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REASSESSMENT OF LIPID COMPOSITION OF THE DIATOM, *SKELETONEMA COSTATUM*

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Abstract—In the present work, we questioned the natural or artifactual origin of the large amounts of free fatty acids recovered in lipids from the diatom *Skeletonema costatum*. Using rapid cell harvest and a very drastic method to extract the lipids, we demonstrated that these lipid patterns rich in free fatty acids were relevant to the degradation of complex lipids. A revised lipid composition of *S. costatum* is given which differs from those previously described by the absence of free fatty acids, while the proportion of polar lipids is substantially increased. Membrane lipids are mostly represented and storage lipids are less abundant, since we analysed the cells during their exponential growth phase. However, the fatty acid composition is in agreement with previous data. Polyunsaturated fatty acids (PUFA) consist essentially of 20:5 ω3, 16:3 ω4 and 16:4 ω1, while 16:1 ω7 is the main monounsaturated FA. This confirms that, with regard to its lipid composition, the diatom, *S. costatum*, provides a good diet for molluscs, particularly through its high proportion of ω3 PUFA. Using this extraction procedure, sterols were found to be identical to previous results but a new compound, a 18:1 fatty alcohol, was detected. The consequences of this reassessement on the lipid compositions of diatoms so far published and their application to the lipid diet of mollusc larvae in aquaculture are discussed.

INTRODUCTION

Lipids of diatoms have been extensively studied in the past, therefore much data are available on their fatty acid and lipid composition [1, 2]. As is usual in photosynthetic cells, diatoms contain polar lipids. Despite the absence of subcellular fraction studies in diatoms, these lipids are described as membrane-bound and characteristic of the cell compartments by comparison with higher plants and algae [3–5]. Therefore, glycolipids (monogalactosyl-, digalactosyl- and sulfphoquinovosyldiacylglycerol; MGDG, DGDG and SQDG, respectively) and phosphatidylglycerol (PG) are attributed to chloroplast membranes while the phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are considered more characteristic of extrachloroplastic membranes. Diatoms also contain neutral lipids. In plants and algae, this lipid fraction is very diverse. Compounds as different as sterols, fatty alcohols, waxes and acyl lipids (mono-, di- and triacylglycerols; MAG, DAG and TAG, respectively) can be found and, up to now, these lipid classes have also been characterized in diatoms, with the exception of fatty alcohols.

Among neutral lipids, very abundant free fatty acids (FFA) have been consistently reported in diatoms [6–10]. The amount of FFA observed are quite variable according to the strain studied (from 8 to 30% of the total lipids). Such an occurrence of large amounts of FFA seems to us, questionable. Usually, in most living organisms, free fatty acids are only detected in trace amounts. They are intermediary compounds in lipid metabolism, detected in some cases by labelling with ^{14}C and rapidly incorporated in to glycerolipids, such as DAG, leading, for example, to TAG accumulation in oil droplets of lipid-storage cells. In most cases, abundance of FFA is only detected in materials having undergone lipolytic degradation. Apart from natural processes occurring during oil seed germination, the release of such large amounts of FFA are often considered as artifacts, indicating unsuitable conditions for lipid extraction, allowing lipase activity development. Furthermore, it appears that FFA cannot be abundant in the photosynthetic cells because they inhibit electron transfer and are very sensitive to oxidation processes, resulting in degradation products toxic to cells in most cases.

