Long chain *n*-alkyl diols, hydroxy ketones and sterols in a marine eustigmatophyte, *Nannochloropsis gaditana*, and in *Brachionus plicatilis* feeding on the algae

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Abstract

Base-hydrolyzed sterols and long-chain alcohols of a marine eustigmatophyte, *Nannochloropsis gaditana* have been studied. Sterol composition of *N. gaditana* is similar to that of other member of this genus, with predominance of cholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol. The $C_{29:2}$ and $C_{30:1}$ unsaturated aliphatic alcohols were predominant. Saturated and monounsaturated C_{28} to C_{36} *n*-alkyl diols occurred as a mixture of positional isomers with ω16 predominating among the C_{28} alkyl diols, ω18 among C_{30}, C_{32}, C_{34} and C_{36} alkyl diols and ω17 for the odd chain alkyl diols. C_{28}–C_{32} hydroxy ketones were identified for the first time in a marine organism, with a distribution parallelling the alkyl diol mixtures and suggesting a biosynthetic relation between both alcohol classes. *N. gaditana* was fed to *Brachionus plicatilis* and the distribution of alkyl diol positional isomers remained the same in the algae and the rotifer fecal pellets. However, digestion resulted in an increase in C_{30} diols relative to C_{32} diols and in an enrichment of unsaturated long-chain alcohols relative to alkyl diols.
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1. Introduction

Mid-chain alkyl diols and hydroxy ketones were originally identified in deposits rich in organic carbon (de Leeuw et al., 1981; Smith and Eglinton, 1986), and reports of their occurrence have expanded since (Versteegh et al., 1997; Rinna et al., 2002). Alkyl diol isomer distribution in marine sediments is variable, and distinct patterns have been related to freshwater input and to changes in surface water conditions in sedimentary records from the penultimate deglaciation and from the two last centuries (Versteegh et al., 2000; Pinturier et al., 2002).

Marine eustigmatophytes are generally considered as the source of mid-chain hydroxy alkanols and hydroxy acids in marine sediments (Gelin et al., 1997; Versteegh et al., 2000; Volkman et al., 1999). The occurrence of even-chain alkyl diols, hydroxy alkanones and ketoaldehydes in a temperate zone fern comprising the same alkyl diol homologues as those of marine eustigmatophytes, points to an alternative possible source for these compounds buried in marine sediments (Jetter and Riederer, 1999).

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Fecal pellet rainfall constitutes a major vector for organic matter deposition to sediments. While algae are being grazed and their remains are packed into fecal pellets, algal lipids are altered; and the extent of these changes on any potential paleoproxy needs to be comprehended. To our knowledge a sole study addressed the microbial biodegradation of long-chain alcohols of eustigmatophytes (Grossi et al., 2001) and the impact of digestion processes on alkyl diol composition still needs to be apprehended. This line of thinking prompted us to follow alcohol compositions of the marine eustigmatophyte Nannochloropsis gaditana while the algae was grazed by a herbivore, and packed into fecal pellets. N. gaditana is widely used as live feed in aquaculture (Huertas et al., 2000). Although this species was observed to bloom in Mediterranean coastal waters (Lubián et al., 1985), its global distribution is not known. N. gaditana was isolated from coastal waters in the Mediterranean Sea in Spain, Morocco and Italy, from the East coast of the US (New York and Connecticut States), from South Africa and from Australia (L.M. Lubián, personal communication). This wide occurrence makes N. gaditana a potential contributor to sedimentary organic matter in coastal areas worldwide. This is the first report on alcohol composition of the eustigmatophyte N. gaditana.

The selected herbivore, Brachionus plicatilis, is commonly used in studies on lipid changes associated with herbivory (Ederington et al., 1995; Fernández-Reiriz and Labarta, 1996; Lubzens et al., 1985; Nichols et al., 1989).

2. Experimental

2.1. Cultures and feeding experiments

The eustigmatophyte alga N. gaditana was grown from a strain of the culture collection held at the University of Barcelona. The first culture was developed in large volume (5 l) as food for the feeding experiment with the rotifer B. plicatilis. This first culture, referred to "N. gaditana #1", was developed in a 12:12h light–dark cycle, at 18 °C. After 2 weeks, 1 l of the culture was filtered on a pre-combusted GF/F filter, while the rest was used for feeding the rotifer. B. plicatilis was obtained from a stock culture from the University of Barcelona. It was added to the N. gaditana culture flask and was grown for several generations (2 weeks). After this period, the rotifers were sieved through 60 μm mesh nets and rinsed with filtered seawater. They were then re-suspended into seawater and filtered onto GF/F filters. Fecal pellets emitted by B. plicatilis at the bottom of the culture flask were hand picked with a Pasteur pipette, re-suspended into seawater and filtered onto GF/F filters.

A second culture of N. gaditana was grown later, in order to implement the results obtained previously for which the GC–mass spectrometry (GC–MS) scan range was not propitious to the semi-quantification of isomers of alkyl diols and hydroxy alkenones. This culture is referred to as "N. gaditana #2" in the text, and was developed following the method described above.

2.2. Lipid extraction and separation

Samples of N. gaditana #1, B. plicatilis and its fecal pellets, retained on GF/F filters, were analyzed. Lipids were hydrolyzed with 5N KOH in purified Milli-Q water, during 36 h in the dark. After the reaction, the internal standard, β-epicoprostanol was added to the extract. Neutral lipids were extracted by 3×40 ml of diethyl ether and combined extracts were vacuum evaporated to 0.5 ml.

Neutral lipids were separated by adsorption chromatography on a column (12.5 cm × 2.5 mm i.d.) filled with 2 g of alumina activated at 120 °C overnight. A first fraction was eluted by 5 ml of n-hexane/CH₂Cl₂ (95:5) and 7 ml of n-hexane/CH₂Cl₂ (1:2) to remove hydrocarbons and methyl esters that may interfere with the analysis of alcohols. The second fraction was eluted with 10 ml of CH₂Cl₂/methanol (1:1) and contained most of the alcohols and sterols. These second alcohol fractions were derivatized with bis-(trimethylsilyl)-trifluoroacetamide in iso-octane, at 80 °C for 75 min. The obtained trimethylsilyl (TMSi) ethers of total alcohols, sterols and other hydroxyl substituted compounds were evaporated to dryness to remove the remaining reagent and were re-dissolved in iso-octane for analysis.

