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Effects of auxosporulation on distributions of C_{25} and C_{30} isoprenoid alkenes in *Rhizosolenia setigera*

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Abstract

The effect of life cycle on the distributions of C_{25} and C_{30} highly branched isoprenoid (HBI) alkene lipids has been investigated for the marine diatom *Rhizosolenia setigera*. The concentrations of the C_{30} compounds are largely independent of the cell volume, though the ratios of the individual isomers possessing five and six double bonds show a dependence on the position of the cell during its life cycle, especially during auxosporulation. In contrast to the C_{30} pseudo-homologues, the C_{25} isomers are not always detected in cultures of *R. setigera*. The biosynthesis of the C_{25} HBIs would appear to result from the onset of auxosporulation, with further changes to their distributions taking place after this phase, including the formation of more unsaturated isomers. The results of this investigation may be used in part to explain the large variations in these lipids reported previously.

Keywords: *Rhizosolenia setigera*; Microalgae; Diatoms; Highly branched isoprenoids; Alkenes; Life cycle; Auxosporulation; HBIs

1. Introduction

C_{25} and C_{30} highly branched isoprenoid (HBI) alkenes are unusual secondary metabolites that are derived from diatoms and are commonly used as biological markers in sediments and other geochemical environments (Robson and Rowland, 1986; Rowland and Robson, 1990). Several of the isomeric C_{25} alkenes also possess a cytostatic effect on a human non-small-cell broncopulmonary cell line (NSCLC-N6) (Rowland et al., 2001a). Volkman and co-workers (1994) were the first to determine biological sources of these isoprenoids, namely the marine diatoms *Haslea ostrearia* (C_{25}) and *Rhizosolenia setigera* (C_{30}). Some representative structures of C_{25} (haslenes) and C_{30} (rhizenes) HBIs are shown in Figs. 1 and 2. Although an account (in terms of structures and distributions) of the C_{25} HBIs produced by *H. ostrearia* would appear to be well defined, the situation with *R. setigera* is less clear. The culture of *R. setigera* investigated by Volkman et al. (1994) was found to contain three C_{30} pentaenes ($C_{30:5}$) and two C_{30} hexaenes ($C_{30:6}$), with no detection of any C_{25} alkenes. In contrast, Sinninghe Damsté et al. (1999a) showed that a strain of *R. setigera* isolated from Vineyard Sound, MA, USA contained only a single C_{25} pentaene in addition to two novel *n*-alkenes, with no C_{30} homologues. The structure of the $C_{25:5}$ in this strain was subsequently shown to be **3**, previously found in cultures of *H. ostrearia* (Sinninghe Damsté et al., 1999b). During the course of our own studies, we have observed both C_{25} and C_{30} HBI alkenes within the same cultures of *R. setigera,* together with considerable variations in their distributions (Rowland et al., 2001b). It is of course possible that these differences may be attributable to changes in phenotypic variables employed during the culturing experiments (e.g. light, salinity, temperature, nutrients, etc.) or to the use of different strains of diatoms belonging to the same species. Indeed, we have noted some variation in distributions with temperature and salinity, and also with the origin of the diatom strain. Significantly, however, we have also observed variations in distributions under 'controlled' conditions (constant temperature, salinity, light cycle, etc.) using a

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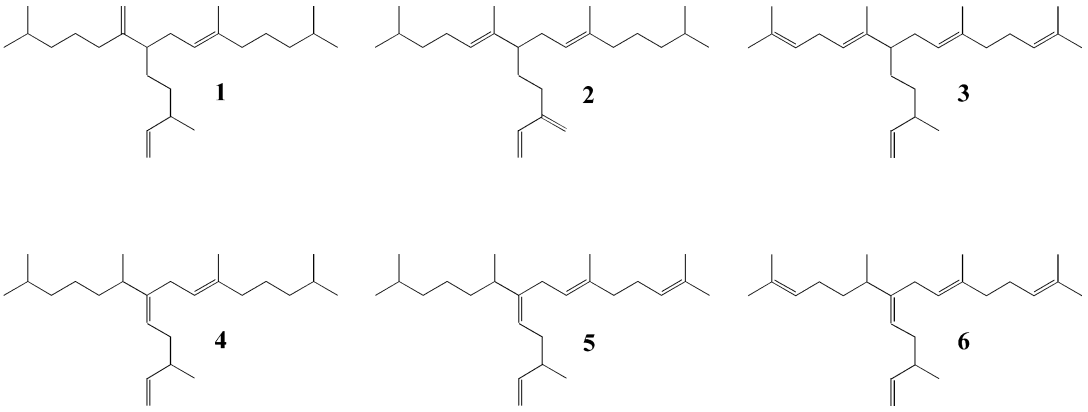

Fig. 1. Representative structures of C_{25} HBI alkenes isolated from various marine diatoms.

