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LIPIDS OF FOUR SPECIES OF FRESHWATER DINOFLAGELLATES

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Key Word Index—*Peridinium lomnickii*; *P. cinctum*; *Woloszynskia coronata*; *Ceratium furcoides*; Dinophyceae; sterols; stanones; steryl esters; alkyl esters; triacylglycerols.

Abstract—The steroidal derivatives, fatty acid alkyl esters and triacylglycerols have been identified in two cultured dinoflagellates, *Woloszynskia coronata* and *Ceratium furcoides*, and natural populations of two species, *Peridinium lomnickii* and *P. cinctum*. Within each species, the same sterol moieties occurred in free and esterified forms, but the species differed in the proportion of 4α-methyl and desmethyl sterols. Both groups occurred in the two *Peridinium* species, whereas *W. coronata* contained only 4α-methylsterols and *C. furcoides*, uniquely among dinoflagellates analysed to date, contained only desmethylsterols, although these were analogues of 4α-methylsterols present in the other species. 4α-Methyl steroidal ketones were present in *P. lomnickii* and *W. coronata*; cholest-4-en-3-one and 5α-cholestan-3-one were detected in *C. furcoides*, but no steroidal ketones were detected in *P. cinctum*. Methyl and ethyl esters of fatty acids (C_{12}–C_{18}) were detected in three species, in *P. cinctum* the ethyl esters were dominant, while phytyl esters occurred only in the *Peridinium* spp. The molecular composition of triacylglycerols containing only saturated and monoenoic acyl groups was determined by GC-MS. Lower carbon number constituents from *W. coronata* contained a C_4 acyl group, previously found only in animal milk fats.

INTRODUCTION

Dinoflagellates are major primary producers in the oceans, being surpassed in importance only by the diatoms [1]. The lipid, particularly the sterol, compositions of marine dinoflagellates have received much attention [2]. The abundance of 4α-methylsterols and the unique nature of certain of their structures distinguish most dinoflagellates from other algae and they have been used as a taxonomic tool [3, 4]. The predominance of 4α-methylsterols over 4-desmethylsterols in most dinoflagellate species is consistent with the presumed primitive status of the Dinophyceae; the large variety of sterol structures therein has also prompted interest in biosynthetic mechanisms [2, 5–7]. 4α,23,24-Trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol; **4f**), the principal sterol of several marine dinoflagellates [8–10], was used as a sediment marker of past dinoflagellate blooms in the Black Sea [11] and, together with other 4α-methylsterols, has been used as an indicator of dinoflagellate inputs to other marine sediments [12, 13].

Dinoflagellates also occur widely in the phytoplankton of freshwater environments and may predominate, as in Lake Kinneret (Israel) [14]; there are few reports, however, on the lipid composition of freshwater species [15]. The distributions of 4α-methylsterols and 4α-methylstanones isolated from a naturally occurring freshwater dinoflagellate have been correlated with the corresponding distributions in the underlying surficial sediment [16], thus demonstrating clearly a dinoflagellate origin. Previously, 4α-methylsterols in recent [17] and ancient [18] lacustrine sediments had been attributed to *in situ* methanotrophic bacterial activity. To obtain more information about freshwater dinoflagellates, two cultured species, *Woloszynskia coronata* (Woloszynska) Thompson and *Ceratium furcoides* (Levander) Langhans, and two naturally occurring species, *Peridinium lomnickii* Woloszynska and *Peridinium cinctum* fa. *westii* (Lemm.) Lef., have been studied.

RESULTS AND DISCUSSION

Lipid concentration data presented in Table 1 refer mainly to specific compound classes determined by GC-MS analysis, as detailed below.

Hydrocarbons

n-Alkanes showed a carbon preference index (CPI) close to unity and occurred in low abundance, except for *P. lomnickii* in which the distribution ranged from C_{15} to C_{31}, maximizing at C_{17} and C_{27} and showing a CPI value of 1.9. Hydrocarbons of the remaining three species were dominated by heneicosahexaene ($C_{21:6\omega3}$), as previously recorded in marine dinoflagellates [19]; low levels of squalene were also present.

Steroidal derivatives

Free sterols were major lipid components of each of the four species analysed (3.1–5.7 mg/g dry wt), with steryl esters also a significant lipid fraction (up to 4 mg/g, or more; see Table 1). The difficulty in accurately determining absolute levels of lipids in organisms limits the reliability of comparisons with data obtained in other laboratories, but similar levels of total sterols have been reported for the saponified extracts of marine dinoflagel-

Table 1. Lipid composition of freshwater dinoflagellates

	Composition*(μg/g dry alga)			
	P.l.	P.c.	C.f.	W.c.
Solvent extractable lipids	71 350	268 500	330 000	318 200
Hydrocarbons (total)†	4400	2900	3000	2000
n-Alkanes	640		150	
Steryl esters	815	6100	1000	4000
Phytyl esters	150		ND	ND
Methyl + ethyl esters	NA	150‖	70	50
Steroidal ketones	430	ND	30	60
Triacylglycerols	3460	16 500‖	5400	15000
Sterols	3080	5500	5700	4000
Alcohols‡	4470	16500	3000	10000
Acidic/Polar lipids§	2100	250	2000	3600
Monocarboxylic acids	630	tr	tr	1360

*Abbreviations: P.l. = *Peridinium lomnickii*, P.c. = *Peridinium cinctum*, C.f. = *Ceratium furcoides*, W.c. = *Woloszynskia coronata*, tr = trace ($< 10\ \mu g\ g^{-1}$), ND = not detected.

†Major constituents $C_{21:6}$ (except P.l.) and squalene.

‡Including C_{16} and C_{18} *n*-alkanols and phytol.

§Lipids retained on silica gel/KOH column for P.l. and W.c.; components of methanol eluate remaining at origin during TLC, for P.c. and C.f. (see Experimental).

‖ α-Tocopherol and its methyl ether also present.

lates [6, 10]. These levels are generally higher than those found in other algal types [20–22].

Although all of the identified sterols (Table 2) have been previously found in marine dinoflagellates [2], many have not been found in freshwater algae while dinostanol (**4h**) and 4α,23,24-trimethylcholesta-5,22-dien-3β-ol (**5f**) are not universally present in marine species. The former is a minor constituent in *Gonyaulax diagenesis* [23] and the four known species of *Heterocapsa* [24] but is a major sterol in *G. polygramma* [25] and *Gymnodinium wilczeki* [10]; the latter is a major sterol of *Crypthecodinium cohnii* [5] and a trace constituent in several other species [2]. Peridinosterol (4α,23*R*,24*R*-trimethylcholest-17(20)-en-3β-ol; **4g**) is a rare sterol which has been isolated from two binucleate species, *P. foliaceum* [26] and *Exuviaella mariae-labourae* [2].

