Growth and Photosynthesis of *Nanochlorum eucaryotum*, a New and Extremely Small Eucaryotic Green Alga

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Z. Naturforsch. **37 c**, 115–119 (1982); received October 19, 1981

Nanochlorum eucaryotum, Growth, Photosynthesis, Cytochrome f, Chlorophyll-Protein Complexes

Nanochlorum eucaryotum is a very small species of unicellular coccoid green algae (1.5 µm). The growth of *Nanochlorum* under different conditions of salinity, pH and light intensities was studied. Optimal growth rates were observed with normal sea water salinity and low light conditions at pH 7.0. The contents of chlorophylls, carotinoids, soluble proteins and the chlorophyll a to chlorophyll b ratio were measured. The light saturating curves of *Nanochlorum* cells grown under light intensities of 100 lx, 2000 lx and 10000 lx reveal a very narrow capacity of light adaptation. When cultured under higher light intensities, *Nanochlorum* was not able to reach high photosynthetic activities but underwent a photoinhibition of photosynthesis. The contents of cytochrome f, P-700 and ribulosebisphosphate carboxylase were low and comparable with those of low light adapted Chlorella cells. The analysis of the chlorophyll-protein complexes shows that about 80% of total chlorophyll is bound in the light harvesting chlorophyll protein complexes. All results indicate that *Nanochlorum* is a low light adapted marine organism with very narrow ecological flexibility.

Introduction

Some months ago, we discovered and isolated what seems to be a new green alga. The most important features of this autotrophically growing alga, named *Nanochlorum eucaryotum*, are its extremely small cell size (up to 0.8–1.1 µm in width and 1.2–2.2 µm in length), its very reduced cellular organization (one nucleus, one chloroplast, one mitochondrium and very little cytoplasma) and its very low DNA content (6.0×10^{-14} *g* DNA per cell) [1]. To our knowledge, it is the smallest eucaryotic photophytotrophic organism, and there is no green alga that contains less DNA. In the present study, we investigated the growth of *Nanochlorum* under controlled conditions as well as the structure of its photosynthetic apparatus.

Materials and Methods

The cells were grown under conditions described previously [1]. The growth factor µ was evaluated by the following formula:

$$\mu = \frac{A_{680} - A_{750} \text{ of the culture after 3 days}}{A_{680} - A_{750} \text{ of the starting culture}}.$$

The pigment analysis was carried out according to Bauer and Wild [2]. The cell volume was calculated on the basis of the cell size, which was measured with a calibrated ocularmicrometer fixed on a ZEISS light microscope. The number of cells per volume cell suspension was counted in a Neubauer chamber. The content of soluble protein was measured with the Bio-Rad protein assay (Fa. Bio-Rad laboratories). The photosynthetic CO_2 fixation and O_2 evolution were determined with the methods as described previously [2]. The determination of the content of P-700 and Cyt f has already been published [3]. Before measuring the RuBP carboxylase activity, the algae were harvested by centrifugation (20 min at $5000 \times g$). The pellet was resuspended in the following medium: 0.05 M Tricin buffer, 0.075 M KCl, 0.35 M mannitol, adjusted with KOH to pH 8.0. The concentrated cell suspension was transferred to a duran glas flask, ¾ filled with a mixture of glass beads (0.2 mm and 1 mm in diameter), and homogenized under cooling with CO_2 snow in a homogenizer (type MSK Braun, Melsungen). The further preparation was carried out according to Wild *et al.* [4]. The chlorophyll protein complexes were sepa-

Abbreviations: A, absorbance; Chl, chlorophyll; CP I, P-700-chlorophyll a-protein; Cyt f, cytochrome f; LHCP, light-harvesting chlorophyll a/b protein; FC, free chlorophyll; P-700, reaction center of photosystem I; RuBP, ribulosebisphosphate; susp, suspension.

Reprint requests to Prof. Dr. A. Wild.

0341-0382/82/0100-0115 $ 01.00/0

rated by SDS gel electrophoresis (PAGE) as previously published [5]. Gel scanning was carried out at 653 nm and at 672 nm.

Results and Discussion

In our experiments with *Nanochlorum eucaryotum*, we tried to determine whether the very small cell size is either caused by environmental conditions, or whether it is strongly fixed by genes. It is well known that the cell size and the complexity of cell structure of many algae are reduced under extreme limitating conditions [6]. Looking for limitating factors of growth, we checked the growth rate under various conditions. In all experiments, it was impossible to observe any alterations of cell size caused by environmental conditions.

