Nanochlorum eucaryotum: a Very Reduced Coccoid Species of Marine Chlorophyceae

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Cell Division, Chlorococcales, Chloroplast, DNA Content, *Nanochlorum*

Nanochlorum eucaryotum was isolated from a sea water aquarium housing different sponge species, cucumarias, small crustaceans and annelids. This bright green marine alga differs from all other known coccoid species. Its most prominent features are its very small cell size (1.5 µm) and its reduced cellular organization. Its cell contains one nucleus, one chloroplast, one mitochondrium and small vacuoles. Sometimes a Golgi apparatus can be seen. No other subcellular features have been observed. The cell wall is thin and smooth and does not contain any material of high electron density; only dividing cells show a rougher surface. The cells split into two daughter cells. No sexual reproduction has been observed in this organism. We have analyzed the ultrastructural cell organization, the amount of total DNA and RNA per cell, the pigment composition, the growth requirements and the sensitivity towards different inhibitors (chloramphenicol, cycloheximid, penicillin, lysozym and cellulase). The results afford the introduction of a new species; a new family or suborder of coccoid green algae is discussed.

Diagnosis

Cellula ovata et singularis, nucleo vero, membrana tenui et glabra aut verrucosa in casu divisionis. Unus chromatophorus viridi colore fulgens semperque parietalis, cellulam explens ultra dimidiam partem. Pyrenoides non observatae.

Propagatio fit divisione cellulae in duas pares cellulas. Diametrus cellulae 1.5 µm (0.8–2.2 µm).

The unicellular eucaryotic green alga is commonly surrounded by a smooth and thin cell wall. The chloroplast is bright green and parietal, occupying more than 50% of the cell. No pyrenoid is observed. In all cases only two daughter cells are formed during cell division. Dividing cells often have a rougher cell wall. The cell size varies between 0.8 µm and 2.2 µm and was found to be 1.5 µm on the average.

Introduction

In the past some species of very small eucaryotic algae were detected that were almost as small in size as procaryotes. Ettl [1] described an asymmetric green flagellate, and Manton [2] introduced a very reduced monadoid chrysophyceae. In addition to monadoid algae, coccoid forms of the genus *Chlorella* were isolated, which show a very small cell size (1.5 µm–3 µm) [3, 4]. Professor Zahn from the Institut of Physiological Chemistry at the University of Mainz was the first, who observed a very small, bright green, eucaryotic green alga that differs from all other known coccoid species.

After isolation, we are now able to cultivate the cells under controlled conditions and to characterize its growth requirements. This report introduces the new species, named *Nanochlorum eucaryotum*, and makes some proposals for its taxonomy.

Abbreviations: Chl, chlorophyll; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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Materials and Methods

Isolation and culturing

Nanochlorum has been isolated from a sea water aquarium housing different sponge species, cucumarias, small crustaceans and annelids collected from the Adriatic Sea (Rovinj, Yugoslavia). The cells were separated from other organisms by differential centrifugation and were transferred in the culture medium, technical sea water supplemented by the following nutrient solution: 5 mM KNO_3, 0.5 mM KH_2PO_4, 16.4 mM $FeSO_4$ and AZ solution according to Strotmann [5]. The pH was maintained at 7.0.

The cells were grown for fifteen days on a shaker under permanent light of an intensity of 2000 lx at 20 °C. The cultures reached the steady state phase at a cell density of 1.5×10^{11} cells per litre that corresponded to a chlorophyll content of about 10 mg per litre. These cultures were contaminated with about 5% unidentified organisms: colorless bacteria and zooflagellates, green algae greater than 3.5 µm and diatoms. These cultures were centrifuged 30 min at 100 *g* at 4 °C for dissipating the contamination which was 0.5–1% after this procedure. These cultures were used for measurements.

SEM

The cell suspension was centrifuged 15 min with 20 000 × *g*. The pellet was transferred to a fixation solution containing 2% OsO_4/Michaelis buffer with 24% saccharose (pH 7.2) for 1.5 hours. Fixed cells were dehydrated by using a series of alcohols steps up to acetone and critical point dried. Critical point drying from CO_2 and the following coating with gold was carried out with the sputter Coater Polaron E 5100 (2 × 2′). The Cambridge Mark II A at 20 KV was used for observations.

