Fatty Acid Composition in the Lipids of some Marine Chlorococcales and Eustigmatales

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The fatty acid composition in the lipids of six coccoid microalgae species (≈2 μm in diameter) was investigated. In addition to analyses of ultrastructure and pigment content, lipid composition is shown to be a chemotaxonomic tool in the classification of algae. The four species of Chlorococcales (Chlorophyceae) – the marine species as well as the fresh water species, both kept in artificial seawater – contain a fatty acid composition resembling that of *Chlorella spp.* and of green leaves of higher plants. The fatty acid pattern is characterized as lacking in C20 acids but containing large amounts of C16 and C18 polyunsaturated fatty acids, hexadecatrienic acid (16:3) and α-linolenic acid (18:3ω3) in particular. In contrast the two species of Eustigmatales (Eustigmatophyceae) investigated here show a deficiency of the C16 and C18 polyunsaturated fatty acids but contain large amounts of eicosapentaenic acid (20:5).

Introduction

The lipids of green leaf cell membranes are highly uniform in composition. The fatty acid pattern is characterized as lacking in C20/22 acids but containing large amounts of C16 and C18 polyunsaturated fatty acids (PUFA), 16:3 and 18:3ω3 in particular [1]. In contrast, fatty acid compositions of algae, which often contain C20/22 acids and more than three double bonds per molecule vary greatly [2–4].

In comparing reports on algal lipids, it becomes evident that the fatty acid composition of only the Chlorococcales thus far examined, *i.e.* the fresh water species, resemble that of green leaves of higher plants in general, whereas the chlorophyceen seaweeds are able to synthetize the C20 PUFA in addition. For a long time Chlorococcales were believed to grow in freshwater habitats only [5], however several strains of coccoid members were recently isolated from marine habitats. Most of them belong to the nanoplankton, with a cell size of ≤5 μm in diameter. Due to their small number of morphological characteristics, their taxonomy is uncertain. Following the investigation of ultrastructure and pigmentation of morphologically similar green coccoid microalgae, some required reclassification into entirely different taxonomic classes [6].

In our previous study, the ultrastructural, biochemical and physiological characterizations of some newly isolated, unidentified strains of green microalgae were compared to those of the genus *Nannochloris* [7]. One part of that investigation, concerning the acyl lipids in particular, is reviewed in this paper. The purpose of the study was to find out whether the fatty acid composition of the marine chlorococcacean species is influenced by their environment or proves to be a stable feature within the taxonomic unit, and therefore may be used as a chemotaxonomic tool to explore taxonomical questions in algae systematics.

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulpholipid (sulphoquinovosyldiacylglycerol); PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; *N.e., Nanochlorum eucaryotum; N.o., Nannochloris oculata; NL1, Chlorella nana; VmO, Oocystic marssonii; NL2, Nannochloropsis sp.; NL3, Nannochloropsis sp.*

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Materials and Methods

Plant material

Nanochlorum eucaryotum (strain Mainz 1981/1; Institut für Allgemeine Botanik, University of Mainz, F.R.G.), *Nannochloris oculata* (strain CCAP 251/6; Culture Collection of Algae and Protozoa, Cambridge, U.K.). The following strains were identified by ultrastructural characteristics using TEM-methods (Menzel, 1988): *Oocystis marssonii* (strain 1984/Vm-O); *Chlorella nana* (strain 1982/NL-1), *Nannochloropsis sp.* (strain 1982/NL-2), and *Nannochloropsis sp.* (strain 1982/NL-3) were originally received as unidentified isolates from Lewin, U.S.A. (1982). All samples were taken from subcultures maintained for some years at the Institut für Allgemeine Botanik, University of Mainz, F.R.G.

Culture

Algae strains were all grown autotrophically in artificial seawater [11] in aerated tubes at 22 °C. Illumination conditions were at 2.5 Wm^{-2} of white light (Osram TL 40 W/25 fluorescent tubes), at intervals of 16 h light to 8 h dark for 9 days.