According to the large amount of literature already on diatom lipids [6–8, 11], additional surveys are not warranted. In the present work, we try to verify previous studies in which large amounts of FFA in diatom lipids

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Table 1. Compositon of lipid classes (% total lipid) from *Skeletonema costatum*

Neutral lipids			Glycolipids				Phospholipids		
TAG	Sterols	Alcohol	FFA	MGDG	DGDG	SQDG	PC	PG	PE
(a) 5.4	1.1	3.1	0.0	47.4	9.5	10.8	10.5	7.6	4.6
(b) 5.6	1.2	3.2	14.8	43.0	6.8	7.9	8.9	5.1	3.5

(a) Boiling water directly on cell pellet.
(b) Room temperature.

were reported. Consequently, in order to assess the natural or artifactual presence of FFA in diatom lipids, we reexamined the lipid composition of the diatom, *Skeletonema costatum*. This species is well documented and used extensively in aquaculture to feed larvae of molluscs and crustacea [12–16]. As in other diatoms it also contains large amounts of FFA. Its acyl lipids are characterized by high contains of long-chain, polyunsaturated fatty acids (PUFA), such as eicosapentaenoic (20:5 ω3) and docosahexaenoic acid (22:6 ω3). These fatty acids are very important in the diet of marine organisms because they appear to control the growth of molluscs and crustacea during their larval stages [12–18]. Using careful centrifugation to harvest the cells and immediate fixation by boiling water before lipid extraction, the present work demonstrates that FFA are absent from lipids of *S. costatum* and that the previously published data probably result from artifacts. A revised lipid composition is given and discussed with regard to diatom lipids and the use of this algal mat in aquaculture diets.

RESULTS AND DISCUSSION

Using classical techniques of high-speed centrifugation followed by extraction of lipids at room temperature according to Bligh and Dyer [19], we were surprised to recover substantial amounts of FFA. Because this might be due to lipase activities, we performed several assays. The best results were obtained after centrifugation of cultures at low speed (1000 *g*) then rapid addition of boiling distilled water onto the cell pellet, to cause lipase inactivation (see Experimental). Using these conditions, no FFA were detected in lipid extracts (Table 1). In the neutral lipid fraction, TAG were the only source of fatty acids; neither DAG nor MAG were detected. The amount of neutral lipids was very low among total lipids from the cultures during their exponential-growth phase (*ca* 10%), while polar lipids (glyco- and phospholipids) were predominant (67 and 23% of total lipids, respectively) (Table 1). Glycolipids were represented mainly by MGDG, which accounted for 70% of the glycolipids while DGDG and SQDG represented only 14 and 16%, respectively. For phospholipids, the proportions of PC, PG and PE were 46, 34 and 20%, respectively. In addition to TAG, which represented *ca* 56% of the total weight of the neutral lipids compounds (Table 1), sterols (1.1% of total lipids) and a long-chain alcohol (3.1% of total lipids) were also found.

During the exponential growth phase the dominant fatty acids in *S. costatum* were PUFA (77%), while saturated and monounsaturated acids represented only 10 and 13%, respectively (Table 2). PUFA were mainly 20:5 ω3, 16:3 ω4 and 16:4 ω1, which accounted for *ca* 61% of the total fatty acids. 16:1 ω7 was the main monounsaturated fatty acid, while the saturated acids were 14:0 and 16:0. PUFA were identified by comparison of the retention times with those of authentic standards and natural extracts of known composition. Glycolipids and phospholipids were very rich in PUFA (89 and 70%, respectively). TAG contained 41% monounsaturated and 38% PUFA (Table 3) and the highest proportion of saturated fatty acids (21%).

Fatty acid families were not equally distributed in all lipid classes (Table 3). Besides saturated fatty acids (11%), phospholipids contained ω3 (61%) and ω7 (14%) PUFA. In glycolipids, ω3 acids were abundant (41%),

Table 2. Relative proportions (%) of fatty acid classes in total lipids from *Skeletonema costatum*

Class	Fatty acid	Percentage	Total
Saturated	14:0	5.4	
	16:0	4.2	
	18:0	0.3	9.9
Monounsaturated	16:1ω7	9.6	
	16:1ω5	1.6	
	18:1ω9	1.1	
	18:1ω7	0.2	
	20:1ω9	0.2	
	22:1ω9	0.3	13.0
Polyunsaturated	16:2ω4	4.5	
	16:3ω4	13.1	
	16:4ω4	0.1	
	16:4ω1	12.5	
	18:2ω6	1.0	
	18:2ω3	0.5	
	18:4ω3	4.4	
	20:3ω6	0.1	
	20:5ω3	35.3	
	22:6ω3	5.6	77.1

