Sterol Composition of *Phaeodactylum tricornutum* as Influenced by Growth Temperature and Light Spectral Quality

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ABSTRACT: In a detailed sterol analysis of the marine diatom *Phaeodactylum tricornutum,* free sterols as well as esterified and glycosylated conjugates were found. When the alga was grown under standard conditions (i.e., at 13°C under white light), 64% of total sterols were steryl glycosides. In all sterol classes, except steryl esters, (24*S*)-24-methylcholesta-5,22*E*-dien-3β-ol (epibrassicasterol) was the major (80 to 99%) sterol component. Eight other sterols were identified. Growth under different light spectral quality (red, blue, yellow, and green) at 13 and 23°C was examined. At 23°C, a dramatic decrease in total sterol content was observed, especially under blue light. The distribution of sterols between free and conjugated forms as well as sterol profile inside each class was found to be strongly dependent on the light spectral quality at both temperatures.

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Cultures of microalgae are extensively used as food for the commercial rearing of marine invertebrates such as oysters. Diatoms, which are abundant components of the phytoplankton, constitute a major nutritional source in aquaculture (1). As dietary polyunsaturated fatty acids and sterols are of great importance for growth and development of marine invertebrates, investigations have focused on these compounds. *Phaeodactylum tricornutum*, a marine pennate diatom (class Diatomophyceae), can satisfy these dietary requirements. Polar lipids of this diatom are characterized by a high content in C_{20}-polyunsaturated fatty acyl chains, mainly 20:5n-3, which is a major compound in stationary phase (2,3). The biosynthetic pathways of these polyunsaturated fatty acids in *P. tricornutum* have been recently investigated (4,5). Sterols are also an important aspect of lipids in marine nutrition. Bivalve mollusks are unable to synthesize *de novo* the sterols they need as membrane constituents and precursors of steroid derivatives (6), but are capable of using sterols from dietary sources such as phytoplankton (7). Few reports have dealt with sterols of *P. tricornutum* (3,8,9). The major sterol, which accounted for 91% of total sterols, was 24-methylcholesta-5,22-dien-3β-ol (3), with an α-oriented (or 24*S*) methyl group at C-24 of the side chain (i.e., epibrassicasterol) (8). The occurrence of 9% Δ7-sterols was reported (3), but has not been confirmed (9). We therefore decided to reinvestigate the sterol composition of *P. tricornutum*, with special emphasis on steryl conjugates. Moreover, since conditions of algal growth can affect its sterol composition (9 and references cited), we examined the influence of light spectral quality and temperature on the sterol profile of *P. tricornutum*. We show that this diatom contains free sterols (FS) as well as steryl esters (SE), steryl glycosides (SG), and acylated steryl glycosides (ASG). These different sterol classes were analyzed for their sterol composition. The nature of sugar(s) and fatty acyl chains of steryl conjugates was not determined. Our results indicate that profound alterations in the total amount as well as in the sterol profile of each sterol class of *P. tricornutum* occur in response to changes in both light spectral quality and culture temperature.

MATERIALS AND METHODS

Algal material and culture conditions. A unialgal culture of the diatom *P. tricornutum* Bohlin was obtained from the Culture Collection of the Laboratoire de Biologie et Biotechnologies Marines (University of Caen Basse-Normandie, France) (10). Under our growth conditions, this strain consists in colonial fusiform cells, with rare single or triradiate cells. A 150 mL-aliquot of stock culture was transferred in 2.5 L of enriched (soil extract and vitamins) sea water medium ES-Tris II (11) supplemented with 0.2 mM sodium metasilicate in 4-L flasks under aseptic conditions. Cultures were incubated at 13 or 23°C (±0.5) with a 12/12 h light/dark cycle provided from cool white, red, blue, yellow, and green 60 W lamps (Osram) at a light intensity of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ (12). All cultures were bubbled with 0.22 μm-filtered air. Cells were harvested at the beginning of stationary phase by centrifugation and lyophilized.

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Abbreviations: ASG, acylated steryl glycosides; FS, free sterols; GC, gas chromatography; MS, mass spectrometry; SE, steryl esters; SG, steryl glycosides.

Sterol analysis. Sterols were extracted from dried samples with dichloromethane/methanol (2:1, vol/vol). After solvent evaporation, lipid extracts were applied to silica gel plates (0.25 mm, Durasil-25UV$_{254}$; Macherey-Nagel, Düren, Germany) to separate FS, SE, SG, and ASG (13). Sterols from SE, SG, and ASG were obtained as previously described (12,13). After acetylation, FS and sterols from conjugates were identified by gas chromatography (GC) using a chromatograph equipped with a flame-ionization detector and a glass fused silica capillary column (30 m × 0.32 mm) coated with a 0.25 μm film of CP-Sil5 (Chrompack, Middleburg, The Netherlands). Column was operated at head pressure of 10 psi of helium. The oven temperature was raised from 260 to 300°C at 2°C/min and held at 300°C for 10 min. Injector and detector temperatures were held at 310°C. Sterol identities were confirmed by GC/mass spectrometry (MS) (14,15) and quantified by GC using 5α-cholestane as an internal standard.