Lipid extraction and isolation

Cells were harvested after 9 days by centrifugation (10 min, 8000 × *g*). Following treatment with boiling *iso*-propanol to avoid lipid degradation by lipases, the pellet (50 mg dry wt) was homogenized by cooling with CO_2-snow in a homogenizer with glass beads. Further extraction, separation and identification generally agrees with Hartmann *et al.* [19]. The resulting fatty acid methyl esters were chromatographed isothermally (200 °C) using GLC methods on a Shimadzu GC-9 A and a Dani 3800, both equipped with flame ionization detectors (FID). Nitrogen was used as carrier gas (flow rate 2 ml/min). Separation was carried out on WCOT fused silica capillary columns (0.32 mm i.d.) with stationary phase either 50 BP Superox (0.25 μm film thickness, 30 m length) or FFAP (0.2 μm film thickness, 25 m length). Identification and quantitative determination were based on standard reference methylesters and the internal standard method. The data shown are mean values for at least two experiments.

Results

In comparing the total lipid compositions in Table I, it is observable that characteristic fatty acid patterns divide the examined algae, in accordance with taxonomical classification (Fig. 1), into two groups. Thus, the four Chlorococcales are noted for a high level of 16:3, 18:2 and α-linolenic acid (18:3ω3), but they do not contain fatty acids with more than 18 carbon atoms or three double bonds. In contrast to this, the absence of C16 and C18 PUFA together with the occurrence of large amounts of eicosapentaenoic acid (20:5) in the two Eustigmatales is noticeable. Further investigations of lipid fractions were conducted with only two of the Chlorococcales *(Nanochlorum eucaryotum, Nannochloris oculata)* and the two Eustigmatales *(Nannochloropsis spp.)*.

Table I. Fatty acid composition of total lipids. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	*N.o.*	*NL1*	*VmO*	Eustigmatales *NL2*	*NL3*
14:0	1.5	1.1	1.1	1.5	6.8	5.7
16:0	21.0	23.1	21.8	19.8	33.8	26.7
16:1	6.8	2.1	2.3	2.5	31.8	26.5
16:2	8.7	3.4	1.8	2.4	–	–
16:3	12.5	8.3	6.3	5.1	–	–
18:0	0.7	6.1	1.1	0.8	0.9	1.4
18:1	5.3	15.8	18.8	17.3	14.2	10.1
18:2ω6	20.2	17.1	23.4	26.6	1.4	2.1
18:3ω3	22.9	23.1	22.6	22.5	–	–
20:4	–	–	–	–	1.6	4.9
20:5	–	–	–	–	7.6	21.8
Total fatty acid content (nmol/mg dry wt):	252.1	138.2	377.7	390.1	1043.9	460.8

The glycolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) represent the main part of the lipid classes in all species investigated here, while the glycolipid sulphoquinovosyldiacylglycerol (SQDG) and the phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) exist in all cases only in small quantities.

In MGDG, *Nanochlorum eucaryotum* and *Nannochloris oculata* (Chlorococcales) contain high

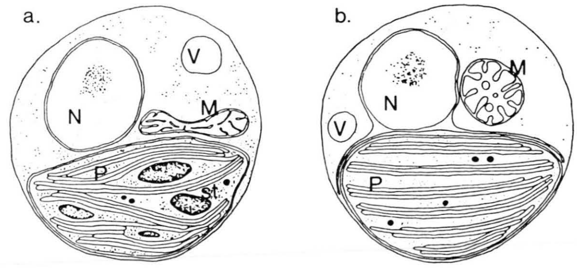

Fig. 1. Scheme of ultrastructural details. a. *Nannochloris*-type (Chlorococcales) with characteristic pigments chlorophyll *a*, chlorophyll *b*, and lutein. b. *Nannochloropsis*-type (Eustigmatales) with characteristic pigments chlorophyll *a*, violaxanthin and esterified vaucheriaxanthin.

levels of C16 and C18 PUFA with a preference for three double bonds. In this fraction, α-linolenic acid is the dominant fatty acid constituting ca. 30%. In the two *Nannochloropsis spp.* (Eustigmatales), however, representatives of the C18 acids play a minor role, where half of the fatty acid content is represented by 14:0, 16:0 and *cis*-16:1, and eicosapentaenoic acid (20:5) constitutes 40–50% of the total fatty acid content (Table II).