TEM

For TEM observations, the cells were centrifuged 10 min with 16 000 × *g* and fixed in 2.5% glutaraldehyd in 0.1 M cacodylatbuffer/sea water 1:1 (pH 6.8) at 4 °C during the night. The postfixation was carried out in 1% OsO_4/0.1 M cacodylatbuffer (pH 7.2) for 1.5 hours at 4 °C. The samples were washed with buffer medium, dehydrated in a graded series of ethylalcohol and embedded in Araldit. The thin sections were poststained with lead citrate and examined with a Hitachi H-600 electron microscope at 75 kV.

The light microscopic observations were carried out with a LEITZ microscope and a Neubauer chamber. The sell size was determined with a calibrated ocularmicrometer.

DNA/RNA content

For the determination of the DNA content, the organisms were extracted and treated according to Barth and Willershausen [6] by crushing with prewashed carborundum (particle size ca. 0.01 mm previously washed with 3 M HCl and 0.2 M EDTA, pH 8.0). The content of RNA and protein was determined as described previously [7].

Biochemical analysis

The spectra were monitored with an AMINCO DW 2 spectrophotometer. The pigment extracts were prepared according to Wild and Egle [8] and identified by the method of Hager and Stransky [9].

In order to test the sensitivity of *Nanochlorum* towards some inhibitors of the protein synthesizing system, chloramphenicol and cycloheximid were added to the nutrient medium with a final concentration of 50 µM. The capacity to synthesize procaryotic cell wall components was tested with penicillin G (concentration 2500 E/ml) and lysozym (500 µg/ml). The inhibition was determined by means of the chlorophyll content, 5 and 20 days after the application.

Results

No internal cell organization is visible in the light microscope (Fig. 1). The cells are spheroidal and sometimes double forms can be seen. The cell size ranges from 0.8–1.5 µm in width and from 1.1–2.2 µm in length.

SEM

The SEM observation shows that the cells of *Nanochlorum* are roundish or spherical on an average diameter of 1.5 µm. In Fig. 2, some rod bacteria appear beside the cells of *Nanochlorum*. The cell surface is smooth and sometimes small granulae can be seen (Fig. 3). These surface structures especially appear after application of cellulase during culturing (Fig. 4), although smooth forms remain visible. Dividing cells are larger and lengthened and show an equatorial groove (small arrow in Fig. 2), which becomes deeper towards the end of the propagation. In Fig. 2, two different phases are marked by a small and a large arrow. The fraction of dividing cells was found to be below 5%.

TEM

The TEM observation reveals that the cells of *Nanochlorum* contain one nucleus, one mitochondrium and one chloroplast, which fills more than half of the cell (Fig. 5). Sometimes the cytoplasma

