Factors influencing the distribution of polyunsaturated terpenoids in the diatom Rhizololenia setigera

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Factors influencing the distributions of polyunsaturated terpenoids in the diatom, *Rhizosolenia setigera*

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Abstract

Polyunsaturated highly branched isoprenoid (HBI) hydrocarbon distributions of laboratory cultures of five strains of the planktonic diatom *Rhizosolenia setigera* (Brightwell) are shown herein to be highly variable. Some strains produced both haslenes with from three to five double bonds and rhizenes. The haslenes comprised not only Δ5 alkenes but also those with C7(20) unsaturation, including hasla-7(20),9*E*,*Z*, 23-trienes and hasla-7(20),9*E*,*Z*-13, 23-tetraenes. The rhizenes contained C7(25) unsaturation and the vinyl moiety common to all algal haslenes so far characterised. The effects of temperature and salinity on HBI composition, along with isotopic content, were determined in strain CS 389/A. Increase in growth temperature from 18 to 25 °C increased the degree of unsaturation in the haslenes and *E* to *Z* isomerisation in the triene. There was also an increase in unsaturation in the rhizenes at the highest growth temperature, with hexaenes dominant over the pentaenes but in the rhizenes, *Z* to *E* isomerisation increased. Increased salinity from 15 to 35 psu increased cell growth and rhizene production but decreased haslene production. Unsaturation in haslenes was not changed by increased salinity but unsaturation in the rhizenes decreased. These may reflect growth rate differences. The carbon isotopic compositions of the haslenes and rhizenes were similar to that of the major sterol at 18 °C, but the major HBI isomers were 3–4 per mil depleted relative to phytol released by saponification from chlorophyll a. This suggests biosynthesis of HBIs from a different isotopic pool of isopentenyl biphosphate to that from which phytol is biosynthesised. At 25 °C, further isotopic differences were observed. The variables controlling HBI distributions in *R. setigera* are still not fully understood and rationalisation of the environmental controls on the sedimentary distributions of the HBIs from *R. setigera* may only be possible once such factors are established.

Keywords: *Rhizosolenia setigera*; Bacillariophyceae; Microalgae; Diatoms; Isoprenoid alkenes; Highly branched isoprenoids; C_{30} alkenes; Rhizenes; Haslenes

1. Introduction

The only highly branched isoprenoid (HBI) hydrocarbons reported to date in the diatom *Rhizosolenia setigera* (Brightwell) are either a C_{25} pentaene (I) or C_{30} alkenes (Volkman et al., 1994, 1998; Sinninghe Damsté et al., 1999a). We have proposed the name 'haslenes' for the HBI C_{25} alkenes (Rowland et al., 2001), because they were first reported (Volkman et al., 1994) from the diatom *Haslea ostrearia* (Simonsen). We now suggest the name 'rhizenes' for the corresponding C_{30} HBIs, as they were first reported from *R. setigera* (Volkman et al., 1994).

The single haslapentaene (**I**) identified thus far in *R. setigera*, has been characterised by NMR spectroscopy and comparison with data for the same compound in *H. ostrearia* (Wraige et al., 1997; Sinninghe Damsté et al., 1999b) and the double bond positions of four rhizenes have recently been established (Belt et al., 2001). However, the factors controlling the distributions of HBIs in *R. setigera* are at present unknown. These are important since HBIs are quite commonly reported in oceanic sedimenting particles (reviewed in Belt et al., 2000a) where they may be useful indicators of specific diatom inputs. Whilst a number of benthic diatoms are known to produce haslenes (e.g. several *Haslea* spp. and

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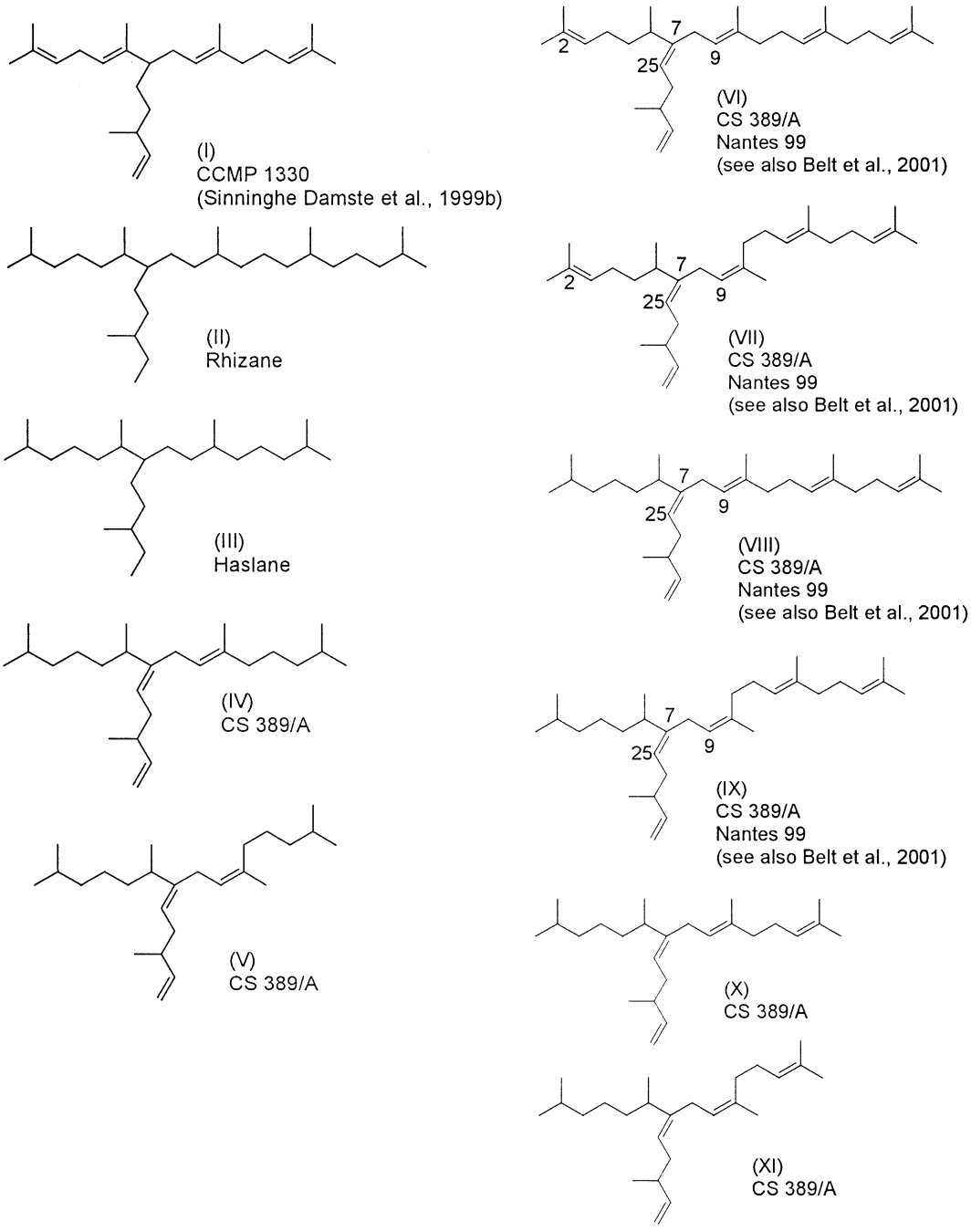

Pleurosigma intermedium; Belt et al., 2000a), *R. setigera* is to date the only marine planktonic diatom known to produce either haslene or rhizene HBIs.