The sample N. gaditana #2 was analyzed for lipids from N. gaditana #2 and were extracted ultrasonically for 15 minutes in the following sequence of solvents, 3×5 ml of CH₃OH and 3×5 ml of CH₂Cl₂. The internal standard was added to the combined extracts. The extract was vacuum-concentrated to a few milliliters and dried overnight on pre-extracted MgSO₄. The dried extract was filtered on pre-extracted glass wool and saponified as described above. Neutral lipids were vacuum-evaporated and separated by adsorption chromatography on a 180 mm × 6 mm column packed with silica 40 (0.063–0.200 mm, Merck, Darmstadt, Germany) as adsorbent. The silica was pre-extracted, activated at 120 °C over-night and desactivated with 5% Milli-Q water. Hydrocarbons and methyl esters were eluted with 12 ml of n-hexane and 19 ml of ethyl acetate/n-hexane (1:9, v/v). Alcohols and sterols were eluted with 22 ml of ethyl acetate/n-hexane (3:17, v/v). A last fraction was eluted with 24 ml of CH₂Cl₂/CH₃OH (1:1, v/v) to check for the recovery of long-chain hydroxylated compounds. The hydroxylated compounds present in these fractions were converted to their TMSi ethers by reaction with bis-(trimethylsilyl)-trifluoroacetamide (80 °C, 1 h).

2.3. GC and GC–MS analyses

Samples of *N. gaditana* #1, *B. plicatilis* and its fecal pellets were dissolved in *iso*-octane and analyzed by gas chromatography (GC) in a HRGC 3500 Carlo Erba gas chromatograph equipped with a flame ionization detector and a splitless injector. A CPSIL-8CB column (50 m × 0.25 mm i.d., coated with methylphenylsilicone 95:5) was used to separate TMSi ethers. After 1 min at 60 °C, the oven temperature was raised to 150 °C at 10 °C min⁻¹ and then to 320 °C at 3 °C min⁻¹. The final temperature was held for 23 min. Hydrogen was used as carrier gas at a flow of 50 cm s⁻¹. Detector and injector temperatures were 330 °C and 280 °C, respectively. Samples were injected in the splitless mode (solvent *iso*-octane, hot needle technique) keeping the split valve closed for 35 s. TMSi ethers of alcohols of *N. gaditana* #2 were injected in a Varian 3400 gas chromatograph in splitless mode at 300 °C. A 25 m capillary column (0.25 mm i.d.) coated with a 0.25 μm thick stationary phase of 5% phenylmethyl polysiloxane (HP-5 Hewlett Packard) was used for the separation of TMSi ethers, using hydrogen as the carrier gas. The oven was kept at 70 °C for 1 min, heated to 150 °C at 15 °C min⁻¹, then to 310 °C at 4 °C min⁻¹, and finally held at 310 °C for 50 min. The flame ionization detector was fed with air (300 ml min⁻¹) and hydrogen (30 ml min⁻¹) and was maintained at 300 °C. Nitrogen was used as make up gas (30 ml min⁻¹). The FID detector response was digitized by a Nelson 900 interface and processed with a Nelson 2600 software package (Perkin Elmer). Relative concentrations of sterols were calculated from the GC-FID response areas.

Analyses by GC-MS of the TMSi ethers were performed with a Fisons 8000 gas chromatograph coupled to a quadrupole mass analyzer Fisons MD-800. Samples were injected in splitless mode at 300 °C into a capillary column similar to that used for the GC analyses and the oven was identically programmed. Helium was the carrier gas. Fragmentation was obtained in electron impact mode (70 eV). Ion source and transfer line were kept at 300 °C. Spectra were recorded and processed using the Masslab software (THERMO Instruments). Spectra of *N. gaditana* #1, *B. plicatilis* and its fecal pellets were obtained by scanning from *m/z* 50 to 550 every second. In order to obtain the molecular ions of alkyl diols and hydroxy ketones, GC–MS analysis of *N. gaditana* #2 was performed by scanning from *m/z* 50 to 700 every second.

3. Results

3.1. Lipids of Nannochloropsis gaditana

3.1.1. Sterols

The hydroxyl compounds present in the neutral lipid fraction were dominated by cholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol. Both components accounted for about 75% of sterols and aliphatic alcohols in both analyzed cultures with a slight dominance of cholest-5-en-3β-ol (Table 1). The data given in Table 1 are given in percent of sterols, long chain alcohols, diols and hydroxy ketones. A comparable composition was reported in other eustigmatophytes, with variable abundances of the C_{29} sterol (Volkman et al., 1999). 24-Ethylcholest-5-en-3β-ol is indeed absent or less abundant than cholest-5-en-3β-ol in previously described marine species, whilst it is the dominant sterol in freshwater ones (Volkman et al., 1992, 1999). Other minor sterols were identified such as Δ^5- and Δ^7-stenols, with or without a methyl or an ethyl substituent at C-24 (Table 1). They accounted for less than 1% of total sterols. Two isomers of 24-ethylcholesta-5,24(28)-dien-3β-ol were eluting before and after 24-ethylcholest-5-en-3β-ol. They were attributed to *E* and *Z* isomers of the $\Delta^{24(28)}$ bond after examination of the mass spectra and retention time data. (Volkman et al., 1992). These sterols are also present in other species of the *Nannochloropsis* genus (Volkman et al., 1992, 1999; Grossi et al., 2001).

3.1.2. n-Alcohols

Although both cultures of *N. gaditana* were saponified, a higher proportion of short chain alcohols, phytol, C_{16} and C_{18} alkanols was observed in *N. gaditana* #1 than *N. gaditana* #2. Differences in phytol abundance may result from variable chlorophyll cellular content as well as from difference in the analytical methods (Grossi et al., 1996). Phytol and short-chain alcohols contributed to less than 1% of total alcohols in both *N. gaditana* cultures. This agrees with the low content in short-chain *n*-alkanols reported in saponified extracts of marine and freshwater eustigmatophytes (Versteegh et al., 2000; Volkman et al., 1992). As the focus of this paper is long-chain alcohols and sterols, only the alkanols with 28 or more carbon atoms will be further considered.

Long-chain *n*-alkanols were present in the GC chromatograms of both cultures of *N. gaditana*. The series also comprised mono- and di-unsaturated aliphatic alcohols with carbon numbers from 29 to 34. The alkenol $C_{32:1}$ was the predominant series member for both cultures, its relative abundance reached 2.6% and 0.8% of sterols and long-chain alcohols. The distribution of long-chain *n*-alkanols and unsaturated alcohols resembles that reported in other marine species of *Nannochloropsis*, in which the variable relative proportion of $C_{32:2}$ was sometimes higher (Volkman et al., 1992).