The growth rates of marine and limnic organisms depend on the salinity of the environmental medium [7]. Therefore, we tested, the growth of *Nanochlorum* under different salt concentrations (between 0.1% and 15%). As plotted in Fig. 1, *Nanochlorum* shows its optimal growth rate at a salt concentration of 3.5%–4%; this value corresponds to the osmotic pressure of normal sea water [8]. The figure also shows that *Nanochlorum* is able to grow under very reduced osmotic pressure as well as under conditions of threefold higher salinity than sea water. It is only in a medium containing more than 12% salt that the growth rates drops below one and the cells die.

Fig. 2 illustrates the dependence of growth on the pH. The optimal growth conditions were found at

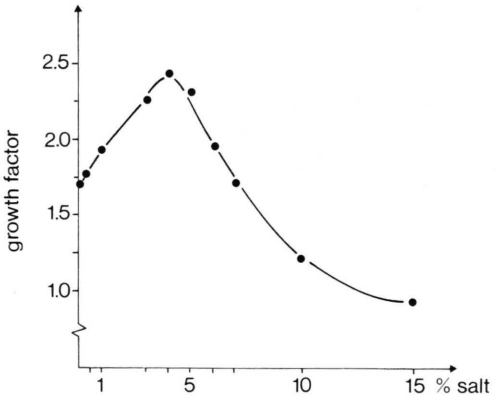

Fig. 1. Growth dependence of *Nanochlorum eucaryotum* on different salt concentrations in the medium.

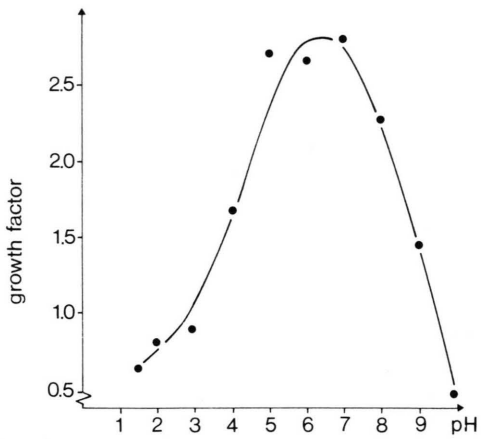

Fig. 2. The pH optimum curve of the growth of *Nanochlorum eucaryotum.*

pH between 5 and 7; but even at pH 4, respectively pH 9, the growth rate is higher than one. The pH of the growth optimum of *Nanochlorum* significantly differs from the pH of free sea water, which is reported to be in the range from 7.4–8.5 [9]. This result suggests that *Nanochlorum* does not belong to the free living nanoplancton.

Fig. 3 demonstrates the growth dependence of *Nanochlorum* on different light intensities during culturing. Curve A shows the time course of growth

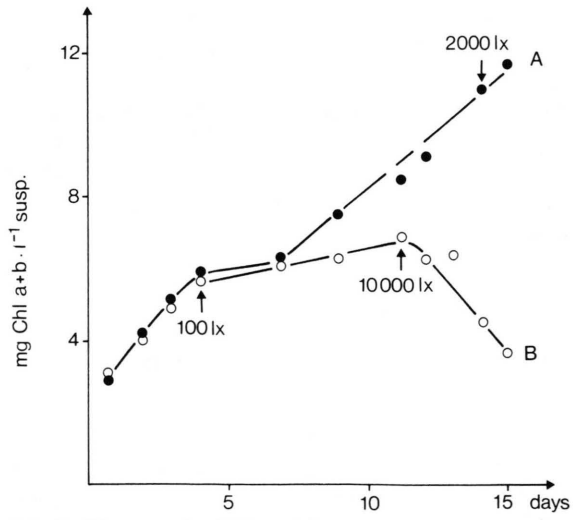

Fig. 3. The growth of *Nanochlorum eucaryotum* under different light intensities. Curve A (●—●) shows the greening of the culture under a light intensity of 2000 lx. In curve B (○—○) the light intensities have been changed.

without changing the light intensity. The growth inhibition between the fourth and the seventh day was caused by changing the culture medium in order to avoid limitation by the nutrients. Between the seventh and tenth day, the culture illuminated with 2000 lx grew with a higher rate than the 100 lx culture (culture B). A transfer of the culture B to a higher light intensity (10 000 lx) led to a stop in growth eventually to the death of the cells. From this experiment it can be concluded that *Nanochlorum* is not able to exist under permanent high light conditions.