Table 3. Relative proportions (%) of fatty acid in lipids classes from *Skeletonema costatum*

	Phospholipids			Glycolipids			Neutral lipid
Fatty acid	PC	PG	PE	MGDG	DGDG	SQDG	TAG
14:0	3.5	5.1	2.1	1.0	1.7	30.8	9.2
16:0	2.6	13.8	8.9	0.5	1.7	1.7	9.7
16:1ω7	15.6	15.4	6.8	4.0	3.5	2.2	26.8
16:1ω5	—	9.5	6.3	—	—	—	—
16:2ω4	5.0	2.4	1.3	4.3	18.9	0.6	1.6
16:3ω4	3.8	1.6	—	29.1	7.8	1.2	5.8
16:4ω4	—	—	—	—	—	1.1	—
16:4ω1	2.5	1.6	1.8	27.4	7.8	—	8.2
18:0	—	—	—	0.1	0.9	0.2	1.7
18:1ω9	—	0.8	1.5	0.3	0.9	0.2	7.1
18:1ω7	—	—	—	—	—	—	2.3
18:2ω6	2.9	—	—	—	—	—	5.1
18:3ω3	1.7	—	—	0.5	—	—	—
18:4ω3	6.0	0.7	1.5	2.5	—	23.5	2.3
20:1ω9	—	—	—	—	—	—	1.6
20:3ω6	—	—	—	—	—	—	1.2
20:5ω3	49.8	35.4	35.1	30.2	56.8	38.1	11.8
22:1ω9	—	—	—	—	—	—	3.3
22:6ω3	6.6	13.7	34.7	0.1	—	0.4	2.3

while ω4 and ω1 PUFA represented 28 and 20%, respectively. The fatty acid composition of TAG was more heterogeneous; there was some predominance of ω7 acids (30%) but absence of ω5 acids. ω5 fatty acids existed only in phospholipids, while the ω9 family was detected mainly in TAG. Some fatty acids, which were detected in low quantities, appeared to be specific of a class of lipid (Table 3). This was the case for 20:1 ω9, 20:3 ω6 and 22:1 ω9 (0.84% of total fatty acids) which were present only in TAG. SQDG was the only class containing 16:4 ω4 (0.1% of total fatty acids) 16:1 ω5 and 22:6 ω3 appeared to be specific for phospholipids.

Apart from glycerolipids, the neutral lipid fraction also contained sterols and a fatty alcohol. Analysis of the sterol fraction revealed the presence of four compounds, together with some minor ones which were unidentified (Table 4). Cholesterol (41.6%) and 24-methylenecholesterol (32.5%) were the most important, while the fucosterol content was very low (2.8%). The fatty alcohol fraction obtained after TLC contained a single long-chain fatty alcohol, *cis*-vaccenyl alcohol, (18:1 ω7).

Table 4. Sterol composition of *Skeletonema costatum* (% total sterols)

Sterols	%
Cholesterol	41.6
22-(*E*)-Dehydrocholesterol	18.5
24-Methylenecholesterol	32.5
Fucosterol	2.8
Unidentified	4.6

The major finding of our study is the absence of any free fatty acids in the lipids from *S. costatum*. Our method of lipid extraction shows a discrepancy (Table 5) between those previously described for *S. costatum* [6, 7] and,

Table 5. Published compositions of neutral lipid classes from *Skeletonema costatum*

