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LIPIDS IN MARINE DIATOMS OF THE GENUS *THALASSIOSIRA*: PREDOMINANCE OF 24-METHYLENECHOLESTEROL

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Abstract—The major sterols in the marine diatoms *Thalassiosira pseudonana* (clones 3H and CS-20c) and *Thalassiosira eccentrica* (CS-17) have been identified as 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol), 24-ethylcholesta-5,24(28)*E*-dien-3β-ol (fucosterol) and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol (28-isofucosterol) There are no previous reports of fucosterol and isofucosterol occurring together in diatom species Small amounts of cholesterol, 24-methylcholesterol and 24-ethylcholesterol were also detected. The only abundant sterol in *Thalassiosira oceanica* (CS-67) was 24-methylenecholesterol; its predominance (>75%) appears to be characteristic of the sterol compositions of *Thalassiosira* The greater complexity of the sterol distribution in *T pseudonana* compared with *T oceanica* supports the recent reclassification of these two algae as separate species The fatty acids of *Thalassiosira* are typical of most diatoms, with a predominance of 16.0, 16:1ω7 and 20:5ω3. Small amounts of 18:4ω3 and 22.6ω3, not usually reported in diatoms, were also found.

INTRODUCTION

Diatoms are abundant components of the phytoplankton in coastal and upwelling marine environments and hence they are a major source of lipids in the diet of marine animals found there, and of the organic matter in many marine sediments [1–5] Species of the diatom genus *Thalassiosira* are common in coastal plankton blooms, at times forming dense gelatinous masses which can be harmful to filter-feeding shellfish [6] Some lipid constituents, particularly sterols and fatty acids, are useful markers for following the flow of diatom-derived lipids through marine food chains [1–5] As an aid to such studies, we analysed the sterols and fatty acids of three species of marine diatoms *Thalassiosira pseudonana* Hasle et Heimdal (clones 3H and CS-20c), *T. oceanica* Hasle (CS-67) and *T eccentrica* (Ehr) Cleve (CS-17) (= *Coscinodiscus eccentricus* Ehr.) to determine whether these diatoms might contain distinctive lipids that could be useful in chemotaxonomic investigations We also compared the sterols of *T pseudonana* (= *Cyclotella nana* Hustedt) with three previous analyses reported in the literature [7–9], each of which had presented surprisingly different results

Light microscopy is of limited value for identifying small, weakly silicified diatom cells. However, with the more widespread use of transmission and scanning electron microscopy, which have made it possible to define characteristic structures of the diatom valve [10, 11], many of the uncertainties about the taxonomic position of diatom species have now been clarified A re-examination of Hustedt's type material of *Cyclotella nana* by electron microscopy showed that it did not belong to the genus *Cyclotella* but to *Thalassiosira* [12] Also, various clones of *C nana* were recognized as different species and reclassified as *Thalassiosira pseudonana* Hasle et Heimdal (= *C. nana*, Guillard's clone 3H), *T. guillardii* Hasle (= *C nana*, clone 7–15) and *T. oceanica* Hasle (= *C nana*, clone 13–1), using slight differences in the morphology and location of strutted and labiate processes on the diatom valve as diagnostic features [10, 12]. *Cyclotella nana* has been cultured successfully in several laboratories and used extensively in physiological studies. Physiological differences between oceanic, coastal and estuarine isolates of *C. nana* have been reported [13], but it is probable that the isolates were in fact different species.

RESULTS AND DISCUSSION

The sterols of two clones of *Thalassiosira pseudonana* were examined in this study. To determine whether the sterol patterns found were typical of diatoms of the genus *Thalassiosira*, the sterols of *T eccentrica* and *T oceanica* were also determined (see Experimental for details of the culturing conditions). Compositional data were also obtained for lipid classes and fatty acids in *T. pseudonana* and *T. oceanica*