The haslenes in estuarine sediments appear to allow resolution of the contributions of the benthic species to the total diatom flux (Cooke et al., 1998) and some haslenes also appear to possess cytostatic effects against human lung cancer cells in vitro (Rowland et al., 2001). It would therefore be interesting to know whether the rhizenes are equally geochemically specific and/or bioactive. Here, we report the haslenes and rhizenes of five strains of *R. setigera* and investigate the effects of growth temperature and salinity on haslene and rhizene production in one strain. Furthermore, we have investigated the effects of these variables on the carbon isotopic composition of the haslenes and rhizenes in this strain. Such data may eventually help to allow the environmental distributions of HBIs in sediments and sedimenting particles to be understood.

2. Results and discussion

R. setigera (Brightwell) is a common diatom often found in the plankton of coastal and marine waters

(Round et al., 1990). The taxonomy of the genus is however, somewhat uncertain (Round et al., 1990; Volkman et al., 1994, and references therein) and has been revised by Sundström (1986) and also been subject to several other modifications, including the transfer of the only two freshwater *Rhizosolenia* species to a new genus *Urosolenia* (Round et al., 1990).

The HBIs of three strains of *R. setigera* have been reported previously (Volkman et al., 1994, 1998; Sinninghe Damsté et al., 1999a) and, in contrast to the production of haslenes only in the benthic diatom *H. ostrearia*, the HBIs of *R. setigera* varied from rhizenes characterised by GCMS and hydrogenation to the parent rhizane (**II**) in two Australian strains CS-62 and CS-389/1 grown at 20 and 18.5 °C and 70–80 μE m^{-2} s^{-1} white light and 28 psu salinity (Volkman et al., 1994, 1998), to haslapentaene (**I**) only, in strain CCMP1330 grown at 4, 12 and 20 °C in North Atlantic seawater (Sinninghe Damsté et al., 1999a). Since the distributions in the previous reports were rather different from one another, we decided to examine further the hydrocarbons of strain CCMP 1330 and four other strains of *R. setigera*.

2.1. *R. setigera CS strain 389/A*

2.1.1. *HBI identifications and partial structural characterisation of rhizenes*

A 10 l culture of *R. setigera* was grown from an inoculum obtained from the Huon estuary, Tasmania, Australia. The wet paste obtained after centrifugation was extracted by allowing it to stand in chloroform (24 h). This yielded 220 mg of total chloroform extractable lipids. Microscale hydrogenation (Adam's catalyst) of a small aliquot of the total lipids of a culture grown at 18 °C (35 psu salinity) produced rhizane (**II**) and haslane (**III**) as the only HBIs, identified by GCMS and GC retention indices compared to the synthetic alkanes (Robson and Rowland, 1986, 1988). A small amount of an unidentified C_{30} alkane (RI $2543_{HP\text{-}5}$) with two degrees of unsaturation (ca. 10% relative to **II**) was also present (cf Prahl et al., 1980).

The total chloroform extract was further fractionated by open column chromatography on silica and fractions containing respectively, two C_{25} trienes, three C_{30} pentaenes, and three C_{30} hexaenes (identified by GCMS) obtained by elution with successive volumes of pentane. GCMS, ^{1}H and ^{13}C NMR spectroscopy revealed that the C_{25} trienes were identical to those recently identified in *Pleurosigma intermedium* (Belt et al., 2000a) viz. hasla-7(20),9*E*/*Z*,23-trienes (**IV** and **V**). Both *E* (major) and *Z* isomers were present. These Δ7(20) haslenes are therefore different to those of *H. ostrearia* and indeed of *R. setigera* strain CCMP 1330 (Sinninghe Damsté et al., 1999a) which contained a Δ5 double bond (**I**).

The presence of inseparable mixtures of three C_{30} alkenes in each of the pentaene and hexaene fractions precluded full structural characterisations by NMR, but a number of features were nonetheless clear. Namely, the rhizenes all contained a vinyl moiety (Δ28)—present in all haslenes and rhizenes identified in diatoms thus far—a number of trisubstituted double bonds and, consistent with the presence of the haslatrienes (**IV** and **V**), Δ7(25) unsaturation (^{1}H NMR: δ 5.71, 1H, *ddd*, H-28; 4.9, 2H, *m*, H-29; ^{13}C NMR DEPT,COSY,COLOC; δ 144.6, C-28; 112, C-29;142, C-7). Full structural characterisation of four of the six alkenes (**VI**–**IX**) was possible upon isolation of two pairs of isomers from a further strain of *R. setigera,* Nantes 99 (vide infra; Belt et al., 2001).

Thus, this study has shown that *R. setigera* is able to biosynthesise simultaneously, haslenes *and* rhizenes, whereas previously only one or the other was reported, and that whilst *H. ostrearia* produces haslenes with Δ5 and Δ6(17) double bonds, *P. intermedium* and *R. setigera* apparently produce haslenes with Δ7(20) unsaturation. Interestingly, haslenes and rhizenes have been reported to co-occur in some marine sediments and the present findings raise the possibility that in such cases both may have derived from *R. setigera*.

2.1.2. *Effects of salinity and temperature on HBI distributions*

To investigate the controls on the concentrations and the apparent variability of haslene and rhizene distributions in *R. setigera,* small scale cultures of strain CS 389/A were grown at two salinities (18 °C; 15 and 35 psu) and three temperatures (10,18, 25 °C; 35 psu). Each culture was grown for three growth cycles then a single sample taken from each after the third growth cycle (i.e. four samples) before culturing and harvesting in triplicate after a fourth cycle (i.e. a further 12 samples). This multiple cycle culturing was designed to allow the algae to acclimate somewhat to each set of new culture conditions. Cell concentrations in the fourth growth cycle were monitored by a calibrated fluorescence method (days 0, 2, 4, 6, 7; Fig. 1) and by microscopy (days 0, 7: mean cell counts). Cells were harvested at day 7. Cultures were filtered and filters extracted with chloroform with ultrasonication after addition of *n*-docosane as internal standard for quantification by GCMS. Recovery of the internal standard was 137±17% ($n=12$). Extracts were also saponified, re-extracted into hexane and after silylation the non-saponifiable lipids were examined by GCMS and by GC-isotope ratio monitoring-MS (GC-irm-MS). The absolute concentrations of HBIs ranged from about 5 to 20 pg $cell^{-1}$. The cell concentrations in the day 7 samples were used to estimate these concentrations.