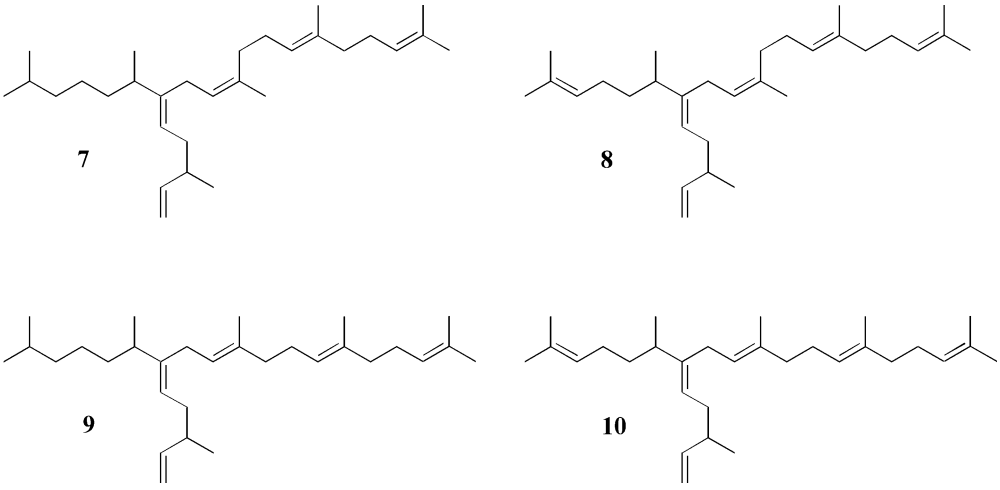

Fig. 2. Structures of C_{30} HBIs isolated from *R. setigera*.

single strain, indicating that other factors are also important (Rowland et al., 2001b). In addition, Sinninghe Damsté and co-workers (2000) have reported that distributions of the HBI $C_{25:5}$ and C_{25} and C_{27} *n*-alkenes produced by *R. setigera* were quite variable for experiments performed at the same temperature.

In this report, we describe an investigation of the distribution of C_{25} and C_{30} HBI alkenes biosynthesised by *R. setigera* as a function of the position of the cells through their life cycle. Our observations reveal a relationship between cell size and HBI content, including a dramatic change in the distribution of alkenes during the regeneration of their original size through a sexual cycle (auxosporulation). This is a necessary phase of the life cycle, since vegetative multiplication, involving the formation of new valves within the parent frustule, results in consequential formation of increasingly smaller cells. After a sufficiently large number of such divisions, a critical point in cell size is reached and sexual reproduction is induced with formation of an auxospore (an expandable zygotic cell). This forms the basis of a new generation of large daughter cells. Since the sexual reproductive phase and auxospore formation is relatively short (ca. 1 week) compared to the total life cycle (as much as several months or years), the observation of such events is more achievable in laboratory cultures (rather than in natural populations) since more homogeneous samples are attainable. Further information relating to diatom life cycles can be found elsewhere (e.g. Round et al., 1990).

2. Results and discussion

2.1. Distribution of HBIs in RS-1

A strain of *R. setigera* was isolated for the first experiment (RS-1) from Le Croisic, France. From a single cell, an inoculum was obtained (1 month equilibration time) which was used to generate a series of batch cultures corresponding to six consecutive cycles of growth. Following extraction and derivatisation, total ion current