Total lipids

Compositional data for major lipid classes in *T. pseudonana* (Clone CS-20c) and *T. oceanica* are shown in Table 1. Polar lipids predominated in both species. Sterols represented 7.6 and 6.8% of the total lipids, respectively. Hydrocarbons were minor constituents in both algae; they consisted mainly of *n*-heneicosahexaene (*n*-$C_{21\ 6}$) with small amounts of squalene. *Thalassiosira pseudonana* (clone CS-20c) contained large amounts of triacylglycerols and minor amounts of free fatty acids and diacylglycerols. In contrast, *T oceanica* contained mainly free fatty acids, which are rarely abundant in

actively growing cells, with small amounts of triacylglycerols and diacylglycerols (Table 1) These data are consistent with the observation that the *T pseudonana* culture was healthy when extracted, whereas cells in the *T. oceanica* culture had clumped to the bottom of the culture flask, indicating that the cells had become senescent. Both algae were cultured under similar conditions so this difference in growth behaviour may reflect physiological differences between them

Sterols

Over 95% of the total sterols in each of the four cultures occurred as non-esterified (free) sterols. Saponification of the total extract yielded only a slight increase in the amount of sterols. Of the eight sterols detected in the three species, the structures of six were identified Compositional data are given in Table 2 together with data from other analyses of *Thalassiosira* reported in the literature In each species the major sterol was 24-methylenecholesterol, which represented over 75% of the total sterols in the two clones of *T pseudonana* and in *T. eccentrica* It was the only major sterol in *T. oceanica* This sterol is found in a few diatoms [14], but rarely as the major component. Exceptions include *Nitzschia alba* (100%) [15], *Chaetoceros simplex* (43%) [16], and *Skeletonema costatum* (40%) [17] It is also the major sterol (58%, Table 2) in *Thalassiosira weissflogii* (Grunow) Fryxell et Hasle (= *T fluviatilis* Hustedt) [18] It appears from these data that high concentrations of this sterol are characteristic of species of *Thalassiosira* Further, the simplicity of the sterol distribution of *T oceanica* compared with *T pseudonana* supports the recent reclassification of these algae as separate species [10]

The sterol compositions of the two clones of *T pseudonana* are very similar, even though one isolate was from North America and the other from Australia. Each isolate contains the same sterols, although clone 3H contains slightly more C_{29}-sterols and relatively less 24-methylenecholesterol Such small variations can occur when phytoplankton are grown under slightly different culture conditions or when held for different periods in batch culture [17] The distribution of sterols in *T eccentrica* is very similar to that of *T pseudonana* (Table 2)

Minor sterols found in all three species included cholesterol and 24-methylcholesterol Cholesterol is common in diatoms and is the major constituent in some species [14] 24-Methylcholesterol is also common, but rarely represents more than 30% of the total sterols [14] It is thought to be formed by reduction of the $\Delta^{24(28)}$-double bond in 24-methylenecholesterol [20] The absence of 24-methylcholesta-5,22*E*-dien-3β-ol in *Thalassiosira* contrasts with its occurrence in most other diatoms, in some species this sterol represents over 90% by weight of the total sterols [14]

Two C_{29}-sterols in *T. pseudonana* were identified by coinjection with standards and from their mass spectra (as both acetate and TMSi-ether derivatives) as 24-ethylcholesta-5,24(28)*E*-dien-3β-ol (fucosterol) and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol (28-isofucosterol) In both clones, fucosterol was more abundant than 28-isofucosterol Although these isomers are readily separated on capillary columns, GC-MS data are essential for quantification due to coelution of 24-ethylcholesterol with fucosterol on non-polar columns It is unusual for the two isomers to occur together in the same species, the present work is the first such report for a diatom There are only a few reports of C_{29}-sterols with $\Delta^{5,24(28)}$-unsaturation in diatoms 28-Isofucosterol was reported as a trace constituent in *Thalassionema nitzschioides* [17], but has not been reported in other diatoms Fucosterol was reported to be the principal sterol in *Nitzschia closterium* [21], but this was later shown to be a misidentification. Of the 11 diatom species studied by Orcutt and Patterson [8], five were reported to contain fucosterol but no supporting data were presented

Fucosterol and isofucosterol are formed from 24-methylenecholesterol by transmethylation from *S*-adenosylmethionine, which isomer is produced depends on the mechanism by which hydrogen is rearranged to quench the enzyme bound intermediate carbonium ion [20]. Higher plants and green algae produce the 24(28) *Z*-isomer 28-isofucosterol, whereas brown algae produce the 24(28) *E*-isomer fucosterol [20] The presence of both isomers in *T pseudonana* is unusual and may indicate that there is little stereospecific control over the hydrogen rearrangement reaction

