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Identification of the alga known as "marine *Chlorella*" as a member of the Eustigmatophyceae

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An alga known as "marine *Chlorella*", obtained from Suisan Center (Makishima-machi, Nagasaki, Japan), has been critically investigated in culture and identified on the evidence of ultrastructure and biochemistry as *Nannochloropsis oculata* (DROOP) HIBBERD, a member of the Eustigmatophyceae. Cells are spherical to slightly ovoid, 2-4 μm in diameter, and contain an ovoid or cup-shaped chloroplast. The chloroplast is bounded by two double membranes, the outer representing the CER and showing connections with the nuclear envelope, and the inner representing the chloroplast envelope. Chloroplast lamellae consist of three appressed thylakoids. Girdle lamellae and pyrenoids are absent. Starch was not detected. Optimum growth, with a specific growth rate of 0.9 day^{-1}, occurred at a temperature of ca. 25°C, salinity of 15-30‰, light intensity greater than 12 klux, and initial pH of ca. 8. Predominant photosynthetic pigments were chlorophyll *a*, carotene, violaxanthin, and vaucheriaxanthin ester. Chlorophyll *b* and lutein were not detected.

Key Index Words: *Eustigmatophyceae*; *growth*; *marine* Chlorella; Nannochloropsis oculata; *pigment*; *ultrastructure*.

A small, planktonic, unicellular alga known as "marine *Chlorella*" has been widely used as food for the rotifer *Brachionus plicatilis* in the culture of many types of fish (WATANABE *et al.* 1978a). Because it is an excellent food for use in the culture of juvenile marine fish, the alga has been the subject of many nutritional studies, which have been revealed to have a high content of eicosapentaenoic acid (WATANABE *et al.* 1978b). However, there have been few studies on its biology and biochemistry. In the present report the growth, pigment composition, and ultrastructure of this alga are examined and the results applied to its taxonomic assignment.

Materials and methods

Materials

A sample of the alga used for culture of rotifer *Brachionus plicatilis* was obtained as a nearly pure culture from Suisan Center, Nagasaki City Institute of Fisheries (Makishima-machi, Nagasaki, Japan) in July, 1981, and purified by plating out suitable dilutions. Two other unicells, *Monodopsis subterranea* (PETERSEN) HIBBERD (obtained from the Sammlung von Algenkulturen, Göttingen No. 848-1 as *Monodus subterraneus*) and *Nannochloropsis oculata* (DROOP) HIBBERD (obtained from the Algal Culture Collection at the University of Texas No. 2164 as *Nan-*

nochloris oculata) were used for purposes of comparison in the identification of photosynthetic pigments (WHITTLE and CASSELTON 1975, ANTIA *et al.* 1975).

Culture conditions

The alga was cultured in a medium containing 0.5 g KNO_3, 0.1 g Na_2HPO_4, 15 mg EDTA-Na-Fe, 1 m*l* of Arnon's solution A_5 (WATANABE 1960), 5 m*l* of vitamin mixture S-3 (PROVASOLI *et al.* 1957), and 0.5 μg of vitamin B_{12} per liter of either natural sea water or Jamarin S artificial sea water (Jamarin Laboratory, Osaka) that had air enriched with 0.1-0.5% carbon dioxide bubbling through it. Culture vessels were oblong and flat. Standard conditions of culture were 25°C and continuous illumination (white fluorescent lamp, 6-7 klux). These conditions were altered for certain experiments. For light intensity and pH experiments, glass Erlenmeyer flasks containing unbubbled medium were used. For salinity experiments, Jamarin S artificial sea water containing MBM (WATANABE 1960) and vitamins was used. To determine vitamin requirements, Provasoli's ASP 2 (PROVASOLI *et al.* 1957) was used.

Culture density was estimated by measuring optical density in a spectrophotometer at 700 nm. The specific growth rate was determined during exponential growth. The growth rate during non-exponential conditions was calculated from average growth during the culture period.

Electron microscopy

Cells were washed with 50 mM phosphate buffer containing 0.25 M sucrose (pH 7.2), fixed with 2% glutaraldehyde in the same buffer for 9 hr at ca. 4°C, post-fixed with 2% OsO_4 in the same buffer without sucrose for 24 hr at ca. 4°C, embedded in agar, dehydrated in a graded ethanol series (50% to 100%), transferred to acetone, and embedded in Spurr's resin (SPURR 1969). Following polymerization, sections were cut with a Porter-Blum MT-2 Ultramicrotome, stained with lead citrate for 10 min., and viewed with a JEM 200 CX electron microscope at 100 kV.