These observations provoked the question how the photosynthetic apparatus is organized in this organism. A comparison of different physiological parameters of *Nanochlorum* with those of low light adapted *Chlorella* cells reveals many similarities. Table I summarizes these results: there are also interesting differences especially in respect to the chlorophyll content. The content of chlorophyll per cell is thirteen times lower in *Nanochlorum* than in *Chlorella.* This difference is not surprising if one takes into account the different cell sizes of these two algae. In contrast, it is astonishing that the chlorophyll content per cell volume is four times higher in *Nanochlorum* than in *Chlorella.*

The light saturating curve gives much information about the ability of a plant to adapt to different light intensities during growth. Fig. 4 shows three

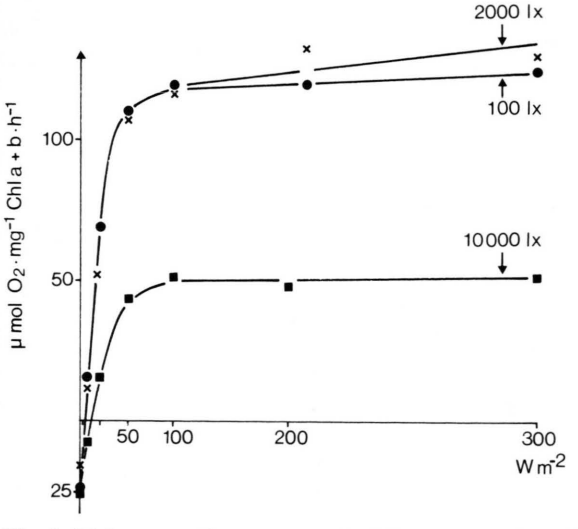

Fig. 4. Light saturating curves of different cultures of *Nanochlorum eucaryotum.* Cells were precultivated at 2000 lx and transferred to different light intensities for 36 h.

Table I. Different physiological parameters of *Nanochlorum eucaryotum* and *Chlorella fusca* C. 1.1.10 grown under low light conditions.

Parameter	*Nanochlorum*	*Chlorella fusca*
cell volume ml	1.33×10^{-12}	65.47×10^{-12}
mg Chl a + b per cell	4.7×10^{-11}	6.1×10^{-10}
mg Chl a + b per ml cell volume	35.34	9.32
mg Chl a + b per g dry weight	24.9	31.1
Chl a/Chl b	2.4	2.2
mg carotine per g dry weight	8.2	6.4
mg carotine per mg Chl	0.3	0.2
soluble protein per g dry weight	130.0	206.0
soluble protein per mg Chl	5.2	7.0

light saturating curves of *Nanochlorum* grown under 100 lx, 2000 lx and 10 000 lx for 36 hours. It is evident that *Nanochlorum* is not able to adapt to high light conditions. According to several authors [10, 11], high light cultures of plants that are able to adapt to high light conditions reach higher photosynthetic activities at saturating illumination than the low light cultures. The contrary is observed with *Nanochlorum.* Culturing under high light conditions damaged the cells, and they showed very low photosynthetic activity. On the other hand cells of *Nanochlorum* grown under 100 lx show the same rate of photosynthesis as those cultures cultivated under 2000 lx. Measuring the CO_2 uptake, one attains comparable photosynthetic activities (Table II) as based on the rates of O_2 evolution. When the O_2 evolution was recorded at very high light intensity (325 W/m²) for a time of twenty minutes, the photosynthetic rates decreased markedly, although the conditions of measurement were not limiting. In algae two types of adaptive reaction to the factor light can be distinguished. The "*Chlorella* Typ" is

Table II. Different components of the photosynthetic apparatus of *Nanochlorum eucaryotum.*

Chl/Cyt f	963
Chl/P-700	1200
RuBP carboxylase activity *in vitro* in µmol CO_2 mg^{-1} Chl a + b h^{-1}	85.5
In vivo CO_2 fixation rate in µmol CO_2 mg^{-1} Chl a + b h^{-1}	101

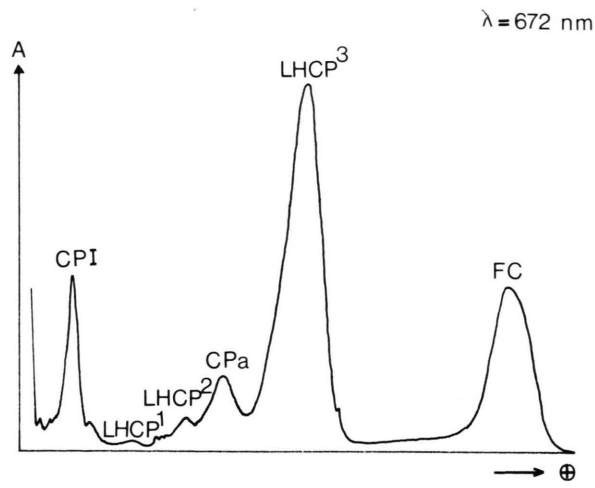

Fig. 5. Distribution of chlorophyll-protein complexes of *Nanochlorum eucaryotum* by scanning unstained gels *in situ* at $\lambda = 672$ nm.