Cell growth was faster at 35 psu salinity than at 15 psu (Fig. 1) and at 35 psu total HBI production (mean 19.4±

0.6 pg cell^{-1}) was twice that at 15 psu (10.23±0.57 pg cell^{-1}; Fig. 2a). In contrast, haslene production was greatest at the lower salinity (Fig. 2b).

The ratio of rhizenes to haslenes was, however, much higher at 35 psu (Fig. 2c) due to the higher absolute concentrations of rhizenes. Whilst unsaturation in the *E,Z* Δ7(20) haslatrienes (**IV**, **V**) was unchanged by salinity (Fig. 3a and b)—as was also the case for the Δ6(17) triene in *H. ostrearia* (Wraige et al., 1998)—unsaturation within the rhizenes was changed and was higher at the lower salinity (Figs. 2d and 3a). Whether these variations can be attributed directly to the differences in salinity, or actually reflect the difference in growth rates, will require further investigation at further, intermediate, salinities.

At 35 psu salinity, *R. setigera* did not grow well after 4 days at 10 °C (Fig. 1) but at 18 and 25 °C growth was good to 7 days (Fig. 1). Variation of growth temperature from 10 to 18 to 25 °C produced a change in the total HBI production, which maximised at 18 °C (19.4± 0.6 pg cell^{-1}; Fig. 2e). [The mean value at 10 °C (Fig. 2e) should be treated as a maximum since the measurement was based on cell counts determined at 7 days where growth had decreased (Fig. 1).] Thus maximum HBI production in *R. setigera* occurred at 35 psu, 18 °C. This may reflect the conditions to which the strain had become best acclimated in culture previously.

Within the HBIs, maximum haslene production was observed at 25 °C, and there was a steady increase in haslene production with increasing temperature (Fig. 2f). In contrast, rhizene production maximised at 18 °C, leading to a maximum in the rhizene/haslene ratio at this temperature (Fig. 2g).

Unsaturation in the haslenes also varied with temperature; haslatrienes (**IV** and **V**) were accompanied by haslatetraenes (**X**, **XI**) at 25 °C (Fig. 4c). This variation of the degree of unsaturation in the haslenes of *R. setigera* with growth temperature in strain CS 389/A contrasts with the previous observation of the invariance in the single haslapentaene (**I**) produced by *R. setigera* strain CCMP 1330 with variation of temperature between 4 and 20 °C (Sinninghe Damste et al., 1999a). However, the degree of unsaturation in the two strains CCMP 1330 vs CS 389/A was quite different (viz. 5 vs 3 and 4 double bonds, respectively) and furthermore, haslenes in the CCMP 1330 strain had a Δ5 double bond whereas those of strain CS 389/A had 7(20) unsaturation. The variation of unsaturation with temperature in this strain is similar to, though less pronounced than, that observed in the Δ5 and Δ6(17) haslenes of *H. ostrearia* which varied from diene production at 5 °C to tetraene and pentaene production at 25 °C (Rowland et al., 2001). Isomerisation of the *E* to *Z* haslene isomers also increased with increasing temperature (Fig. 4).

Strain CS 389/A cultured herein, produced both the haslenes and rhizenes at all temperatures. In contrast, strain CS 389/1 cultured at 18.5 °C, produced only rhizenes (Volkman et al., 1994), suggesting that co-production of haslenes and rhizenes is not a general phenomenon even in *R. setigera* strains from the same

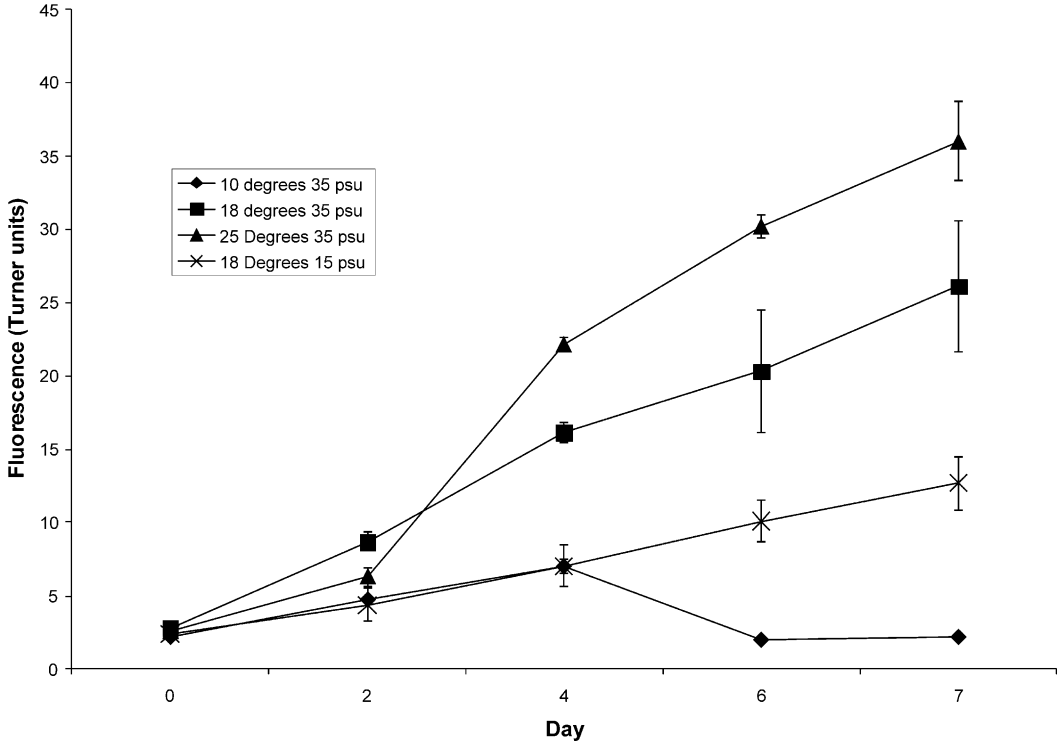

Fig. 1. Growth curves of *Rhizosolenia setigera* strain CS 389/A at 15 and 35 psu, 10, 18 and 25 °C ($n=3\pm1$ standard deviation).