Pigment analysis

Pigments were extracted by treating the cells, which were first washed with artificial sea water, with 85% acetone. Extracted pigments were separated on columns of sucrose using 0.5% n-propanol in petroleum ether (b.p. 30-60°C) as the developing solvent. Each fraction was eluted diethyl ether and further separated using thin-layer chromatography (TLC) with cellulose plates (Merck) and a ranning solvent of petroleum ether : n-propanol (96 : 4 v/v) or petroleum ether : acetone : n-propanol (90 : 10 : 0.45 v/v). Rf values were calculated and absorption spectra examined. Chlorophyll *c* was sought using a spectrophotometer after separation of the extracted pigments by TLC with cellulose plates and a running solvent of chloroform : petroleum ether (1 : 3 v/v). Carotenoids were extracted for quantitative analysis using acetone : methanol (7 : 3 v/v), saponified for removal of chlorophyll, and estimated spectrophotometrically after separation by TLC.

Analysis of fatty acids

Lipids were extracted from cells with a methanol chloroform mixture and saponified in the usual manner (KATES 1972). Fatty acids were isolated from the saponification mixture using petroleum ether, methylated, then analysed by gas chromatography (Shimadzu GC-3BF).

Test for starch

Cells were decolorized with methanol, incubated in 0.2%I_2/2%KI for 20 min., then examined with an optical microscope using opal glass slides.