Ceratium furcoides is notable for the presence of gorgosterol (22,23-methylene-23,24-dimethylcholest-5-en-3β-ol; **2i**) and dihydrogorgosterol (**1i**), not previously detected in freshwater algae, but obtained from an aposymbiotic dinoflagellate isolated from a sea anemone, *Aiptasia pulchella* [27]. *C. furcoides* is also notable for the absence of 4α-methylsterols although it contains the desmethyl analogues of many of them. Conversely, the absence of 4-desmethylsterols in *W. coronata* is unusual. However, marine species containing low levels of 4-desmethylsterols (<15% total sterols) include *Gymnodinium wilczeki* [10], *Amphidinium* spp. [28] and *Heterocapsa* spp. [24]. The absence of 4α-methylsterols in *Ceratium furcoides* and the low level of dinosterol in *W. coronata* imply that the absence of dinosterol in bottom sediments need not reflect an absence of dinoflagellates in the phytoplankton.

Biosynthesis of sterols

The large variety of sterol structures found in dinoflagellates has prompted interest in their mechanisms of biosynthesis. The major sterols are usually 4α-methyl ring-saturated compounds with additional side-chain methyl groups at C-23 and C-24 (e.g. dinosterol), but the desmethylsterols also present are often unsaturated and have unsubstituted side-chains (e.g. cholesterol; **2a**). There appears, therefore, to be a dichotomy in dinoflagellate sterol biosynthetic pathways, one route leading to the 4α-methylsterols and the other route, in which side-chain alkylation is relatively unimportant, leading to the 4-desmethylsterols [5]. The high proportion of C_{27} desmethylsterols in *P. lomnickii* and *P. cinctum* is indicative of this dichotomy.

At least two mechanisms have been proposed for the formation of the 23,24-dimethyl side-chain found in dinosterol and other dinoflagellate sterols: (i) whereby alkylation commences with the introduction into a Δ^{24}-side-chain (b) of a methyl substituent at C-24 [5] and (ii) in which bioalkylation of an isolated double bond occurs, even in the absence of a 24-methyl substituent, giving rise to a 23-methyl-Δ^{22}-sterol [7]. Of the two proposed pathways for side-chain bioalkylation in dinoflagellates, that starting with introduction of the C-24 methyl best accounts for the sterols found in *P. lomnickii*, *P. cinctum*, *C. furcoides* and *W. coronata*. 4α-Methyl-5α-cholestan-3β-ol (**4a**) could be formed by reduction of the presumed precursor, 4α-methyl-5α-cholest-24-en-3β-ol (**4b**). Similarly dinostanol (**4h**) could be biosynthesized by reduction of the Δ^{22}-double bond in dinosterol, or, alternatively, by quenching of the carbonium ion intermediate produced in the postulated biosynthesis of dinosterol [5]. Two pathways have been suggested for the formation of peridinosterol (**4g**): (a) double bond migration from the Δ^{22}-double bond of dinosterol and (b) isomerization of a 23-desmethylgorgosterol precursor to a $\Delta^{20(22)}$-23,24-dimethyl intermediate followed by double bond migration to the $\Delta^{17(20)}$-position [29].

The relative sequence of side-chain bioalkylation and ring system saturation in dinoflagellate sterol biosynthesis

Table 2. Sterols of four freshwater dinoflagellates

Systematic name*	Structure	Abundance† P.l.	P.c.	C.f.	W.c.
Desmethylsterols					
Cholesta-5,22-dien-3β-ol				tr	
5α-Cholest-22-en-3β-ol				3	
Cholest-5-en-3β-ol	**2a**	tr	52	73	
5α-Cholestan-3β-ol	**1a**	20	2	17	
5α-Cholest-7-en-3β-ol	**3a**	1			
24-Methyl-5α-cholest-22-en-3β-ol				tr	
24-Methyl-5α-cholest-24(28)-en-3β-ol	**1d**			tr	
24-Methylcholest-5-en-3β-ol	**2e**		1		
24-Methyl-5α-cholestan-3β-ol	**1e**	1		1	
23,24-Dimethyl-5α-cholest-22-en-3β-ol				tr	
24-Ethylcholesta-5,22-dien-3β-ol				tr	
24-Ethyl-5α-cholest-22-en-3β-ol				tr	
24-Ethylcholest-5-en-3β-ol		5			
24-Ethyl-5α-cholestan-3β-ol		2			
22,23-Methylene-23,24-dimethylcholest-5-en-3β-ol	**2i**			4	
22,23-Methylene-23,24-dimethyl-5α-cholestan-3β-ol	**1i**			tr	
Total %		29	55	98	0
4α-Methylsterols					
4α-Methyl-5α-cholest-8(14)-en-3β-ol	**6a**		tr		
4α-Methyl-5α-cholestan-3β-ol	**4a**	17	2		40
4α,24-Dimethyl-5α-cholest-22-en-3β-ol	**4c**	3	1		
4-Me $C_{29:1}$			tr		
4α,24-Dimethyl-5α-cholestan-3β-ol	**4e**	7	3		19
4α,23,24-Trimethylcholesta-5,22-dien-3β-ol	**5f**	13	10		38
4α,23,24-Trimethyl-5α-cholest-22-en-3β-ol	**4f**	18	13		3
4α,23,24-Trimethyl-5α-cholest-17(20)-en-3β-ol‡	**4g**		14		
4α,23,24-Trimethyl-5α-cholestan-3β-ol	**4h**	13	tr		
Total %		71	43	0	100
Total absolute concentration (μg/g dry weight)		3080	5500	5700	4000

*Identifications made by combination of comparison of mass spectra with those of standards, mass spectral interpretation, relative retention times and behaviour on hydrogenation (e.g. assignment of compound as 4α-methyl-5α-cholest-8(14)-en-3β-ol was based on resistance to hydrogenation of the hindered double bond. Configurations at C-23 and C-24 in **4h** from P.l. shown to be *R* by cochromatography with a standard.

†Abundances are given as % of total sterols.

