Total, Free and Conjugated Sterolic Forms in Three Microalgae Used in Mariculture

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Total, free and conjugated forms (steryl esters, steryl glycosides and acyl steryl glycosides) of sterols from three microalgae that are extensively used in mariculture (*Tetraselmis chuii, Nannochloropsis salina* and *Skeletonema costatum*) were examined. The results revealed that cholesterol is the only common fraction detected in all investigated species and distributed in free and all conjugated forms. However, the total sterol content of *T. chuii* was about 325 μg/g dry wt, most of it was concentrated amongst 24-methylcholesta-5,24-diene-3β-ol and 24-methylcholest-5-en-3β-ol. On the other hand, the majority of the fractions were distributed in the free form. The total sterol content of *N. salina* was about 180 μg/g dry wt, cholesterol was the major fraction that was detected. Nevertheless, the dominant distribution forms were esterified. While in *S. costatum*, the total sterol content was 76 μg/g dry wt, approximately most fractions are quantitatively alike and dominated in the free form. Furthermore, our study shows clearly that most sterols are not distributed regularly within each form, a result that encouraged us to suggest a distribution of specific sterol fraction as a free or conjugated can be used as a serving tool in chemotaxonomic studies.

Key words: Mariculture, Microalgae, Sterols

Introduction

Microalgae are used in mariculture as living feeds for all growth stages of mollusks, for the larval stages of crustaceans, some fish species, and for zooplankton used in mariculture food chains (Brown *et al.*, 1997). Although a number of unicellular algae used in mariculture have been screened for their lipids, emphasis was on their fatty acids (Mohammady, 2000b), sterols being considered far less attractive than other lipids for animal requirements. With the knowledge that *de novo* sterol biosynthesis does not occur in bivalve mollusks and oysters (Holden and Patterson, 1991; Patterson *et al.*, 1994a), bivalve mollusks need sterols as membrane constituents and precursors of steroid derivatives (Holden and Patterson, 1991). Changes in the sterol composition of microalgae are very important characteristics that can be used to determine the nutritive requirements in microfeeders such as oysters (Patterson *et al.*, 1994a), mollusks (Fabregas *et al.*, 1997), and bivalves (Véron *et al.*, 1998). Patterson *et al.* (1994a) reported that sterols of microalgae, steryl esters, as well as steryl glycosides, are negligible both in quantity and in quality. *Tetraselmis*, *Nannochloropsis* and *Skeletonema* are desirable as a potential food source in mariculture (Patterson *et al.*, 1994a,b; Véron *et al.*, 1998; Mohammady *et al.*, 2000). In this connection, Brown *et al.* (1997) concluded that both *T. chuii* and *S. costatum* are a good nutritional source for prawn larvae (*Penaeus* spp.) while *N. salina* is a good nutritional source for zooplanktons (*Brachionis plicatilis* and *Artemia* spp.).

Our purpose here was to determine the total sterols along with profile sterol weights, as well as their distribution in free and conjugated forms of three algal species, *T. chuii*, *N. salina* and *S. costatum*, that are extensively used in mariculture.

Materials and Methods

Biological material

Three species belonging to different classes and widely used in mariculture were examined for their sterol contents and distribution. *Tetraselmis chuii* Bucher, (Prasinophyceae) was obtained from UTEX, LB232; *Nannochloropsis* (*Monallantus*) *salina* Hibberd (Eustigmatophyceae) was obtained from the Mariculture Center in Eilat and originally from the Solar Energy Research Institute (SERI) Culture Collection in Golden, Colorado,

USA; *Skeletonema costatum* (Greville) Cleve (Bacillariophyceae) was locally isolated and obtained from the marine fishpond at Institute of Oceanography and Fisheries, Alexandria, Egypt.