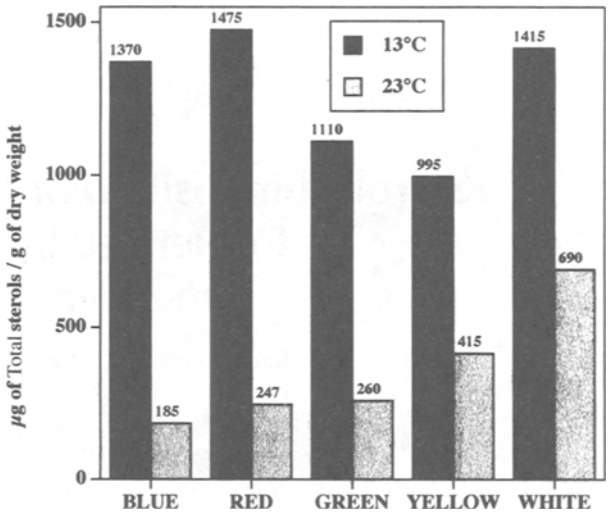

FIG. 1. Total sterol content in *Phaeodactylum tricornutum* grown at 13 and 23°C under different light spectral qualities.

RESULTS

Effects of light spectral quality and growth temperature on total sterol content. In Figure 1 are shown total sterol amounts of *P. tricornutum* grown at 13 and 23°C under different light spectral qualities. When expressed as μg/g dry weight, the total sterol content of cells grown at 13°C appears to be roughly similar (between 1100 and 1500) in all the illumination situations, with slightly lower amounts for cells grown under yellow and green light.

When grown at 23°C instead of 13°C, a dramatic decrease in total sterol amounts of cells was found, especially under blue light, with a 7-fold reduction. Such a temperature shift triggered a net inhibition of sterol biosynthesis, which was observed not to slow down the cell growth (data not shown). A similar but less important (1.4- to 3.7-fold) decrease in the total sterol content in cells of the chrysophyte *Ochromonas danica* was also reported to occur for growth temperature above 23°C (16).

Analysis of the different sterol classes. When grown at 13°C under white light, *P. tricornutum* was found to contain FS, but also SE, SG, and ASG. In contrast to most higher plant cells, FS were not the major compounds. The predominant sterol class was SG with 64% of total sterols (Table 1). FS represented 33% and ASG and SE together, only 3%. Such a repartition of sterols between the different classes was

TABLE 1
Distribution of Free and Conjugated Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Different Light Illuminations[a]

		Growth temperature							
		13°C				23°C			
		Sterol composition				Sterol composition			
Light spectral quality		FS	SE	SG	ASG	FS	SE	SG	ASG
Blue	%	20	2	76	2	73	12.5	5.5	9
	μg/g	275	30	1040	25	135	25	10	15
Red	%	60	2	22	16	80.5	14.5	4	1
	μg/g	885	30	325	235	200	35	10	2
Green	%	25	3.5	51	20.5	8.5	36	41	14.5
	μg/g	275	40	565	230	20	95	110	35
Yellow	%	35	7	18	40	34	14	50	2
	μg/g	350	70	180	395	140	60	205	10
White	%	33	0.5	64	2.5	45	2.5	31	21.5
	μg/g	470	5	905	35	310	15	215	150

[a]FS, free sterols; SE, steryl esters; SG, steryl glycosides; ASG, acylated steryl glycosides.

found to be strongly dependent on the type of illumination. Whereas SG remained the main sterol conjugates under green and blue light, a marked increase in FS was observed under red light and in ASG under yellow light. In all situations, SE were minor forms of sterols.

When the culture temperature was raised to 23°C, a completely different distribution of sterols was observed. Under white light as well as under red and blue light, FS were the major compounds. SG were found to represent 50% of total sterols under yellow light and similar amounts of SG and SE (41 and 36%, respectively) were present in cells grown under green light.