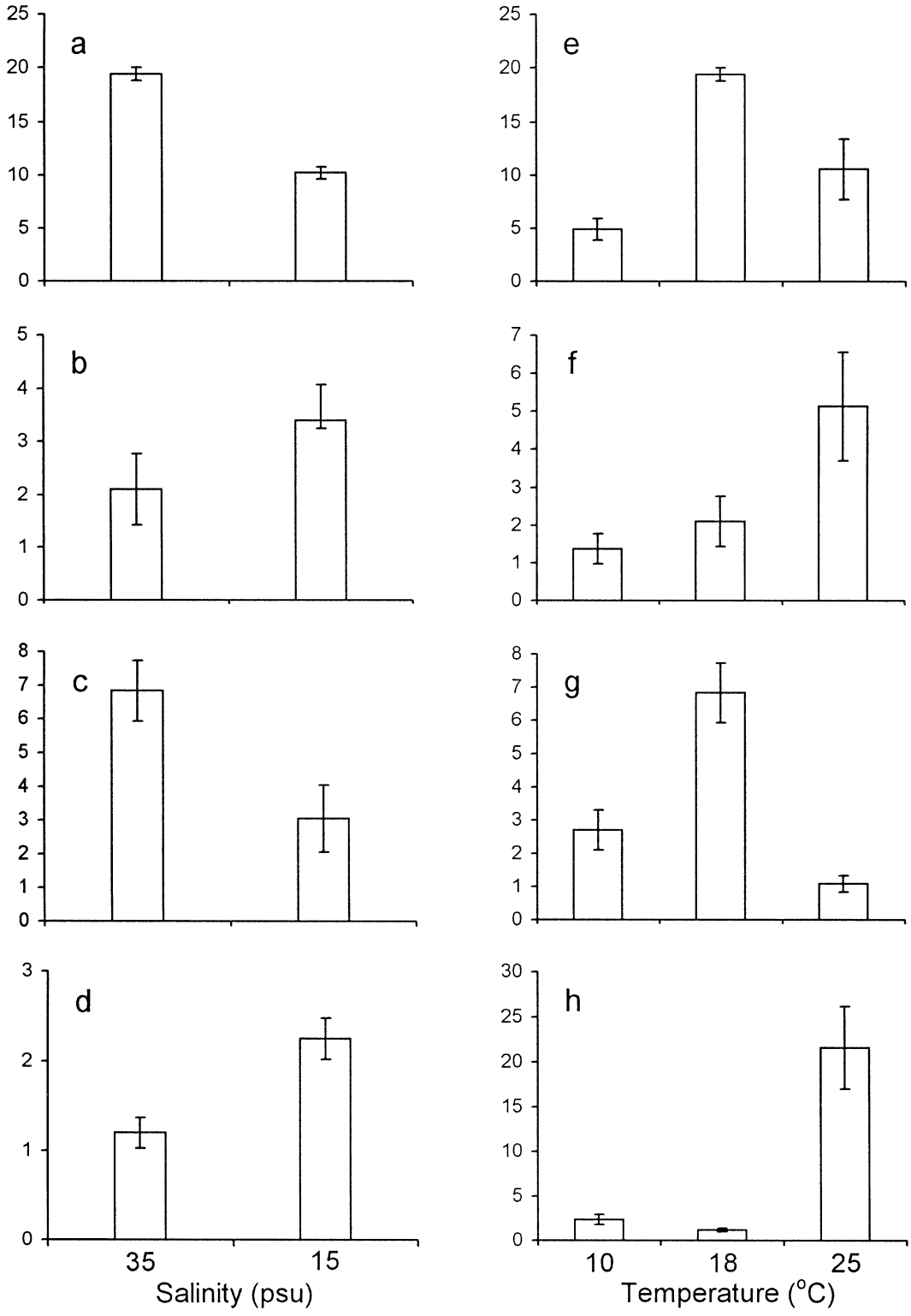

Fig. 2. Histograms illustrating variations of haslene and rhizene production (pg cell^{-1}) at different salinities and growth temperatures. Error bars indicate standard deviations of means of triplicate culturing experiments: a and e: total HBI concentrations; b and f: total haslene concentrations; c and g: rhizenes:haslenes ratio; d and h: rhizahexaenes:rhizapentaenes ratio.

general location. Nonetheless, 18 °C was the optimum temperature for rhizene production in strain CS 389/A (Fig. 2g). As with the haslene distributions, the degree of unsaturation in the rhizenes of strain CS 389/A changed reproducibly with temperature; the proportion of hexaenes to pentaenes increasing markedly with an

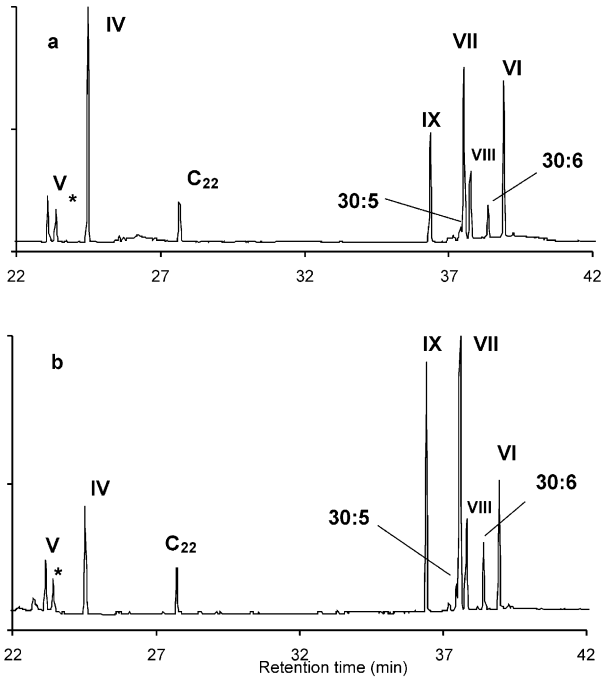

Fig. 3. GCMS total ion current chromatograms of total chloroform extracts of *Rhizosolenia setigera* strain CS 389/A at (a) 15 psu and (b) 35 psu salinity, 18 °C. Roman numerals refer to HBI structures shown in text. C_{22} = *n*-docosane internal standard. * = *n*-Henicosahexaene. 30:5, 30:6 = unknown C_{30} pentaene and hexaene, respectively.

increase in temperature from 18 to 25 °C (Figs. 2h and 4a–c). Again, this is somewhat similar to the change observed in the Δ5 and Δ6(17) haslenes of *H. ostrearia* (Rowland et al., 2001). However, opposite to the haslenes, Z to E isomerisation occurred in the rhizenes (Fig. 4).

2.1.3. Effects of temperature on the isotopic compositions of HBIs

The $^{13}C/^{12}C$ isotope ratios (in the δ notation relative to PDB standard) of phytol, haslene (**IV**), five of the six rhizenes and cholest-5, 24-dienol (Barrett et al., 1995) in *R. setigera* CS 389/A grown at 18 and 25 °C were measured by GC-irm-MS. Certified reference materials [*n*-hexadecane (*n*-C_{16}), *n*-icosane (*n*-C_{20}), *n*-tetracosane (*n*-C_{24}) and *n*-dotriacontane (*n*-C_{32})] each of known isotopic composition, were co-chromatographed with each sample during each GC-irm-MS analysis. The isotope value of the *Z*-haslatriene (**V**) could not be obtained due to insufficient GC resolution from *n*-henicosahexaene. The δ per mil value of each analyte was measured. Then, to allow for within-run instrument drift, differences between the measured and certified values of the nearest eluting *n*-alkane reference were added to the measured value of each analyte to give a range. The means and standard deviations for the ranges obtained in replicate experiments were then calculated and are shown as box and whisker diagrams in Figs. 5 and 6.