Culture conditions

One culture of each species was grown axenically in 5 l enriched seawater medium according to Boussiba *et al.* (1987) supplemented by 0.2 mM sodium metasilicate for diatom in a controlled culture room under 12 h/12 h light/dark cycle; the irradiance was provided by white fluorescent tubes at 100 μmol photons m^{-2} s^{-1}. The cultures were continuously agitated by bubbling with sterile air, which was also the source of CO_2. Temperature was 18 ± 1 °C. All cultures were harvested toward the beginning of the stationary growth phase by centrifugation, and the pellets were immediately lyophilized.

Sterol isolation and analysis

1 g of dried biomass of each species was extracted in a Soxhlet apparatus for 4 h with diethyl ether. The half amount of total lipid was analyzed for total sterols according to Nadal (1971). However, the remaining amount of total lipid was fractionated according to Véron *et al.* (1996a) by preparative thin layer chromatography (TLC) developed in the first dimension in hexane/ethyl acetate 92:2 (v/v) to separate the steryl esters (SE), and in a second dimension in dichloromethane/methanol/water 90:10:0.5 (v/v) to separate free sterols (FS), steryl glycoside (SG) and acyl steryl glycoside (ASG) fractions. FS, SE, SG and ASG bands were located according to the R_f values of standards. Spots of standards were visualized with the Libermann-Burchard reagent. The different bands were scraped off and eluted with dichloromethane for FS and SE and with 2:1 (v/v) dichloromethane/methanol for SG and ASG. SE were saponfied by a 1-h reflux with methanolic KOH (6% w/v) and 0.5% (w/v) pyrogallol. SG and ASG were separately hydrolyzed by a 4-h reflux with ethanolic H_2SO_4 (1% v/v). Sterols were recovered by partition into hexane and acylated at room temperature in the dark for 48 h using acetic anhydride in anhydrous pyridine. Acetyl derivatives were purified by TLC on silica gel plates developed in dichloromethane with cholesteryl acetate as the standard. The location was determined as described above. Sterols generated from preceding fractions were identified by capillary gas liquid chromatography (GLC). Abundances of the individual components were determined by comparison of their GLC peak areas with those of 5α-cholestane used as an internal standard.

Statistical analysis

All analyses were made in three replicas and the standard deviations (SD) were obtained.

Results and Discussion

Sterol amounts and composition

Total sterol and the profile contents of the three species studied are shown in Fig. 1, A, B and C; most fractions detected were often distributed among the investigated algae. In the prasinophyte *T. chuii* (Fig. 1, A), seven sterols were separated and identified, their total weight was 325 μg/g dry wt, the majority of them was concentrated amongst 24-methylcholesta-5,24-diene-3β-ol and 24-methylcholest-5-en-3β-ol. However, each one weighed more than 139 μg/g dry wt; so these two fractions were over 86% of the total sterol contents. However, brassicasterol, stigmasterol, cholesterol, 22-dehydrocholesterol and β-sitosterol were also detected, but in small amounts. In the eustigmatophyte *N. salina* (Fig. 1, B), six sterol fractions were detected, their total amount was about 180 μg/g dry wt. Cholesterol was the dominant fraction that weighed more than 145 μg/g dry wt, so it represented about 80% of the total sterols weight. However, the other fractions (24-methylcholesta-5,24-diene-3β-ol, campesterol, stigmasterol, 22-dehydrochlesterol and β-sitosterol) were detected in small amounts. Whereas in the centric diatom *S. costatum* seven sterols were weighed about 76 μg/g dry wt, most of them are recognized in approximate concentrations (Fig. 1, C).