24-Ethylcholesterol was a minor constituent in both clones of *T pseudonana* and few algae contain large quantities It predominates in the diatom *Asterionella glacialis* [14], the Prymnesiophyte *Pavlova lutheri* [18], and we have recently shown it to be the major sterol in two Raphidophycean flagellates, *Heterosigma akashiwo* and *Chattonella antiqua* [22] 24-Ethylcholesterol is probably formed by reduction of the side chain double bond in one or both of the $\Delta^{5,24(28)}$-unsaturated sterols

Species of *Thalassiosira*, including *T eccentrica*, are common in Peruvian coastal waters [19, 23, 24], and thus they are a likely source of high concentrations of 24-methylenecholesterol found in Peruvian coastal sediments [24] Gagosian *et al* [24] found that 24-methylenecholesterol was a major sterol in anchoveta faeces obtained from sediment traps deployed off Peru. Since these samples also contained many frustules of *T eccentrica* [23] this indicates that some of the dietary sterol is excreted by the anchoveta

Table 1 Relative concentrations of lipid classes in *T pseudonana* (clone CS-20c) and *T oceanica* (clone CS-67)*

	Composition (%)	
	T pseudonana	*T oceanica*
Hydrocarbons	1 3	4 0
Triacylglycerols	32 1	3 8
Free fatty acids	0 4	22 6
Sterols	7 6	6 8
Diacylglycerols	0 8	6 8
Polar lipids	57 8	56.0

*Determined by thin layer chromatography-flame ionisation detection

Comparison of sterol data for T pseudonana *with previous literature reports*

Different results have been reported in previous analyses of the sterols in species referred to as *T pseudonana* or *Cyclotella nana* (Table 2) Berenberg and Patterson [7]

Table 2 Percentage composition of sterols in diatoms of the genus *Thalassiosira*

		T pseudonana					Other species		
		Clone 3H	Clone CS-20C				*T. oceanica*	*T. eccentrica*	*T. weissflogii*‖
*RR*f*	Sterol	This work		Ref. [7]	Ref. [8]	Ref [9]	This work		Ref. [19]
1.0	Cholesterol	1 0	0 2	0 5	1.7	—	1.0	2.1	25
1 12	24-Methylcholesta-5,22 E-dien-3β-ol	ND	ND	—	—	100	ND	ND	—
1.31	24-Methylcholesta-5,24 (28)-dien-3β-ol	75.1	86.1	83.0	—	—	99.0	76.4	58
1.34	24-Methylcholesterol	3.6	3 5	9.0	36.0	—	Tr	7 6	6
1.63	24-Ethylcholesterol	1 0	0 5	4 0	—	—	—	1.0	11
1 63	24-Ethylcholesta-5,24(28)E-dien-3β-ol	10.5	5.9	Tr	5.7	—	Tr	8.3	—
1 67	24-Ethylcholesta-5,24(28)Z-dien-3β-ol	6.3	2.8	ND	—	—	—	2 6	—
	Others	2.5†	1 0	3 5‡	56.6§	—	Tr	2.0	—

*Retention time relative to cholesteryl acetate = 1 0

†Unknowns *RR*f 1 54 (1 0%), *RR*f 1.80 (1 5%). See Experimental.

‡24-Methylcholesta-5,24 (25)-dien-3β-ol (misprinted as 24-methyl-5,22 (25)-cholestadienol in Table 2 of Ref [7]

§Includes several sterols reported to have Δ^7-unsaturation See text

‖Analysed as *Thalassiosira fluviatilis*

ND· Not detected, —. Not reported, Tr Trace <0 5%.

analysed *T. pseudonana* supplied by Mr Dean Day from the University of Delaware. Their data are very similar to ours. They also identified the major sterol as 24-methylenecholesterol which we have suggested is characteristic of the sterol distribution in this alga when cultured under optimal conditions. Berenberg and Patterson [7] found slightly more 24-methylcholesterol and smaller amounts of C_{29}-sterols (Table 2) in their sample than we did in the two clones we analysed Fucosterol was only a trace constituent and 28-isofucosterol was not detected, but the proportion of 24-ethylcholesterol was higher. These small differences suggest that there was slightly more reduction of sterols with $\Delta^{5,24(28)}$-double bonds to the corresponding Δ^5-unsaturated sterol in their culture, and overall a smaller amount of C_{28}-sterols undergoing further alkylation at C-24.

