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Comparison of neutral lipid profile of various trilaminar outer cell wall (TLS)-containing microalgae with emphasis on algaenan occurrence

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Abstract

The neutral lipid profiles of nine species of thin trilaminar outer wall (TLS)-containing freshwater and marine microalgae from the class of Chlorophyceae were studied with emphasis on the relationship between the lipid content and the occurrence of insoluble non-hydrolysable biopolymer (i.e. algaenan). All the freshwater microalgae produce a highly aliphatic algaenan. In sharp contrast, no algaenan was isolated from the two marine microalgae, *Chlorella marina* and *Chlorella minutissima marina*, supporting the absence of a close relationship between the presence of TLS and the occurrence of algaenan. High molecular weight straight-chain hydrocarbons (C_{23}–C_{29}) were identified in most of the algaenan-producing microalgae and in the algaenan-devoid *C. minutissima marina*, whereas only low molecular weight hydrocarbons were detected in algaenan-producing *Scenedesmus subspicatus* and in algaenan-devoid *C. marina*. Sterols, phytol and fatty alcohols were the major constituents of the polar fraction of the neutral lipids of all the microalgae investigated. High molecular weight saturated or mono-unsaturated alcohols were detected in *C. emersonii* and in all the microalgae belonging to the genus *Scenedesmus*. High amounts of saturated C_{30} and C_{32} α,ω-diols were also detected in *S. subspicatus*, *S. armatus* and *S. pannonicus*. Three classes of lipids were encountered in very small amounts in the medium polarity fraction of the neutral lipids of the microalgae investigated: (i) Monoesters composed predominantly of saturated C_{16} or C_{18} fatty acids and saturated C_8, C_{16} or C_{18} alcohols and (ii) long-chain methyl ketones from C_{25} to C_{31} were detected in several species and (iii) methyl esters of fatty acids ranging from C_{16} to C_{28} were identified in all the microalgae. Attempts to use the neutral lipid composition and particularly the unusual long-chain lipids, as specific indicators of the occurrence of algaenan in TLS-containing microalgae were unsuccessful.

Keywords: *Chlorella emersonii*; *Chlorella marina*; *Chlorella minutissima*; *Chlorella minutissima marina*; *Scenedesmus armatus*; *Scenedesmus communis*; *Scenedesmus pannonicus*; *Scenedesmus subspicatus*; *Tetraedron minimum*; Chlorophyceae; Algaenan; Trilaminar outer cell walls; Neutral lipids; Long-chain hydrocarbons; Long-chain alcohols; Long-chain methyl ketones

1. Introduction

Insoluble non-hydrolysable biopolymers, unusually resistant to drastic non-oxidative chemical treatments, termed algaenans have been encountered in cell walls of several green freshwater and marine microalgae from the class Chlorophyceae, Dinophyceae, Eustigmatophyceae (Derenne et al., 1992; Gelin et al., 1999). The characteristic feature of algaenan-producing microalgae is the membrane-like trilaminar structure (TLS) of their outer wall which exhibits two electron-dense sublayers sandwiching one sublayer with low electron density when they are examined by transmission electron microscopy (TEM). Combination of TEM observations and chemical analyses of algaenans

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isolated from several microalgae have shown that such biopolymers build up the trilaminar outer walls and have generally a highly aliphatic structure. Nevertheless, no strict relationship between TLS and algaenan has been demonstrated and the lack of algaenan has been noted in several TLS-containing species (Brunner and Honegger, 1985).

Regarding the highly aliphatic structure of the algaenans which predominantly generate, upon pyrolysis, *n*-alkenes and *n*-alkanes up to C_{30} (e.g. Derenne et al., 1992; Gelin et al., 1999; Blokker et al., 1998a), the involvement of long-chain lipids in the biosynthesis of the resistant outer cell wall can be considered. Thus, recent studies on the freshwater green microalga *Botryococcus braunii*, an alga which biosynthesizes high amounts of a well-characterized algaenan, showed that a structural relationship exists between the algaenan and several lipids extracted from this alga (Metzger and Largeau, 1999). A comparative study between the lipid composition of TLS-containing microalgae and the occurrence of algaenan could help to understand the relationship between the algal lipids and the presence (or the absence) of a resistant biopolymer in the trilaminar outer wall. Furthermore, such a study could provide some information about chemical structure of the algaenan precursors. The work described here, was designed to determine if any patterns could be discerned in lipid composition due to the presence or absence of algaenan in TLS-containing microalgae. The neutral lipid composition of nine green freshwater and marine TLS-containing microalgae from the class Chlorophyceae (*Chlorella emersonii*, *C. marina*, *C. minutissima*, *C. minutissima marina*, *Scenedesmus armatus*, *S. communis*, *S. pannonicus*, *S. subspicatus* and *Tetraedron minimum*) are reported and the presence of algaenans is examined.