Sterol distribution

Our data revealed that cholesterol is not only the regular fraction that was found in our investigated algae, but also it is distributed in all sterol forms. Fig. 2 shows that free sterols were dominant in both *T. chuii* and *S. costatum.* However, in *N. salina* the steryl esters are the major constituents (Table I). In *T. chuii* cholesterol and brassicasterol were distributed in all sterol classes and dominated in both steryl glycosides and acyl steryl glycosides. However, the other sterol fractions are

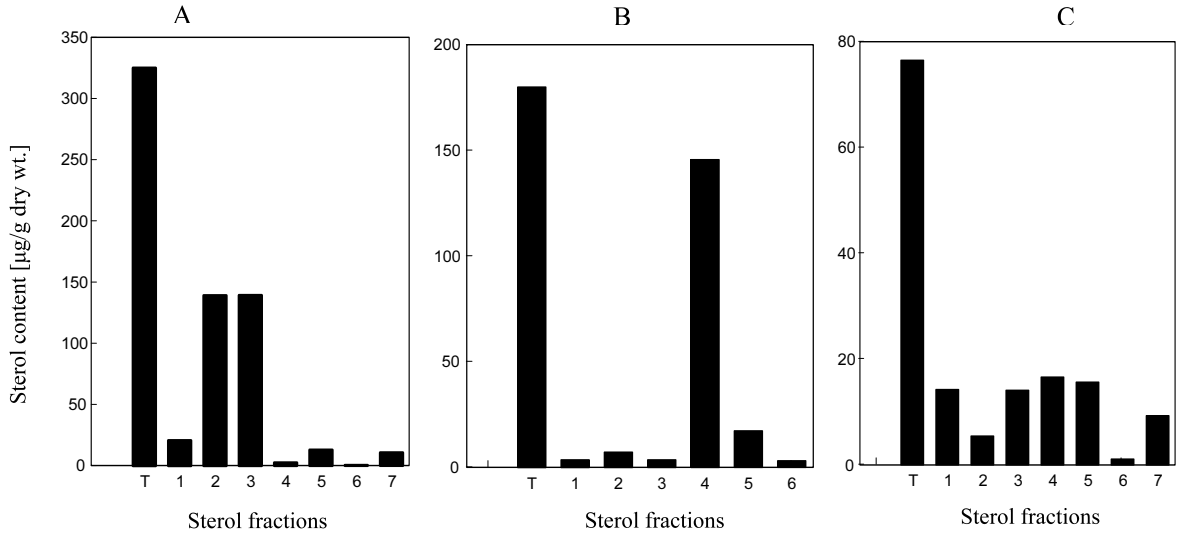

Fig. 1: A) Sterol contents in *Tetraselmis chuii* (T, total; 1, brassicasterol; 2, 24-methylcholesta-5,24-diene-3β-ol; 3, campesterol; 4, stigmasterol; 5, cholesterol; 6, 22-dehydrocholesterol; 7, β-sitosterol). B) Sterol contents in *Nannochloropsis salina* (T, total; 1, 24-methylcholesta-5,24-diene-3β-ol; 2, campesterol; 3, stigmasterol; 4, cholesterol; 5, 22-dehydrocholesterol; 6, β-sitosterol). C) Sterol contents in *Skeletonema costatum* (T, total; 1, brassicasterol; 2, campesterol; 3, stigmasterol; 4, cholesterol; 5, 22-dehydrocholesterol; 6, 5α-cholestan-3β-ol; 7: β-sitosterol).

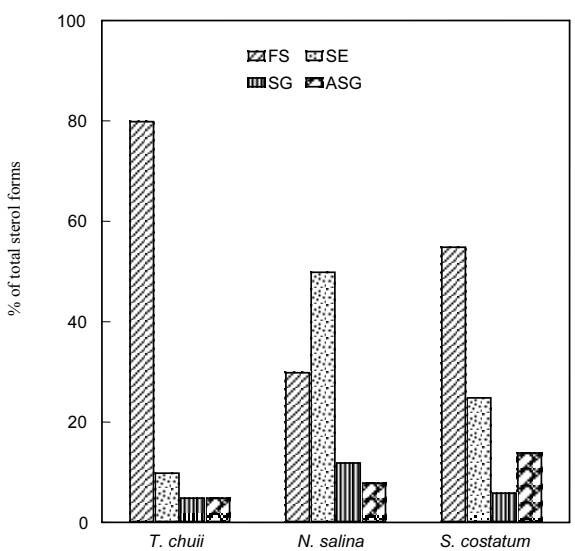

Fig. 2. Distribution of free and conjugated sterols among the investigated algal species. (FS, free sterols; SE, steryl esters; SG, steryl glycosides; ASG, acyl steryl glycosides.)