3.1.3. Mid-chain n-alkyl diols

Saturated and monounsaturated C_{28}–C_{36} alkyl diols were identified from their GC–MS spectra, and

Table 1
Percentage of base-hydrolyzed alcohols of two cultures of *Nannochloropsis gaditana*, of *Brachionus plicatilis* fed *N. gaditana*, and of its fecal pellets

	N. gaditana #1	*N. gaditana* #2	*B. plicatilis*	Fecal pellets
Sterols				
Cholest-5-en-3β-ol	45.6	40.1	43.9	39.1
5α(H)-Cholestan-3β-ol	0.5	n.d.	2.0	1.2
Cholesta-5,7-dien-3β-ol	0.2	0.3	4.9	2.2
Cholest-7-en-3β-ol	0.1	0.2	3.4	1.6
24-Methycholest-5-en-3β-ol	0.2	n.d.	n.d.	n.d.
24-Ethylcholesta-5,24(28)Z-dien-3β-ol	0.8	n.d.	n.d.	n.d.
24-Ethylcholest-5-en-3β-ol	28.8	34.8	21.8	16.4
5α(H)-24-Ethyl-cholestan-3β-ol	n.d.	n.d.	2.4	1.0
24-Ethylcholesta-5,24(28)E-dien-3β-ol	0.2	0.5	0.4	0.9
24-Ethylcholesta-5,7-dien-3β-ol	n.d.	n.d.	3.0	1.0
24-Ethylcholest-7-en-3β-ol	0.2	0.1	4.1	1.7
24-Ethyl-cholestadien-7,24(28)-3β-ol	n.d.	n.d.	0.3	n.d.
Sum of sterols	76.6	76.0	86.2	65.1
Long chain n-alkan-1-ols and unsaturated alcohols				
C_{28}	0.2	0.1	0.2	0.2
$C_{29:2}$	1.3	0.9	0.7	1.8
$C_{29:1}$	0.8	0.3	0.5	1.3
C_{30}	0.4	0.0	0.2	1.5
$C_{30:1}$	2.0	0.3	2.1	4.3
$C_{31:1}$	0.2	0.6	0.4	0.8
C_{31}	0.2	0.1	n.d.	0.1
$C_{32:2}$	0.1	0.1	0.9	0.7
$C_{32:1}$	2.6	0.8	4.5	11.6
C_{32}	0.1	0.1	n.d.	n.d.
C_{34}	0.1	n.d.	n.d.	n.d.
Sum of long chain alcohols	8.0	3.3	9.5	22.4
n-Alkyl diols and hydroxy alkenones				
C_{28}	n.d.	tr	n.d.	n.d.
$C_{29:1}$	tr	tr	n.d.	n.d.
C_{29}	tr	n.d.	n.d.	n.d.
C_{30}	2.3	0.9	0.8	2.2
K_{30}	0.1	0.6	n.d.	n.d.
C_{31}	1.3	1.1	1.1	2.0
K_{31}	tr	tr	n.d.	n.d.
$C_{32:1}$	0.9	3.8	0.2	0.4
C_{32}	7.7	10.1	1.4	4.7
$K_{32:1}$	tr	tr	n.d.	n.d.
K_{32}	0.3	0.1	0.2	0.2
C_{33}	0.3	0.3	n.d.	0.2
$C_{34:1}$	0.1	0.3	n.d.	n.d.
C_{34}	1.1	0.8	0.2	0.9
C_{35}	0.3	0.3	0.1	0.5
$C_{36:1}$	0.2	1.5	0.1	0.3
C_{36}	1.0	1.1	0.2	1.2
Sum of alkyl diols and hydroxy ketones	15.6	20.9	4.3	12.6

n-Alkan-1-ols, unsaturated alcohols and alkyl diols are labeled C_x or $C_{x:y}$, and hydroxy ketones are labeled K_x or $K_{x:y}$. The numbers x and y correspond to the number of carbon atoms and of double bonds, respectively. tr: trace levels; n.d.: not detected.

contributed to 15.2–20.2% of sterols, long-chain alco-hols, diols and hydroxy ketones in both cultures of *N. gaditana* (for alkyl diol general structures, see Versteegh et al., 1997). The major alkyl diol was C_{32}-alkane-1,15-diol, whose spectrum has already been described (Volkman et al., 1992). Diol mass spectra showed major ions at m/z corresponding to cleavage α to either side of the mid-chain OTMSi group. In each chromatographically-resolved peak, several ion pairs due to α cleavage were present, showing the coelution of various isomers. The series of alkyl diols was composed by homologues with an alkyl chain from C_{28} to C_{36}, with C_{32} the dominant series member (Fig. 1). The chromatogram showed signals before the peaks of saturated diols, they correspond to monounsaturated homologues (Fig. 1). Both cultures of *N. gaditana* contain monounsaturated alkyl diols with carbon atom ranging from 29 to 36 carbon atoms and m/z values indicated that the double bond was located between the mid-chain hydroxy-group and the methyl end of the alkyl diol. C_{28} and C_{29} saturated alkyl

diols and monounsaturated $C_{29:1}$ were characterized on GC–MS traces but they were present in too small quantities to be properly quantified by GC.

The relative proportions of alkyl diol positional iso-mers could be estimated from peak areas integrated on the ion currents specific of cleavage α to OTMSi-ethers at various positions (Volkman et al., 1999). For instance, in the elution zone of the C_{32} diol, peaks at m/z $355+373$, $341+387$, $327+401$, $313+415$ and $299+429$ were integrated. Each peak area provides the relative abundance of the isomer, with the mid-chain OTMSi in position 14, 15, 16, 17 and 18, respectively, to the C_{32} isomer mixture. Abundances of alkyl diols isomers are represented in Fig. 2. For even-chain alkyl diols, the mid-chain hydroxyl group is mainly located at position ω16 (C_{28} diol), and at ω18 (C_{30}–C_{36} diols). Together with these major isomers, up to four minor isomers coeluted with the mid-chain OTMSi group in position ω14, ω15, ω17, ω19 or ω20. Up to four different isomers were also present in the mixture coeluting to form the
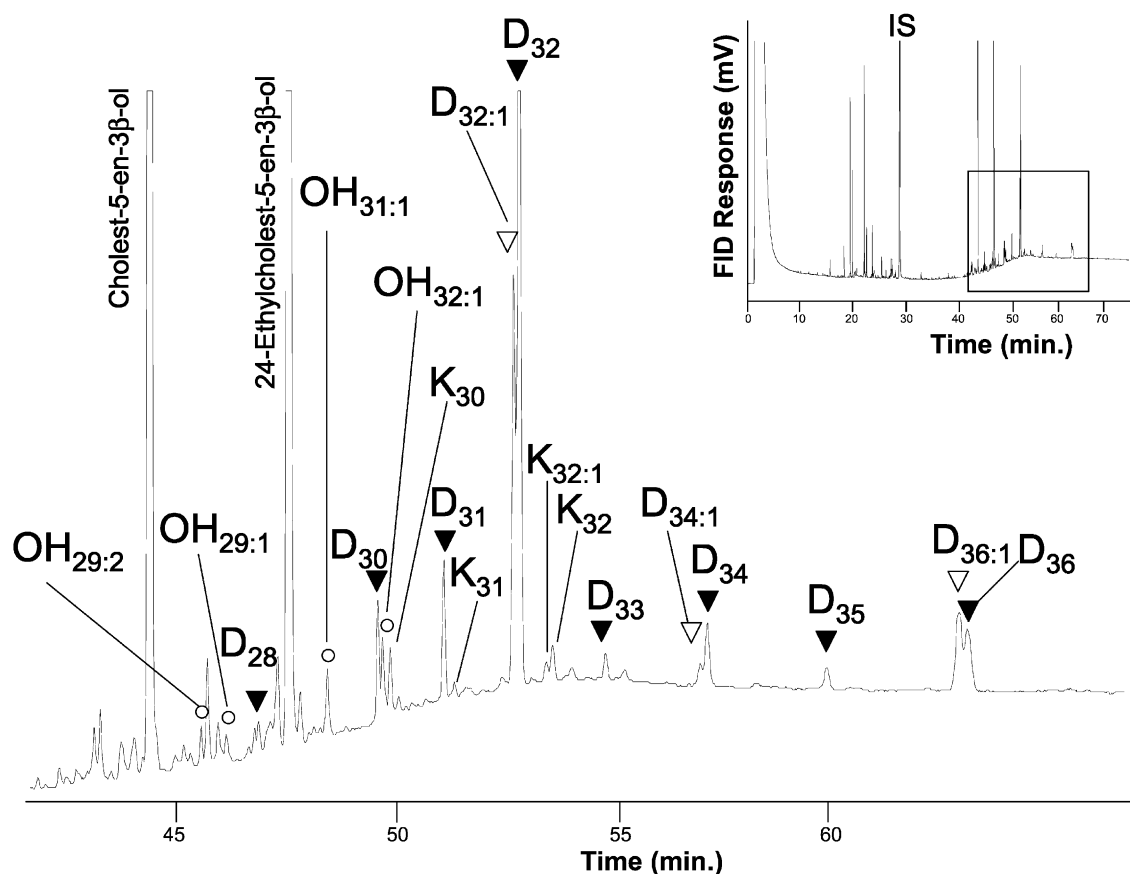