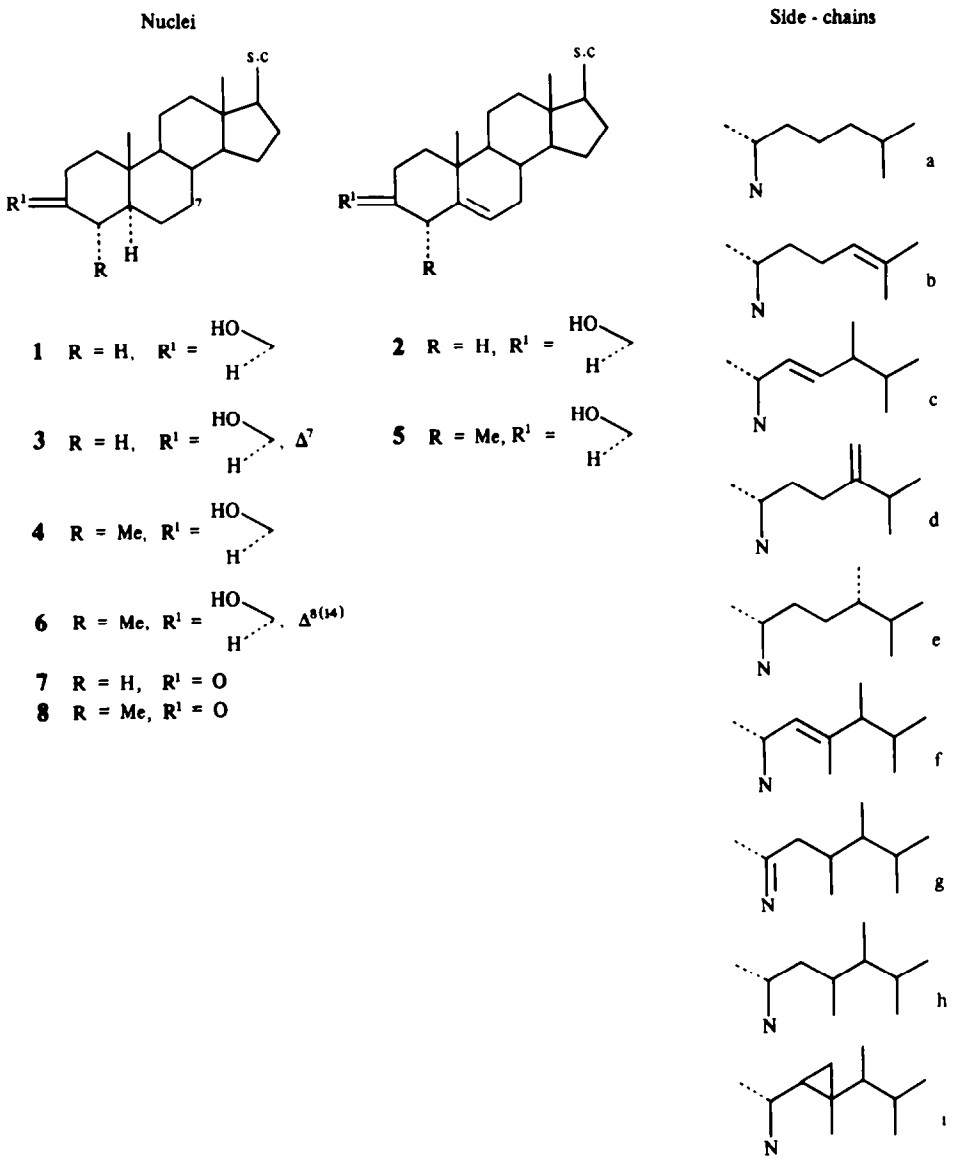
‡Assignment confirmed by cochromatography with a standard (donated by Dr. W. C. M. C. Kokke).

is unclear. The presence of 4α,23,24-trimethylcholest-5,22-dien-3β-ol (**5f**) in *P. lomnickii*, *P. cinctum* and *W. coronata* shows that, for these dinoflagellates, the 23,24-dimethyl-Δ^{22}-side-chain could be formed before complete saturation of the ring system. 5α-Cholestan-3β-ol (**1a**), present in *P. lomnickii*, *P. cinctum* and *C. furcoides*, could be formed by demethylation of a 4α-methyl-5α-cholestanol precursor. However, the presence of 5α-cholest-7-en-3β-ol (**3a**) in *P. lomnickii* suggests that an alternative pathway may be operating, in which ring system saturation, via various redox steps, occurs after loss of the 4α-methyl group, as in the production of 5α-cholestanol in animal tissues [30].

The presence of 4α-methylgorgostanol (**4i**) in certain dinoflagellates [26, 31] indicates that perhaps cyclopropanation precedes demethylation at C-4 for gorgosterol-/gorgostanol formation. However, this sequence is as yet unknown and 23,24-dimethylcholest-5,22-dien-3β-ol (**2f**) appears to be the most likely precursor for gorgosterol [27]. The ring saturated analogue of this precursor was identified in *C. furcoides* which contains the desmethyl analogues of many of the typical dinoflagellate 4α-methylsterols.

Chemotaxonomy

The sterol composition of marine dinoflagellates has potential taxonomic value [2, 3], showing some agreement with groupings based on morphological observations [32]. The sterol composition of these freshwater species is correlated with relevant data for marine dinoflagellate species that may have taxonomic affinities with the former (Table 3). The sterol composition of *P. cinctum* resembles that of the non-photosynthetic marine dinoflagellate *P. foliaceum* [26] in respect of the presence of peridinosterol, major cholesterol content and the proportions of desmethyl- and 4α-methyl-sterols (Table 3). The freshwater order Peridiniales consists of eight families [33], among which the Woloszynskiaceae, to which *Wolozynskia coronata* belongs, were believed to be related



to the Gymnodiniaceae. Marine dinoflagellates of the order Gymnodiniales [32] include a family, Gymnodiniaceae, containing the genera *Amphidinium*, characterized by the presence of $\Delta^{8(14)}$-4α-methylsterols, e.g. amphisterol (**6d**) [28], and *Gymnodinium*, two species of which contain widely different proportions of nuclear-saturated 4α-methylsterols and desmethylsterols [10, 34]. *Woloszynskia coronata*, which contains no $\Delta^{8(14)}$-4α-methylsterols or desmethylsterols, was assigned to the family Lophodiniaceae, within the order Gymnodiniales by Parke and Dixon [32]. This order is therefore not homogeneous based on sterol composition.