not regularly distributed inside each sterol class. The eustigmatophyte *N. salina* contained essentially cholesterol which was detected in all forms, 54% as free sterols and over 80% of the remaining forms. Whereas, 22-dehydrocholesterol was also detected in free and all combined forms but in less amounts, while other fractions are not regularly distributed in each sterol class. In the centric diatom *S. costatum*, both cholesterol and β-sitosterol are the only fractions distributed in free and all combined forms.

The possibility of changing sterol concentration of microalgae is a very important characteristic that can be used to determine the physiological state of a microalgal population, as a population biomarker, or to define the nutritive requirements in microfeeders such as mollusks (Fabregas *et al.*, 1997). Optimal amounts of sterols for animal growth and survival remain to be determined (Véron *et al.*,1996b). However, sterol metabolism, as in higher plants, is influenced by culture conditions (Wright *et al.*, 1980), culture age (Mohammady, 2000a), light intensity and composition (Ballantine *et al.*, 1979; Véron *et al.*, 1996b; Mohammady and Noaman, 2001) as well as the nutrient medium (Orcutt and Patterson, 1975). Qualitative and/or quantitative differences in free sterols depend on species or culture conditions (Grunwald, 1980). In our study, culture conditions were standardized to compare results. These conditions are not always optimal for the growth of our se-

Table I. Sterol distribution forms among the investigated microalgae (data expressed as percentage ± SD).

Sterol fraction	Sterol distribution[a]											
	FS			SE			SG			ASG		
24-Methylcholesta-5,22-diene-3β-ol (brassicasterol)	2 ± 0.3[b]	–[c]	17.5 ± 2[d]	3.5 ± 0.1[b]	–[c]	9.5 ± 2[d]	40 ± 2[b]	–[c]	–[d]	69 ± 1[b]	–[c]	16 ± 1[d]
24-Methylcholesta-5,24-diene-3β-ol	46.5 ± 0.1	–	–	29 ± 0.1	2.5 ± 1	–	–	2.5 ± 1	–	10.5 ± 1	3.5 ± 0.3	–
24-Methylcholest-5-en-3β-ol (campesterol)	47 ± 0.1	3.3 ± 3	–	29.5 ± 1	4 ± 0.5	16.5 ± 1	–	5.5 ± 2	–	–	–	9.5 ± 3
24-Ethylcholesta-5,22-diene-3β-ol (stigmasterol)	0.5 ± 0.2	–	19.5 ± 2	4 ± 0.6	3 ± 0.2	13.5 ± 1	–	2.5 ± 0.2	–	–	–	–
Cholest-5-en-3β-ol (cholesterol)	1 ± 0.5	54 ± 1	17.5 ± 3	11.5 ± 3	81.5 ± 1	5 ± 0.1	34 ± 1	81.5 ± 1	63 ± 0.5	20.5 ± 1	85.5 ± 1	14 ± 1
Cholesta-5,22-diene-3β-ol (22-dehydrocholesterol)	–	13 ± 2	17.5 ± 1	–	7 ± 0.3	11.5 ± 2	26 ± 1	5.5 ± 1	–	–	7.5 ± 1	22.5 ± 1
24-Ethylcholest-5-ene-3β-ol (β-sitosterol)	3 ± 0.2	–	6 ± 0.4	8.5 ± 0.1	2 ± 0.4	4 ± 0.2	–	2.5 ± 0.3	18.5 ± 0.1	–	3.5 ± 2	28 ± 2
5α-Cholestan-3β-ol	–	–	2 ± 0.2	–	–	–	–	–	–	–	–	–

[a] FS, free sterols; SE, steryl esters; SG, steryl glycosides; ASG, acyl steryl glycosides.
[b] *T. chuii.*
[c] *N. salina.*
[d] *S. costatum.*
–: Not detected.

lected species since their photosynthetic pigments are different. Similarly, the optimal growth temperature is different for each alga.