Fig. 1. GC–MS Total Ion Current (TIC) of the sterols and long-chain alcohols of *Nannochloropsis gaditana*. (IS: internal standard, β-epicoprostanol) and enlarged section. The partial TIC shows the TMSi ethers of sterols and long-chain hydroxy compounds. TMSi ethers are labeled as follows: Open circles: *n*-alken-1-ols or *n*-alkadien-1-ols [$OH_{x:y}$], open triangles: unsaturated alkyl diols [$D_{x:y}$], filled triangles: alkyl diols [D_x], K_x, hydroxy ketones. The numbers *x* and *y* stand for the number of carbon atoms and of double bonds, respectively.

peaks of odd-chain alkyl diols, with ω17 isomers predominating for odd-chain C_{29}–C_{35} alkyl diols. Unsaturated $C_{29:1}$ and $C_{35:1}$ alkyl diols were identified at trace level, and locations of the mid-chain OTMSi group were tentatively determined at position ω18 and ω17. Positional isomers of alkyl diols detected in *N. gaditana* are compared to the alkyl diols of other marine eustigmatophytes in Table 2.

A compound of molecular mass 508 bearing a mid-chain hydroxyl group eluted before the C_{30} diol and its structure could not be fully elucidated. Its abundance was less than 1% of sterols and long-chain alcohols, and a similar unknown compound was also described in other species of *Nannochloropsis* (Volkman et al., 1999).

3.1.4. Hydroxy ketones

Mid-chain keto-alkan-1-ols (hydroxy ketones or keto-ols) were detected at trace levels in the cultures of *N. gaditana*. They elute on the downslope of peaks of saturated alkyl diols and were detected in the carbon range of C_{30}–C_{32}. The C_{32} hydroxy ketone dominated over the other homologues and was abundant enough to be quantified on GC traces. Spectra of hydroxy ketones from *N. gaditana* are presented in Fig. 3. The prominent fragment at m/z 130 is produced by secondary McLafferty rearrangements of methyl end fragments and is a typical feature of their fragmentation. In the higher m/z range the fragment that corresponds to loss of a methyl group from the molecular ion $[M-15]^+$ is clearly visible, whereas the molecular ion itself is not always visible (Fig. 3). In these spectra, McLafferty rearrangements on both sides of the keto group yield even ions in the m/z range between 300 and 382. Even fragments of m/z values 300, 314 or 328 are yielded by McLafferty on the methyl side of the hydroxyl ketone with an H in the γ position of the keto group (de Leeuw et al., 1981). These fragments are present together with their corresponding $[M-15]^+$
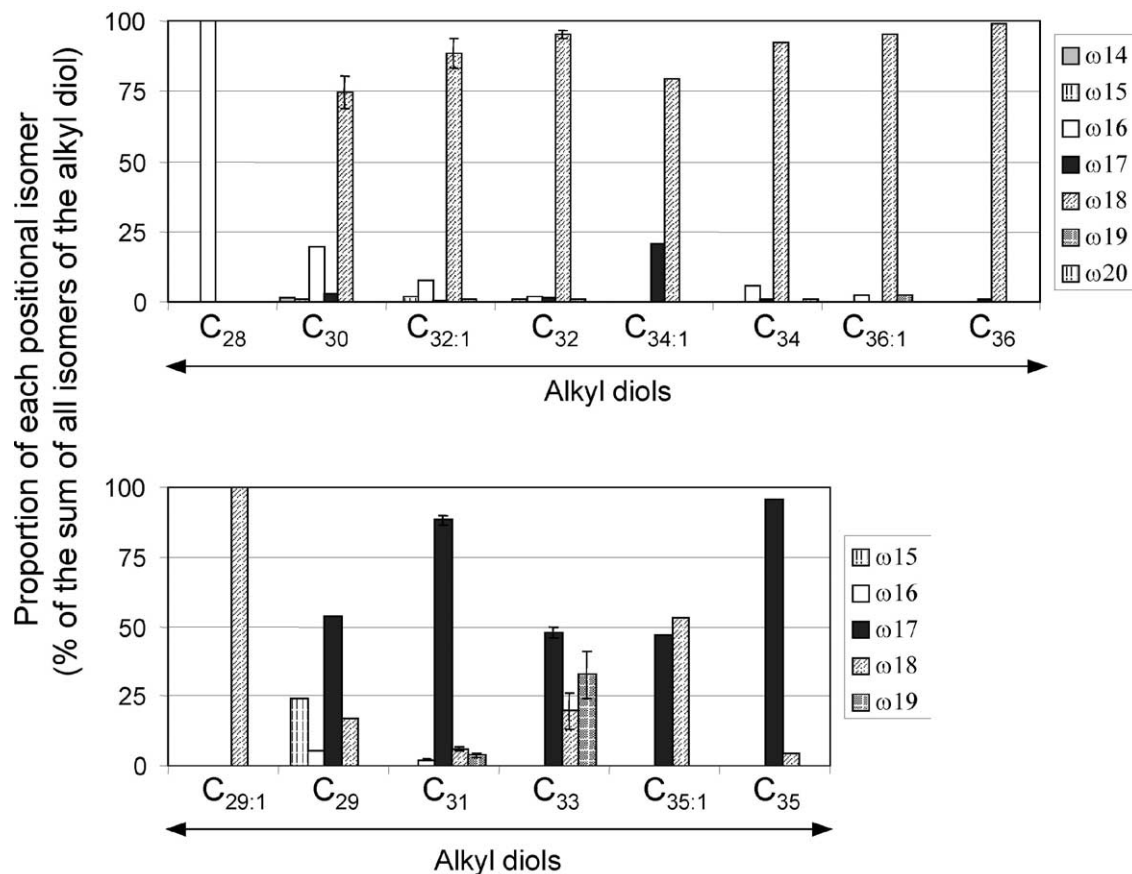