In DGDG, the two Chlorococcales contain higher 16:0 and lower C16 PUFA amounts as compared to MGDG. The 18:2/18:3 ratio is in favour of the 18:2 acids. In *Nannochloropsis spp.*, the 16:0, 16:1 and 20:5 acids represent about one third each of the fatty acid content (Table III).

SQDG in general shows the most simple fatty acid content particularly for the two Chlorococcales. 16:0 is the major component (≈60%) whereas the unsaturated representatives of the C16 series are almost entirely absent. The unsaturated acids of the C18-series represent about 10% each of the remainder. In the *Nannochloropsis spp.*, the 16:0 is dominant (44%) but *cis*-16:1 is also well represented at more than 30%. 14:0, 18:1 and 20:4 comprise the remainder, while MGDG's dominating 20:5 exist only in minimal amounts or are absent (Table IV).

In PC, the two Chlorococcales contain a fatty acid composition similar to that in DGDG. In the two *Nannochloropsis spp.*, however, the composition of PC deviates noticeably from that of DGDG, containing high amounts of 18:1 acid (25–30%), which plays a relatively minor role in all other lipids of the species. Whereas α-linolenic acid could not be detected in any of the lipid fractions of the *Nannochloropsis* species, they contain gamma-linolenic acid (18:3ω6) in PC alone in small amounts together with minute amounts of arachidonic acid (20:4) and eicosapentaenoic acid (20:5) (Table V).

In PG, 16:0 is once again a major component in both of the two Chlorococcales as well as of the two Eustigmatales. The complete absence of the C16 PUFA and of *cis*-16:1, the latter replaced by *trans*-16:1ω13 in the two Chlorococcales, is noteworthy.

Table III. Fatty acid composition of DGDG fraction. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	Chlorococcales *N.o.*	Eustigmatales *NL2*	Eustigmatales *NL3*
14:0	0.5	0.9	6.2	9.3
16:0	25.6	32.8	31.9	28.2
c16:1	1.5	0.8	38.7	32.0
t16:1ω13	–	–	–	–
16:2	8.8	6.1	–	–
16:3	11.1	9.6	–	–
18:0	–	2.7	–	–
18:1	3.8	5.7	2.1	1.7
18:2ω6	26.5	21.5	1.2	0.8
18:3ω3	22.3	20.0	–	–
20:4	–	–	0.9	0.6
20:5	–	–	19.0	27.4
Total fatty acid content (nmol/mg dry wt):	38.2	36.5	27.1	28.7

Table II. Fatty acid composition of MGDG fraction. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	Chlorococcales *N.o.*	Eustigmatales *NL2*	Eustigmatales *NL3*
14:0	–	–	13.5	16.3
16:0	3.3	4.3	16.6	13.8
c16:1	2.0	1.8	17.6	14.2
t16:1ω13	–	–	–	–
16:2	16.3	15.1	–	–
16:3	28.4	29.1	–	–
18:0	–	0.3	0.8	–
18:1	2.5	2.7	2.0	1.1
18:2ω6	16.9	13.5	0.8	1.9
18:3ω3	30.7	33.2	–	–
20:4	–	–	5.4	3.3
20:5	–	–	43.3	48.8
Total fatty acid content (nmol/mg dry wt):	58.3	58.4	57.6	48.5

Table IV. Fatty acid composition of SQDG fraction. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	Chlorococcales *N.o.*	Eustigmatales *NL2*	Eustigmatales *NL3*
14:0	–	–	4.7	7.2
16:0	68.5	56.6	42.8	46.1
c16:1	–	–	37.5	39.3
t16:1ω13	–	–	–	–
16:2	–	–	–	–
16:3	–	–	–	–
18:0	–	2.8	–	–
18:1	3.9	13.7	4.9	2.4
18:2ω6	15.2	15.6	–	–
18:3ω3	12.5	11.3	–	–
20:4	–	–	8.7	5.1
20:5	–	–	1.3	–
Total fatty acid content (nmol/mg dry wt):	12.8	13.8	16.9	25.1