Sterols are either esterified with fatty acids or conjugated with sugars, which are often acylated. The most common transformation of sterols in plants is the conjugation. Nevertheless, the relative amounts of individual sterols are hereditary (Grunwald, 1975). In contrast to higher plants, in which the constituents of steryl esters are the same as those in free sterols in most cases, the situation in planktonic microalgae is quite variable (Véron *et al.*, 1998). In the present work, the green alga *T. chuii*, a member of the Prasinophyceae (the closest class to higher plants), had a composition in steryl esters similar to its free sterols. The Prasinophyceae hold a group of divergent taxa that are currently considered paraphyletic (Melkonian and Surek, 1995; Danilov *et al.*, 1996), and the heterogeneity of the class is reflected by the variety of their sterols (Véron *et al.*, 1998). The major constituents are 24-methylcholesta-5,24-diene-3β-ol and 24-methylcholest-5-en-3β-ol. According to Volkman *et al.* (1994) four chemotaxonomic groups may be distinguished in the Prasinophyceae. Furthermore, important variations exist in sterolic composition within genera holding a great number of species, such as *Pyramimonas* or *Tetraselmis.* In this latter genus, which is considered the most evolved of the class, Patterson *et al.* (1993) distinguished four groups according to their sterolic composition, including one with cholesterol as its main constituent (90%–95% of total sterols). However, our results on *T. chuii* agree with Véron *et al.* (1998) in which the two sterols, 24-methylcholesta-5,24-diene-3β-ol and 24-methylcholest-5-en-3β-ol, are dominated in roughly equal proportions. Volkman *et al.* (1981, 1992) and Patterson *et al.* (1994a) examined several isolates of *Nannochloropsis* and showed that all strains contained over 50% cholesterol. In the present work, cholesterol represents about 80% of total sterols in *N. salina*, most are conjugates. However, Patterson *et al.* (1994a) estimated them at a very low percentage in their eustigmatophyte isolates. On the other hand, the most abundant sterols of the centric diatom *S. costatum* were detected in the free form, a result which agrees with Véron *et al.* (1998). However, Gladu *et al.* (1991) reported the diversity of sterolic profiles in the Bacillariophyceae emphasizing the heterogeneity of the class. Recent molecular sequence data suggested that centrals are paraphyletic in agreement with the greater variability of their sterolic profiles (Sorhannus *et al.*, 1995). More attention should now be paid to conjugates when analyzing the heterogeneity of sterolic composition in algal classes since some useful biomarkers can be more abundant in combined forms than in free sterols (Véron *et al.*, 1998). The occurrence of both steryl glyco-

sides and acyl steryl glycosides in the algal classes suggests that an UDPG-sterol (β-D-glucosyltransferase) and a steryl glycoside (acyltransferase) are likely involved in the synthesis of these compounds as was demonstrated previously in the chlorophyte *Prototheca zopfii* by Hopp *et al.* (1978). The biological functions of SG and ASG appeared as hormones or precursors and their metabolic function as storage pools or transportable forms of sterol (Eichenberger, 1977). Wojciechowski (1991) suggested that the major role of SG and ASG, under the influence of phytohormones or environmental factors, is the control of membrane equilibrium and glycoside sterols could also be a kind of second messenger. Dyas and Goad (1993) proposed that steryl esters in plants seem to be a storage pool of sterols which can be used when *de novo* synthesis of free sterols is not sufficient or for transport through different cellular compartments and the influence of conjugates in the organization and in the fluidity of membranes seems probable mainly for acylated forms (SE and ASG). However, Jensen-Pergakes *et al.* (2001), Wentzinger *et al.* (2002) and Laule *et al.* (2003) reported that conjugation of sterols and fatty acids is a critical homeostatic response by all eukaryotic cells to an excess of either resource. The intracellular esterification reaction is mediated by enzymes known collectively as *O*-acyltransferases and provides an important storage depot and detoxification process in order to overcome the membrane perturbations that accrue from elevated sterols or free fatty acid levels. Thus the uptake, synthesis and conjugation of these metabolites are subject to multiple levels of regulation.