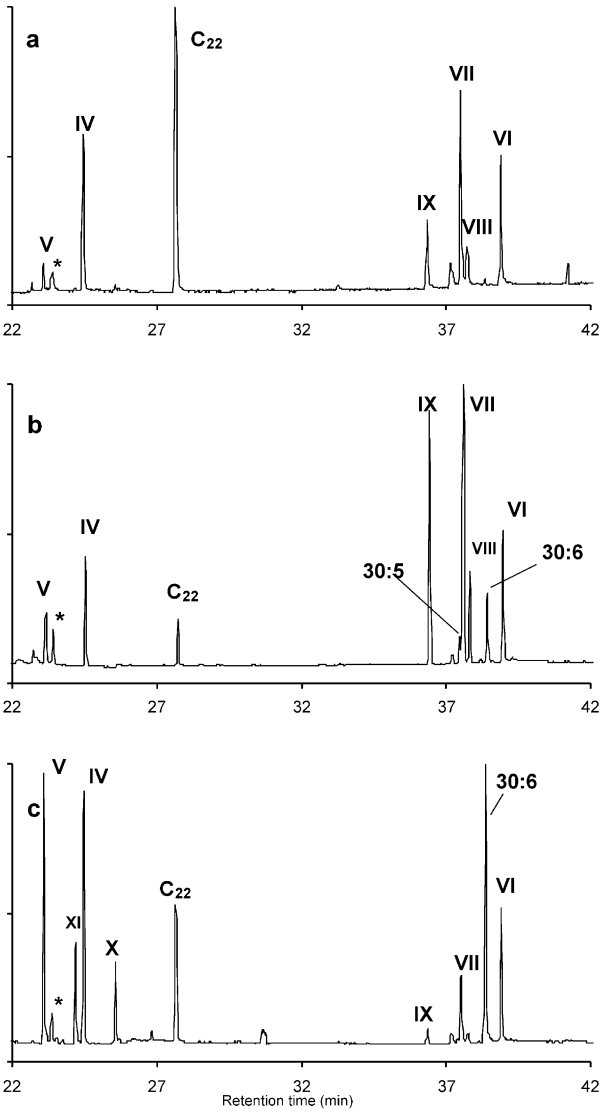

Fig. 4. GCMS total ion current chromatograms of total chloroform extracts of *Rhizosolenia setigera* strain CS 389/A at 35 psu salinity, (a) 10 (b) 18 and (c) 25 °C. Roman numerals refer to HBI structures shown in text. C_{22} = *n*-docosane internal standard. * = *n*-Henicosahexaene. 30:5, 30:6 = unknown C_{30} pentaene and hexaene, respectively.

Thus, any major differences in δ between the individual lipids which emerge are unlikely to be artefactual. The mean within-run variations from the certified values were: [($n = 10$) *n*-C_{20}, 0.4 ± 0.3 per mil; *n*-C_{24}, 0.3 ± 0.3 per mil; *n*-C_{32}, 0.4 ± 0.2 per mil].

At 18 °C, the *E*-haslatriene (**IV**) was about 3–4 per mil depleted in ^{13}C compared to phytol. This applied to both the algae cultured for three generations (Fig. 5a) and the final fourth generation samples (Fig. 5b). At 25 °C the result was the same (Fig. 5a). A previous study showed that haslapentaene (**I**) in *R. setigera* strain CCMP 1330 grown at 12 °C was 4.5 per mil depleted compared with phytol (Schouten et al., 1998). This is probably not significantly different from the 3–4 per mil determined in the present study.

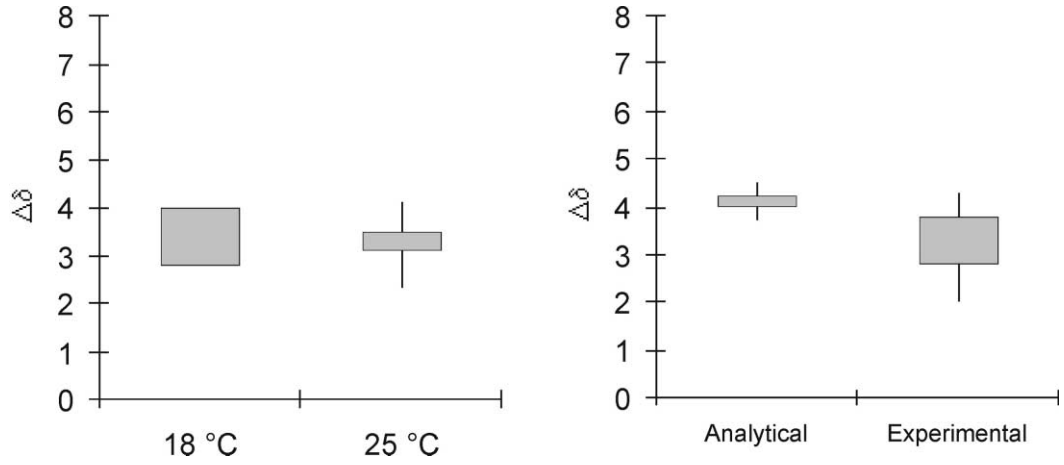

Fig. 5. Bar and whisker diagrams illustrating the influence of growth temperature on the Δδ ^{13}C isotope values of hasla −7(20), 9*E*,23-triene (**IV**) compared with phytol TMSi ether (isotopically corrected) in *Rhizosolenia setigera* CS 389/A. (a). Culture grown for three growth cycles at 18 and 25 °C. Analyses made in duplicate (18 °C) and triplicate (25 °C). (b). Culture grown for four growth cycles at 18 °C in triplicate. The results of triplicate analysis of a single sample ('Analytical') and full experimental triplicate ('Experimental') are shown. The data indicate a 3–4 per mil depletion in ^{13}C in **IV** compared with phytol.

The isotopic compositions of the haslene (Fig. 5) and cholest-5,24-dienol (desmosterol) in the present strain grown at 18 °C, were about equal (Fig. 6f), again as found previously (Schouten et al., 1998). Schouten et al. concluded from these similarities, that (**I**) was biosynthesised, like the sterol, in the cytosol of the diatom cell and not in the plastid. Our data support the contention that the haslene and the sterol in *R. setigera* CS 389/A were biosynthesised from a similar isotopic pool of isopentenyl diphosphate in the culture grown at 18 °C; perhaps in the cytosol.