Lipid class (% total lipids)					
Neutral lipids	Diacylglycerols + sterols	Free fatty acids	Triacylglycerols	Hydrocarbons	Reference
36.4	0.7	17.4	4.5	0.3	[8]
59.3	13.0	28.9	11.6	5.8	[11]
12.7	1.7	8.5	1.7	0.8	[7]
57.0	15.0	12.0	14.0	16.0	[6]
9.6	1.1	0.0	5.4	nd	Present work*

nd: Not detected.
* See also Table 1.

more generally, on diatom lipids [20]. The use of boiling water prior to lipid extraction inhibits lipase activities which would induce hydrolysis of 'complex lipids' to FFA. In plants, FFA are transitory molecules resulting from the turnover of lipids and are present in very low amounts in intact living cells. However, various stresses, such as drought or protoplast preparation, can induce lipase activities or make cells more fragile and accessible to endogenous lipases [21]. Under such stress conditions, when extraction is either performed without lipase inhibition or is prolonged, it always results in high levels of FFA. Furthermore. the presence of an external silica skeleton on diatoms with apertures around cells can lead to intracellular components being completely destroyed if excess centrifugation is applied to the culture. This would allow intracellular lipases to become activated. We observed that any delay between cell harvesting and the addition of boiling water, even during the short time needed to resuspend the pellet, resulted in some FFA in lipids. This confirms the presence of high levels of lipases, at least in *S. costatum*, but probably also in most diatoms having a FFA-rich lipid pattern.

Inhibition of the artifactual occurrence of free fatty acids in diatoms modifies the lipid pattern with the predominance of polar lipids (glyco- and phospholipids) as opposed to neutral lipids. This is in agreement with the results of Volkman [7] but contrasts with those of Lee *et al.* [6]. Some differences between the proportions of the lipid classes can also be explained by culture conditions [22], because in algal cultures the accumulation of neutral lipids is linked with the stationary growth-phase [9, 23].

As far as total fatty acids are concerned, the ratios between PUFA, monounsaturated and saturated fatty acids (77:13:10) in *S. costatum* were close to those obtained by Chuecas and Riley [24] but different from those of others [7, 23, 25, 26]. The different ratios previously published might originate from the strain used or the status of the culture, or even from the procedure used to store the lipid extracts before analysis. The importance of PUFA is further confirmed by analysis of each lipid class. Although the major fatty acids of classes are quite different from those published by Brockerhoff *et al.* [27], they corroborate the hypothesis that membrane lipids are generally polyunsaturated. However, the 22:6 $\omega 3$ distribution confirms that it is an important constituant of phospholipid membranes [28], while 20:5 $\omega 3$ is largely represented in most classes and seems to be ubiquitous.

In addition to TAG, the neutral lipids of *S. costatum* comprise a fatty alcohol and sterols. The presence of a fatty alcohol has not been reported before; this might be explained by the use of boiling water which may help its extraction. The biological significance of *cis*-vaccenyl alcohol is unknown. The four sterols identified are the same as those identified by Titsa-Tzardis *et al.* [29] and Ballantine *et al.* [30] but their proportions are quite different. A high amount of 24-methylenecholesterol was observed instead of cholesterol.

Most analyses of lipids from diatoms now have to be considered with care. Diatoms which are predominant in the diet for larvae of molluscs and crustacea [12] provide growing organisms with PUFA amongst which, the $\omega 3$ family appears to be essential for the survival and growth of shrimps and bivalves [12–14, 17, 18]. Thus, it is essential to have accurate data on their lipid composition for better control of hatcheries. Furthermore, it would be of interest to know whether FFA or complex lipids favour the nutritional value of the algal mat, in order to promote a better use of cultures with or without lipase-activation by ageing.

EXPERIMENTAL

Culture. Cultured cells originated from a natural population of *S. costatum* extensively grown for mollusc nutrition at the marine station of Bouin (Vendée-France). They were collected then grown in natural seawater enriched with Provasoli medium [31] and sodium silicate (300 μM) in plastic flasks. Light intensity (62 μE m^{-2} sec^{-1}) was provided with a 12 hr/12 hr photoperiod, at 16°. After 3 months of growth adaptation, lipid analysis was carried out. Cells were gently harvested by low-speed centrifugation (1000 *g* for 20 min) during the exponential growth-phase.