(TIC) chromatograms of the non-saponifiable lipid fractions obtained from each cycle demonstrated the presence of phytol, *n*-$C_{21:6}$ (henicosa-3,6,9,12,15,18-hexaene) and four C_{30} HBI alkenes as the main identifiable components. The C_{30} HBI alkenes, which consisted of two pentaenes ($C_{30:5}$) and two hexaenes ($C_{30:6}$), were identified as isomers **7**–**10** (Fig. 2) by comparison of their GC and MS properties with authentic compounds (Belt et al., 2001). A small amount of C_{25} triene **4** could also be detected. Fig. 3 shows a partial TIC chromatogram corresponding to cycle 1 which illustrates the relative amounts of these compounds including the $C_{30:5}$ and $C_{30:6}$ isomers. Subsequent cycles resulted in extremely similar chromatograms, though the $C_{25:3}$ observed in cycle 1 could not be detected. For all cycles, the major rhizenes were the pentaene isomers ($C_{30:5}/C_{30:6}$ = 12 ± 1.4).

A summary of the cell dimensions and HBI distributions can be found in Table 1. As expected, cell dimensions (mean cell width and volume) decreased slightly with consecutive cycles as a result of cell division. The mean concentration of the C_{30} HBI alkenes (10.7 ± 1.7 pg cell^{-1}) is reasonably close to that found by Rowland et al. (2001b) (17.3 pg cell^{-1}; 18 °C) but rather higher than that reported by Volkman et al. (1994) (1.6 pg cell^{-1}). However, an exact agreement may not be expected due to the differences in culture conditions and/or strains. Perhaps of greater significance is the fact that the mean concentration of HBIs per unit cell volume was found to be essentially invariant throughout the six cycles (Table 1). Although the observation of C_{30} HBI alkenes was in general agreement with those made by Volkman and co-workers (1994), the virtual absence of any C_{25} alkenes seemed surprising since we have previously reported both C_{25} and C_{30} alkenes from *R. setigera* isolated from the same location and cultured under similar experimental conditions (Rowland et al., 2001b). However, in terms of addressing our original aim, we considered that although the six cycles studied in RS-1 certainly represent numerous individual replications or generations, the study may have been limited in terms of a broader range of physiological changes. Since a more comprehensive study involving a whole life cycle, including the sexual regeneration was considered impractical, we decided to concentrate on this last and quite distinct physiological event (viz. auxosporulation). We therefore conducted a second experiment (RS-2) to examine the effect of this sexual reproductive phase on HBI alkene structures and distributions.

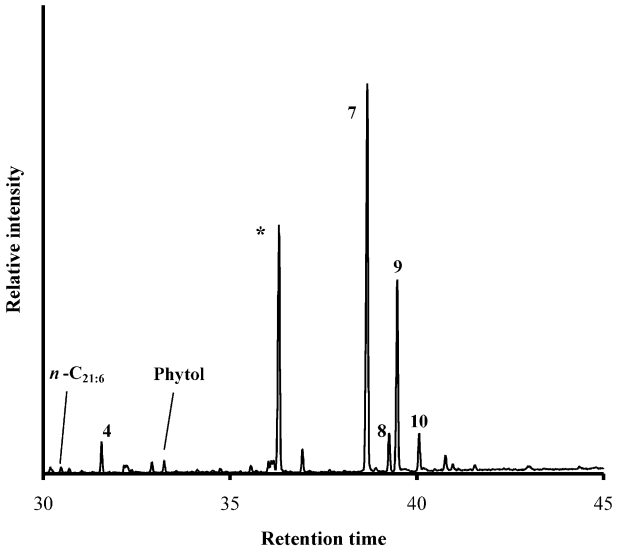

Fig. 3. Partial TIC chromatogram of a non-saponifiable lipid fraction from RS-1 (cycle 1). The peak marked * is due to the internal standard. The peak numbers refer to the C_{25} and C_{30} structure numbers in Figs. 1 and 2.

2.2. Distribution of HBIs in RS-2

In a second experiment (RS-2), a further inoculum was generated from a single *R. setigera* cell, though in contrast to RS-1, the cells were believed to be close to the onset of auxosporulation (ca. 4.7 μm width). Indeed, during the third cycle, auxospores could be detected using light microscopy. Significant numbers of initial or "daughter" cells were seen in cycle 3 (14.0%) with the proportion increasing (61.4%) during cycle 4 (Table 2). By cycles 5 and 6, virtually all cells could be considered

Table 1
Cell dimensions and C_{25} and C_{30} HBI concentrations obtained from six consecutive cycles of a culture of *R. setigera* (RS-1)