The absence of 4α-methylsterols in *Ceratium furcoides* is of interest from a taxonomic viewpoint as *Ceratium* species form a family within the order Peridiniales in both freshwater [33] and marine [32] classifications. Analysis of further species within the family seems necessary to assess the significance of this unique sterol composition.

Steroidal ketones

4α-Methylsteroidal ketones identified in *P. lomnickii* and *W. coronata* corresponded in distribution to the analogous 4α-methylsterols of the respective organisms (Table 4), except that the ketone analogue of 4α,23,24-trimethylcholesta-5,22-dien-3β-ol (**5f**) was absent. In *C. furcoides*, which contained no 4α-methylsterols, two desmethylsteroidal ketones, cholest-4-en-3-one and 5α-cholestan-3-one (**7a**), showed a similar abundance ratio to the related sterols, cholest-5-en-3β-ol and 5α-cholestan-3β-ol, respectively. Steroidal ketones were absent in the sample of *P. cinctum*, collected during the seasonal bloom in March.

The 4α-methylsteroidal ketone dinosterone (4α,23,24-trimethyl-5α-cholest-22-en-3-one; **8f**) was previously noted in *Crypthecodinium cohnii* [5] and also in *Thoracosphaera heimii*, in which 4,24-dimethyl-5α-

Table 3. Comparison of sterol distributions in marine and freshwater dinoflagellates

	4α-Methylsterols†											Desmethylsterols†							
Taxon*	4a	4c	4d	4e	4f	4g	4h	5f	6a	6d	Other	1a	2a	2c	2d	2f	Other	%4α-methyl	Reference
Order Prorocentrales																			
Family Prorocentraceae																			
Prorocentrum cordatum		1	5		29						Note 1		4		35	12	**1f, 2c**	44	[10]
Order Gymnodiniales																			
Family Gymnodiniaceae																			
Amphidinium carterae									26	43	**6f**		8					86	[28]
Amphidinium corpulentum									27	58	**6f**		5					94	[28]
Gymnodinium wilczeki				4	48		24				Note 2		2					93	[10]
Gymnodinium simplex				22									tr	40		10	Note 3	24	[34]
Family Lophodiniaceae																			
Woloszynskia coronata	40			19	3			38										100	This work
Order Peridiniales																			
Family Peridiniaceae																			
Glenodinium sp.				15	79													100	[28]
Heterocapsa niei				24	49		21						tr				**2e**	95	[24]
Heterocapsa triquetra				10	61		14						4				**2e**	86	[24]
Peridinium foliaceum				x	*y*	6					**4i**		35				**2e, 2i, 1i**	35	[26]
Peridinium cinctum	2	1		3	13	14		10				2	52					43	This work
Peridinium lomnickii	17	3		7	18		13	13				20	tr				See Table 2	71	This work
Family Gonyaulacaceae																			
Gonyaulax polygramma	3	7	1		16		25					1	17	3		5	**1c, 1f, 2h, 1h**	55	[25]
Family Ceratiaceae																			
Ceratium furcoides												17	73				See Table 2	0	This work

*After Parke and Dixon [32]; freshwater species after Bourrelly [33].

† Structures given in Fig. 1. Figures as % of free sterols. For *P. foliaceum*, $x + y = 33\%$. tr = trace.

Note 1. 4α,23,24-Trimethyl-5α-cholest-24(28)-en-3β-ol or 4α-methyl-24-ethyl etc (8%).

Note 2. 4α,23,24-Trimethyl-5α-cholest-7-en-3β-ol (15%).

Note 3. 27-Nor-24-methylcholesta-5,22-dien-3β-ol (24%).

Table 4. Steroidal ketones of three freshwater dinoflagellates

Identity*	Structure	Abundance† P.l.	C.f.	W.c.
Cholest-4-en-3-one			100	
5α-Cholestan-3-one	**7a**		34	
4α-Methyl-5α-cholestan-3-one	**8a**	70		100
4α,24-Dimethyl-5α-cholest-22-en-3-one	**8c**	30		
4α,24-Dimethyl-5α-cholestan-3-one	**8e**	35		91
4α,23,24-Trimethyl-5α-cholest-22-en-3-one	**8f**	100		35
4α,23,24-Trimethyl-5α-cholestan-3-one	**8h**	40		
Total absolute concentration (μg/g dry wt)		430	30	60

*Based on comparison of mass spectra with reference spectra and/or published spectra, or by mass spectral interpretation and relative retention time.

†As % of most abundant component.

cholestan-3-one (**8e**) co-occurred [4]. The occurrence in *P. lomnickii* and *W. coronata* of a suite of 4α-methylsteroidal ketones is unprecedented. Desmethylsteroidal Δ^4-3-ketones have previously been isolated from the marine dinoflagellate, *Pyrocystis lunula* [2].

3-Keto steroids are intermediates in one of the pathways by which Δ^5-3β-hydroxy sterols are converted into the corresponding stanols in sediments [35] and also in invertebrate organisms [36]. The interconversion of 5α-cholestan-3-one and 5α-cholestan-3β-ol in algal mats has also been reported [37]. The common biosynthetic origin of the 4α-methylsterols and 4α-methylsteroidal ketones in these organisms is demonstrated by their similarity in distribution. Side chain alkylation may occur before or after formation of the 3-keto group, although the presence of 4α,23,24-trimethylcholesta-5,22-dien-3β-ol in both *P. lomnickii* and *W. coronata* suggest that the former is more likely.

Steryl esters

The molecular composition of the steryl esters, determined by capillary column GC-MS [38], is presented in Table 5. The two *Peridinium* species contained both 4α-methyl- and 4-desmethylsteryl esters, the latter being relatively more abundant in *P. cinctum* than in *P. lomnickii*. In the cultured algae, *W. coronata* contained only 4α-methylsteryl esters whereas *C. furcoides* contained none of the latter, consistent with the relative abundance of 4α-methyl and desmethylsterol moieties in the free sterols and steroidal ketones of these species (see above), thus demonstrating a common biosynthetic origin.