Changes in culture conditions can produce major variations in the fatty acid compositions of unicellular algae, but generally the compositions of sterols are not markedly changed [17] As sterols are major structural constituents of membranes, there would have to be significant changes in physiological condition before the membrane composition would be altered markedly. On the other hand, fatty acids occur in many different structural and storage lipids, so their abundance would be dependent on the condition of the alga, as shown in the lipid data for *T pseudonana* and *T. oceanica* (Table 1)

Orcutt and Patterson [8] reported that *T. pseudonana* contained 24-methyl-5α-cholesta-7,22-dien-3β-ol (39.4%), 24-methylcholesterol (36%) and 24-methyl-5α-cholesta-7,24(28)-dien-3β-ol (14.6%). These Δ^7-unsaturated sterols were not found in our study or in that of Berenberg and Patterson [7]. The apparent absence of any 24-methylenecholesterol is particularly striking. Orcutt and Patterson analysed the same clone 3H as we did (Guillard, R. R. L., personal communication), so the two sets of data should have been similar. It seems unlikely that such very different results could be due to differences in culturing conditions, unless some feature of their culture led to a blockage in the conversion of Δ^7-unsaturated sterols to Δ^5-unsaturated sterols. Their cultures were bubbled with carbon dioxide, but it is not known whether this could have affected the sterol distributions. Orcutt and Patterson [8] also reported high concentrations of Δ^7-unsaturated sterols in four other diatoms, including *Phaeodactylum tricornutum*, which has been the subject of two studies [17, 25], neither of which reported Δ^7-unsaturated sterols. High concentrations of Δ^7-unsaturated sterols have not been reported in other diatoms [14], nor are they present in significant amounts in diatomaceous sediments [e.g 3, 5, 24], which suggests that they are not common in diatoms under favourable growth conditions. It is possible that 24-methylenecholesterol was misidentified as 24-methyl-5α-cholesta-7,22-dien-3β-ol, which has a similar retention time on both polar and non-polar columns [26] However, the two sterols are readily distinguishable from their mass spectra and it was stated by Orcutt and Patterson [7] that the sterols from *T. pseudonana* were analysed by GC-MS.

Kanazawa *et al.* [9] reported that the only sterol in *C. nana* was 24-methylcholesta-5,22*E*-dien-3β-ol. The culture used in that study was supplied by Dr H. Takano from material isolated from Tokyo Bay From electron microscope studies [Sheet 5, Ref. 6], it appears that the species analysed was also *T. pseudonana* (Takano, H., personal communication). 24-Methylenecholesterol is an intermediate in the formation of 24-methylcholesta-5,22*E*-dien-3β-ol, but it is difficult to explain why the latter should be the only sterol reported in that study or why this should be the only report of 24-methylcholesta-5,22-dien-3β-ol in any species of *Thalassiosira* (Table 2).

Other lipids

The fatty acid compositions of free fatty acids and triacylglycerols in *T. pseudonana* and *T oceanica* are

presented in Table 3 Major constitutents are 16.0, 16 1ω7 and 20.5ω3 fatty acids which is typical of most diatoms [8, 27], as is the low proportion of C_{18}-fatty acids One interesting feature is the presence of high concentrations of 18·4ω3 and the presence of 22:6ω3, neither of which are commonly reported in diatoms; they may have been missed in earlier studies because of the low resolution gas chromatography techniques in use then.