	Biomass (cell ml^{-1})	*n*-$C_{21:6}$ (pg cell^{-1})	Phytol (pg cell^{-1})	Total HBI (pg cell^{1})	Total C_{30} (pg cell^{-1})	$C_{30:5}$ (pg cell^{-1})	$C_{30:6}$ (pg cell^{-1})	$C_{30:5}/C_{30:6}$	Mean width (μm)	Mean volume (μm^3)	Total HBI (fg μm^{-3})	Total C_{30} (fg μm^{-3})
Cycle 1	11 640	0.09	0.16	10.40	9.99	8.90	1.10	8.12	18.7	1309	7.9	7.6
Cycle 2	12 400	0.00	3.74	11.70	11.70	10.67	1.03	10.33	18.1	1279	9.1	9.1
Cycle 3	10 120	0.11	0.48	9.60	9.51	8.86	0.64	13.82	17.2	964	10.0	9.9
Cycle 4	9360	0.16	4.20	13.36	13.36	12.45	0.91	13.70	16.6	1254	10.7	10.7
Cycle 5	10 720	0.12	0.09	9.33	9.31	8.65	0.66	13.12	15.9	1151	8.1	8.1
Cycle 6	14 800	0.00	2.49	10.12	10.12	9.38	0.74	12.65	15.0	1090	9.3	9.3

Table 2
Cell dimensions, populations and C_{25} and C_{30} HBI concentrations obtained for 11 consecutive cycles of a culture of *R. setigera* (RS-2)

	Biomass	*n*-$C_{21:6}$	Phytol	Total HBI	Total C_{25}	Total C_{30}	C_{25}/C_{30}	$C_{25:3}$	$C_{25:4}$	$C_{30:5}$	$C_{30:6}$	$C_{30:5}$/$C_{30:6}$	Daughter cell	Mean volume	Mean volume	Mean volume	Total HBI	Total C_{25}	Total C_{30}
	(cell ml^{-1})	(pg $cell^{-1}$)	(pg $cell^{-1}$)	(pg $cell^{-1}$)	(pg $cell^{-1}$)		(pg $cell^{-1}$)	(pg $cell^{-1}$)	(pg $cell^{-1}$)	(pg $cell^{-1}$)	(pg $cell^{-1}$)		(% of total cells)	(μm^3)	mother (μm^3)	daughter (μm^3)	(fg μm^{-3})	(fg μm^{-3})	(fg μm^{-3})
Cycle 1	25440	0.00	0.00	1.87	0.03	1.85	0.02	0.03	0.00	0.89	0.96	0.93	0	133	133	–	14.1	0.2	13.9
Cycle 2	24320	0.00	0.00	2.20	0.02	2.19	0.01	0.02	0.00	1.00	1.19	0.84	0.7	116	116	–	19.0	0.1	18.9
Cycle 3	10560	0.00	0.00	3.88	0.00	3.88	0.00	0.00	0.00	3.02	0.87	3.48	14.0	373	117	1945	10.4	0.0	10.4
Cycle 4	5800	0.25	0.19	18.64	0.44	18.20	0.02	0.44	0.00	14.84	3.36	4.42	61.4	1379	132	2164	13.5	0.3	13.2
Cycle 5	6480	0.55	0.37	20.70	2.40	18.30	0.13	2.06	0.34	13.45	4.85	2.78	92.6	1890	128	2031	11.0	1.3	9.7
Cycle 6	5560	0.90	0.49	25.64	4.68	20.96	0.22	3.79	0.89	15.03	5.93	2.53	100	2091	–	2091	12.3	2.2	10.0
Cycle 7	3070	0.74	1.05	30.51	7.45	23.06	0.32	6.04	1.41	18.04	5.02	3.59	100	2288	–	2288	13.3	3.3	10.1
Cycle 8	4520	0.82	0.70	29.87	9.60	20.27	0.47	5.73	3.86	15.29	4.99	3.07	100	2353	–	2353	12.7	4.1	8.6
Cycle 9	2930	1.54	1.16	47.54	17.51	30.03	0.58	10.79	6.72	22.19	7.84	2.83	100	2353	–	2353	20.2	7.4	12.8
Cycle 10	4140	0.98	0.62	33.20	9.53	23.68	0.40	6.13	3.40	17.40	6.27	2.77	100	2359	–	2359	14.1	4.0	10.0
Cycle 11	3680	1.42	1.07	48.38	25.14	23.23	1.08	11.23	13.09	15.64	7.60	2.06	100	2293	–	2293	21.1	11.0	10.1