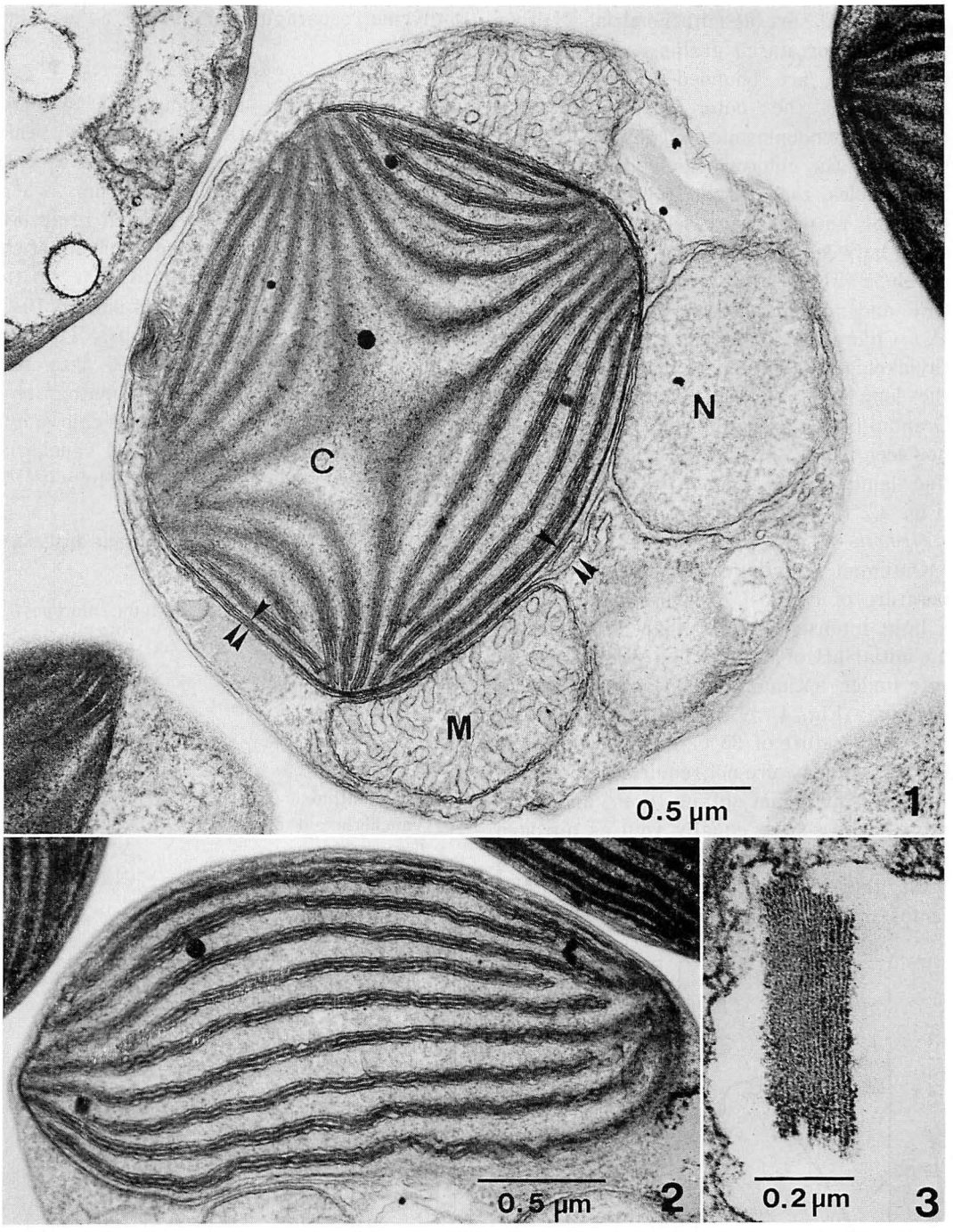
Results

Optical microscopic observations

Live cells are spherical to slightly ovoid and measure 2-4 μm in diameter. They usually contain one chloroplast. Testing with I_2/KI failed to detect starch. Vegetative multiplication takes place by binary fission. No motile cells were seen.

Electron microscopic observations

Cells are enclosed in a thin wall and contain an ovoid or cup-shaped chloroplast, a



Figs. 1-3. Electron micrographs of "marine *Chlorella*". Fig. 1. Section of whole cell. The chloroplast is enclosed by two double membranes: the outer one is the CER (double arrow head) while the inner one is the chloroplast envelope (single arrow head). Direct continuity between the CER and the nuclear envelope is observed. C, chloroplast; N, nucleus; M, mitochondrion. Fig. 2. Section of chloroplast. Bands of three associated thylakoids extend across the entire chloroplast length with no girdle lamellae. Fig. 3. Section showing fine structure of lamellate vesicle in cytoplasm.

nucleus, and several mitochondria. Neither pyrenoids nor starch grains were observed. Chloroplasts are bounded by two double membranes, the outer representing the chloroplast endoplasmic reticulum (CER) and the inner the chloroplast envelope (Fig. 1). No vesicles that might represent a perplastidal network were seen in the narrow space between the CER and the chloroplast envelope (Fig. 1). Continuity between the CER and nuclear envelope is apparent (Fig. 1). Chloroplast lamellae consist of three thylakoids running approximately parallel to the long axis of the chloroplast (Fig. 2). Granum-like stacks or girdle lamellae were not seen (Figs. 1, 2). Vesicles containing fine lamellae are common in the cytoplasm (Fig. 3).

Growth

Optimum growth was obtained at a temperature of ca. 25°C, a salinity of 15-30‰, a light intensity greater than 12 klux, and an initial pH of ca. 8. The specific growth rate under optimum conditions was about 0.9 day^{-1} (Fig. 4). No growth was apparent at a temperature of 35°C or at a salinity of 0‰. Vitamins were not required. Potassium nitrate, ammonium sulfate, urea, and casamino acids served equally well as nitrogen sources. The alga did not grow in the dark with glucose, galactose, fructose, maltose, lactose, acetic acid, citric acid, ethanol, glycine, asparagine, or alanine as a carbon source.

Photosynthetic pigments

The absorption spectrum of the total pigment extract in diethyl ether showed peaks at 661 nm, 470 nm, and 429 nm. No peaks or shoulders at 642 nm or 452 nm, where chlorophyll *b* would be expected to absorb, were found. The absorption spectrum of the cell suspension was similar to that of the extract except that the peaks shifted 10-20 nm toward longer wave lengths. The total pigment extract was separated into four fractions by sucrose column chromatography. The fractions were identified as chlorophyll *a*, carotene(s), violaxanthin, and vaucheriaxanthin ester by co-chromatography with the

Table 1. Photosynthetic pigment analysis of "marine *Chlorella*".

Pigment	Percent total chlorophyll or carotenoid
Chlorophyll *a*	100
Chlorophyll *b*	ND*
Chlorophyll *c*	ND
Carotene(s)	11
Violaxanthin	51
Vaucheriaxanthin ester	26
Lutein	ND
Other carotenoids	12