2. Results and discussion

2.1. Ultrastructural study and algaenan isolation

The ultrastructures of most of the studied microalgae have been described elsewhere. When examined by TEM the outer walls of *C. emersonii* (Afi et al., 1996), *C. marina* (Rascio and Casadoro, 1981), *C. minutissima* (Dempsey et al., 1980), *S. armatus* (Largeau et al., 1990), *S. communis* (Derenne et al., 1991), *S. pannonicus* (Pickett-Heaps and Staehelin, 1975) and *S. subspicatus* (Corre, 1998) exhibit a thin trilaminar

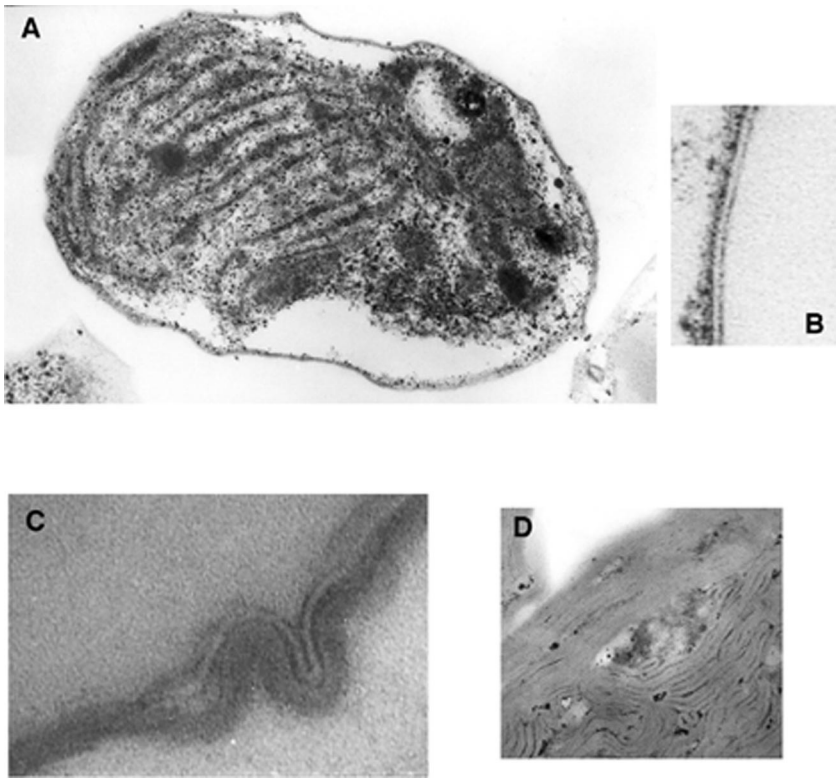

Fig. 1. Transmission electron microphotographs of (**A**) (×34,000) *C. minutissima marina*. Detail showing the trilaminar organisation of the outer cell walls of (**B**) *C. minutissima marina*; and (**C**) *T. minimum*; (**D**) (×38,000) algaenan isolated from *S. communis*, only the electron-lucent zone of the TLS is recognisable.

structure (10–20 nm thick). The presence of a TLS was also observed here in *C. minutissima marina* and *T. minimum* (Fig. 1).

All the TLS-containing microalgae so far examined were reported to produce algaenan (Derenne et al., 1991, 1992, 1996; Gelin et al., 1999; Largeau et al., 1990). However, it has been shown that the usual process used for the isolation of algaenans (process 1) (Berkaloff et al., 1983) could lead to the formation of insoluble non-hydrolysable artifactual melanoidin-like polymers, due to the condensation of sugars and amino acids during saponification and/or acid hydrolysis (Allard et al., 1997). As a consequence, algaenans obtained by this process contain substantial amounts of insoluble contaminants and their yields are greatly overestimated. Moreover, in some cases insoluble non-hydrolysable residue was obtained from microalgae although they actually did not produce any algaenan (Gelin et al., 1999). To avoid these artifacts a new method for the isolation of algaenans has been developed (process 2) leading to pure or much more less contaminated algaenans (Allard et al., 1998).

Using the latter isolation process (process 2) algaenan was obtained from all the freshwater species examined. The yields of algaenan isolated from these freshwater microalgae range from 1 to 6% of the dry biomass (Table 1).

In sharp contrast, when the lipid-free biomass of the two marine microalgae were treated using process 2 no final residue, i.e. algaenan, was detected. *C. marina* was previously reported to contain an amorphous algaenan providing large amounts of aromatic moieties upon pyrolysis (Derenne et al., 1996). However, this algaenan was isolated using process 1 and likely consists of artifactual melanoidin-like polymers. This implies that the aromatic moieties present in the pyrolysate of this insoluble non-hydrolysable residue probably derive from hydrolysable lipids which were incorporated into the recalcitrant structure of melanoidin-like polymers during the isolation process.