to be present as a result of auxosporulation (92.6 and 100% daughter cells, respectively). Cells from a further five cycles were harvested to allow for a further comparison with the results from RS-1. In contrast to the relatively modest volume changes that were observed for cells belonging to consecutive cycles in RS-1 (Table 1), much greater differences were observed as a result of auxosporulation (Table 2; Fig. 4). Thus, mean cell volumes were determined for mother (125 ± 8 μm^3) and daughter (2210 ± 160 μm^3) cells using mean cell volumes (total cells) and relative populations (Table 2). Cell volumes for cycles 6–11 were all similar, with little variation from a mean value of 2290 ± 100 μm^3 despite consecutive cell division. Not surprisingly, since cell volumes and growth rates (indicated by the cell concentrations at the end of each exponential phase) are inextricably linked, an inverse relationship was found between these for the pre- and post-auxosporulation phases.

When the non-saponifiable lipid fractions for each cycle were analysed by GC-MS, some differences were observed between cycles and with the results obtained from RS-1 (vide infra). A partial TIC chromatogram from cycle 1 (Fig. 5) indicated the presence of the same $C_{30:5}$ and $C_{30:6}$ HBI alkenes present in RS-1, though phytol and *n*-$C_{21:6}$ were not detected. In addition, there was a trace amount of the C_{25} triene **4** which was mostly absent in the first experiment (RS-1) and there was a greater concentration of the pair of C_{30} hexaenes compared to the pentaenes. Thus, the $C_{30:5}/C_{30:6}$ ratio was 0.93, which compares with a mean ratio of 12 ± 1.4 in RS-1. Similar observations were made in cycle 2 (Table 2). During cycle 3, when auxospores and daughter cells were first detected using light microscopy, the small amount of $C_{25:3}$ observed in the first two cycles was absent and the C_{30} pentaenes were the dominant isomers ($C_{30:5}/C_{30:6}$ ratio increased sharply to 3.48). In cycle 4, when over 60% of the culture corresponded to daughter cells, the $C_{30:5}/C_{30:6}$ ratio increased further (4.42). In addition, phytol, *n*-$C_{21:6}$ and the C_{25} triene **4** were also detected and the total concentration of HBIs (pg $cell^{-1}$) increased noticeably (Table 2). This total concentration increased further during cycles 5 and 6-11 (100% daughter cells) though the C_{25} HBI concentrations increased at a greater rate compared with those for the C_{30} HBIs (C_{25}/C_{30} increased from cycles 4 to 11, Table 2). In addition, a C_{25} tetraene (**5**) was detected during cycle 5 while a C_{25} pentaene (**6**) was detected in cycle 11. Finally, the $C_{30:5}/C_{30:6}$ ratio underwent further changes, with a gradual reduction between cycles 4 and 11.

The dramatic changes in cell dimensions and concentrations of HBIs (pg $cell^{-1}$) that accompanied auxosporulation (cycles 3–5) prompted us to consider more closely the HBI concentrations on a unit cell volume basis (1 μm^3). Using this approach, the total concentration of C_{30} HBIs was found to be reasonably constant (Fig. 6a) with a mean value of 12.0 ± 2.9 fg μm^{-3} fol-