Steryl esters have been found in almost all marine dinoflagellate species examined for such compounds [2]. Saponification techniques showed sterol constituents sometimes differing from those of the free sterols [25, 28]. In these four freshwater species the distribution of esterified sterols, obtained from the combined abundance of esters containing a given sterol (data of Table 5), may be compared with the composition of the respective free sterols (Table 2). A close correspondence in distribution of major sterols is observed in *C. furcoides*, in which cholest-5-en-3β-ol and 5α-cholestan-3β-ol show an abundance ratio of *ca* 4:1 in both esterified and free sterols, and in *W. coronata*, in which 4α-methyl-5α-cholestan-3β-ol and 4α,23,24-trimethylcholesta-5,22-dien-3β-ol occur in almost equal amounts in both esterified and free sterols. In the two *Peridinium* species, which contain both desmethyl- and 4α-methylsterols, the former are more abundant in the esters than in the free sterols of both species, but show a similar composition. Considerable differences in composition between free and esterified 4α-methylsterols occur in both *P. lomnickii* and *P. cinctum*. In the latter species, peridinosterol (**4g**) and dinosterol (**4f**), the major free sterols, were undetectable among the esters in which Δ^5-dinosterol (**5f**) was dominant.

Steryl esters are presumed to serve as metabolic storage forms of sterols in the algal cell [39]. The prominence of esterified 4α-methylsterols in Black Sea sediments [13] probably reflects a major input from dinoflagellates to these sediments.

Acyclic monoesters

Methyl and ethyl esters of fatty acids in the range C_{12}–C_{18} were detected in *C. furcoides*, *W. coronata* and *P. cinctum* (Table 6). Although base or acid-catalysed transesterification of long-chain esters may occur in the presence of excess methanol or ethanol [40], neither of these solvents was used in the isolation of esters from *P. cinctum*, in which ethyl esters predominated and unsaturated acyl units were detected in addition to the saturated groups common to all three species. Ethanol was not used during extraction of *C. furcoides* and *W. coronata* suggesting that the ethyl esters (and probably the methyl esters) from these species are also not artefacts. There are few previous reports of methyl and ethyl esters in organisms, however C_{14}–C_{24} ethyl esters were isolated from a liverwort, *Conocephalum conicum* [41], and $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ ethyl and methyl esters were detected in the phycomycete *Rhizopus arrhizus* [42]. Certain algae belonging to the Prymnesiophyta contain methyl and ethyl esters of di- and tri-unsaturated C_{36} acids structurally related to co-occurring ketones [43]. Ethyl esters have been reported in the 50 million year old lacustrine Messel oil shale, in which 4α-methylsterols characteristic of dinoflagellates are also abundant [44].

Phytyl esters of even carbon number C_{14}–C_{18} fatty acids were inferred in *P. lomnickii* by mass spectral interpretation and confirmed, in the cases of the C_{16} and

Table 5. Composition of steryl esters isolated from dinoflagellates

Compound*	Abundance (% of total) P.l.	P.c†	C.f.	W.c.
Desmethylsteryl esters				
$C_{27}\Delta^5$-14:0	tr	6	2	
C_{27}-14:0	5	1	tr	
$C_{27}\Delta^5$-16:0	tr	19	43	
C_{27}-16:0	13	2	12	
$C_{27}\Delta^5$-18:1		37	28	
C_{27}-18:1	11	1	11	
$C_{27}\Delta^5$-18:0		tr		
C_{27}-20:1	16			
Unidentified			4	
Total	45	66	100	0
4-Methylsteryl esters				
4-Me C_{28}-14:0	3	1		4
4,24-di Me-14:0	2	2		1
4,23,24-tri Me $\Delta^{5,22}$-14:0		7		7
4,23,24-tri Me Δ^{22}-14:0	2	tr		
4,23,24-tri Me-14:0	3			
4-Me C_{28}-16:0	7	1		27
4,24-di Me Δ^{22}-16:0	tr	tr		
4,24-di Me-16:0	5	2		4
4,23,24-tri Me $\Delta^{5,22}$-16:0	1	5		19
4,23,24-tri Me Δ^{22}-16:0	7	tr		
4,23,24-tri Me-16:0	6			
4-Me C_{28}-18:1	3	tr		11
4,24-di Me-18:1		1		2
4,23,24-tri Me $\Delta^{5,22}$-18:1		11		25
4,23,24-tri Me Δ^{22}-18:1	2	tr		tr
4,23,24-tri Me-18:1	3			
4-Me C_{28}-20:1	9			
Unidentified	2	4		
Total	55	34	0	100

*Shorthand notation in the form alkyl-acyl, e.g. 4,23,24-tri Me $\Delta^{5,22}$-18:1 refers to 4,23,24-trimethyl-cholesta-5,22-dien-3β-ol esterified to a monoenoic n-C_{18} fatty acid. Identifications by comparison of mass spectra with those of standards or by spectral interpretation. tr = trace (< 1%).

†Unidentified 4-methylsteryl esters with shorter acyl chains also present.

C_{18} esters, by comparison of mass spectra and GC co-elution [45] with standards. The C_{14}, C_{16} and $C_{18:1}$ phytyl esters and dihydrophytyl-C_{18} were also detected in *P. cinctum* but phytyl esters were not detected in the other species. Phytyl esters have previously been reported in two marine *Peridinium* spp. [46] and also in moss species [47, 48] and in higher plants [49, 50].

Triacylglycerols

Triacylglycerols containing 32–52 acyl carbon atoms were detected by GC analysis of the lipid fraction showing appropriate TLC mobility; the carbon number distribution (Table 7) was obtained from peak areas detected by flame ionization. Direct insert EI probe mass spectra of these triacylglycerols showed acyl fragment ions attributable to 12:0, 14:0, 16:1, 16:0 and 18:1 acyl units with additional diagnostic fragment ions $[M-RCO_2]^+$ resulting from loss of one acyl group [51]. In *P. lomnickii* acyl ion intensity decreased in the order 14:0, 16:0, 18:1, 16:1 and 12:0, whereas in *P. cinctum* the order of intensity was 18:1, 16:0, 18:2, 14:0; $[M-RCO_2]^+$ ions in the probe mass spectra of the former showed m/z values indicative of fragments containing 28:0, 30:0, 32:0, 32:1 and 34:1 acyl carbon atoms:double bonds, in order of decreasing ion intensity, whereas in *P. cinctum* the order was 34:1, 32:0, 32:1, 30:0, 36:2 and 28:0. Probe mass spectra of the triacylglycerols from *W. coronata* showed a base peak of m/z 71, in part attributable to a C_4-acyl group, based on the presence of additional ions at m/z 145, 186 and 199 diagnostic for this group [51]; the remaining acyl ions, $[RCO]^+$, decreased in intensity in the order 14:0, 16:0, 18:1, 16:1 and 18:0 and the $[M-RCO_2]^+$ ions, showing a bimodal distribution, decreased in intensity in the order; 18:0, 30:0, 20:0, 32:0, 32:1, 34:1 and 28:0. Triacylglycerols from *C. furcoides* were not analysed by probe mass spectrometry. Variation between species in chain length of the major saturated acyl

group in triacylglycerols, as indicated by the probe mass spectral data above, has also been observed in transesterified fatty acids from three marine species, in one of which 12:0 and 14:0 predominated while 16:0 was dominant in the other species [52, 53].