At 25 °C, however, the Δδ values of the haslene (ca. 3–4 per mil; Fig. 5a) and the sterol (1–2 per mil; Fig. 6f) were somewhat different; certainly in the 4th generation samples for which the most comprehensive data set was obtained (Figs. 5a and 6f). In fact, the Δδ value of the sterol at both 18 and 25 °C, closely matched the value of the dominant alkene at each temperature (Fig. 6a, d, and f). Thus, at 18 °C, the dominant rhizenes were the *E*-pentaene (Figs. 4b and 6a; **IX**) and hexaene (Figs. 4b and 6c; **VII**) and the Δδ values of both these (and the *E*-haslatriene) and the sterol were about 3–4 per mil in the 3rd generation and in the 4th generation samples (Figs. 5a,b and 6a, c, and f). At 25 °C, the dominant alkene was an unknown hexaene (30:6 Figs. 4c and 6d), and whilst there was some spread in the data, the mean range of Δδ values of both this and the sterol were lower than those of the haslene (Fig. 5a) and the other rhizenes.

These data suggest that the biosynthesis of the dominant alkenes and sterol are coupled at both temperatures, probably from an isotopically similar pool of isopentenyl diphosphate.

Interestingly, the Δδ values of some of the individual alkenes varied quite considerably. Thus whilst the values for the C_{30} rhizapentaene (**VIII**; Fig. 6b) and rhizahexaenes (**VII**; Fig. 6c; **VI**; Fig. 6e) were similar to that of the haslenes (ca. 3–4 per mil) at both temperatures, these varied somewhat from those of the unknown hexaene 30:6 (Fig. 6d) . (Although there also appeared to be a difference in the Δδ values of the pentaene (**IX**; Fig. 5a) between 18 and 25 °C (Fig. 6a) this probably reflected the very low abundance of the pentaene at the higher temperature (0.15 ± 0.07 pg cell^{-1}; Fig. 4c), which made measurement much less accurate and prone to influences from co-eluting compounds such as bis(2-ethylhexyl)phthalate, a contaminant which was present in the procedural blank and had a similar retention index to the alkene).

Clearly, the isotope studies support our earlier conclusion that biosynthesis of the HBIs in *R. setigera* strain CS 389/A is complex. The following interpretations must therefore be viewed as speculative. Overall the isotopic measurements suggest:

1. Biosynthesis of phytol from an isotopically different pool of IPP to the haslenes, rhizenes and sterol at 18 °C, in the plastid, in agreement with a previous report for a Δ5 haslene in a different *R. setigera* strain.
2. Particularly close coupling of the biosynthesis of the major alkenes and the sterol at both temperatures, but some differences in isotopic fractionation of carbon even within the different C_{30} alkenes, especially between VI,VII and the unknown $C_{30:6}$ (Fig. 5c, d and e) at the highest growth temperature.

We propose, at both 18 and 25 °C, biosynthesis of phytol in the chloroplast from geranyl diphosphate probably via a non-mevalonate route from CO_2 as is true of phytol production in some other diatoms (Cvejic and Rohmer, 2000).

At 18 and 25 °C, we envisage biosynthesis of haslenes probably outwith the plastid, either via a mevalonate

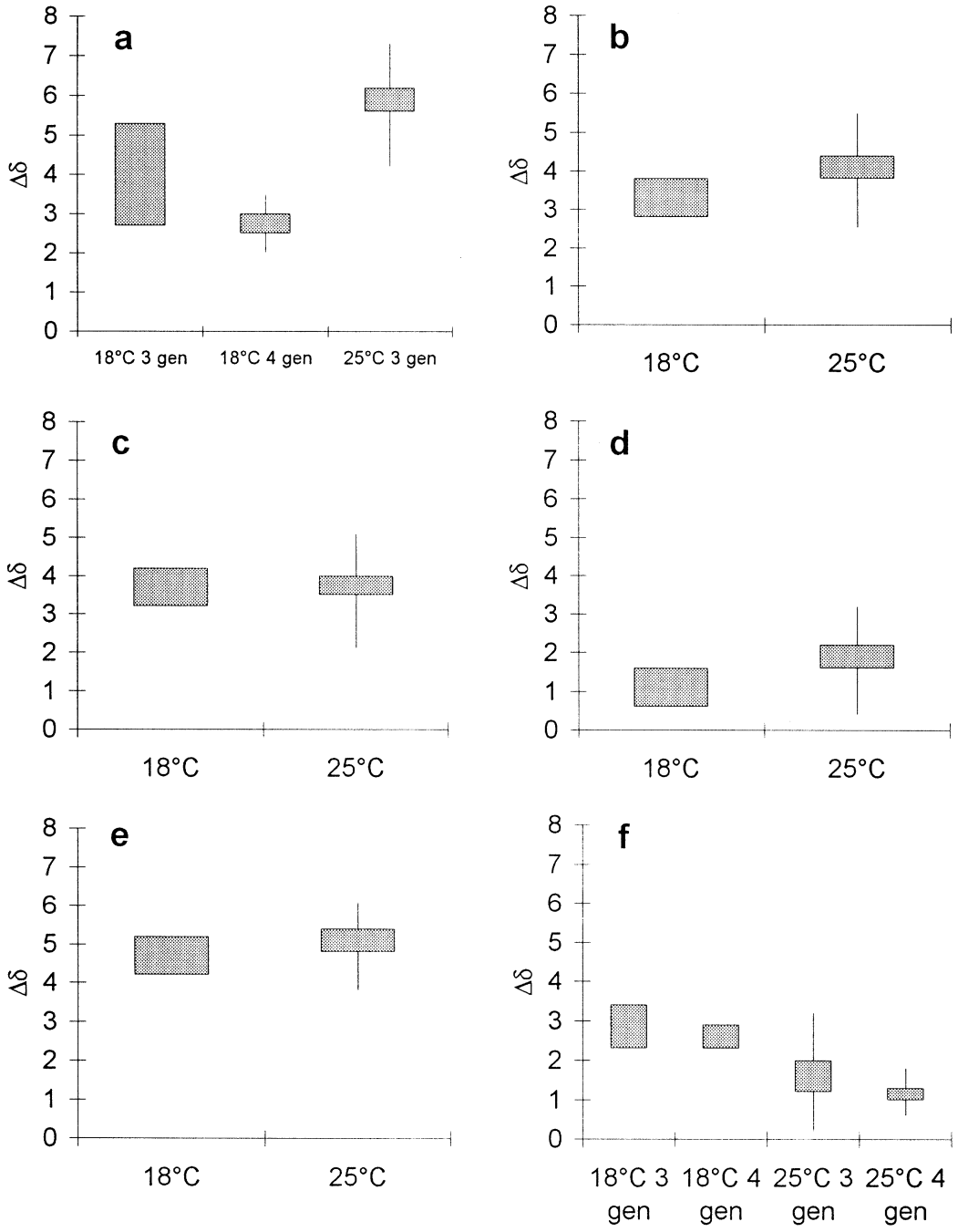

Fig. 6. Bar and whisker diagrams illustrating the influence of growth temperature on the $\Delta\delta$ ^{13}C isotope values of alkenes (**VII–IX**, Unknown 30:6) and cholesta-5,24,-dienol (TMSi ether, isotopically corrected) compared with phytol TMSi ether (isotopically corrected) for *Rhizosolenia setigera* CS 389/A cultures grown for three (3 gen) and four (4 gen) generations at 18 and 25 °C. (a) Rhizapentaene **IX**; (b) Rhizapentaene **VIII**; (c) Rhizahexaene **VII**; (d) unknown C_{30} hexaene 30:6; (e) Rhizahexaene **VI**; (f) cholest-5,24-dienol.