These data compare well with total fatty acid data reported by Fisher and Schwarzenbach [27] for *T. pseudonana* (clone 3H) and *T oceanica* (clone 13-1) cultured in f_2 medium As these authors used chemical ionisation mass spectrometry to identify the fatty acids, double bond positions were not identified and individual lipid classes were not examined Our data confirm the presence of 18 4, 16 4 and 22.6 fatty acids noted by these authors Fisher and Schwarzenbach [27] found that 14 0 was a major component in both species of diatom at four different growth stages, but in our analysis of *T pseudonana* (Clone CS-20c) this fatty acid is much less abundant The concentration of 14 0 fatty acid varies considerably among different lipid classes (Table 3), and it seems that its abundance is sensitive to changes in culturing conditions

Each of the cultures was examined for unusual lipids that have been found in many marine sediments, including diatom-rich sediments off Peru where *Thalassiosira* is abundant These included very long chain ketones that have only been found in some Prymnesiophytes [28], C_{30} and C_{32}-diols and keto-ols [29], and unusual C_{25} highly branched isoprenoid alkenes [4] None of these lipids was detected One unusual feature was the presence of minor amounts (< 1% of total lipids) of alkyl (wax) esters in *T eccentrica*, but a detailed study was not carried out.

CONCLUSIONS

The widely divergent sterol data reported in earlier studies of the diatom *Thalassiosira pseudonana* highlight the need for accurate taxonomic identification of the species under study, including clonal designation, careful attention to the culturing conditions and rigorous identification of the lipid compounds isolated Whatever the reasons for differences reported in earlier work, our study clearly establishes that a high concentration of 24-methylenecholesterol is characteristic of at least three diatoms of the genus *Thalassiosira*, and that clones of the same species isolated from different parts of the world can have

Table 3 Relative abundances of fatty acids in *T pseudonana* (clone CS-20c) and *T oceanica* (clone CS-67)

	Composition (%)			
	T. pseudonana		*T oceanica*	
Fatty acid*	Free fatty acids	Triacyl-glycerols	Free fatty acids	Triacyl-glycerols
14 0	3 5	6 1	18 4	10 0
15 0	0 6	1 0	1 6	1 1
16 0	18 4	26 9	23 0	23 7
18 0	1 6	1 4	0 3	1 0
20 0	1 1	0 3	ND	ND
16 1ω7	21 0	32.0	30 0	25 8
16 1ω5	1 6	Tr†	Tr	0 2
16 1ω13*t*	Tr	ND‡	0 5	ND
18 1ω9	2 0	1 5	0 5	5 0
18 1ω7	0 1	0 2	4 0	Tr
16 2ω7	1 1	1 8	0 7	NAD§
16 2ω4	0 7	1 4	1 4	NAD§
16 3ω4	7 1	4 8	3 5	6 4
16 4ω1	2 3	0 8	0 8	3 9
18 2ω6	1 8	1 3	1 0	1 1
18 3ω3	0.8	0 7	1 1	0 9
18 4ω3	5 8	4 0	1 8	5 2
20 4ω6	1 1	0 9	Tr	Tr
20 5ω3	23 9	12 1	11 0	13 4
22 6ω3	3 5	1 5	1 4	0 8
Others	2 0	1 3	0 4	1 5

*Fatty acids are designated number of carbon atoms number of double bonds The position (*n*) of the ultimate double bond from the methyl group is given by ωn All double bonds in polyunsaturated fatty acids are methylene interrupted

†Tr = Trace < 0 5%

‡ND Not detected

§NAD Not accurately determined due to low abundance and overlap with other components

similar lipid compositions when cultured under similar conditions.

EXPERIMENTAL

Cultures *T eccentrica* (clone CS-17 isolated by B Grant from Port Phillip Bay, Victoria, Australia) was obtained from the CSIRO Algal Culture Collection. It was grown in a 2 l flask at 17 5° in aerated f_2 medium [13] under a 12 hr light:12 hr dark cycle at a light intensity of 50 $\mu E/m^2$ sec (Philips De Luxe, TL 20W/47) The cells were harvested after 21 days at a cell density of $8\,4 \times 10^3$ cells/ml

T pseudonana (Clone 3H isolated from Long Island Sound, New York, U S A) was obtained from the culture collection of Dr R R. L Guillard and cultured under white light using f_2 culture medium bubbled with air at $18 \pm 1°$ The cells were harvested during exponential growth *T pseudonana* (Clone CS-20c isolated from the Swan River, Western Australia by R E. Davis and D J Nicol) was obtained from the CSIRO Algal Culture Collection. It was cultured in the same manner as *T eccentrica* in 2 l flasks containing f_2 medium and harvested after 35 days *T oceanica* (Clone CS-67 isolated from the Port Hacking River, New South Wales, Australia by J C. Eyles) was also obtained from the CSIRO Algal Culture Collection and cultured in fE_2 medium (f_2 modified by the addition of 15 mg/l of Na EDTA) [30] under the same conditions as *T. pseudonana* (Clone CS-20c) EDTA was added since oceanic diatoms appear to be more adversely affected by high concentrations of trace metals than species from coastal areas

The taxonomy of all cultures was confirmed by a combination of light and electron microscopy [11].