Table V. Fatty acid composition of PC fraction. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	*N.o.*	Eustigmatales *NL2*	*NL3*
14:0	1.2	1.6	1.6	2.0
16:0	28.8	28.3	13.9	15.4
c16:1	1.7	3.0	34.2	33.4
t16:1ω13	–	–	–	–
16:2	3.2	1.8	–	0.9
16:3	4.5	3.9	–	–
18:0	–	1.6	0.6	0.4
18:1	6.7	13.1	25.4	30.8
18:2ω6	32.8	26.8	9.9	7.4
18:2ω9?	–	–	1.8	1.3
18:3ω3	21.2	20.0	–	–
18:3ω6	–	–	3.9	3.0
20:4	–	–	4.6	3.1
20:5	–	–	3.1	2.3
Total fatty acid content (nmol/mg dry wt):	18.4	52.5	45.0	48.4

For the two *Nannochloropsis spp.*, *cis*-16:1 and *trans*-16:1ω13 are present in small, approximately equal quantities (≤10%), and there are no representatives of the C18 acids with the exception of 18:1 (14%). In this plastidic phospholipid, 20:5 is once again present at a high proportion of about 30% (Table VI).

Discussion

Keeping in mind that data for lipid composition in several algae classes are quite sparse, characteristic features seem significant for some taxonomic units. For example, whereas representatives of Rhodophyta and Chromophyta contain C20 PUFA in general, many of the green algae do not [3, 4]. Taxonomy of the Chlorophyta is presently being revised, while several approaches using different systematic characteristics as the key criterion exist [8]. In looking for a natural phylogenetic system, fatty acid composition will possibly prove to be a further useful tool in exploring the relationships between Chlorophyta. In comparison to the other classes of green algae more data for lipid research in Chlorophyta are available especially from the orders of the Volvocales and the Chlorococcales. Apart from some disagreements regarding the occurrence of 16:4 and 18:4 acids, the special position of the thus far investigated Chlorococcales, containing the simplest fatty acid composition within the green algae and the only identical characteristics to higher plant leaves, is confirmed by all authors [4].

Table VI. Fatty acid composition of PG fraction. The values represent mol% of the fatty acid methylesters.

Fatty acids	Chlorococcales *N.e.*	*N.o.*	Eustigmatales *NL2*	*NL3*
14:0	–	–	–	–
16:0	25.6	41.6	48.3	39.7
c16:1	–	–	4.5	6.3
t16:1ω13	27.3	8.8	2.5	7.3
16:2	–	–	–	–
16:3	–	–	–	–
18:0	–	–	–	–
18:1	14.0	15.8	14.7	14.1
18:2ω6	12.4	14.6	–	–
18:3ω3	20.6	19.3	–	–
20:4	–	–	–	4.8
20:5	–	–	32.5	27.9
Total fatty acid content (nmol/mg dry wt):	92.9	75.3	1.5	3.1

In our investigation, the fatty acid pattern proved to be important for the classification of *Nanochlorum eucaryotum*. This strain was described by Wilhelm *et al.* [9] as a novel marine algae species with an uncertain taxonomic position. Whereas some features were chlorococcacean, others suggested an isolated position in the sense of a "pre-eucaryote" [10–12]. Its similarity in characteristic fatty acid profile confirms the membership of *Nanochlorum eucaryotum* in the Chlorococcales [7, 13].

In our investigation, however, we analyzed only one small part of the lipid metabolism of the coccoid algae. Changing culture conditions influences fatty acid contents by decreasing or increasing their amounts. But even the fresh water species *Oocystis marssonii*, when grown in a saline environment, did not synthetize C20 PUFA but kept its characteristic chlorococcacean fatty acid pattern.