Furthermore, sterols are progressively more used in physiological, chemotaxonomic, and phylogenetic studies (Mohammady *et al.*, 2000, 2002). In the present work, our study shows clearly that, although most sterols are frequently found in the investigated algae, they are not distributed regularly within different sterol classes. A result that encouraged us to suggest a distribution of specific sterol fraction as a free or conjugated can be used as a serving tool in chemotaxonomic studies.

Ballantine J. A., Lavise A., and Morris R. J. (1979), Sterols of the phytoplankton – effects of illumination and growth stage. Phytochemistry **18**, 1459–1466.

Boussiba S., Vonshak A., Cohen Z., Avissar Y., and Richmond A. (1987), Lipid and biomass production by halotolerant microalga *Nannochloropsis salina*. Biomass **12**, 17–24.

Brown M. R., Jeffrey S. W., Volkman J. K., and Dunstan G. A. (1997), Nutritional properties of microalgae for mariculture. Aquaculture **151**, 315–331.

Danilov R., Marin B., Hoef-Emden K., and Melkonian M. (1996), Systematics of the genus *Tetraselmis* Stein (Chlorodendrales, Chlorophyta). Proceedings of the First European Phycological Congress. Eur. J. Phycol. **31**, 27–32.

Dyas L. and Goad L. J. (1993), Steryl fatty acid esters in plants. Phytochemistry **34**, 17–29.

Eichenberger W. (1977), Steryl glycosides and acylated steryl glycosides. In: Lipids and Lipid Polymers in Higher Plants (Tevini M. and. Lichtenthaler H. K., eds.). Springer, Berlin, pp. 169–182

Fabregas J., Aran J., Morales E. D., Lamella T., and Otero A. (1997), Modification of sterol concentration in marine microalgae. Phytochemistry **46**, 1189–1191.

Gladu P. K., Patterson G. W., Wikfors G. H., Chitwood D. J., and Lusby W. R. (1991), Sterols of some diatoms. Phytochemistry **30**, 2301–2303.

Grunwald C. (1975), Plant sterols. Anu. Rev. Plant Physiol. **26**, 209–236.

Grunwald C. (1980), In: Secondary Plant Products (Bell E. A. and. Charlwood B. V., eds.). Springer, Berlin, pp. 221.

Holden M. J. and Patterson G. W. (1991), Absence of sterol biosynthesis in oyster tissue culture. Lipids **26**, 81–82.

Hopp H., Romero P. A., Daleo G. R., and Pont Lezica R. (1978), Steryl glucoside biosynthesis in the alga *Prototheca zopfii*. Phytochemistry **17**, 1049–1052.

Jensen-Pergakes K., Guo Z., Giattina M., Sturley L. S., and Bard M. (2001), Transcriptional regulation of the two sterol esterification genes in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **183**, 4950–4957.

Laule O., Furholz A., Chang H. S., Zhu T., Wang X., Heifetz P. B., Gruissem W., and Lang M. (2003), Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Plant Biol. **100**, 6866–6871.

Melkonian M. and Surek B. (1995), Phylogeny of the Chlorophyta: congruence between ultrastructural and molecular evidence. Bull. Soc. Zool. Fr. **120**, 191–208.

Mohammady N. G. (2000a), Sterol composition during the exponential and stationary growth phases of some blue-green algae. Proc. 1st Int. Conf. Biol. Sci. (ICBS). Fac. Sci., Tanta Univ. Egypt. **1**, 83–88.

Mohammady N. G. (2000b), Effect of salinity on the fatty acid composition of *Nannochloropsis oculata* during exponential and stationary growth phases. Egypt. J. Phycol. **1**, 151–155.