Table 1
Yield and elemental analysis (wt%) of algaenans isolated from microalgae[a]

	Yield	C	H (H/C)	O (O/C)	N (N/C)
C. emersonii	3.1	73.8	10.5 (1.7)	12.1 (0.12)	0.6 (0.007)
C. minutissima	6.0	73.7	10.2 (1.7)	12.9 (0.13)	0.8 (0.009)
S. armatus	2.4	66.8	8.5 (1.5)	16.2 (0.18)	0.9 (0.012)
S. communis	2.6	72.9	9.2 (1.5)	10.1 (0.10)	1.1 (0.013)
S. pannonicus	3.0	70.3	9.7 (1.7)	13.9 (0.15)	0.4 (0.005)
S. subspicatus	1.0	73.3	9.9 (1.6)	12.0 (0.12)	0.4 (0.005)
T. minimum	3.5	74.3	9.8 (1.6)	11.6 (0.12)	0.6 (0.007)

[a] As % of the dry biomass.

In all the above freshwater species the TLS is partly preserved following drastic hydrolyses. The trilaminar organization of the outer wall is not retained and only the electron-lucent zone is recognizable in isolated algaenan when observed by TEM (Fig. 1 D). However, it has been reported that the morphology of TLS was retained following the drastic chemical treatments used for isolating algaenans of most of the microalgae so far examined (e.g. Derenne et al., 1992; Gelin et al., 1999). This could suggest that the two electron-dense zones which are recognizable by TEM observations in algaenans obtained by process 1 are likely artifactually preserved by melanoidin-like polymer formation on the native electron-dense sublayers. Indeed these latter, binding heavy metals, are hydrophilic and probably built of proteins and/or polysaccharides. This observation suggests that algaenans are located in the hydrophobic inner electron-lucent sublayer in agreement with the aliphatic nature of this biopolymer.

2.2. Bulk chemical feature of algaenans

The elemental composition of the algaenans isolated from all the studied freshwater microalgae is rather similar (Table 1) and indicates a high carbon content (atomic ratio H/C ca. 1.6). This value corroborates the highly aliphatic structure of the algaenans produced by these microalgae.

The FTIR spectra of the algaenans show the same general features (Fig. 2) indicating (i) the highly aliphatic character of the residues revealed by the strong absorption at 720 cm^{-1} (skeletal vibration of $(CH_2)_n$ chains), (ii) the low branching level of the alkyl moieties indicated by the high ratio of the intensities of the absorption bands at 1465 cm^{-1} (CH_3 and CH_2 asymmetric bending) and 1370 cm^{-1} (CH_3 asymmetric bending), (iii) the presence of hydroxyl groups characterised by the absorption centered at 3400 cm^{-1}, and (iv) the presence of carboxyl groups characterised by the absorption band at 1710 cm^{-1}. This band disappears when algaenans are treated at alkaline pH (washed with 0.1 N NaOH) giving rise to two bands at 1585 and 1380 cm^{-1} corresponding to symmetric and asymmetric carboxylate stretching vibrations. (v) The presence of carbonyl groups indicated by the absorption band at about 1735 cm^{-1}. This band displays a low intensity and appears only as a shoulder in the spectra of some algaenans (Fig. 2B, D, E, G). The presence of carbonyl groups could indicate the polyester nature of the algaenans isolated from the microalgae studied. A structure containing linear polyester chains cross-linked by ether linkages has been proposed by Blokker et al. (1998a, 1998b) for the algaenans obtained from *S. communis* and *T. minimum*. However, in sharp contrast with the FTIR spectra of *S. commu-*

nis and *T. minimum* algaenans reported by Blokker et al. (1998a), our spectra do not exhibit intense absorption bands in the region corresponding to the C–O stretching vibrations of ether groups. Consequently, it appears that the contribution of ether bridges to the structure of the algaenans isolated from the microalgae