Effects of light spectral quality and growth temperature on the sterol composition of each class. Sterol analysis. Nine sterols were identified in *P. tricornutum*. The major compound was by far 24-methylcholesta-5,22-dien-3β-ol (**4**) as already reported (3,9). The configuration at C-24 of this sterol was previously assigned to 24α (or 24*S*) (8); this compound therefore corresponding to epibrassicasterol. The second major sterol was found to be cholest-5-en-3β-ol (cholesterol) (**2**). Sterols with an ethyl group at C-24 such as (24ξ)-24-ethylcholest-5-en-3β-ol (**8**) and (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**) were present in very low amounts, indicating that in *P. tricornutum* the enzyme involved in the second methylation step was not very active. Minor sterols, 5α-cholestan-3β-ol (cholestanol) (**3**), cholesta-5,22*E*-dien-3β-ol (22-dehydrocholesterol) (**1**), (24ξ)-24-methylcholesta-5,24(24^1)-dien-3β-ol (24-methylenecholesterol) (**5**), (24ξ)-24-methylcholest-5-en-3β-ol (**6**), and 24-ethylcholesta-5,24(24^1)*Z*-dien-3β-ol (isofucosterol) (**9**), were also identified in agreement with previous data (9). The C-24 stereochemistry was not determined in this work, but as *P. tricornutum* belongs to the order Pennales and contains both epibrassicasterol and isofucosterol, two sterols with a 24α-oriented alkyl group, all other 24-alkylsterols of this diatom likely have the same configuration (17). Diatoms from the order Pennales would be unique among algae, since most other algae contain sterols with a 24β-configuration (18).

Effects of light spectral quality at 13°C (Tables 2 to 6). Whatever the spectral nature of light illumination, epibrassicasterol (**4**) was the major compound of FS (between 68 and 95% of total FS). Cholesterol (**2**) accounted for only 2 to 5%, except for cells grown under red light (25%). Such cells were also found to contain 7% (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**).

SE were characterized by a lower content in epibrassicasterol (**4**) compared to FS. Cholesterol (**2**) was the major sterol in SE for cells grown under white and blue light and 22-dehydrocholesterol (**1**), the major one under yellow and green light. Significant amounts of 24-ethylsterols were present under all light conditions.

SG and ASG were mainly constituted of epibrassicasterol (**4**), except for ASG from cells grown under red light in which cholesterol (**2**) was predominant. The C-24 alkylated sterols (**6**), (**7**), (**8**), and (**9**) were not detected as glycosylated conjugates, but other compounds with similar relative retention times have been found. They are designated as "unknown" in Tables 2 to 6. According to MS data, these compounds might correspond to hydroxylated derivatives of epibrassicasterol and cholesterol. However, conclusive determination of their structure requires nuclear magnetic resonance analysis.

Whatever the light spectral quality, 24-methylenecholesterol (**5**), a biosynthetic intermediate in the synthesis of epibrassicasterol (8), was never detected as a free compound.

Effects of light spectral quality at 23°C (Tables 2 to 6). In FS, epibrassicasterol (**4**) remained the major sterol, except for cells grown under green light. Cholesterol (**2**) was the second best represented compound.

Cholesterol (**2**) was present as the main sterol in SE. Significant amounts of 24-methylenecholesterol (**5**), were also detected.

The sterol composition of SG was dominated by epibrassicasterol in cells grown under white, red, green, and yellow light, but by cholesterol, under blue light. In ASG, epibrassicasterol (**4**), cholesterol (**2**), and 24-methylenecholesterol (**5**) were the major compounds.

TABLE 2
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Blue Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	5[a]	63	3.5	13	27.5	86.5	70.5	44
Epibrassicasterol (**4**)	89	9	95	48	66.5	2.5	12	35.5
24-Methylenecholesterol (**5**)	—	8.5	—	5	—	6	6.5	10.5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	—	—	—	—	0.5	—	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	—	10	—	—	3.5	—	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	3.5	4	—	—	2	5	—	—
Isofucosterol (**9**)	2.5	6.5	—	—	—	—	—	—
Unknown	—	—	1.5	34	—	—	11	10

[a]Percentage of total sterols in each sterol class; —, not detected; trace amounts (<0.5%); Retention times of steryl acetates relative to 5α-cholestane/cholesta-5,22*E*-dien-3β-ol (22-dehydrocholesterol) (**1**) = 1.57; cholest-5-en-3β-ol (cholesterol) (**2**) = 1.64; 5α-cholestan-3β-ol (cholestanol) (**3**) = 1.67; (24*S*)-24-methylcholesta-5,22*E*-dien-3β-ol (epibrassicasterol) (**4**) = 1.75; 24-methylcholesta-5,24(24^1)-dien-3β-ol (24-methylenecholesterol) (**5**) = 1.80; (24ξ)-24-methylcholest-5-en-3β-ol (**6**) = 1.86; (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**) = 1.97; (24ξ)-24-ethylcholest-5-en-3β-ol (**8**) = 2.09; [24(24^1)*Z*]-24-ethylcholesta-5,24(24^1)-dien-3β-ol (isofucosterol) (**9**) = 2.15.