comparable to the light adaptation reaction of higher plants [10]. The other type, found in *Cyclotella,* shows an inverse correlation between the activities of photosynthesis and light intensity [12, 13]. Frankzisket [14] reported that filamentous green algae living in the skeleton of reef corals show a photoinhibition of photosynthesis at light intensities higher than 500 lx. The behaviour of *Nanochlorum* under different light intensities shows no similarities to the "*Cyclotella* type". As in *Nanochlorum* the inhibition of photosynthesis under higher light intensities is very drastic and a clear adaptive reaction to different low light regimes (*e.g.* 100 lx and 2000 lx) is not visible, one cannot speak of an ability to adapt to the environmental factor light at all. This also supports the hypothesis, that *Nanochlorum* does not occur in the free living plancton.

In order to learn about the causes of the low maximal photosynthetic capacity of *Nanochlorum,* we checked components of the photosynthetic electron transport chain, the chlorophyll-protein complexes and the RuBP carboxylase activity. Table II shows the ratios of Chl/Cyt f, Chl/P-700 and the activity of the RuBP carboxylase. If one takes into account that the ratios Chl/Cyt f and Chl/P-700 can vary under different environmental conditions [3], it can be stated that *Nanochlorum* contains these two components in concentrations which are found in low light adapted *Chlorella* cells.

Table III. Chlorophyll-protein complexes of *Nanochlorum eucaryotum* separated by SDS gel electrophoresis (PAGE).

	FC	$LHCP^3$	CP a	$LHCP^2$	$LHCP^1$	CP I
% of total chlorophyll	20.2	61.7	4.7	3.8	2.4	4.2
Chl a/Chl b	6.0	1.7	71.9	2.0	3.3	89.3

The high content of chlorophylls per cell volume, the low Chl a/Chl b ratio, the low photosynthetic capacity and the ability to grow under extremely low light conditions give evidence that *Nanochlorum* possesses a very effective light harvesting system. The analysis of the chlorophyll-protein complexes indicate that the light harvesting complexes contain about 70%–80% of the total chlorophyll. Figs. 5 and 6 demonstrate a densitogramm recorded at 653 nm and at 672 nm. In this separation, both diagrams give evidence of the dominance of the $LHCP^3$. The complexes that contain the reaction centers occur in very small amounts. Table III represents the proportions of the different chlorophyll-protein complexes of total chlorophyll and the Chl a/Chl b ratios. The free pigment band (FC) originates, in most parts, from the LHCP complexes, as it contains a relatively high chlorophyll b content. The antennae complexes of the two reaction centers, the CP a and the CP I, are represented only in proportions lower than 5%. This correlates with the extremely low P-700 content per chlorophyll (Table II). The very high Chl a/Chl b ratios in the CP a and the CP I prove the purity of the separation.

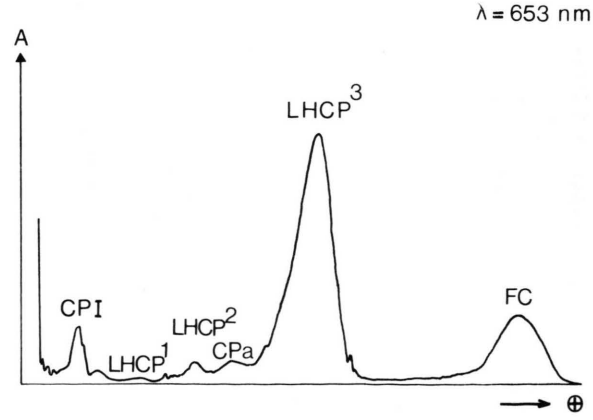

Fig. 6. Distribution of chlorophyll-protein complexes of *Nanochlorum eucaryotum* by scanning unstained gels *in situ* at $\lambda = 653$ nm.

Summarizing and comparing our results with well known data of *Chlorella,* we can conclude that *Nanochlorum* possesses a photosynthetic apparatus which is very similar to that of *Chlorella.* In contrast to *Chlorella,* however, *Nanochlorum* is strongly adapted to low light conditions. This very narrow ecologic flexibility may be caused by its small genetic pool (low DNA content). The physiology of *Nanochlorum* confirms the supposition that it occurs as an inhabitant of a not yet identified marine organism.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and is part of the first author's thesis.

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