Fig. 2. Relative abundance of positional isomers of alkyl diols identified in *Nannochloropsis gaditana*. The proportion of isomers having the mid-chain hydroxyl in distinct locations was determined by integrating the peaks in the single ion currents corresponding to cleavages α to the mid-chain hydroxyl group. For each saturated or unsaturated alkyl diol, peak areas are expressed as a percentage of the sum of the areas of all isomers of a given homologue. The isomers are identified by the ω nomenclature; ω16 means that the hydroxyl is located on the 16th carbon atom from the methyl end. Error bars indicate the variation between cultures of *N. gaditana*.

ions. Fragments of m/z values 326, 340, 354, 368, 382 occurring with their $[M-15]^+$ homologues, are produced by trimethylsilyl rearrangments and cleavage in the β position of the keto group on the functionalized side of the original hydroxy ketone (de Leeuw et al., 1981). In the spectra of the C_{32} hydroxy ketone only the 15-keto isomer was identified. In contrast, the C_{30} hydroxy ketone signal contains both the predominant 1,13 isomer together with the 1,15 isomer in minor amount. Similarly, the C_{31} hydroxy ketone peak reveals the occurrence of both the 1,15 and 1,14 isomers. The low levels of detected hydroxy ketones were not propitious for semi-quantification of the various isomers using integration of currents typical of the location of the mid-chain keto group. The peak eluting before the C_{32} hydroxy ketone corresponds to the monounsaturated homologue. Its mass spectrum showed intense ions at m/z 352 and 337, as opposed to the intense signals at m/z 354 and 339 in the saturated homologue, which situates the double bond on the alkyl chain between the keto group and the methyl end.

3.2. B. plicatilis *and its fecal pellets*

Alcohols and sterol composition of the rotifer and of its fecal pellets showed similarities and resembled the composition of its diet, *N. gaditana*. The GC traces were dominated by cholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol, with a smaller proportion of the latter with respect to the diet. Small quantities of corresponding stanols accounted for 2.2–4.4% of sterols and long chain alcohols (Table 1).

Alkyl diols and hydroxy ketones were detected in the GC and GC–MS traces from *B. plicatilis* and its fecal pellets. The alkyl diol series comprised the same homologues as in *N. gaditana*, with small differences in relative abundance between the algae and the rotifer (Table 1). For instance, the predominance of C_{32} diols over the C_{30} and C_{31} homologues was less marked in *B. plicatilis* than in the eustigmatophyte. Only the C_{32} hydroxy ketone could be quantified in the rotifer and in the fecal pellets.

The same long-chain alkenols as in the diet were present in both the animal and its fecal pellets in percentages higher than those in *N. gaditana* samples (Table 1).

Table 2
Occurrence of alkyl diols in marine eustigmatophytes

	Alkyl diols													
	C_{28}	$C_{29:1}$	C_{29}	C_{30}	C_{31}	$C_{32:1}$	C_{32}	C_{33}	$C_{34:1}$	C_{34}	$C_{35:1}$	C_{35}	$C_{36:1}$	C_{36}
Phytoplantonic bloom (Morris and Brassell, 1988)	1,13		1,13	1,13 1,14 1,15	1,15	1,15	1,15							
Nannochloropsis oculata (Volkman et al., 1992)				1,13 1,15	1,15	1,15	1,15			1,15				
Eustigmatophyte CS-246 (Volkman et al., 1992)				1,13 1,15	1,15	1,15	1,15			1,15				
Nannochloropsis salina (Volkman et al., 1992)			1,13 1,15	1,15	1,15	1,15				1,15				
Nannochloropsis salina (Gelin, 1996)			1,13	1,13 1,15	1,15	1,15	1,15	1,17		1,17		1,19	1,19	1,19
Nannochloropsis salina (Gelin et al., 1997)	1,13			1,13 1,14 1,15	1,14		1,16			1,17				1,19
Nannochloropsis gaditana (this work)	1,13	1,12	1,12 1,13 1,14 1,16	1,13 1,14 1,15 1,16	1,13 1,14 1,15 1,16	1,14 1,15 1,16 1,17 1,18	1,14 1,15 1,16 1,17 1,18	1,15 1,16 1,17	1,17 1,18	1,15 1,16 1,17 1,18 1,19	1,18 1,19	1,17 1,18 1,19	1,18 1,19 1,20 1,21	1,18 1,19 1,20

This table compares the literature reviewed by Versteegh et al. (1997, Table 1) to the results of the present study. For each alkyl diol, positional isomers are indicated by positions of the hydroxy groups in the identified isomers.

4. Discussion

4.1. Alcohols of N. gaditana *and other eustigmatophytes*

Marine and freshwater strains of eustigmatophytes have cholest-5-en-3β-ol or 24-ethylcholest-5-en-3β-ol as the dominant sterol, respectively. In contrast to the marine *Nannochloropsis salina*, *N. gaditana* showed both sterols in comparable amounts, 56±5% and 42±3% of total sterols, respectively. Other detected sterols accounted for less than 3% of total sterols. As underlined by Volkman et al. (1999), sedimentary C_{29} sterols may partly originate from eustigmatophytes, especially if alkyl diols are also present.

N. gaditana encompasses three classes of long-chain hydroxy compounds: saturated and unsaturated alkyl diols, alken-1-ols and saturated and unsaturated hydroxy ketones. Both former classes of lipids have been described in other eustigmatophytes where they mostly occur as ester-linked and sulfate- or amide-bound polar lipids, and they are also constituents of macromolecular algenans (Gelin et al., 1997; Volkman et al., 1992). The predominant alkyl diols in *N. gaditana* are similar to that of other marine eustigmatophytes (Table 2). A $C_{32:1}$ alkyl diol has been reported in a similar proportion to the saturated C_{32} homologue in *N. salina* (Grossi et al., 2001; Volkman et al., 1999), while the $C_{36:1}$ alkyl diol is rarely mentioned (Table 2). In *N. gaditana* the dominant mid-chain hydroxy isomer of each alkyl diol compares well with that of other marine eustigmatophytes. The alkyl diols isomers cover a wider isomerism range than in the other marine species (Table 2), however additional isomers only occurred at trace levels.