Table 6. Distribution of fatty acid methyl and ethyl esters in freshwater dinoflagellates

	Relative abundance†		
Compound*	P.c.	C.f.	W.c.
12:0, Me			22
12:0, Et			3
14:0, Me	6	20	13
14:0, Et	34	2	4
16:0, Me	28	100	100
16:0, Et	83	3	10
18:2, Me	3		
18:2, Et	10		
18:1, Me	16	–	
18:1, Et	100		
18:0, Me	14	15	12
18:0, Et	7	tr	3
Total concentration (μg/g dry wt)	150	70	50

*Shorthand notation of chain length, unsaturation and whether methyl or ethyl ester.

†As % most abundant constituent; tr = trace (< 1%).

Capillary gas chromatography-mass spectrometry (C-GC/MS) was used to study the molecular composition of algal triacylglycerols. The results are given in Table 7. C-GC-MS analysis of triacylglycerols containing saturated or monoenoic acyl groups enables molecular species containing these groups to be determined [45]. However, the technique is not applicable [54] to the triacylglycerols containing polyenoic acyl groups which occur in aquatic organisms [55], including diatoms [56, 57], but these were minor constituents in three marine dinoflagellates [52, 53] suggesting that this limitation of C-GC-MS may be of minor importance in the present study. Recognition of polyenoic acyl groups via saponification was avoided because of the inevitable loss of molecular structure. In the absence of suitable standard compounds, quantitative analysis of molecular composition, based on corrected relative acyl ion intensities, was not possible, nor could positions of the acyl chains on the glycerol unit be assigned.

Similar molecular species were found in triacylglycerols containing 44–50 acyl carbon atoms isolated from *P. lomnickii*, *C. furcoides* and *W. coronata*; the dominant acyl groups in this molecular weight range, 14:0, 16:0 and 18:1, being those also found in the steryl esters of these organisms. The C_4-acyl group, detected only in *W. coronata* by probe mass spectrometry, occurs solely in triacylglycerols containing 32–38 acyl carbon atoms, in which the other acyl groups consisted of pairings of 14:0, 16:0, 18:1 and 18:0 (Table 7). The triacylglycerols from *P. cinctum* were not examined by C-GC-MS. However the probe mass spectral data indicates a predominance of

Table 7. Molecular composition of triacylglycerols from three dinoflagellates

	% Composition†			Acyl combinations‡		
C.N.:U*	P.l.	C.f.	W.c.	P.l.	C.f.	W.c.
32:0			3			4/14/14
34:0			11			4/14/16
36:1			18			4/14/18:1
36:0			9			4/16/16
						4/14/18
38:1			10			4/16/18:1
38:0			1			4/16/18
40:0	3		tr	12/14/14		NA
				12/12/16		
42:0	17		tr	14/14/14		NA
44:0	17	5	2	14/14/16		14/14/16
46:1	10	5	5	14/16/16:1	14/16/16:1	14/14/18:1
				14/14/18:1	14/14/18:1	
46:0	15	9	7	14/16/16	14/16/16	14/16/16
48:1	18	25	8	14/16/18:1	14/16/18:1	14/16/18:1
48:0	6	8	12	16/16/16	16/16/16	16/16/16
50:2		8	4	NA	14/18:1/18:1	14/18:1/18:1
					16/16:1/18:1	
50:1	12	31	6	NA	16/16/18:1	16/16/18:1
52	2	9	3	NA	NA	16/18:1/18:1

*Carbon number (excluding glycerol skeleton): number of double bonds: C_{52} constituents unresolved by GC; also C_{50} in P.l.

†Species identification, see footnote to Table 1; tr = trace (< 1%).

‡Saturated, except where stated otherwise. NA not analysed. The order of the acyl groups does not reflect their position on the glycerol chain.

constituents possessing 48–52 acyl carbon atoms and one or two double bonds.

Triacylglycerols occur widely in organisms in which they serve as storage lipids. However constituents containing a C_4-acyl moiety only appear to have been detected in animal milk fats [55]. During analysis of a triacylglycerol fraction isolated from contemporary bottom sediment of Windermere, a freshwater lake, constituents having 34, 36 and 38 acyl carbon atoms also contained a C_4-acyl group (P. A. Cranwell, unpublished data), based on the criteria given above, again suggesting that triacylglycerols containing a C_4-acyl group may occur more widely than previously recognised.

CONCLUSIONS

Comparison of marine and freshwater dinoflagellates

The lipid composition of these freshwater dinoflagellates shows features in common with marine species:

(1) High abundance of sterols compared with other organisms.
(2) The presence of esterified sterols and steroidal ketones, as in most marine species analysed for these constituents.
(3) Occurrence of phytyl esters in species (two freshwater, two marine) belonging to the genus *Peridinium*.
(4) Dinosterol (**4f**) may be only a minor constituent or even absent, as noted for marine species [2].
(5) Triacylglycerols constituted 3–5% of extractable lipids, comparable with the value (7%) found in a marine species [53]. The chain length of the major acyl constituent shows considerable variation between species, as in marine dinoflagellates [52, 53].
(6) Hydrocarbon distributions in which 21:6 ω3 was dominant and *n*-alkanes only trace constituents, except for *P. lomnickii*.

Features in the lipids differing from those in marine species are:

(1) The absence of cholesterol in *W. coronata* refutes a suggestion [3], based on marine species, that this is the only sterol consistently found in all dinoflagellates, if only as a minor constituent.
(2) The absence of 4α-methylsterols, or derivatives thereof, in *C. furcoides* appears unprecedented in marine dinoflagellates.

Dinoflagellate lipids as biological markers in freshwater sediments

The dominance of 4α-methylsterols only in dinoflagellates among marine primary producers has led to their use as biological markers of dinoflagellate input to recent marine sediments [11–13]. The sequence of diagenetic reactions which modify sterols in sediments, generating steroidal hydrocarbons, has been widely studied [58], consequently the 4α-methylsteroidal hydrocarbons in ancient sediments [59, 60] may reflect dinoflagellate input to their original depositional environment.