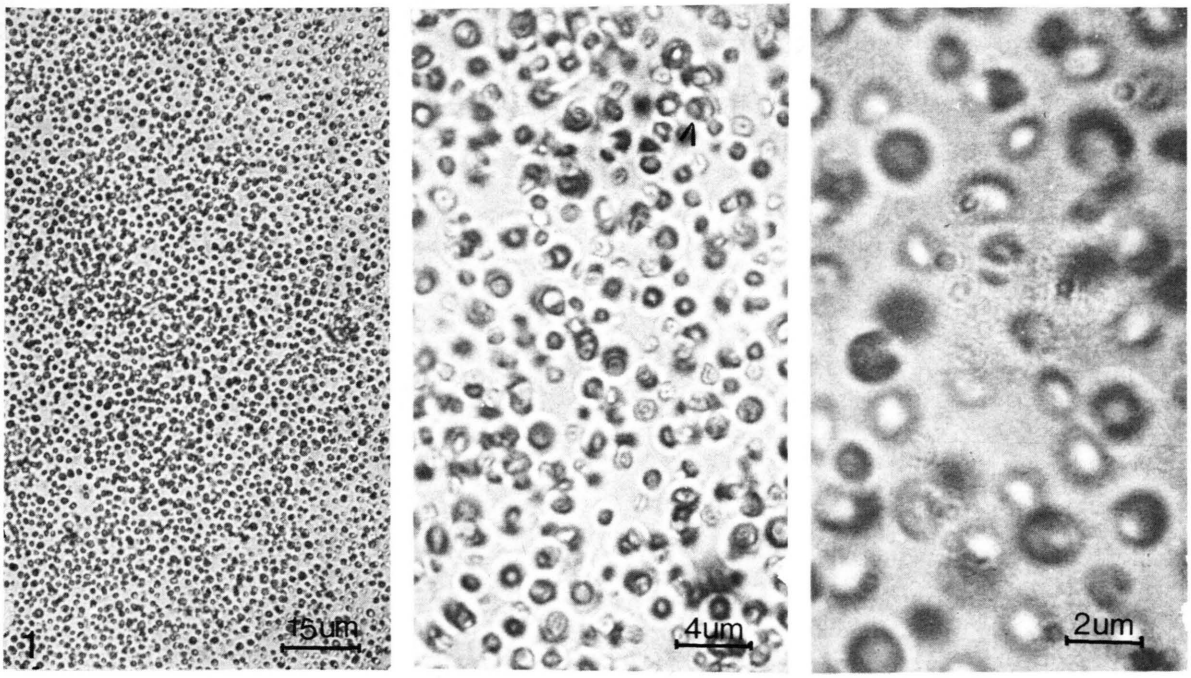


Fig. 1. Cells of *Nanochlorum* under the light microscope. The cells show a cell size of 1.5 µm on the average. No subcellular cell organization is visible.

includes little vacuoles. Figs. 6 and 7 show two representative cells of *Nanochlorum* grown under a light intensity of 2000 lx. Usually, the chloroplasts do not contain large amounts of starch. Yet in some cases, starch granulae can be seen. No pyrenoid has been observed. If one examines cells of cultures grown under extremely weak light conditions (500 lx), the internal organization of the chloroplast and the other organells remains unchanged (Fig. 8). In *Nanochlorum* the cytoplasm occupies a very small fraction of the cell volume only (Figs. 5, 6, 7).

The chloroplast is surrounded by a double membraned envelope. Osmiophyilic bodies, the plastoglobuli, regularly, in the chloroplast are found. It's thylacoids are grouped into band of three to five lamellae without grana (Fig. 9).

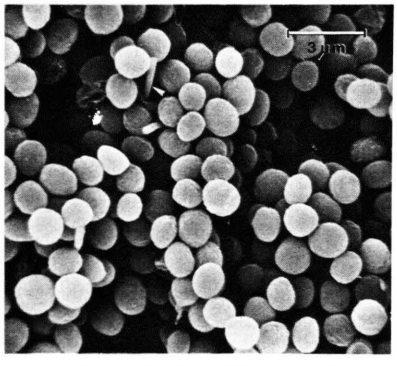


Fig. 2. Cells of *Nanochlorum* under the scanning electron microscope. The cells show a smooth surface. Beside the cells of *Nanochlorum* some rod bacteria appear in the pellet (arrow).

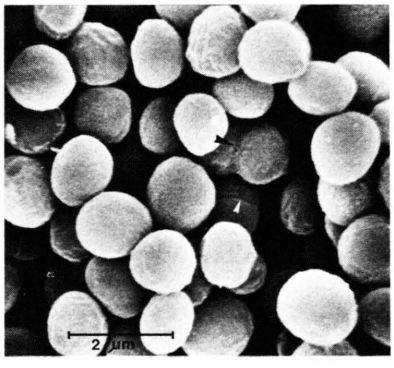


Fig. 3. Dividing cells of *Nanochlorum* under the scanning electron microscope. The arrows indicate two different phases of propagation. Some cells show a rougher surface.