*Not detected

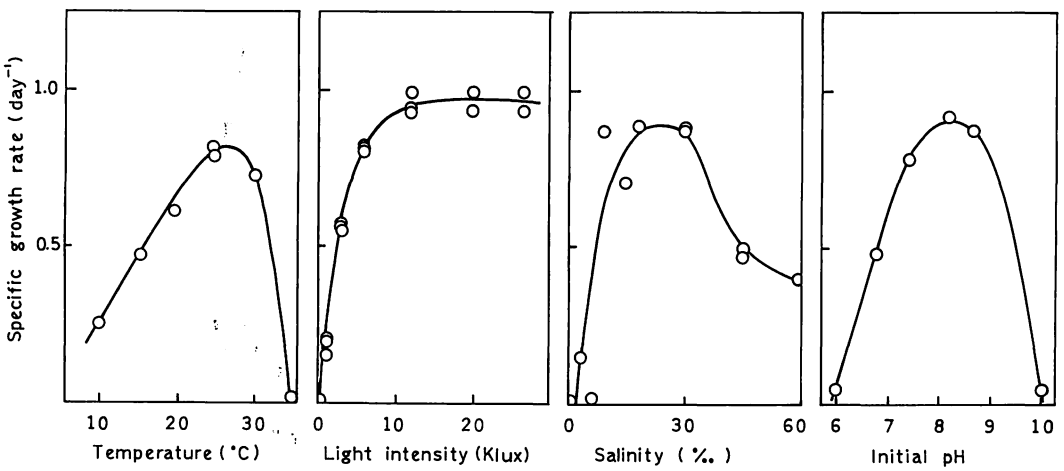


Fig. 4. Effect of culture conditions on the growth rate of "marine *Chlorella*"

Table 2. Fatty acid composition of "marine *Chlorella*".

Fatty acid	Percent total fatty acid
14 : 0	5.0
16 : 0	17.8
16 : 1	24.9
18 : 0	tr
18 : 1	4.9
18 : 2ω6	3.2
18 : 3ω3	1.2
20 : 3ω3 20 : 4ω6	4.2
20 : 5ω3	37.1

pigments of *Monodopsis subterranea* and *Nannochloropsis oculata*. The major xanthophyll was violaxanthin. Chlorophyll *b*, chlorophyll *c*, and lutein were not detected (Table 1).

Fatty acids

The major fatty acids were eicosapentaenoic acid (20 : 5ω3), palmitoleic acid (16 : 1), and palmitic acid (16 : 0). A small quantity of the stearate family of acids were represented (Table 2).

Discussion

Since this alga grows well at a salinity of 15 to 30‰ but does not grow at a salinity of 0‰, and since the pH of optimal growth (ca. 8) agrees with that of sea water, it appears to be a marine form that grows well at moderate temperatures (ca. 25°C) and high light intensity (12-27 klux). The proportions of fatty acids, with 20 : 5ω3, 16 : 1, and 16 : 0 as the major fatty acids, are similar to those of "marine *Chlorella*" that have been reported previously (WATANABE *et al.* 1978b).

As is evident from its biochemistry and ultrastructure, however, this alga cannot be assigned to the Chlorophyceae and hence is incorrectly placed in the genus *Chlorella*. The alga lacks chlorophyll *b* and lutein, which are always present in Chlorophyceae. Chloroplasts are surrounded by two double membranes rather than one as in Chlorophyceae, and the lamellae consist of three thylakoids rather than one. Starch, which is the storage product of all Chlorophyceae, is absent. The pigments and cytological features are similar to those of *Nannochloropsis*

Table 3. Comparison of main taxonomic characteristics between *Nannochloropsis oculata* (Millport No. 66) and "marine *Chlorella*".

Characteristics	*Nannochloropsis oculata* (Millport No. 66)	"marine *Chlorella*"
Cell dimension	2-4 μm*	2-4 μm
Cell shape	globose*	globose or slightly ovoid
Chloroplast	single, ovoid or cup-shaped parietal**	single, ovoid or cup-shaped
Propagation	binary fission*	binary fission
Pyrenoid	rarely observed**	not observe
Chloroplast ER	present (continuous with the nuclear envelope)**	present (continuous with the nuclear envelope)
Thylakoid arrangement	3-thylakoid lamellae**	3-thylakoid lamellae
Girdle lamellae	absent**	absent
Lamellate vesicles	in chloroplast**	in cytoplasm
Predominant photosynthetic pigments	chlorophyll *a* carotene violaxanthin vaucheriaxanthin ester**	chlorophyll *a* carotene violaxanthin vaucheriaxanthin ester