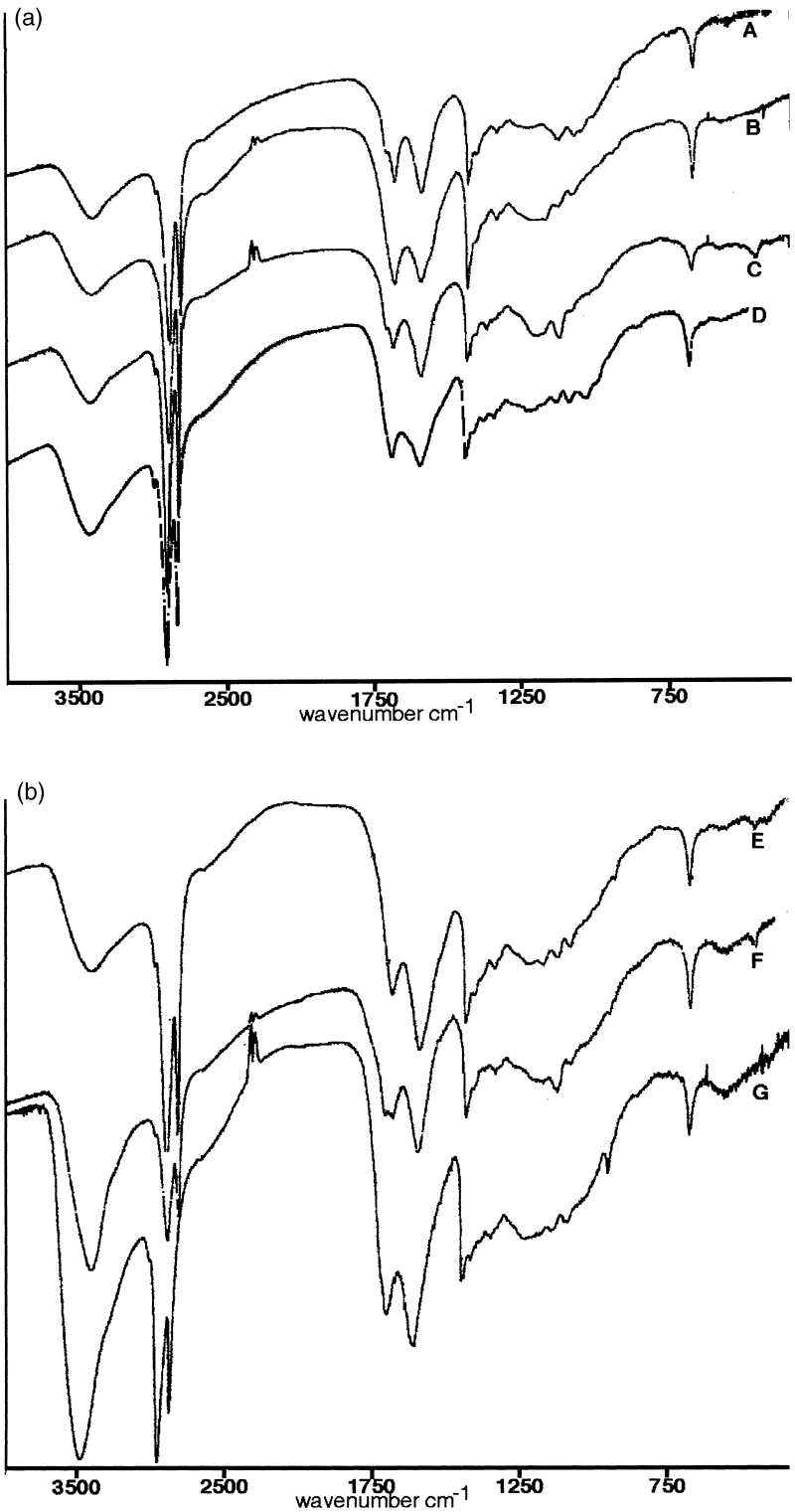

Fig. 2. FTIR spectra of algaenans isolated from (a) (**A**) *C. emersonii*, (**B**) *C. minutissima*, (**C**) *S. communis*, (**D**) *S. pannonicus*. (b) (**E**) *T. minimum*, (**F**) *S. subspicatus*, (**G**) *S. armatus*.

studied here is minor. (vi) All the spectra show a broad absorption band centered at 1620 cm^{-1}. This band is not shifted by pH changes and cannot therefore be assigned to COOH, NH and/or NH_2 groups. Considering the low contribution of absorption bands in the range 3000–3100 cm^{-1} (=CH stretching vibrations) and the absence of absorption bands due to out of plane =CH deformation vibrations of alkenes or aromatic compounds, the absorption band at 1620 cm^{-1} cannot be assigned to carbon–carbon double bonds. This latter band probably arises from carbonyl groups forming strong hydrogen bonds with parts of the molecular structure. This band could be assigned to the amide I band of a protein fraction which, deeply penetrating the lipid region, establishes hydrophobic interactions with lipids. Indeed the high strength of hydrogen bonds within a hydrophobic domain could account for the unusual low frequency of the amide I band (Banuelos et al., 1995). The algaenans show a low nitrogen content (Table 1) leading to an estimation of 1–6% residual proteins. If we assume that, in algaenans, nitrogen originates from proteins, a possible explanation is that the absorption band centered at 1620 cm^{-1} could correspond to the amide I band of proteins embedded in the hydrophobic environment of the aliphatic algaenan. This could protect proteins against the effects of chemical degradation occurring in the aqueous phase. This is, however, no more than a hypothesis and remains to be confirmed.