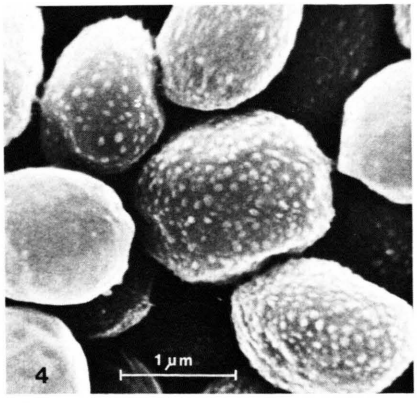


Fig. 4. Cells of *Nanochlorum* under the scanning electron microscope after treatment with cellulase. The surface is rough, in most cases, and shows little granulae.

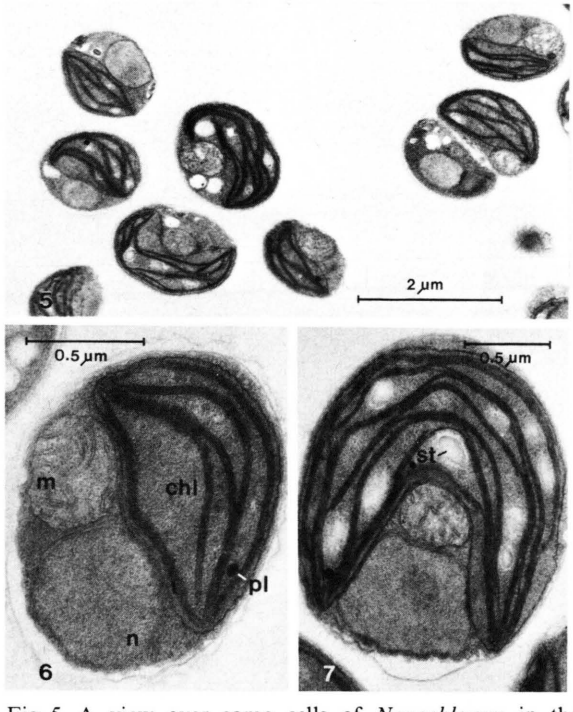
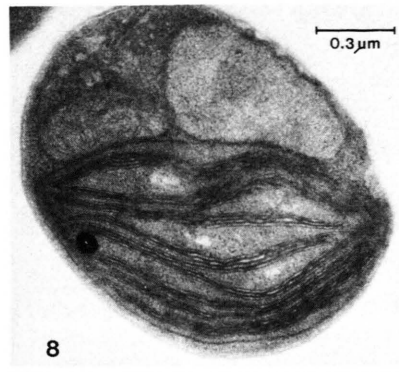


Fig. 5. A view over some cells of *Nanochlorum* in the transmission electron microscope. Single cells have a diameter of about 1.5 µm, whereas dividing mother cells are 1.8 µm in size.

Fig. 6. One single cell of *Nanochlorum* grown at 2000 lx with one chloroplast, one mitochondrium and one nucleus. The chloroplast contains no starch granulae.

Fig. 7. One single cell of *Nanochlorum* grown at 2000 lx. The chloroplast contains some starch granulae, but no pyrenoid is visible.



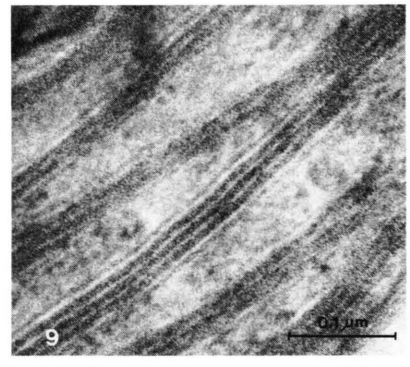


Fig. 8. One single cell of *Nanochlorum* grown at 500 lx. The subcellular structure is the same as in cells grown at 2000 lx light intensity.

Fig. 9. Thylacoid bands in the chloroplast of *Nanochlorum*. The band consists of 5 stacks of thylacoids that is typical for green algae.

The cell wall of *Nanochlorum* is thin (10–30 nm) and, in most cases, consists of two layers, which do not contain material of high electron density. In some cases, the cell wall shows up to five layers (Fig. 10).