In one freshwater environment a direct dinoflagellate input to the sediment has been demonstrated using 4α-methylsterols [16]. Recent studies on Lake Kinneret, the phytoplankton of which has been dominated by a dinoflagellate, *P. cinctum*, for many years [14], also show that input from this source is evident in the sedimentary lipids, notably in the distributions of 4α-methylsterols and possibly in those of the methyl and ethyl esters [61].

The above data facilitate a re-appraisal of ancient lacustrine deposits such as the Eocene Messel shale in which 4α-methylsterols, the corresponding ketones and steranes had previously been found to be unusually abundant, relative to their 4-desmethylsterol analogues [18, 60], but for which no source was then known. Laboratory interconversions of the sedimentary 4α-methylsterols and steroidal ketones demonstrate their parallel distributions, reflecting a common biosynthetic origin [62]. Analysis of freshwater dinoflagellates has shown that the 4α-methylsteroidal ketones present in the Messel shale may reflect direct dinoflagellate input, alternatively they may result from microbial oxidation of the sedimentary 4α-methylsterols that are probably derived from dinoflagellates, as recently proposed elsewhere [63].

EXPERIMENTAL

Woloszynskia coronata (clone 1117/2, Culture Centre of Algae and Protozoa, Cambridge) was inoculated into medium (20 l.) modified from that described by Jaworski *et al.* [64], by the addition of soil extract (0.5%) and incubated at 20° under continuous illumination from daylight fluorescent lamps, the output being attenuated by plastic netting to give an irradiance of 28 $\mu E \cdot m^{-2} s^{-1}$ at the base of the flasks. Cells in late log-phase were harvested after 35 days by centrifuging at 2200 r.p.m. The wet alga was extracted with $CHCl_3$–MeOH as described for *P. lomnickii* [45]. The weight of cellular material was determined after extraction by drying at 90°.

Ceratium furcoides (clone L258, FBA algal culture collection), recently recognized as a species distinct from *C. hirundinella* [65], was inoculated into medium [64] (2 × 20 l.) and incubated as described above. Cells in late log-phase (12.5×10^4/ml) were concentrated by filtration and the concentrate was centrifuged to obtain a pellet. Lipids were extracted with $CHCl_3$–MeOH using ultrasonication.

Peridinium cinctum fa. *westii* was collected from Lake Kinneret (Israel) during the initial period of its annual dominance [14]. Inspection by optical microscopy and phase contrast microscopy of an aliquot of lyophilized cells suspended in water showed *P. cinctum* to constitute >99% of phytoplankton and to contain low levels of attached bacteria. Lipids were extracted by ultrasonication with CH_2Cl_2.

Peridinium lomnickii was collected from the stratified water column of Priest Pot, a eutrophic lake of the English Lake District, by pumping water from a layer 1.2 m below the surface, corresponding with a maximum chlorophyll *a* concentration associated with this alga. Microscopic examination showed that this dinoflagellate comprised >95% of the sample. Details of the extraction procedure have been published [45]; free fatty acids were removed prior to separation of neutral lipids.

Lipids were separated by alumina column chromatography into three fractions by successive elution with hexane, hexane–Et_2O (9:1) and MeOH. The hexane eluate contained hydrocarbons; fractions 2 and 3 were re-separated by TLC on silica gel to give fractions containing steryl esters, methyl and ethyl esters of fatty acids, ketones, triacylglycerols, alcohols and fatty acids, respectively.

These components were analysed by gas chromatography using open-tubular Flexsil columns (25 m or 50 m × 0.3 mm) wall coated with an apolar liquid phase OV-1 or SE-30. Fatty acids were converted into methyl esters and alcohols were treated with BSTFA to produce the corresponding trimethylsilyl ethers prior

PHOTO 26/2-6

to GC analysis. Abundances of the individual components were determined by comparison of their GC peak areas with those of C_{18} and C_{28} *n*-alkane standards, or, in the case of alkanols and sterols, with *n*-hexadecanol and cholesterol (derivatized as trimethylsilylethers), respectively. Equal FID response factors were assumed for lipid components and the relevant standards. Constituents were identified by GC/MS analysis using a fused silica capillary column (14 m × 0.32 mm i.d.) coated with DB1 (0.1 μm), mounted in a Carlo Erba 5160 chromatograph fitted with an on-column injector and coupled to a Finnigan 4000 quadrupole filter mass spectrometer operating in the EI mode. The ion source was operated at 40 eV with an ionization current of 350 μA; mass spectral data were acquired and edited using an Incos 2300 data system.