Lipids As the lipids of the different species were analysed in different laboratories, the procedures differed in minor respects. *T pseudonana* (Clone 3H) was harvested by filtration on to glass fibre pads, washed briefly with distilled H_2O and extracted with CH_2Cl_2 using sonication. Free sterols were isolated by CC on silica gel and converted to acetate derivatives for GLC and GC-MS analysis as described previously [24] The other species were also harvested by filtration onto glass fibre pads, washed with saline and extracted with *iso*-PrOH (10 ml) and CH_3Cl–MeOH (2 1, 4 × 10 ml) with sonication The extract was washed with milli-Q H_2O and reduced to dryness.

Concentration data for major lipid classes in the two species were obtained from TLC- flame ionisation detection using an Iatroscan TH-10 Mk III analyser [31]. Sterols were isolated by CC on silica gel and converted to trimethylsilyl ethers (TMSi-ether) derivatives for capillary GLC and GC-MS analysis, using methods described previously [22]. Identifications based on retention index measurements [26] were confirmed by coinjection with standards and from characteristic mass spectra. Cholesteryl acetate MS *m/z* (rel int): 368 [M − HOAc] (42), 353 (4), 260 (1), 255 (1), 247 (4), 213 (3), 147 (89), 81 (100), 24-methylcholesta-5,24(28)-dien-3β-yl acetate. 380 [M − HOAc] (56), 365 (10), 338 (1), 296 (25), 281 (11), 253 (15), 228 (7), 213 (24), 145 (100), 81 (100), 24-ethylcholesta-5,24(28)*E*-dien-3β-yl acetate 394 [M − HOAc] (34), 379 (4), 296 (86), 281 (21), 273 (2), 253 (12), 228 (10), 213 (28), 147 (81), 145 (86), 81 (100), 24-ethylcholesta-5,24(28)*Z*-dien-3β-yl acetate 394 [M − HOAc] (6), 379 (1), 296 (100), 281 (21), 253 (8), 228 (6), 213 (16), 211 (10), 147 (31), 145 (44), 81 (58) Note the higher abundance of *m/z* 296 in 28-isofucosterol, which is characteristic of the 24(28)*Z*-isomer [32] Mass spectra of the TMSi-ether derivatives also agreed well with standards [3]

Two minor sterols in *T pseudonana* were not identified One eluted at RR_t 1 54 (as the acetate) but its mass spectrum resembled a diunsaturated C_{27}-sterol MS *m/z* (rel. int): 366 [M − HOAc?] (34), 296 (2), 281 (4), 253 (32), 227 (2), 213 (11), 159 (37), 147 (94), 145 (92), 81 (100) The second eluted at RR_t 1 80 and appears to be a diunsaturated C_{28}-sterol MS *m/z* (rel int) 380 [M − HOAc?] (2), 312 (5), 281 (7), 243 (3), 229 (7), 95 (100) Both eluted much later than other C_{27} and C_{28}-sterols reported in the literature [26]. Sterols with Δ^7-unsaturation and 5α-stanols were below the level of detection (i e $< 0\,5\%$ of sterols) in each of the algae

Fatty acids were converted to methyl esters with BF_3–MeOH, and analysed on a 25 m × 0 25 mm I.D methyl silicone fused silica capillary column (BP1, SGE Australia), and on a 25 m × 0.25 mm I D. carbowax fused silica capillary column (BP20, SGE, Australia), using an HP 5890 gas chromatograph Fatty acids are designated as total number of carbon atoms number of double bonds followed by the position of the ultimate double bond from the aliphatic (omega) end of the molecule The suffixes *c* and *t* denote *cis* and *trans* geometry Double bonds are methylene interrupted.

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