Mohammady N. G. and Noaman N. H. (2001), Effect of different light spectra on growth, pigment, protein and sterol composition of *Nostoc muscorum* and *Phormidium fragile* (Cyanobacteria). Azhar J. Microbiol. **52**, 70–79.

Mohammady N. G., Khaleafa A. F., Shalaan S. H., and Abd-El- Salaam E. (2000), Sterol and fatty acid composition of chlorophytes in relation to some other green plants. Egypt. J. Phycol. **1**, 169–180.

Mohammady N. G., Khaleafa A. F., Shaalan S. H., and Taha H. M. (2002), Comparative biochemical taxonomy of some *Dunaliella* sp. Egypt. J. Biotechnol. **11**, 248–265.

Nadal N. G. M. (1971), Sterols of *Spirulina maxima*. Phytochemistry **10**, 2537–2538.

Orcutt D. M. and Patterson G. W. (1975), Sterols, fatty acid and elemental composition of diatoms grown in chemically defined media. Comp. Biochem. Physiol. **50B**, 597–583.

Patterson G. W., Tsitsa-Tzardis E., Wikfors G. H., Gladu P. K., Chitwood D. J., and Harrison D. (1993), Sterols of *Tetraselmis* (Prasinophyceae). Comp. Biochem. Physiol. **105**, 253–256.

Patterson G. W., Tsitsa-Tzardis E., Wikfors G. H., Ghosh P., Smith B. C., and Gladu P. K. (1994a), Sterols of Eustigmatophytes. Lipids **29**, 661–664.

Patterson G. W., Tsitsa-Tzardis E., Wikfors G. H., Gladu P. K., Chitwood D. J., and Harrison D. (1994b), Sterols and alkanones of *Isochrysis*. Phytochemistry **35**, 1233–1236.

Sorhannus U., Gasse F., Perasso R., and Baroin Tourancheau A. (1995), A preliminary phylogeny of diatoms based on 28S ribosomal RNA sequence data. Phycologia **34**, 65–73.

Véron B., Dauguet J. C., and Billard C. (1996a), Sterolic biomarkers in marine phytoplankton. I. Free and conjugated sterols of *Pavlova lutheri* (Haptophyta). Eur. J. Phycol. **31**, 211–215.

Véron B., Billard C., and Dauguet J. C. (1996b), Sterol composition of *Phaeodactylum tricornutum* as influenced by growth, temperature and light spectral quality. Lipids **31**, 989–994.

Véron B., Dauguet J. C., and Billard C. (1998), Sterolic biomarkers in marine phytoplankton. II. Free and conjugated sterols of seven species used in mariculture. J. Phycol. **34**, 273–297.

Volkman J. K., Smith D. J., Eglinton G., Forsberg T. E. V., and Corner E. D. S. (1981), Sterol and fatty acid composition of four marine haptophycean algae. J. Mar. Biol. Assoc. UK **61**, 509–527.

Volkman J. K., Barrett S. M., Dunstan G. A., and Jeffrey S. W. (1992), C30–C32 alkyl diols and unsaturated alcohols in microalgae of the class Eustigmatophyceae. Org. Geochem. **18**, 131–138.

Volkman J. K., Barrett S. M., Dunstan G. A., and Jeffrey S. W. (1994), Sterol biomarkers for microalgae from the green algal class Prasinophyceae. Org. Geochem. **21**, 1211–1218.

Wentzinger L. F., Back T. J., and Hartmann M. (2002), Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. **130**, 334–346.

Wojciechowski Z. A. (1991), Biochemistry of phytosterol conjugates. In: Physiology and Biochemistry of Sterols (Patterson G. W. and Nes W. D., eds.). American Oil Chemists' Society, Champaign, Illinois, pp. 361–395.

Wright D. C., Berg L. R., and Patterson G. W. (1980), Effect of cultural conditions on the sterols and fatty acids of green algae. Phytochemistry **19**, 783–785.