic bags under greenhouse conditions.

4. Discussion

In this study three commercial Porphyridium strains were tested for the coproduction of PE and EPS production under various growth conditions.

The OD-measurements were used to calculate the increase in growth. Overall, we found comparable growth pattern in all three Porphyridium strains. The μ ranged between 0,07- 0,09 (k = 0,25-0,31) and were half in comparison to the literature values (k = 0,76-0,84) [9]. This result was also confirmed by other literature [8, 14]. The authors used similar culture conditions (150 mL cultures in 250 mL Erlenmeyer flasks, ASW medium, shaker at 150-170 rpm, and a "cool white" fluorescent lamp at 53-62 μmol photon m^{-2} s^{-1}), but the serial number and quality of the lamp were not named. This might be due to growth limiting factors such as light illumination and spectrum. As a solution the light cycle might be increased from 14:10 to 18:6 for more photosynthesis [9, 23]. Furthermore, the duration of experiments should be longer than 7 days, because of cell acclimation [8, 9, 14].

Further, we confirmed that both, chlorophyll, and carotenoid content are no reliable growth indicators. Porphyridium gains most photosynthetic energy through the PBP [19]. The highest PE productivity was found in *P. p.* SAG 1380-1d. We found a similar PE productivity and percent by dry weight (PE/DW = 3,8 %) by comparison with literature (PE/DW = 2,8-3,3 %), respectively [12, 24]. The authors used similar growth conditions (100 mL culture in 250 mL Erlenmeyer flasks, ASW medium, shaker at 80 rpm, 18 μmol photon m^{-2} s^{-1}, T = 25°C), so that the results are comparable.

The PBP productivities were decreased due to high light intensities of 270-500 μmol photon m^{-2} s^{-1}. Light is the most important factor for PBP synthesis and Porphyridium need less PBP for absorption all photons under a higher light intensity [25]. The light sensitivity of PE was confirmed by literature [25]. The phycoerythrin content were also decreased by 40% under increasing light intensities of 10-120 μmol photon m^{-2} s^{-1} during constant contents of phycocyanin and allophycocyanin [25].

We found an optimal and wide range of N and Mg concentrations in UTEX 161, so it seems that the strain reacts sensitive to N and Mg. We found literature, which showed evidence to our results [14]. The authors used the same strain and similar culture conditions (UTEX 161, 150 mL culture in 250 mL Erlenmeyer flasks, f/2 medium, shaker at 150 rpm, 98 μmol photon m^{-2} s^{-1}, T = 23°C) and they analyzed the effect of different N concentrations on growth, and carbohydrate formation. We found that *P. c.* UTEX 161 metabolized low nitrogen as well as high concentrations [14]. Low concentrations conditioned N limitation in microalgae over time

[14], so that a higher N concentration would be preferred. Furthermore, we did not find comparable studies for Mg sensitivity, so that the result cannot be verified. We found the optimal point for N in SAG 1380-1a and -1d. For that reason, experiments should be done between light intensities 20-200 µmol photon $m^{-2}s^{-1}$, in *P. p.* SAG 1380-1a & -1d. Moreover, the SAG strains did not react to Mg, so it seems that Mg is not necessary for PE production in SAG 1380-1a and -1d. The hypothesis cannot be supported by literature, because of the same reason as above described. For increasing the PBP P_i, we found an application of additional blue, fluorescent light (400-430 nm), which can increase the PBP content by stimulation of PE [8, 15]. The technical practicability will be proved for our incubator.

We found no reliable optimum for EPS productivity, since correlations were low (R^2 = 0,64-0,75). Porphyridium can use secreted EPS as a second energy source [22], for that reason we found negative EPS P_i.

The DoE software calculated the potential production of PE and EPS for all strains. The optimization for PE production shows that the strain UTEX 161 produces mainly PE and PBP according to literature [12, 17]. Therefore, the PE production of UTEX 161 is gradable by comparison of our results, see Section 3.3. The optimization of EPS production was not evaluated, because of low correlations.

The total remove of tPo_4-P was done successfully, because we found slightly less than the calculated tPo_4-P and the results are similar. However, the measurement of tN is not a reliable method, because we found twice as much of tN at the beginning of an experiment as the calculated concentration. It is possible that cell debris and contaminations cause this effect.

The greenhouse cultivation was done partially successful because the cultures were killed by heat. Porphyridium is affected to heat and literatures confirm this issue [6, 20]. Industrial processes are also concerned [21], so that simple and cheap solutions could be used. For low technical costs, Porphyridium should be cultivated seasonal in greenhouses in the spring or autumn in plastic bags at 15-25°C under 40-200 µmol photon $m^{-2}s^{-1}$ and aerated with air. The technical costs increase in winter and summer. In summer, the cultures should be cooled and shaded. During winter, cultures could be heated and illuminated by additional light sources.

The further outlook shows that we will use our optimized growth medium, N15 Mg5 in *P. c.* UTEX 161, for our greenhouse cultivation under low light intensities from 40 to 200 µmol photon m^{-2} s^{-1} in the winter, spring, or autumn. The strains SAG 1380-1a and -1d will be analyze concerning EPS and PBP production under lower light intensities of 20-200 in laboratory.

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