From the similarity in chain length distribution of alken-1-ol and alkyl diols, a biosynthetic pathway hydroxylating alkenols into alkyl diols has been proposed (Volkman et al., 1992). In *N. gaditana*, the position of the mid-chain hydroxyl group of alkyl diols is rather constant with regard to distance from the methyl end. For alkyl diols with an even alkyl chain length between 30 and 36 carbon atoms, the ω18 isomer pre-
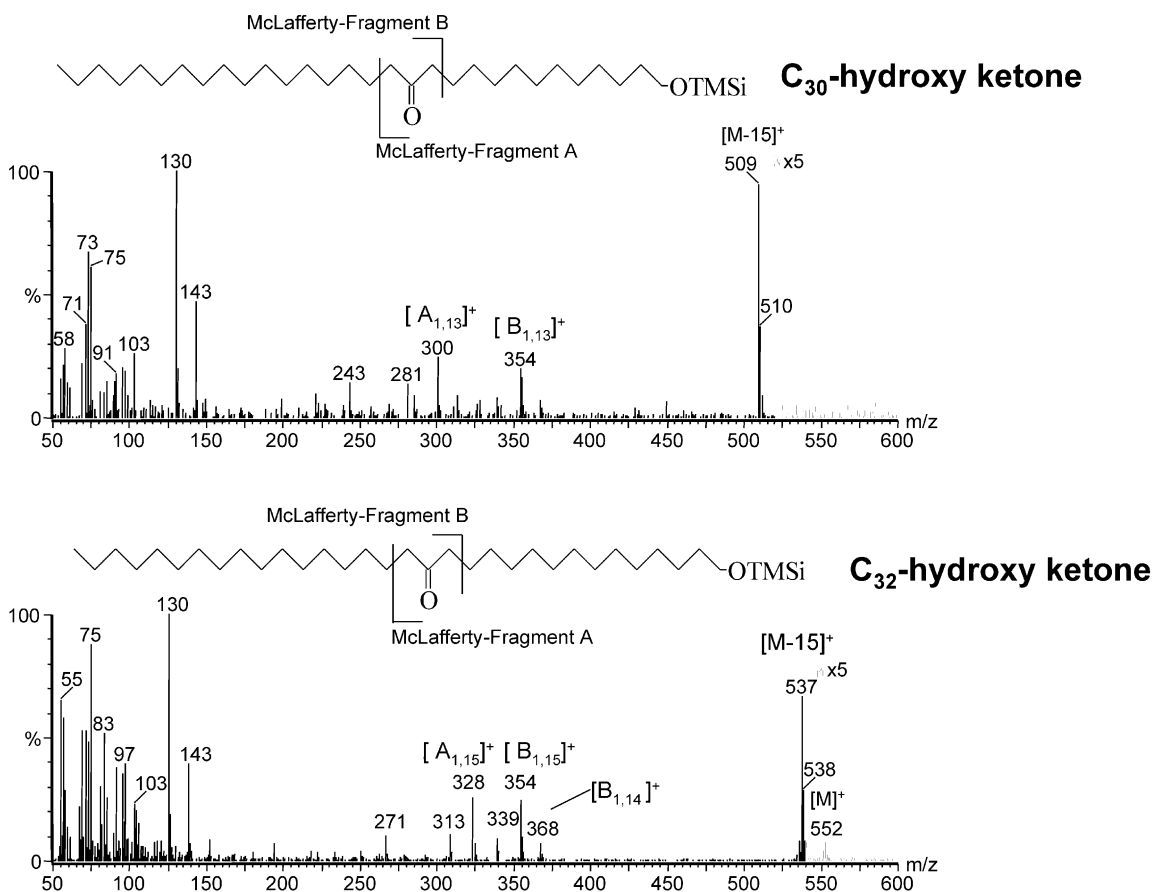

Fig. 3. Mass spectra of the TMSi ethers of hydroxy ketones (E.I; 70 eV). Top: spectrum of the C_{30}-hydroxy ketone (1-hydroxy,13-keto-triacontane), and bottom: spectrum of the C_{32}-hydroxy ketone (1-hydroxy,15-keto-isomer). The signal at *m/z* 368 indicates the coelution of a 1,14-C_{32} positional isomer.

vails, whereas for the C_{28}-alkyl diol the dominant position of the hydroxyl group was ω16 (Fig. 2). This suggests that even alkyl diol precursors might have been a series of alken-1-ols with 28–36 carbon atoms and a double bond at C-16 or C-18 from the methyl end. Since predominant odd-chain alkyl diols mostly belong to the ω17 series, their precursor odd alkenols should have born a double bond at the same distance from the methyl end. The complete series of these hypothetical precursors would comprise odd and even C_{28}–C_{36} alkenols. However, the present appraisal of *N. gaditana* alcohols fails to show such a complete series of alkenols. Although saturated, monounsaturated and diunsaturated alcohols in the carbon range C_{28}–C_{34} showed variable relative patterns from one culture of *N. gaditana* to the other, prevailing homologues are characteristic of marine eustigmatophytes (Gelin et al., 1997; Grossi et al., 2001; Volkman et al., 1992, 1999). This supports long-chain alkenol distribution criteria, as elaborated for the different algal genus by Volkman et al. (1999).

The identification of hydroxy ketones in *N. gaditana* provides the first report of the occurrence of these compounds in water-column living marine organisms, having chain lengths and mid-chain carbonyl group positions, similar to those detected in marine sediments. To date, mid-chain keto-ols have been detected in neither eustigmatophytes nor in particulate material (Versteegh et al., 2000). Noteworthy, the disproportion in alkyl diol and hydroxy ketone amounts, observed in *N. gaditana*, makes the detection of the latter difficult. The

occurrence of hydroxy ketones as cellular constituents of *N. gaditana* suggests that the deposition of algal remains to marine sediments may, at least to some extent, contribute to the sedimentary hydroxy ketones. However, hydroxy ketones and alkyl diols are within the same order of magnitude in marine sediments whereas a marked disproportion is observed in the alga *N. gaditana*. Accordingly, either yet additional unidentified biogenic sources of hydroxy ketones also contribute to these compounds or hydroxy ketones are considerably enriched by prediagenesis with respect to alkyl diols. In this respect, the oxidation of alkyl diols to hydroxy ketones was not observed to occur in the course of anaerobic degradation of *N. salina* (Grossi et al., 2001).