2.3. Lipid composition

Algal cultures were harvested at the end of the linear phase and neutral lipids extracted with chloroform. The lipid content differs markedly according to the algal species ranging from ca. 1% of the dry biomass in the cases of *S. pannonicus* and *S. subspicatus* to 26% in the case of *C. minutissima marina* (Table 2). The lipid extracts were fractionated on alumina column with heptane, toluene and chloroform/methanol (1/1 v/v).

2.3.1. Hydrocarbon distribution

The heptane fraction varies considerably, ranging from ca. 1% of the lipid extract in the case of *C. minutissima*, *S. armatus*, or *C. marina* to 20% in the case of *S. subspicatus* (Table 2). All the microalgae display the marked odd/even hydrocarbon predominance typical of algal hydrocarbon distributions. In term of hydrocarbon distribution the microalgae examined can be separated into two groups. Those with low molecular weight hydrocarbons only and those showing significant amounts of high molecular weight hydrocarbons (Table 2). In the first group, comprising *C. marina* and *S. subspicatus*, the *n*-C_{17} hydrocarbon, either saturated or unsaturated, is the major component. The microalgae of the second group contain, besides the typical *n*-C_{17} hydrocarbon, long-chain hydrocarbons from *n*-C_{23} to *n*-C_{29}. Squalene has been also identified in several species of microalgae from the two groups. The presence of this compound is not surprising regarding the high amounts of sterols identified in the chloroform/methanol eluate of a number of microalgae examined (Table 3).

Data on hydrocarbon composition of TLS-containing microalgae in relation to their ability to produce algaenan are rather rare. The hydrocarbon composition of *C. emersonii*, *S. communis* and *T. minimum* are in good agreement with the data previously reported by Afi et al. (1996) and Gelpi et al. (1970). The latter authors have suggested a close correlation between the presence of high molecular weight hydrocarbons and their contribution to the sedimentary organic matter. More recently, long-chain hydrocarbons with a strong predominance of odd carbon numbers were encountered in two TLS-containing marine eustigmatophytes (Gelin et al., 1997) which produce algaenan, and the role of such high molecular weight unsaturated hydrocarbons as algaenan precursors has been suggested (Gelin et al., 1997). However, our results show that the hydrocarbon composition of the microalgae investigated cannot be easily correlated to the presence of a highly aliphatic algaenan. Indeed, both *C. marina* which does not produce algaenan and *S. subspicatus*, an algaenan-producing microalga, contain only low molecular weight hydrocarbons. On the other hand, *C. minutissima marina*, an algaenan-devoid microalga, contains substantial amounts of high molecular weight unsaturated hydrocarbons.

2.3.2. Free fatty acid and alcohol distribution

The chloroform/methanol eluates were the major fraction of the neutral lipids in all the microalgae examined. They comprised from ca. 10% to more than 40% of the neutral lipids (Table 3). In all the species fatty alcohols were the major constituents of this fraction. The very low amount of fatty acids in neutral lipids is consistent with the fact that in most living organisms fatty acids occur predominantly as polar lipids such as glyco- and phospholipids.

The alcohol patterns display marked variations among the different species. General trends include the predominance of even-chain lengths, except for *T. minimum*, and the presence of significant amounts of terpenoid alcohols, phytol and sterols in agreement with the presence of squalene in the hydrocarbon fraction.

Among all of the microalgae, *C. marina* and *T. minimum* show a quite particular alcohol composition. *C.*

Table 2
Hydrocarbon composition of the neutral lipid extracts[a]

	C. emersonii	*C. minutissima*	*C. minutissima marina*	*C. marina*	*S. communis*	*S. subspicatus*	*S. armatus*	*S. pannonicus*	*T. minimum*
Lipid extract (a)	5	3	26	10	17	1	4	1	4
Heptane eluate									
(b)	5	tr	5	2	9	20	3	12	3
(c)									
C16:1			1						
C16:0			7						
C17:2				28					
C17:1			2	60	79				12
C17:0	20	30	6			43	25	79	17
C18:1			4						
C18:0			3						
C19:1			7						
C19:0		3	4			13			
C21:1			2						
C21:0		8							6
C22:0									3
C23:1			8				5		
C23:0		18							40
C24:0									4
C25:2			12						
C25:1	28		6				25		
C25:0		34						9	9
C26:0									2
C27:2			10						
C27:1	52		11		21		25		
C27:0		4						7	6
C29:2			13						
C29:1			4						
Squalene				12		44	20	5	

[a] (a) As % of the dry biomass; (b) as % of the lipid extract; (c) as % of the heptane eluate.