The nucleus is surrounded by a double membrane. The nuclear envelope persists during nuclear division (Fig. 11). The nucleolus appears as a darker area in one of the nuclei (arrow). In some cases we could observe in the envelope diffusive pores with a membrane distance of about 20 nm. Occasionally we found one Golgi apparatus (Fig. 12). Beside the dictyosome, one tubulus of endoplasmatic reticulum can be seen. Small particles are attached to this tubulus, their size (16–17 nm) suggests that they are ribosomes.

The cells of *Nanochlorum* always divide into two daughter cells. The plasmalemma invaginates after the duplication of the nucleus, the chloroplast and

the mitochondrium (Fig. 13). Between the daughter cells new cell walls are formed, while they are contained still within the parent cells until they finally are set free. Possibly the whole cell wall of the parent cell or parts of it become integrated into the cell wall of the released daughter cells.

DNA/RNA content

A classical criterion to distinguish between pro- and eucaryotic organisms is the amount of genetic information. The DNA content of the *Nanochlorum* cell was found to be $6.01 \pm 0.77 \times 10^{-14}$ g. It contains $1.8 \pm 0.6 \times 10^{-13}$ g RNA and $1.7 \pm 0.8 \times 10^{-13}$ g protein.

Pigments

The *in vivo* spectrum of *Nanochlorum* (Fig. 15) shows a red maximum at 680 nm with a shoulder at

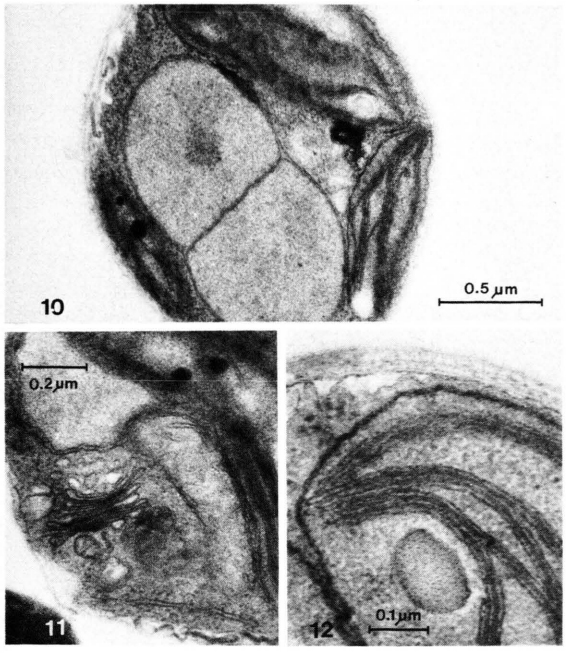


Fig. 10. Dividing nucleus of *Nanochlorum*. During the duplication the envelope of the nucleus remains intact.

Fig. 11. One dictysome in the cytoplasma of *Nanochlorum*, a rather rare observation. Beside the Golgi apparatus one tubulus of ER can be seen.

Fig. 12. The cell wall of *Nanochlorum* sometimes is multilayered.

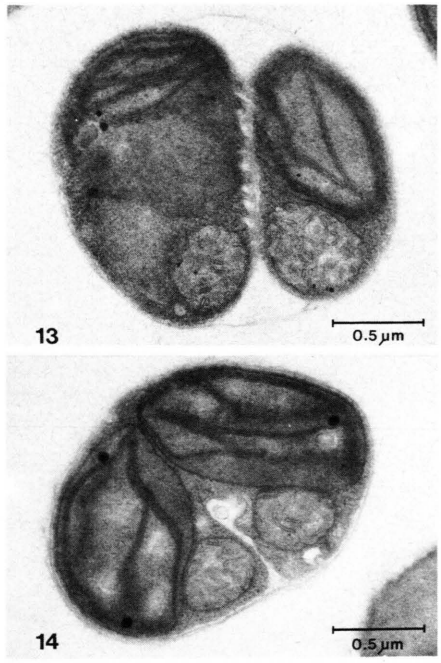


Fig. 13. Propagating cells of *Nanochlorum*. The young cells are held together by the cell wall of the mother cell. Between the daughter cells new cell walls are formed.