Hydroxy ketones in *N. gaditana* parallel the distribution of alkyl diols both in terms of carbon length and even-predominance. The C_{32} hydroxy ketone dominates over the monounsaturated $C_{32:1}$ congener and the double bond is located between the mid chain carbonyl group and the methyl end of the hydroxy ketone, as it occurs for the $C_{32:1}$ alkyl diol. Even hydroxy ketones belonged to the ω18 series whereas C_{31} hydroxy ketones presented mid-chain functionalities both at C-18 and C-17, matching the isomer distribution of alkyl diols (Fig. 4). All these correspondences between homologous distributions of the mid-chain hydroxy and carbonyl groups suggest a common biosynthetic relationship between both lipid classes.

4.2. Fate of algal alcohols throughout herbivore grazing

The alcohols detected in *B. plicatilis* comprise alcohols from the rotifer tissues and from ingested algae in its gut. Alkyl diols, hydroxy ketones and long-chain *n*-alken-1-ols are not constituents of zooplankton tissues, therefore these alcohol classes extracted from *B. plicatilis* more likely stem from eustigmatophytes in the rotifer guts. The lipid pattern of *B. plicatilis* fed on *N. gaditana* may reflect changes affecting algal long-chain alcohols during digestion. Lipids in the fecal pellets are the remains of *N. gaditana* that may be vertically exported, with a possible contribution of constituents of the rotifer. For biomarkers that can be specifically ascribed to the alga, the three sample types (the alga itself, the digestive tracts of *B. plicatilis* and its fecal pellets) afford pertinent insight on how digestion gradually alters the composition of hydroxy compounds.

4.2.1. General grazing effect on alcohol profile

Although alcohols of *B. plicatilis* were not analyzed when the rotifer was fed another diet than was *N. gaditana*, the literature notes which sterols are synthesized by the rotifer and which derive from its diet. It has been shown that cholest-5-en-3β-ol, 5α(H)-cholest-5-en-3β-ol and cholest-7-en-3β-ol are synthesized by *B. plicatilis* whereas C_{28}- and C_{29}-sterols, also present in the rotifer,
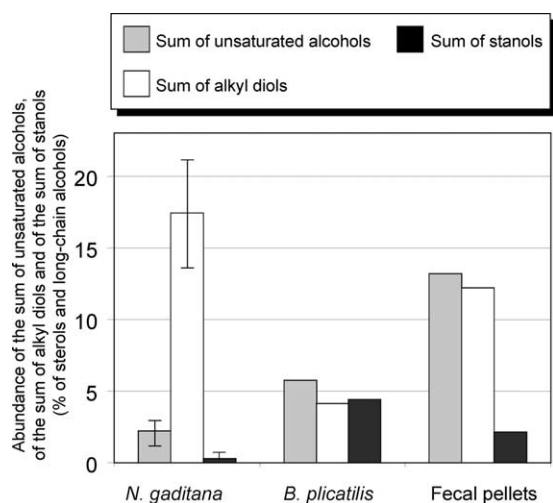
Fig. 4. Evolution of the relative composition of alcohol classes upon digestion of *Nannochloropsis gaditana* by *Brachionus plicatilis*. The sum of unsaturated alcohols, alkyl diols and stanols is represented in% of the sum of sterols and long chain alcohols. Error bars indicate the variation between duplicate cultures of *N. gaditana*.

were sourced from its diet (Teshima et al., 1979). Thus, C_{27} sterols only can be contributed by *B. plicatilis*, in the sample of the rotifer and of its fecal pellets. The ratio values of 5α(H)-cholestan-3β-ol/cholest-5-en-3β-ol and 5α(H)-24-ethyl-cholestan-3β-ol/24-ethylcholest-5-en-3β-ol are higher in *B. plicatilis* and in its fecal pellets than in the alga, indicating that sterols are partially reduced to stanols in the course of the digestion or that these biomarkers are sourced by the rotifer (Fig. 4).

Apart from C_{27} sterols and stanols, not all hydroxy-compounds identified in Table 1 are synthesized by *B. plicatilis*. The proportion between different classes of algal alcohols is slightly altered in the fecal pellets, for instance alkyl diol abundance decreases in relation to that of unsaturated long-chain alcohols (*n*-alken-1-ols and *n*-alka-dien-1-ol) and may reflect differences in stability of these compounds (Fig. 4). Degradation of *N. salina* cells by bacteria also showed a faster decay of alkyl diols relatively to long-chain alkenols (Grossi et al., 2001). The mode of occurrence of these alcohol types may also influence their apparent stability. Acid hydrolysis of *N. oculata* and *N. salina* yielded higher amounts of long-chain alkenols and alkyl diols than base hydrolysis, showing that alkyl diols and unsaturated alcohols occurred as ether or other polar bound lipids (Volkman et al., 1992). In addition, the higher recovery of unsaturated alcohols by acid hydrolysis than by saponification was relatively more pronounced than

for alkyl diols lipids (Volkman et al., 1992). If gut micro flora possesses the enzymatic capability of hydrolyzing non saponificable lipids, they might release an additional pool of alkyl diols or alkenols, which could explain the molecular changes affecting these lipids during the feeding experiment.

Digestion did not significantly change the molecular profile of di- and mono-unsaturated long-chain aliphatic alcohols nor the ratio of saturated to unsaturated alkyl diols of same chain length. This result agrees with observations from anaerobic bacterial-mediated degradation (Grossi et al., 2001). Another parallelism between both studies is the heterogeneous evolution of alkyl diols of distinct chain length. C_{33}–C_{36} saturated and unsaturated diols accounted for about the same proportion of total alkyl diols in the alga, in the rotifer and in its fecal pellets. In contrast, C_{30} and C_{31} alkyl diol proportions increased relative to those of the C_{32} and $C_{32:1}$ congeners in the course of the digestion (Fig. 5). This difference may be due to the release of a pool of alkyl diols with a lower abundance in C_{32} homologues than in base-hydrolyzed lipids. The fecal alkyl diol pattern coincides with sedimentary C_{30}- and C_{32}-alkyl diol proportions (Versteegh et al., 1997), but does not reproduce the low C_{31}-diol abundance observed in sediments. Whether the change in chain length pattern is representative of grazing-related changes or is purely accidental needs further confirmation.
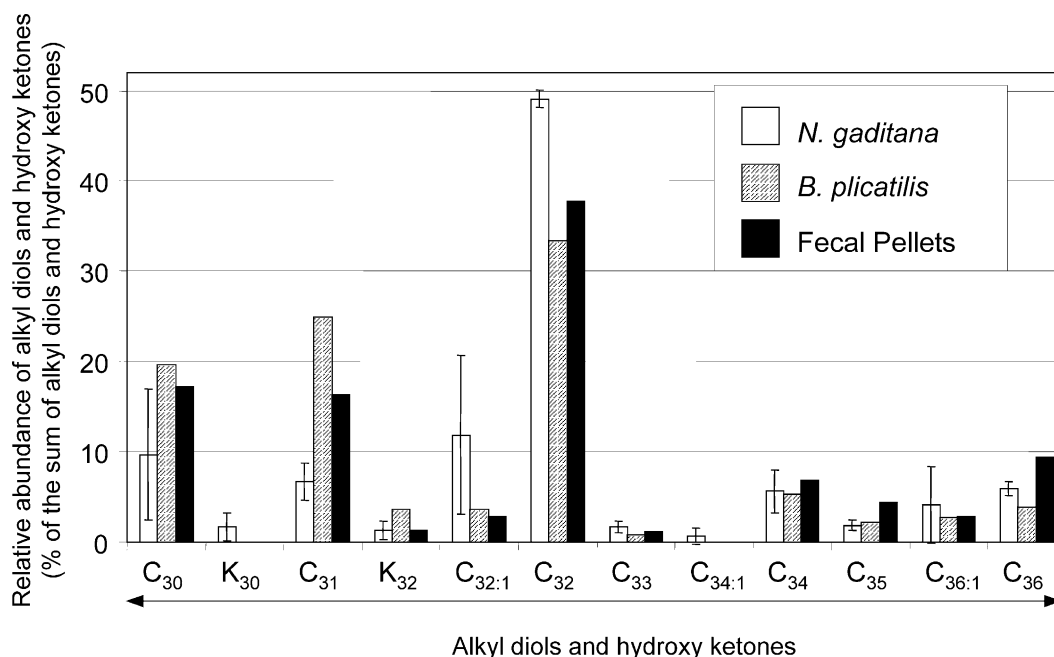