Lipid extraction and fractionation. Boiling H_2O was added immediately to the pellet of cells in order to inactivate lipases. Lipids were further extracted by the method of ref. [19]. Lipid extracts were then stored in C_6H_6–EtOH (1:1) at − 18° under N_2. Before TLC analysis, fractionation of lipids was carried out by adsorption chromatography on silica gel (silica gel 60, 70–230 mesh, Merck) using a modification of the method described in ref. [32]. Silica gel (300 mg) in $CHCl_3$ was used for 10 mg of lipid extract. Neutral lipids were eluted by 10 ml of $CHCl_3$. Elution of glycolipids followed with 15 ml of Me_2CO–MeOH (9:1) and phospholipids were recovered with 10 ml of MeOH. Each fr. was evapd, dissolved in $CHCl_3$–MeOH (2:1) then stored as described above.

Lipid analysis. Neutral lipids were analyzed on precoated PLC plates (silica gel 60 F-254 Merck) prewashed with $CHCl_3$–MeOH (2:1) and dried at 100° for 30 min. Hexane–Et_2O–HOAc (70:30:1) was used as solvent [33, 34]. Glycolipid sepns were performed on the same type of TLC plates, using $CHCl_3$–MeOH–H_2O (65:25:4) [32, 35]. Phospholipids were sepd on Whatman LK5 plates impregnated with boric acid (2.3% in EtOH) with $CHCl_3$–MeOH–H_2O–NMe_3 (30:35:7:35). All compounds were visualized by immersing plates in Me_2CO–H_2O (4:1) containing 0.5% primulin. Plates were then scanned for fluorescence at 366 nm. The CATS evaluation software package (Camag, Switzerland) was further used to quantify lipids. Each lipid class was identified by R_f value and compared with authentic lipid standards (Sigma).

GC. Each spot on the TLC plates was scraped off and submitted to a specific methylation procedure. TAG were added to 250 μl C_6H_6, 550 μl MeOH and 250 μl BF_3 (14% in MeOH) for 45 min at 100°. Glyco- and phospholipids were added to 1 ml BF_3–MeOH and heated for

15 min at 100°. After cooling, 1 ml H_2O and 2 ml pentane were added, the tubes vortexed and further centrifuged (2000 *g* for 5 min). The upper organic phases containing the fatty acid methyl esters (FAME) were collected. FAME were analysed by GC on a WCOT fused silica capillary column (AT-WAX, 60 m long, 0.25 mm i.d., 0.25 μm film thickness, Alltech) using a FID detector. The carrier gas was He and the temp. was prog. from 190° (for 18 min) at 210° at 20° min^{-1}. Samples were injected at 250° with a split-splitless inj. Individual peaks of FAME were identified by comparison of R_ts with those of authentic standards and natural extracts of known composition. FAME quantification was performed using 17:0 as int. standard.

The alcohol fraction was washed ×2 with 3 ml of hexane–Et_2O (1:1) and then centrifuged (2000 *g* for 5 min). The organic phase was collected and evapd. Alcohols were analysed by the same procedure as used for FAME, except that the temp. was constant at 220°.

For sterols, 2 ml of pyridine–Ac_2O (1:1) were added to samples at room temp. for 24 hr, then 30 ml of H_2O were added. Steryl acetates were extracted × 3 with 15 ml of hexane. Excess pyridine was removed by washing with dil. HCl and H_2O. The organic phase was dried (Na_2SO_4) and evapd. Steryl acetates were analyzed by GC using a non-polar silica capillary column (dimethylpolysiloxane, 30 m long, 0.32 mm i.d., 0.25 μm film thickness). The chromatograph was fitted to a MS (EI 70 eV).

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