Table 3
Composition of the choloroform/methanol eluate of the neutral lipids[a]

	C. emersonii	*C. minutissima*	*C. minutissima marina*	*C. marina*	*S. communis*	*S. subspicatus*	*S. armatus*	*S. pannonicus*	*T. minimum*
Chloroform/methanol eluate (a)	10	26	45	40	12	42	29	42	29
Sterols (b)	14	24	35	89	42	14	34	tr	tr
Phytol (b)	27	9	16	11	14	4	25	13	24
Fatty alcohols (c)									
C16:0		29	10		15	14		6	
C18:0		62	46		tr	7		19	
C20:0		6							
C25:1			4						
C26:0	67				24	14	27	25	tr
C27:2			8						
C27:1			13						
C28:1					27				tr
C28:0	43				34	15	40	10	
C29:2			10						
C30:1					tr				
C30:0					tr			7	
C12:0 + C13:0 + C14:0									32
Branched-chain									
C12:0 + C13:0 + C14:0 + C15:0 (d)									68
C30:0 + C32:0 α, ω diols						50	24	30	
Fatty acids (c)									
C16:0	tr		8				9	2	
C18:0	tr	3			tr				
ωC28:0 Hydroxy acid									tr

[a] (a) as % of the lipid ertract; (b) as % of the chloroform/methanol eluate; (c) as % of the sum of fatty alcohols and acids; (d) branched-chain primary alcohols $RCH(R')CH_2OH$.

marina, which does not produce algaenan only contains di- and tri-terpenoid alcohols. *T. minimum* an algaenan-producing microalga, beside significant amounts of phytol, predominantly contains low molecular weight saturated alcohols in the C_{12}–C_{15} range with no odd/even predominance. Furthermore, this microalga is characterized by a high amount of C_{12}–C_{15} branched-chain primary alcohols. The TMSi ethers of these latter show the same typical fragmentation pattern upon mass spectrometry with characteristic ions at *m/z* 75 ($(CH_3)_2$–Si═O + H, base peak), *m/z* (M − CH_3)$^+$·, *m/z* (M − $(CH_3)_3SiOH)^+$· and *m/z* 103 (CH_2═O + $Si(CH_3)_3$, ca. 75% of the base peak). Trace amounts of high molecular weight $C_{26:0}$ and $C_{28:1}$ alcohols were identified together with $C_{28:0}$ ω-hydroxy fatty acid. Upon mass spectrometry, the ω-hydroxy methyl ester, TMSi-ether of the latter shows the characteristic ions at *m/z* 75 (base peak), *m/z* (M − CH_3)$^+$·, *m/z* (M − OCH_3)$^+$·, *m/z* (M − OCH_3–$CH_3OH)^{\cdot +}$, *m/z* 146 (CH_2═$C^+(OSi(CH_3)_3)OCH_3$) and *m/z* 159 (CH_2═CH–C═O + $CH_3(OSi(CH_3)_3)$).

All microalgae belonging to the genus *Scenedesmus* which are algaenan-producers, contain long-chain saturated or mono-unsaturated alcohols from C_{26} to C_{32} as the major components (75–85% of the total alcohols and acids). Furthermore, substantial amounts of C_{30} and C_{32} α, ω-diols are encountered in *S. subspicatus*, *S. armatus* and *S. pannonicus*. These compounds were identified by mass spectrometry as their TMSi-ethers which show characteristic fragments at *m/z* 75 (base peak) *m/z* 149 ($(CH_3)_2Si=O^+Si(OH)(CH_3)_2$), *m/z* (M − $(CH_3)_3SiOH)^+$· and *m/z* (M − 2$(CH_3)_3SiOH)^+$·.

Microalgae belonging to the genus *Chlorella* exhibit much more complex alcohol patterns. As seen above, *C. marina* contains only phytol and sterols. *C. emersonii*, an algaenan-producing microalga, and *C. minutissima marina*, an algaenan-devoid one, both contain predominantly high molecular weight saturated or mono-unsaturated alcohols in the C_{25}–C_{29} range. In contrast, only low molecular weight alcohols were identified in the chloroform/methanol eluate of the algaenan-producing *C. minutissima*.

Fatty alcohols are biosynthetic intermediates between fatty acids and alkanes (or between alkanes and fatty acids) (Schweizer, 1989) and, as expected, a good correlation is observed between alcohol and hydrocarbon chain lengths in most of the microalgae examined. There are few reports on long-chain alcohols in marine (Gelin et al., 1997; Volkman et al., 1992, 1998) or freshwater (Rezanka and Podojil, 1986; Rezanka et al., 1986) microalgae, however, it appears that they have been identified from acid or base hydrolysis of the total lipid extract suggesting that these long-chain alcohols are present as ester-bound rather than as free lipids. Of particular interest is the presence of long-chain alkyl diols in several algae belonging to the genus *Scenedesmus*. These components could be potential building blocks of a highly aliphatic algaenan. Indeed, C_{29}–C_{36} saturated and mono-unsaturated diols with a mid-chain alcohol position were encountered in two algaenan-producing marine microalgae of the genus *Nannochloropsis* and, on the base of structural analysis of the algaenans, they have been suggested as biosynthetic precursors of the polyether-linked algaenans isolated from these microalgae (Gelin et al., 1997).