Fig. 14. Dividing cells of *Nanochlorum* in an early phase of propagation. The semi-autonome organelles are duplicated and the plasmalemma invaginates.

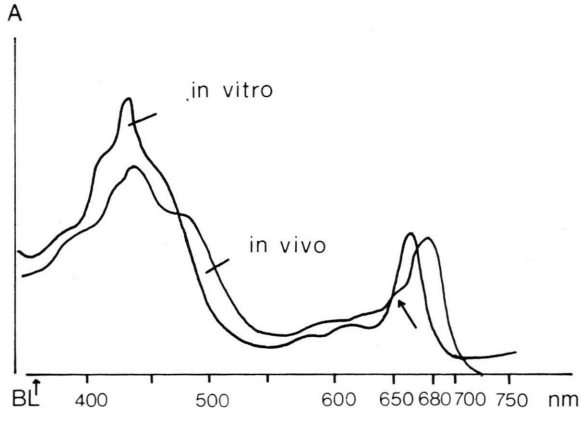


Fig. 15. The *in vivo* and the *in vitro* spectrum of *Nanochlorum*. The shoulder at 650 nm (arrow) in the *in vivo* spectrum is typical for Chl b containing green algae. In the *in vitro* spectrum no traces of pigments of non-green algae can be seen.

Table I. Pigment composition of *Nanochlorum eucaryotum.*

R_f	Pigment	Maximum of Absorbance	III/II value	Colour
0.65	chlorophyll a	430, 662	–	blue-green
0.54	chlorophyll b	452, 642	–	yellow-green
0.92	β-carotene	426, 450, 478	0.35	orange
0.70	lutein	422, 444, 474	0.69	orange
0.39	zeaxanthin	427, 449, 477	0.20	red-orange
0.59	neoxanthin	416, 440, 468	0.83	yellow
0.83	canthaxanthin (?)	474	–	red

Table II. Inhibition of the growth rates in percent of the control by different inhibitors.

Inhibitor	% of control	Applied concentration
lysozym	92	(500 µg/ml)
penicillin	85.1	(2500 E/ml)
chloramphenicol	6.8	(50 µM)
cycloheximid	64.9	(50 µM)
control	100	–

Table III. Comparison of different DNA contents of various pro- and eucaryotic organisms.

Organism	g DNA per cell	Reference
E. coli	4.15×10^{-15}	[21]
Anacystis nidulans	6.15×10^{-15}	[14]
Nanochlorum euc.	6.05×10^{-14}	
Chlorella f.	$1.0-4.0 \times 10^{-13}$	[16]

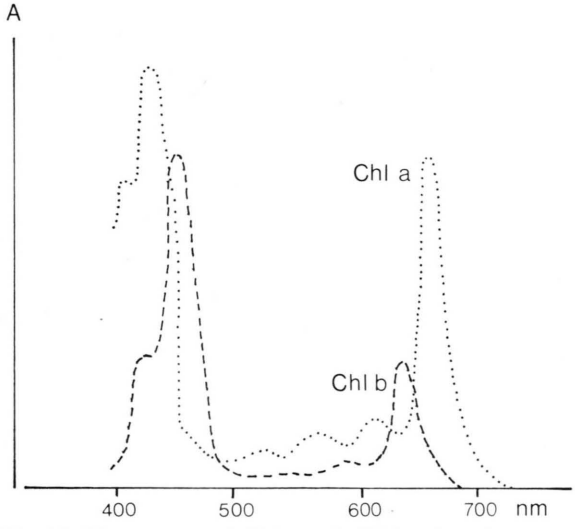


Fig. 16. The spectra of Chl a and Chl b after chromatographic separation. The spectra prove that the shoulder at 650 nm of the *in vivo* spectrum originates from Chl b.

650 nm and a maximum at 440 nm. The red maximum is caused by chlorophyll a and the shoulder at 650 nm by chlorophyll b. Fig. 16 demonstrates the spectra of Chl a and Chl b after separation by chromatography. No traces of any non-green pigments can be observed, in the *in vitro* spectrum (Fig. 15).