Fig. 5. Impact of digestion on the profile of long-chain alkyl diols and hydroxy ketones of *Nannochloropsis gaditana*. Alkyl diol and hydroxy ketone abundances are represented as a percent of the sum of alkyl diols and hydroxy ketones. Error bars indicate the variation between duplicate cultures of *N. gaditana*.
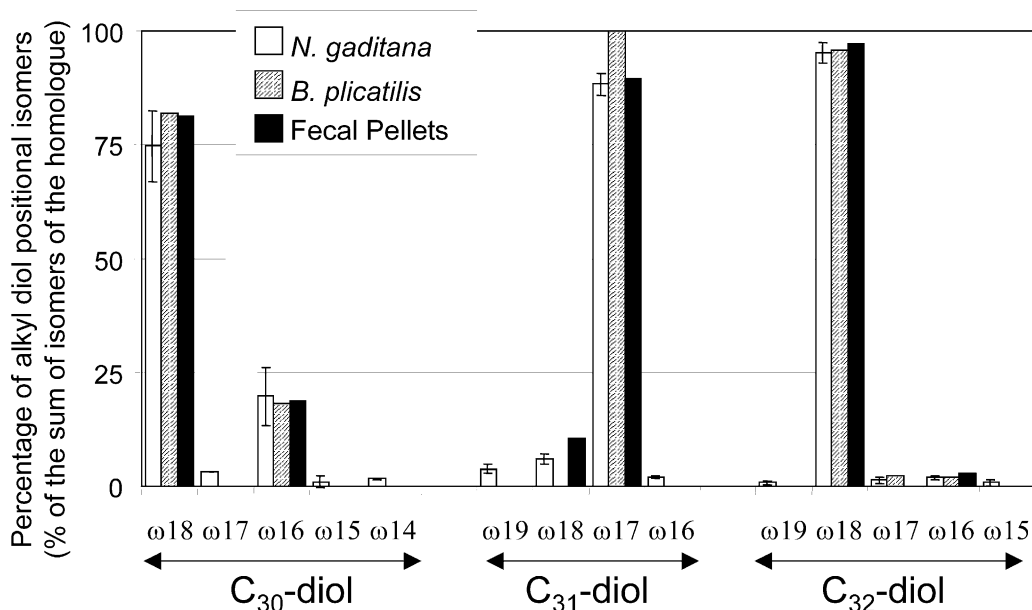
Fig. 6. Impact of grazing on the pattern of alkyl diol positional isomers of *Nannochloropsis gaditana*. For each alkyl diol, isomers with the mid-chain hydroxyl at different locations are represented in percentages of the sum of the isomers. The isomers are identified by the ω nomenclature; ω16 means that the hydroxyl is located on the 16th carbon atom from the methyl end. Error bars indicate the variation between duplicate cultures of *N. gaditana*.

4.2.2. Changes in positional isomer composition of alkyl diols and in "diol index"

The compositional changes in alkyl diol distribution raises the question of the stability of mid-chain positional isomer composition during digestion. The isomer profile of the C_{30} alkyl diols in *B. plicatilis* and in its fecal pellets lies within the range of variation of the cultures of *N. gaditana* even though all minor isomers could not be detected in the rotifer and in the fecal pellets (Fig. 6). Similarly, the isomer profiles of C_{31} and C_{32} alkyl diols are also stable with respect to digestion (Fig. 6). Therefore the alkyl diol isomer pattern is well preserved during digestion, and this stability prompts further assessment of alkyl diol isomer relationships to environmental conditions.

The composition of alkyl diol in marine sediments has shown promises for tracing paleoenvironmental conditions, especially the index defined as (Versteegh et al., 1997, 2000):

$$\text{Diol Index} = 100$$
$$\times [1, 15 - C_{30}\text{diol}]/([1, 15 - C_{30}\text{diol}] + [1, 15 - C_{32}\text{diol}])$$

This index is determined both by the isomer pattern of C_{30} and C_{32} alkyl diols and by the abundance of homologues with 30 and 32 carbon atoms. As previously discussed, the isomer pattern is conserved by digestion whereas the distribution of C_{30} and C_{32} homologues is noticeably altered. Consequently, the

stability of the Diol Index during digestion can be questioned. In order to assess the impact of digestion on this index, its values in *N. gaditana*, *B. plicatilis* and its fecal pellets were calculated and compared. In cultures of *N. gaditana* they are 20 and 6, values that lie within the range of values compiled from algal cultures in the review of Versteegh et al. (1997). In *B. plicatilis* and its fecal pellets, the diol index accounted for 37 and 28, respectively. The slight increase in diol index during digestion is due to changes in the distribution of alkyl diols with 30 and 32 carbon atoms (Figs. 5 and 6). This suggests that the index values may reflect grazing processes as well as environmental conditions of the euphotic zone and prompts further work to decipher the meaning of the diol index values recorded in sediments.

5. Conclusions

Sterols and long-chain alcohols of *N. gaditana* are similar to those of other marine eustigmatophytes, except for the occurrence of long-chain hydroxy ketones (ketools), reported for the first time in a marine organism. When the rotifer *B. plicatilis* was fed with *N. gaditana*, long chain unsaturated alcohols, alkyl diols and hydroxy ketones were observed in the fecal pellets. Changes related to digestion affected the alkyl diol homologous chain length distribution whereas the distribution in positional isomers remained close to that of the alga.

Acknowledgements

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