Unfortunately, as in the case of the hydrocarbons, the alcohol composition of the microalgae studied here cannot be correlated to the presence of algaenan: (i) Long-chain alcohols are present both in algaenan-producing and in algaenan-devoid microalgae, (ii) *C. minutissima*, an algaenan-producing alga was found to produce only low molecular weight alcohols, and (iii) the occurrence of long-chain diols is not widespread in algaenan-producing microalgae.

2.3.3. Toluene eluate

Except for *C. minutissima marina* and *S. communis* the toluene eluates contained only minor amounts of lipids. Three classes of lipids were identified in these eluates, fatty acid methyl and/or ethyl esters, methyl ketones and monoesters. In all the microalgae analysed fatty acid methyl and/or ethyl esters with an even-chain length predominate, comprising from ca. 40 to 100% of the eluate (Table 4). It is generally recognized that the presence of fatty acid esters in a lipid extract results from the esterification of free fatty acids during the extraction. In our case, the extractions involve the use of chloroform stabilised with ethanol and ethyl esters likely result from the esterification of fatty acids. However methyl esters largely dominate this sub-fraction and cannot be related to the presence of methanol during extraction and/or fractionation. Accordingly, fatty acid methyl esters are likely synthesized by microalgae. Except *S. pannonicus* which contains almost exclusively mono-unsaturated C_{28} methyl ester, C_{16} and C_{18} fatty acid methyl esters predominate in all the species of green algae examined. However, it is noteworthy that in all the algaenan-producing algae long-chain fatty acid methyl esters in the C_{20}–C_{28} range were encountered, although in small amounts in some cases, whereas only C_{16} and C_{18} methyl or ethyl esters were identified in the two marine algaenan-devoid microalgae.

Long-chain alkan-2-ones and, more rarely, alken-2-ones in the C_{25}–C_{31} range were identified in small quantities in several algae. The isoprenoid 6,10,14-trimethylpentadecan-2-one, a degradation product of phytol, is the major component of this class of lipids. Methyl ketones have not been reported previously in

Table 4
Composition of the toluene extract of the neutral lipids[a]

	C. emersonii	*C. minutissima*	*C. minutissima marina*	*C. marina*	*S. communis*	*S. subspicatus*	*S. armatus*	*S. pannonicus*	*T. minimum*
Toluene eluate (a)	7	tr	14	4		tr	tr	tr	5
Fatty acid methyl (ethyl)esters (b)(c)									
C16:1			39				3		
C16:0	17	7	43	27*	19	37	19*		30*
C18:3									tr
C18:2				33*	12				tr
C18:1	20	19		39*	30	47	6	tr	39
C18:0	7			tr	10	4	2		tr
C20:1					7				tr
C22:1									tr
C26:0	tr	7				3	9	tr	tr
C28:1					15	2		100	
C28:0	4	5				2	5		24
Methyl ketones (c)									
C25:0					2				
C27:1	tr								
C27:0	tr			tr					tr
C29:1	7								
C29:0	tr			tr					7
C31:1	2								
6,10,14-Trimethyl pentadecan-2-one	29	40	10	tr	5	tr	28	tr	
Monoesters (c)(d)									
C16:0–C8:0		12	3	tr		3	16		
C16:0–C14:0				tr					
C16:0–C16:0	6		tr	tr					
C16:0–C18:0			tr	tr					
C16:0–C20:0				tr					
C18:1–C16:0									tr
C18:1–C18:0									tr
C18:1–C20:0									tr
C18:0–C8:0		9	5			2	12		
C18:0–C16:0	8								

[a] (a) As % of the lipid extract; (b) * indicates the presence of ethyl esters; (c) as % of the toluence eluate; (d) C16:0–C8:0 indicates monoester with C16:O acid and C8:O alcohol.

freshwater algae, and only long-chain alkenones have been found in several species of marine algae (Volkman et al., 1980). However, long-chain methyl ketones up to C_{33} have been identified in the pyrolysates of algaenans and have been assumed to reflect the thermal cleavage of ether bridges (Largeau et al., 1986). Our results show that methyl ketones are biosynthesised by microalgae. The hypothesis that methyl ketones may play a role in the formation of algaenans is unlikely. Indeed, (i) long-chain methyl ketones have been identified in *C. marina*, an algaenan-devoid microalga, and are not encountered in *C. minutissima*, *S. armatus* and *S. pannonicus* which all produce algaenan. (ii) The biosynthesis of algaenan, at least partly, via the condensation of methyl ketones would result in a highly branched biopolymer which is inconsistent with the FTIR data. The presence of methyl ketones in the pyrolysates of algaenans could indicate that they are, at least partly, deeply embedded in the lipid structure of the algaenan and, consequently, protected against solvent extractions and chemical treatments.