Analyzing the pigment composition of *Nanochlorum*, we were able to identify the components represented in Table I. Between β-carotene and the chlorophylls is a red band that shows only one maximum at 474 nm. The R_f-value and the absorption maximum indicate a pigment rather rare in chlorophyceae : canthaxanthin. Detailed further studies should exclude the possibility that this band is a mixture of secondary carotinoids.

Inhibitors

Experiments with different inhibitors show that *Nanochlorum* is very sensitive to cycloheximid, which inhibits 80 S ribosomes, and to chloramphenicol, which blocks the activity of procaryotic, mitochondrial and plastidic ribosomes (Table II). In contrast, the growth of *Nanochlorum* is hardly if at all influenced by the inhibitors of procaryotic cell wall differentiation.

Discussion

The different measurements of cell size by light microscopy, SEM and TEM correspond to such an extent, that we can be shure that *Nanochlorum* has in almost procaryotic cell size. On the other hand morphological data gathered from TEM observation and the sensitivity to cycloheximide place *Nanochlorum* into the eucaryotic algae. The ultrastructure of the chloroplast with its double membraned envelope and its bands of five thylacoids clearly place

Table IV. Comparison of *Chlorella nana, Chlorella minutissima* and *Nanochlorum eucaryotum.*

Characteristic	*Chlorella minutissima*	*Chlorella nana*	*Nanochlorum eucaryotum*
cell size	2.0 µm	2.5 µm	1.5 µm
chloroplast	?	50% of the cell volume	more than 50% of the cell volume
cell wall	?	varying 30–150 nm	thin 30 nm
thylakoids	?	often peripher	regularily central
starch	?	great granula filling the half of the chloroplast	small granula, mostly absent
propagation	?	in two or four daughter cells	only two daughter cells
pH limit of growth	5	?	3.5–9
salt tolerance	1%	?	10–12%
reduction of nitrate	+	?	+
B_1-requirement	–	?	–

Nanochlorum into the taxonomic group of Chlorophyceae, probablely near the Chlorococcales [10, 11]. This placement is supported by further evidence: the content of Chl a and Chl b and the carotinoid pattern showing β-carotene, lutein, zeaxanthin and neoxanthin are reported to be typical of green algae [9, 12, 13]. On the other hand the DNA content is rather low. Table III compares the DNA contents of several organisms. *Nanochlorum* contains tentimes more DNA than the procaryotic blue-green alga *Anacystis,* but only a fraction of the DNA content of *Euglena* or *Chlorella* [14–16]. Thus in respect to the size of the genom and the cell volume, *Nanochlorum* may be a link between pro- and eucaryotic algae. Furthermore the propagation modus is not very typical of Chlorococcales. Firstly: *Nanochlorum,* always releases only two daughter cells, whereas the other Chlorococcales usually form four autospores rsp. zoospores [17, 18]. Secondly: At the present state we cannot exclude that the daughter cells of *Nanochlorum* integrate the whole mother cell or parts of it, whereas in the typical autospore formtion of chlorophyceae maternal material never is used. Thirdly: The variation of environmental conditions like pH, salinity and nutrients does not alter the dividing modus and the number of daughter cells, [19] whereas in other coccoid green algae the number of autospores 'released is subject to environmental conditions [17, 18].

In Table IV we have summarized some features of *Nanochlorum* and other very small coccoid green algae. There is no doubt that *Nanochlorum* is a new species, remarkably different from *Chlorella nana* and *Chlorella minutissima,* the smallest green algae discovered so far [3, 4, 20]. Our results suggest not only the introduction of a new species, but also of a new genus.

The taxonomy of *Nanochlorum* cannot be fixed at this moment. We propose to place *Nanochlorum* into the family of the Oocystaceae, subfamily Chlorelloideae because asexual reproduction and noncenobial organization is obligatory. This taxonomic placement poses some problems, since the question of real autospore formation in *Nanochlorum* is not clear. A new family or suborder must be introduced in case it can be proven that the cell division is not in agreement with the oocystacean modus.

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