*DROOP, 1955 **ANTIA *et al.*, 1975

oculata (DROOP) HIBBERD, an alga which has been assigned to the Eustigmatophyceae on the basis of pigment composition and ultrastructure (ANTIA *et al.* 1975). A comparison with *N. oculata* is made in Table 3. The characteristics agree with two exceptions. First, pyrenoids have been seen in *N. oculata*, although rarely (ANTIA *et al.* 1975), whereas they have not been found in the alga under study. Second, lamellate vesicles, which in the present study were found abundantly in the cytoplasm (in agreement with other eustigmatophytes; [HIBBERD and LEEDALE 1972, HIBBERD 1974]), occur only in the chloroplast of *N. oculata* (ANTIA *et al.* 1975). The pyrenoid of *N. oculata* is similar to the polyhedral pyrenoid characteristic of eustigmatophytes. Its rare appearance suggests that it is a transient feature related to metabolic condition (ANTIA *et al.* 1975). It is possible that a pyrenoid will be detected in the present alga during further study. The discrepancy between the position of the lamellate vesicles cannot be resolved at the present time. HIBBERD (1981) commented that a great deal of uncertainty still remains regarding the small forms classified in the Monodopsidaceae, which includes *Nannochloropsis*, since they are difficult to fix for EM and have relatively few non-ultrastructural anatomical characters.

Additional similarity to the Eustigmatophyceae is found in the composition of fatty acids. Like *N. oculata* (unpublished data) and the related freshwater alga *Monodopsis subterranea* (NICHOLS and APPLEBY 1969) the alga under study contains 20 : 5ω3, 16 : 1, and 16 : 0 as major fatty acids and contains a small quantity of the stearate family of acids.

Since the similarities outweight the discrepancies, it seems appropriate to identify the alga under study as *Nannochloropsis oculata* on the basis of its pigment composition and cytological characteristics.

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丸山　功・中村寿雄・松林恒夫・安藤洋太郎・前田直彦：いわゆる海産クロレラの分類学的性質

水産種苗の初期餌料として重要なシオミズツボワムシの生産に広く用いられる海産クロレラと呼ばれる藻の一つについてその性質を調べた。この藻は通常1個のカップ型または卵型の葉緑体を有する球形あるいはだ円型の細胞であり，主な光合成色素として chlorophyll *a*, carotene, violaxanthin, vaucheriaxanthin ester を含んでいた。電顕観察の結果，核膜と連結したクロロプラストERが葉緑体をとり囲み，葉緑体を囲む膜は葉緑体包膜とクロロプラストERで4枚に観察された。ラメラは三重チラコイドラメラ構造をしていた。この藻は形態，微細構造，色素組成の特徴から現在のところ，真正眼点藻綱 *Nannochloropsis oculata* HIBBERD と同定するのが適当と思われる。(833 福岡県筑後市久富1343番地　クロレラ工業株式会社)

新　刊　紹　介

K. KRAMMER and H. LAMGE-BERTALOT (1986) **Bacillariophyceae I. Teil: Naviculaceae.** *In* H. ETTL, J. GERLOFF, H. HEYNIG and D. MOLLENHAUER 〔eds.〕 Süßwasserflora von Mitteleuropa. Bd. 2. Gustav Fischer Verlag, Stuttgart, New York. 876 pp. 206図版 2976図。(含船郵送料邦貨約18000円)。

本書は "Süßwasserflora von Mitteleuropa" シリーズ24巻中の1つである。今回出版されたのは2巻1号で Naviculaceae を網羅しており，今後他の科についても2号，3号で出版される予定となっている。

内容は大きく2つに分けられており，第1部では，用語，殻の構造，生殖，細胞構造，殻形成，運動，生態，研究方法などが概説され，第2部でそれぞれの種につい記述がなされている。ここで扱われている属は *Navicula*, *Stauroneis*, *Anomoeoneis*, *Frustulia*, *Amphipleura*, *Neidium*, *Scoliopleura*, *Diploneis*, *Pleurosigma*, *Gyrosigma*, *Cymbella*, *Amphora*, *Gomphonema*, *Gomphoneis*, *Didymosphenia*, *Rhoicosphenia*, *Caloneis*, *Pinnularia*, *Mastogloia*, *Diatomella*, *Oestrupia*, *Entomoneis* の22属である。全ての種類が光顕写真で示されており，一部には類似した種類間での相違点が電顕写真によって示されている。

本書は HUSTEDT の Bacillariophyta (1930) 及び Kieselalgen (1930～1966) が元になっており，材料の多くは HUSTEDT のコレクション中から選ばれている。そして特筆すべきことはその中に多くのタイプ標本が含まれ，それが写真によって示されていることである。これは大変貴重なものであり，本書の価値を非常に高めるものであり，利用者にとっては大変有用なものとなるであろう。

(筑波大学生物科学系　出井雅彦)