Monoesters were identified in almost all the microalgae analysed. These compounds show the typical fragmentation pattern upon mass spectrometry with characteristic ions at m/z ($RCOOH^+$, $RCOOH_2^+$, and $(R'-1)^+$). These esters contain saturated or, more rarely, mono-unsaturated C_{16} or C_{18} fatty acids and even-chain length saturated alcohols from C_8 to C_{20}. This type of monoesters are common components of the higher plant epicuticular waxes, but have been rarely detected among Chlorophyceae. Up till now only the freshwater alga *C. kessleri* has been found to contain wax esters up to 50 carbon atoms in trace amounts (Rezanka and Podojil, 1986). Hence these results show that monoesters, although they represent only a very small percentage of the neutral lipids, seem to be more common in Chlorophyceae than had been suggested by Wood (1988).

Although long-chain lipids, such as long-chain diols or ω-hydroxy acids, which might play a role as potential algaenan precursors have been identified in several algaenan-producing algae, no conclusive information was obtained in the present study concerning the relationship between neutral lipid pattern of TLS-containing microalgae and the presence or absence of a highly aliphatic algaenan. Further investigations on lipid composition of the cell walls released during algal reproduction could help to distinguish between intrinsic cell wall components and cytoplasmic lipids. Indeed the preliminary study of the lipids extracted from cell walls of *T. minimum* shows that they predominantly contain saturated C_{26} and C_{28} fatty acids which have not been detected in the neutral lipids of the whole algal biomass.

3. Materials and methods

3.1. General

Elemental compositions were determined by the CNRS analysis center.

FTIR spectra were recorded on a single-beam Brucker 45 spectrometer. The discs were prepared by mixing 1% (w/w) of the sample with KBr.

Analytical GC was performed on a fused silica CPSil5CB capillary column (25 m × 0.22 mm i.d., 0.4 μm film thickness) programmed from 100 to 300°C at 3°C/min. GC-MS analyses were carried out using a HP5890 gas chromatograph (with the same column and conditions as above) coupled to a HP5989 mass spectrometer operated at 70 eV.

Electron microscopy: The samples were fixed for TEM and observed as previously described (Berkaloff et al., 1983).

3.2. Isolation of algaenans

The lipid-free algal biomass was hydrolyzed according to the previously described method (Allard et al., 1998).

3.3. Culture conditions

Axenic strains of *C. marina* (CCAP 211/27), *C. emersonii* (CCAP 211/8P) and *S. communis* (CCAP 276/4b) were obtained from the Institute of Freshwater Ecology (Ambelside, UK). Axenic strains of *S. pannonicus* (Utex 77), *C. minutissima* (Utex 2219) and *C. minutissima marina* (Utex 2341) were obtained from the Culture Collection of Algae Department of Botany (Austin, USA). Axenic strains of *S. subspicatus* and *T. minimum* were supplied by the Pflanzenphysiologische Institut und Botanischer Garten der Universitat Göttingen (Germany). Axenic strain of *S. armatus* was kindly supplied by the Laboratoire de Cryptogamie, Museum National d'Histoire Naturelle (Paris, France).

C. emersonii was grown on a modified CHU 13 medium (Afi et al., 1996), all the *Scenedesmus* species were grown as previously described, (Allard et al., 1998), *C. marina* and *C. minutissima marina* were grown on artificial sea water (Jones et al., 1963), *T. minimum* was grown on a modified CHU 13 medium supplied with soil extract (30 ml $^{-1}$) and *C. minutissima* was grown on a modified CHU 13 medium supplied with proteose peptone (1 g $^{-1}$, DIFCO).

The microalgae were cultured under air-lift conditions at 23°C with continuous illumination (190 μE m^{-2} s^{-1}) and aeration (air–1% CO_2). They were harvested by centrifugation at the end of the linear phase.

3.4. Lipid extraction, fractionation and analysis

The dried biomass was extracted two times with chloroform. The lipid extracts were fractionated by chromatography on activity II alumina column successively eluted with heptane, toluene and chloroform/methanol (1/1 v/v). The heptane and toluene fractions were analyzed by GC and GC-MS. Before GC and GC-MS analysis the chloroforme/methanol fraction was treated with methanol/HCl for 1 h at 60°C to convert fatty acids into methyl esters and then with a mixture of anhydrous pyridine/1,1,1,3,3,3-hexamethyldisilazane/chlorotrimethylsilane (20/2/1 v/v/v) to convert alcohols into their *O*-trimethylsilyl ether derivatives.

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