One must therefore doubt the identity of the strain used for lipid research by Seto *et al.* as *Chlorella minutissima* [14]. *Chlorella minutissima*, a very small fresh water species (1.8–3 μm in diameter), is described with its chlorococcacean ultrastructure by Dempsey *et al.* [15]. In contrast, the published fatty acid composition, lacking α-linolenic acid but containing small amounts of gamma-linolenic acid and high level of eicosapentaenic acid, resembles members of the Eustigmatales rather than the Chlorococcales (Fig. 2). According to Nichols [16], the alternative content of either α-linolenic acid or of gamma-

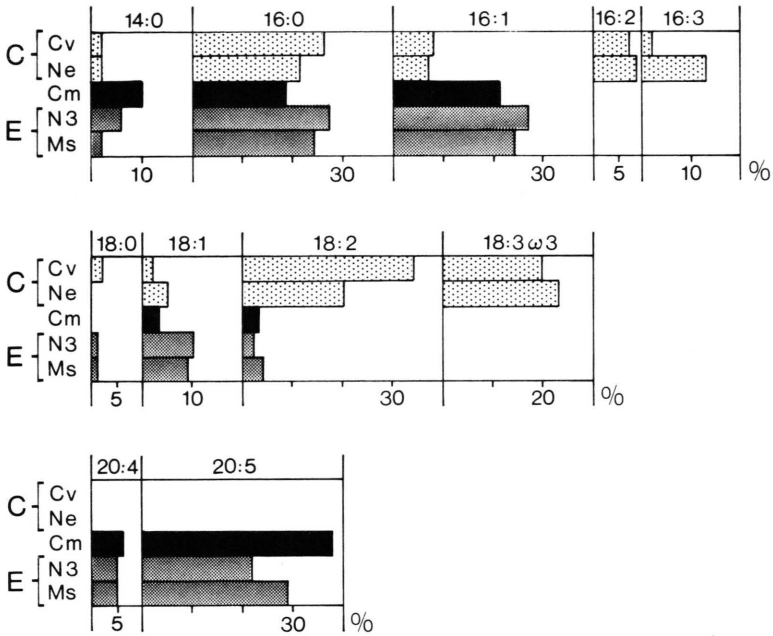

Fig. 2. Fatty acid composition of total lipids. Mol% of the fatty acid methylesters are shown. C, Chlorococcales; E, Eustigmatales; Cv, *Chlorella vulgaris* [4]; Ne, *Nanochlorum eucaryotum* [7]; Cm, *Chlorella minutissima?* [15]; N3, *Nannochloropsis sp.* [7]; Ms, *Monodopsis subterranea* [4].

linolenic acid is of some value in investigating evolutionary relationships and algae systematics. Moreover, the determination of the chlorophyll and main carotenoid species could give an answer as to whether the strain of the author's report belongs to the Chlorophyceae, Eustigmatophyceae or even the Xanthophyceae class. This is presumably another example of a small green species being mistaken for another.

Comparable data on lipid composition concerning Xanthophyceae are not yet available. For the class of the Eustigmatophyceae, literature provides us with fatty acid composition data for only *Monodopsis subterranea*. This strain was investigated for fatty acids as a member of Xanthophyceae [17] but has been, due to its ultrastructure and pigmentation, transferred into the class of the Eustigmatophyceae [6]. In fact, the fatty acid composition of *Monodopsis subterranea* is, in general agreement with our results obtained from the two *Nannochloropsis spp.* (Fig. 2).

In Eustigmatales as well as in Chlorococcales investigated here, fatty acids with the highest degree of unsaturation are found mainly in the dominant lipid of the plastids – the MGDG. Similar observations have been reported in the leaf lipids of higher plants and described for other algae classes [18].

It seems that differences in the plastidic ultrastructure and pigmentation, both important for algae taxonomy, are also confirmed in the fatty acid composition of the plastids. In addition to ultrastructure and pigmentation, lipid composition of the MGDG, DGDG and PC fractions in particular proved to be a useful tool in working on algae systematics in at least some cases. However, under chemotaxonomical aspects, further lipid research on other algae groups using correctly defined species from controlled, comparable culture conditions will be necessary.

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