TABLE 3
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Red Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	25[a]	56	3.5	89.5	15	81	36	48
Epibrassicasterol (**4**)	68	6	93.5	10.5	83	—	64	—
24-Methylenecholesterol (**5**)	—	—	—	—	—	10	—	28.5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	—	—	—	—	—	1	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	7	38	—	—	1	3	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	—	trace	—	—	1	3	—	—
Isofucosterol (**9**)	—	trace	—	—	—	2	—	—
Unknown	—	—	3	—	—	—	—	23

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

DISCUSSION

Evidence is presented here that in *P. tricornutum,* as well as in higher plants (19) and in other types of algae (20), sterols occur as free, ester, and glycosylated forms. Under standard culture conditions (i.e., at 13°C under white light), free sterols are not the major compounds since 64% of total sterols are glycosylated. The occurrence of both SG and AGS suggests that an UDPG-sterol:β-Dglucosyltranferase and an SG:acyltransferase are likely involved in the synthesis of these compounds as demonstrated in the chlorophyte *Prototheca zopfii* (21).

Both the sterol profile inside each class and the distribution of sterols between free and conjugated forms were shown here for the first time to be closely dependent on illumination and growth temperature conditions. The temperature shift from 13 to 23°C was found to trigger a dramatic decrease in free and conjugated sterol biosynthesis. The slowing down of the biosynthesis was attested by the relative accumulation of 24-methylcholesterol (**5**) in SE, resulting in a lower flux of free precursors toward the synthesis of epibrassicasterol (**4**). Whatever the light conditions, an important increase in the relative percentage of cholesterol was also found in all forms of sterols, especially in SE, with a parallel decrease in the amount of epibrassicasterol, indicating a significant inhibition of the first methylation step at C-24.

Sterol glycosylation appears to be very sensitive to environmental changes. Thus, this process was found to be stimulated in cells grown at 13°C under red and blue light, but strongly inhibited at 23°C (a 100-fold decrease in SG under blue light and a 100-fold decrease in ASG under red light). SE synthesis appears to be much less sensitive to environmental changes.

Under blue light, the photosystems of *P. tricornutum* are expected to have their greatest efficiency in regard to carbon fixation and high amounts of chlorophylls *a* and *c* (22), in agreement with the life conditions of this temperate diatom in the euphotic zone of the ocean where blue light prevails. Such culture conditions under nonstressing light illumination could be used in order to study the physiological significance of drastic changes on sterol composition (i.e., shift from SG to FS and dominance of cholesteryl glycosides instead of epibrassicasteryl glycosides at 23°C), in particular their effect on properties of *P. tricornutum* membranes.

Although contents and proportions of algal fatty acids are considered the most important aspects of lipids in animal nutrition, dietary requirements for sterols in Bivalves and other marine invertebrates should be more closely investigated. Op-

TABLE 4
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Green Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
22-Dehydrocholesterol (**1**)	2[a]	43	—	1.5	—	—	—	—
Cholesterol (**2**)	2	8	1	1.5	57.5	52.5	10	62.5
Cholestanol (**3**)	—	—	—	—	—	trace	—	—
Epibrassicasterol (**4**)	93	3.5	98.5	96	42.5	trace	86	30.5
24-Methylenecholesterol (**5**)	—	—	—	—	—	42	—	—
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	0.5	—	—	—	—	—	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	0.5	5	—	—	—	2.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	34	—	—	—	1.5	—	—
Isofucosterol (**9**)	1	6.5	—	—	—	1	—	—
Unknown	—	—	trace	1	—	—	4	7

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

TABLE 5
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Yellow Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
22-Dehydrocholesterol (**1**)	4[a]	66.5	—	1.5	—	—	—	—
Cholesterol (**2**)	2	7.5	2.5	0.5	12	83.5	11	65.5
Cholestanol (**3**)	—	3.5	—	—	—	—	—	—
Epibrassicasterol (**4**)	91.5	3	97.5	96	82	1.5	29.5	3
24-Methylenecholesterol (**5**)	—	—	—	trace	—	6.5	1	5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	1	—	—	—	—	2.5	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	0.5	19.5	—	—	4.5	1.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	—	—	—	1.5	2.5	—	—
Isofucosterol (**9**)	—	—	—	—	—	2	—	—
Unknown	—	—	—	2	—	—	58.5	26.5

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

timal amounts of sterols for animal growth and survival remain to be determined, but we have proven here that algal sterol contents, such as those exemplified by *P. tricornutum*, can be strongly modified by environmental factors. Whether or not sterol conjugates can satisfy the sterol requirement as efficiently as free forms has to be questioned. More suitable algal diets could therefore be provided in hatcheries by manipulating culture conditions.

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TABLE 6
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under White Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	3.5[a]	59	trace	13	21	77	12	20
Epibrassicasterol (**4**)	95	33	99	80	75	3.5	82	59
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	0.5	—	trace	—	trace	4	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	—	—	—	—	2	6.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	8	—	—	2	4	—	—
Isofucosterol (**9**)	—	—	—	—	—	5	—	—
Unknown	—	—	trace	7	—	—	6	21

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

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