route leading to isotopic differences in the IPP compared with that used in phytol biosynthesis, or via a non-mevalonate route using an IPP pool which is isotopically distinct from that used in phytol production (cf. Cvejic and Rohmer, 2000). This might involve condensation of geranyl diphosphate (GPP) and a C_{15} isoprenyl moiety (possibly peruviyl diphosphate, PPP). The involvement of PPP rather than farnesyl diphosphate (FPP) might explain coupling to give the C-7 branched HBI structure, rather than coupling of GPP and FPP which would probably lead to a squalene-type structure.

Biosynthesis of many of the rhizenes at 18 and 25 °C (**VI**, **VII**, **VIII**, **IX**) might be from the same pool of IPP (and hence PPP) as that used for haslene biosynthesis at 18 and 25 °C, but biosynthesis of some C_{30} alkenes (e.g. unknown $C_{30:6}$)—and the sterol at 25 °C, must be from

a somewhat isotopically distinct IPP pool. Given the similarities in the isotopic values of the dominant alkenes and the sterol at both temperatures, we assume alkene synthesis from these IPP pools takes place out with the plastid, even though some terpene secondary metabolites are also believed to be produced in the chloroplast (e.g Cvejic and Rohmer, 2000, and references therein). Production in the cytoplasm is consistent with previous findings that diatoms are able to use different carbon sources in different cell compartments (Cvejic and Rohmer, 2000) and our unpublished findings which indicate that haslenes in *H. ostrearia* are not associated with the fatty acid storage lipids (which are found in the chloroplast).

Whilst more definitive studies of the biosynthetic pathways to the HBIs in *R. setigera* will also require growth of the diatom with isotopically enriched substrates, such as with $^{13}CO_2$ and also studies of the sites of ^{13}C incorporation by ^{13}C NMR, the GC-irm-MS approach used herein provided useful complementary information and did not require perturbation of algal growth with artificially high substrate concentrations or mixotrophic growth conditions. The results of our studies with isotopically enriched substrates will be published separately.

2.2. R. setigera strain CCMP 1330

When North Atlantic (off Massachusetts, USA), strain CCMP 1330 was cultured at 4–20 °C previously, it was shown to produce haslapentaene (**I**) as the only HBI, even when growth temperature was varied. (**I**) was also the only HBI produced when the same strain was cultured at 15 °C in the present study (Fig. 7a). The HBI was identified by comparison of GC and MS data with authenticated samples from *H. ostrearia* (Wraige et al., 1997) and an aliquot of the previous sample isolated from *R. setigera* (Sinninghe Damsté et al., 1999b). The *n*-alkenes reported previously were also present (Fig. 7a).

2.3. R. setigera strain CCMP 1820

The HBIs of this strain have not been reported previously but were found herein (Fig. 7b) to comprise haslapentaene (**I**) as the only HBI in a culture grown at 15 °C . Again, *n*-alkenes were also present (Fig. 7b).

2.4. R. setigera Nantes 1999 and Nantes 2000

Strains of *R. setigera* isolated from the North Atlantic (off southern Brittany, France) contained, like the Tasmanian strain CS 389/A, both haslenes and rhizenes (Fig. 7c and d) and similar to that strain, the haslenes included trienes (**IV** and **V**) with C7(20) unsaturation. The corresponding tetraene [viz. hasla-7(20),9*E*,13, 23-tetraene; **X**] and traces of the corresponding Δ2 pentaene (cf. Belt et al., 2000b) were also present in this strain grown at 15 °C. All compounds were identified by GCMS retention time and mass spectral comparison with previously authenticated compounds from *P. intermedium* (Belt et al., 2000a,b). The rhizenes present comprised two pentaenes and two hexaenes, both with C7(25) unsaturation (**VI**–**IX**) as determined by NMR for strain CS 389/A above. The relative proportions of HBIs, which were very similar in two samples, collected and cultured one year apart, are shown in Fig. 7c and d.

3. Experimental

3.1. Algal cultures

R. setigera strain CS 389/A isolated from the Huon estuary, Tasmania, Australia, was grown at 10, 18 or 25 °C, 35 psu, under white light at 80 μE m^{-2} s^{-1} in fE or fE/2-1 medium as described previously for other strains (Volkman et al., 1994, 1998). Both small scale (250 ml Erlenmeyer flasks) and larger scale samples (10 l, 18 °C) were cultured.

R. setigera strains isolated from France (Nantes 99 and Nantes 00) were isolated from Le Croisic, France and cultured in 4×60 l tanks containing underground saltwater enriched with $NaNO_3$ (8 mg ml^{-1}) and Guillard's medium (f/2, 0.2 ml l^{-1}) at 16–18°C and on the smaller scale at 15°C. Samples for hydrocarbon analysis were obtained by centrifugation. Strains CCMP 1330 and 1820 were purchased from the Guillard Collection USA and grown under the same conditions as the French strains above.

3.2. Lipid extraction and alkene isolation

Alkenes were isolated by extraction of the centrifuged algal paste with chloroform (strain CS 389/A) or hexane, aided by ultrasonication (45 min, Kerry Pulsatron HB172) followed by column chromatogaphy on silica and elution with hexane. Selected samples were saponified by heating (60 °C, 1 h) in methanolic KOH (e.g. Volkman et al., 1998) and derivatised with BSTFA/TMCS (60 °C, 30 min) prior to analyses by GC-MS and GC-irm-MS.

3.3. NMR spectroscopy

NMR spectroscopy was conducted on a JEOL EX 270 multinuclear (^1H, ^{13}C) 270 MHz spectrometer. Data were recorded on the delta scale (ppm) using the resonances of $CDCl_3$ (7.25 ppm ^1H, 77.0 ppm ^{13}C) as internal references.