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REFERENCES

1. Tappan, H. (1980) *The Paleobiology of Plant Protists*. Freeman, San Francisco.
2. Withers, N. (1983) *Mar. Nat. Products* **5**, 87.
3. Loeblich, A. R. and Loeblich, L. A. (1983) *Proc. Joint China–U.S. Phycol. Symp.* 39.
4. Jones, G. J., Nichols, P. D. and Johns, R. B. (1983) *J. Phycol.* **19**, 416.
5. Withers, N. W., Tuttle, R. C., Holz, G. G., Beach, D. H., Goad, L. J. and Goodwin, T. W. (1978) *Phytochemistry* **17**, 1987.
6. Djerassi, C., Theobald, N., Kokke, W. C. M. C., Pak, C. S. and Carlson, R. M. K. (1979) *Pure Appl. Chem.* **51**, 1815.
7. Djerassi, C. (1981) *Pure Appl. Chem.* **53**, 873.
8. Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1982) *Steroids* **40**, 307.
9. Shimizu, Y., Alam, M. and Kobayashi, A. K. (1976) *J. Am. Chem. Soc.* **98**, 1059.
10. Nichols, P. D., Jones, G. J., de Leeuw, J. W. and Johns, R. B. (1984) *Phytochemistry* **23**, 1043.
11. Boon, J. J., Rijpstra, W. I. C., de Lange, F., de Leeuw, J. W., Yoshioka, M. and Shimizu, Y. (1979) *Nature* **277**, 125.
12. Brassell, S. C. and Eglinton, G. (1983) in *Advances in Organic Geochemistry* 1981 (Bjorøy M. ed.) pp. 684–697. Wiley, Chichester.
13. de Leeuw, J. W., Rijpstra, W. I. C., Schenck, P. A. and Volkman, J. K. (1983) *Geochim. Cosmochim. Acta* **47**, 455.
14. Pollingher, U. and Berman, T. (1978) in *Lake Kinneret* (Serruya, C. ed.) pp. 247–253. Junk, The Hague.
15. Cranwell, P. A. (1976) *Freshwater Biol.* **6**, 41.
16. Robinson, N., Eglinton, G., Brassell, S. C. and Cranwell, P. A. (1984) *Nature* **308**, 439.
17. Mermoud, F., Gülacar, F. O., Siles, S., Chassaing, B. and Buchs, A. (1982) *Chemosphere* **11**, 557.
18. Mattern, G., Albrecht, P. and Ourisson, G. (1970) *J. Chem. Soc. Chem. Commun.* 1570.
19. Blumer, M., Guillard, R. R. L. and Chase, T. (1971) *Mar. Biol.* **8**, 183.
20. Patterson, G. W. (1967) *Plant Physiol.* **42**, 1457.
21. Patterson, G. W. (1974) *Comp. Biochem. Physiol.* **47B**, 453.
22. Orcutt, D. M. and Patterson, G. W. (1975) *Comp. Biochem. Physiol.* **50B**, 579.
23. Alam, M., Schram, K. H. and Ray, S. M. (1978) *Tetrahedron Letters* 3517.
24. Alam, M., Sanduja, R., Watson, D. A. and Loeblich, A. R. (1984) *J. Phycol.* **20**, 331.
25. Volkman, J. K., Gagosian, R. B. and Wakeham, S. G. (1984) *Lipids* **19**, 457.
26. Withers, N. W., Kokke, W. C. M. C., Rohmer, M., Fenical, W. H. and Djerassi, C. (1979) *Tetrahedron Letters* 3605.
27. Withers, N. W., Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1982) *Proc. Natn. Acad. Sci. U.S.A.* **79**, 3764.
28. Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1981) *Phytochemistry* **20**, 127.
29. Swenson, W., Tagle, B., Clardy, L., Withers, N. W., Kokke, W. C. M. C., Fenical, W. H. and Djerassi, C. (1980) *Tetrahedron Letters* 4663.
30. Smith, A. G. and Goad, L. J. (1975) *Biochem. J.* **146**, 35.
31. Alam, M., Martin, G. E. and Ray, S. M. (1979) *J. Org. Chem.* **44**, 4466.
32. Parke, M. and Dixon, P. S. (1976) *J. Mar. Biol. Ass. U.K.* **56**, 527.
33. Bourrelly, P. (1970) *Les Algues d'eau douce*, Tome III, 512 pp. Boubee, Paris.
34. Goad, L. J. and Withers, N. W. (1982) *Lipids* **17**, 853.
35. Mermoud, F., Wünsche, L., Clerc, O., Gülacar, F. O. and Buchs, A. (1984) *Org. Geochem.* **6**, 25.
36. Smith, A. G., Goodfellow, R. and Goad, L. J. (1972) *Biochem. J.* **128**, 1371.
37. Edmunds, K. L. H., Brassell, S. C. and Eglinton, G. (1980) in *Advances in Organic Geochemistry* 1979 (Douglas, A. G. and Maxwell, J. R. eds) pp. 427–434. Pergamon Press, Oxford.
38. Lusby, W. R., Thompson, M. J. and Kochansky, J. (1984) *Lipids* **19**, 888.
39. Nes, W. R. and McKean, M. L. (1977) *Biochemistry of Steroids and Other Isopentenoids*, 690 pp. University Park Press, Baltimore.
40. Christie, W. W. (1972) *Topics in Lipid Chem.* **3**, 171.
41. Matsuo, A., Nakayama, M., Hayashi, S. and Nagai, K. (1980) *Phytochemistry* **19**, 1848.
42. Laseter, J. L. and Weete, J. D. (1971) *Science* **172**, 864.
43. Marlowe, I. T., Brassell, S. C., Eglinton, G. and Green, J. C. (1984) in *Advances in Organic Geochemistry*, 1983 (Schenck, P. A., de Leeuw, J. W. and Lijmbach, G. W. M. eds) pp. 135–141. Pergamon Press, Oxford.
44. Habermehl, G. and Hundrieser, H. J. (1983) *Naturwissenschaften* **70**, 566.
45. Cranwell, P. A., Robinson, N. and Eglinton, G. (1985) *Lipids* **20**, 645.
46. Withers, N. W. and Nevenzel, J. C. (1977) *Lipids* **12**, 989.
47. Gellerman, J. L., Anderson, W. H. and Schlenk, H. (1975) *Lipids* **10**, 656.
48. Karunen, P., Mikola, H. and Ekman, R. (1980) *Physiol. Plant.* **48**, 554.
49. Csupor, L. (1970) *Planta Med.* **19**, 37.
50. Suga, T. and Aoki, T. (1974) *Phytochemistry* **13**, 1623.
51. Lauer, W. M. Aasen, A. J., Graff, G. and Holman, R. T. (1970) *Lipids* **5**, 861.
52. Joseph, J. D. (1975) *Lipids* **10**, 395.
53. Harrington, G. W., Beach, D. H., Dunham, J. E. and Holz, C. G. (1970) *J. Protozool.* **17**, 213.
54. Wakeham, S. G. and Frew, N. M. (1982) *Lipids* **17**, 831.
55. Gunstone, F. D. (1967) *An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides*. Chapman and Hall, London.

56. Opute, F. I. (1974) *J. Exp. Botany* **25**, 823.
57. Lee, R. F., Nevenzel, J. C. and Paffenhöfer, G. A. (1971) *Mar. Biol.* **9**, 99.
58. Mackenzie, A. S., Brassell, S. C., Eglinton, G. and Maxwell, J. R. (1982) *Science* **217**, 491.
59. Rubinstein, I. and Albrecht, P. (1975) *Chem. Commun.* 957.
60. Ensminger, A., Joly, G. and Albrecht, P. (1978) *Tetrahedron Letters* 1575.
61. Robinson, N., Cranwell, P. A., Eglinton, G., Brassell, S. C., Sharp, C. L., Gophen, M. and Pollingher, U. (in press).
62. Robinson, N. (1984) Ph.D Thesis, Univ. of Bristol.
63. Habermehl, G. and Hundrieser, H.-J. (1983) *Naturwissenschaften* **70**, 566.
64. Jaworski, G. H. M., Talling, J. F. and Heaney, S. I. (1981) *Br. Phycol. J.* **16**, 395.
65. Canter, H. M. and Heaney, S. I. (1984) *New Phytol.* **97**, 601.