3.4. Gas chromatography–mass spectrometry

Derivatised total chloroform extracts (Figs. 3 and 4), non-saponifiable lipids (Fig. 7) or alkene fractions (for

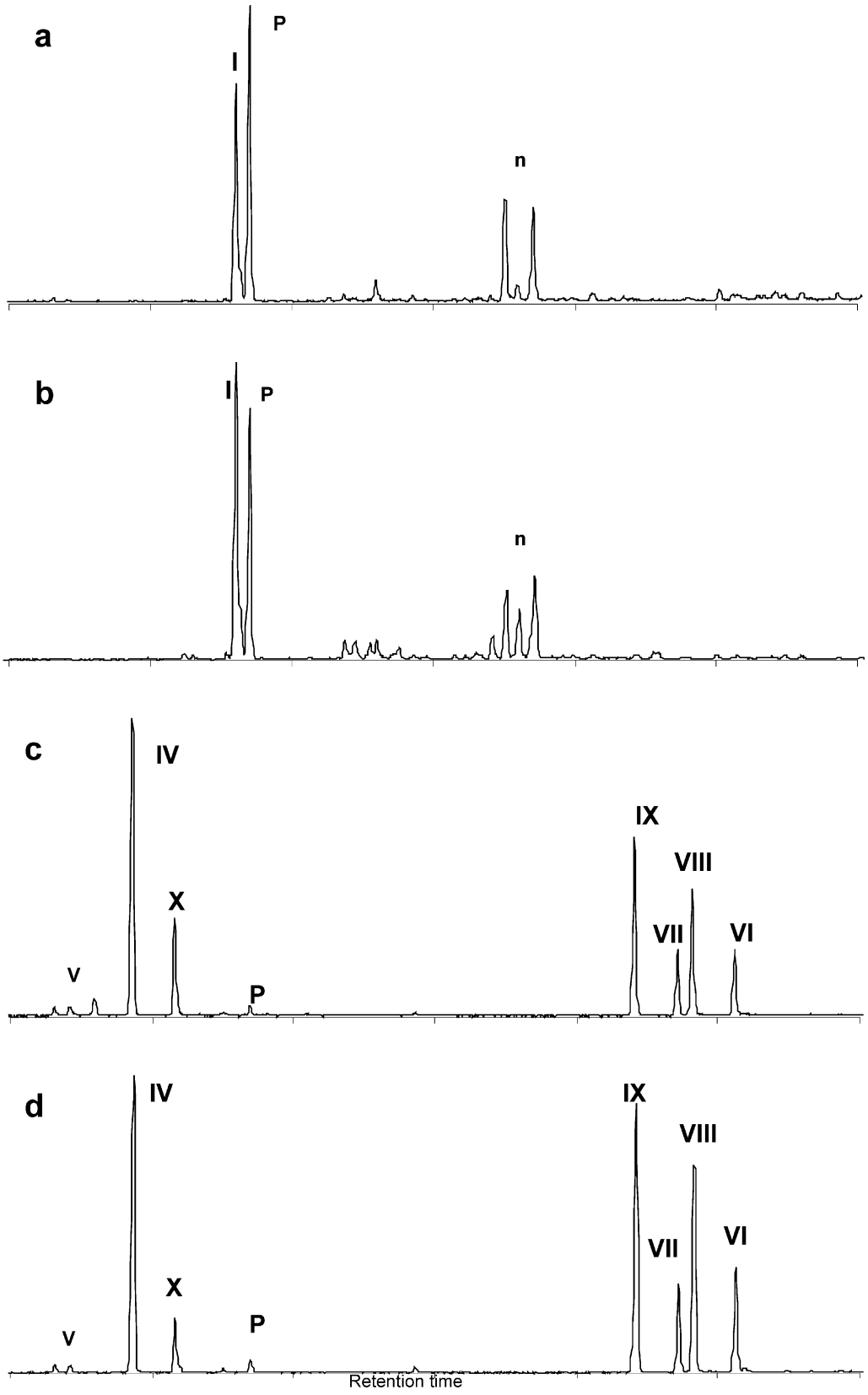

Fig. 7. GCMS total ion current chromatograms of non-saponifiable lipids (TMSi ethers) of *R. setigera* strains CCMP 1330, CCMP 1820, Nantes 99 and Nantes 00. P = phytol, TMSi ether; n = *n*-alkenes. Roman numerals refer to HBI structures shown. (a) *Rhizosolenia setigera* CCMP 1330; (b). *Rhizosolenia setigera* CCMP 1820; (c) *Rhizosolenia setigera* Nantes 1999; (d) *Rhizosolenia setigera* Nantes 2000.

NMR) from strain CS 389/A were examined by gas chromatography–mass spectrometry (GC-MS) performed using a Fisons Instruments MD800 equipped with a Carlo Erba on-column injector. The gas chromatograph was fitted with a 12 m (0.2 mm i.d.) fused silica capillary column (HP-5 Ultra stationary phase). Helium carrier gas was used. The gas chromatograph oven temperature was programmed from 40 to 300 °C at 5 °C min^{-1} and held at the final temperature for 10 min. Mass spectrometer operating conditions were; ion source temperature 250 °C and 70 eV ionisation energy. Spectra (40–650 Daltons) were collected using Fisons Masslab™ software. Alkene fractions from all other strains were examined by gas chromatography-mass spectrometry (GC-MS) performed using a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector fitted with a 12 m (0.2 mm i.d.) fused silica capillary column (HP-1 Ultra stationary phase). Auto-splitless injection and helium carrier gas were used. The gas chromatograph oven temperature was programmed from 40 to 300 °C at 5 °C min^{-1} and held at the final temperature for 10 min. Mass spectrometer operating conditions were; ion source temperature 250 °C and 70 eV ionisation energy. Spectra (35–500 Daltons) were collected using Hewlett Packard Chemstation™ software.

3.5. Gas chromatography–isotope ratio monitoring–mass spectrometry

GC-irm-MS was performed with a Finnigan Delta S mass spectrometer essentially as described previously (Merrit et al., 1995). GC conditions were: oven temperature 40–135 °C at 30 °C min^{-1}, 135–300 °C at 4 °C min^{-1}; 60 m DB-1, helium carrier gas. Isotopic values were determined by integration of the ion currents of m/z 44–46 produced by combustion of chromatographically separated compounds and comparison with CO_2 reference gas. Certified reference materials comprising *n*-hexadecane, *n*-icosane, *n*-tetracosane, *n*-dotriacontane and *n*-hexatriacontane of known isotopic composition were co-chromatographed with the algal lipids derivatised with BSTFA/TMCS. Determinations of the haslene were made in full procedural triplicate, including culturing conditions and in analytical triplicate (Fig. 5b). To correct for the isotopic effect of the derivatisation of phytol and cholest-5,24-dienol with BSTFA/TMCS, both free phytol (Aldrich, 97% mixture of *E* and *Z* isomers; *E*-phytol, −28.02 per mil delta notation relative to PDB standard) and a sample of phytol derivatised with each batch of BSTFA/TMCS were examined by GC-irm-MS. The difference in values was used to calculate the isotopic composition of the TMS group using a published method (Jones et al., 1991).

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