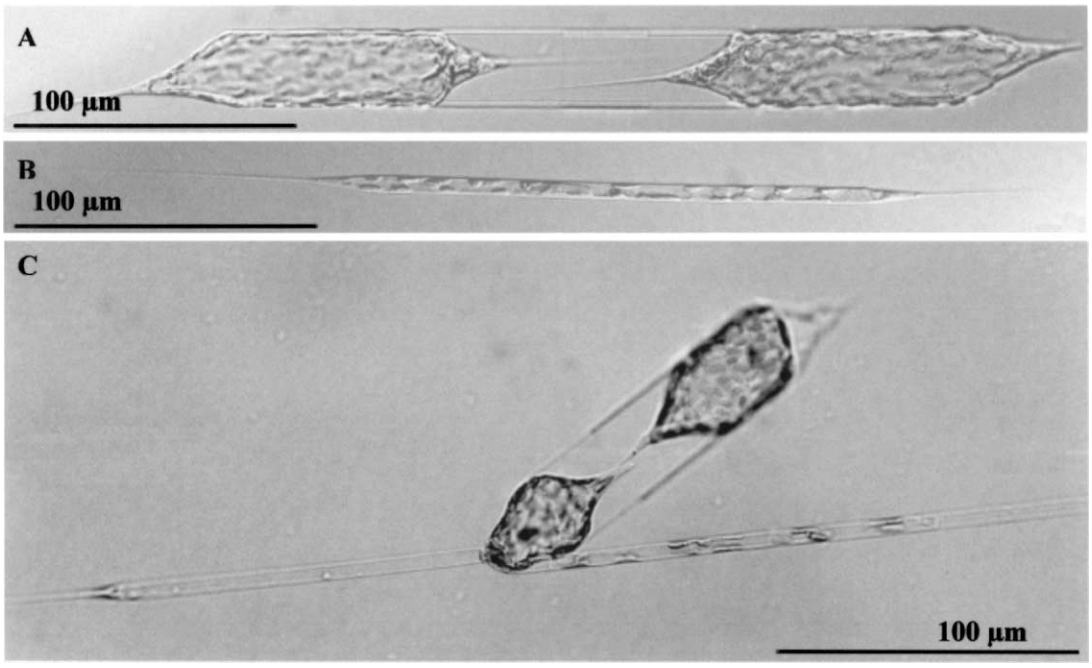

Fig. 4. Photographs of *R. setigera* (RS-2) obtained using light microscopy illustrating the differences in cell dimensions between two daughter cells (A), a single mother cell (B), two evolving daughter cells from their corresponding mother cells (C).

lowing auxosporulation, which compares with that of 9.1 ± 0.9 fg μm^{-3} obtained for RS-1. However, the ratio of the pentaene and hexaene isomers was affected substantially during the period of auxosporulation, with $C_{30:5}/C_{30:6}$ increasing from 0.84 to 4.42 between cycles 2 and 4. Following auxosporulation, this ratio underwent a gradual decrease (Fig. 6b).

Different changes were observed for the C_{25} HBIs. In contrast to the invariance in the total C_{30} alkene concentrations, we observed an increase in the concentration of the C_{25} HBIs (especially $C_{25:4}$) with cycle number following auxosporulation, and this too was reflected in an increasing C_{25}/C_{30} ratio (Fig. 6c and d). While this trend appeared to be initiated by the onset of auxosporulation, the effects taking place during this phase were not as dramatic as those observed for the C_{30} HBIs (viz. changes in $C_{30:5}/C_{30:6}$ ratio, vide infra). Indeed, consistent changes in the C_{25} HBI concentration took place both during and after auxosporulation, being significant in cultures containing only daughter cells, including the observations of pentaene **6** and tetraene **5** as the major isomer during cycle 11 (Fig. 5).

Thus, we have demonstrated that distributions of C_{25} and C_{30} HBI alkenes biosynthesised by *R. setigera* are strongly dependent on the position of the diatom in its life cycle, with the most noticeable changes taking place as a result of auxosporulation. These observations can be summarised as follows: (1) C_{30} HBIs (rhizenes) are only biosynthesised with five or six degrees of unsaturation. (2) The total rhizene concentrations (measured on a unit volume basis) remain constant during different stages of the life cycle, but the degree of unsaturation ($C_{30:5}/C_{30:6}$ ratio) is highly variable, especially during the period of auxosporulation. (3) C_{25} HBIs (haslenes) are biosynthesised with between three and five degrees of unsaturation. (4) Unlike the rhizene pseudo-homologues, haslenes are not always observed; their production would appear to be mainly stimulated by the onset of auxosporulation, though their concentrations and unsaturation continue to increase after this phase.

While these results can be used in part to explain the large variations in C_{25} and C_{30} HBI distributions observed in other cultures of *R. setigera*, it is not clear at this stage if similar or related effects apply to other lipid classes. Relationships between carbon content or chlorophyll *a* levels as functions of cell dimensions (including volume) have been reported (Mullin et al., 1966; Durbin, 1977), though to our knowledge, such studies have not been carried out on individual lipids. However, it has been reported recently that cell size (amongst other factors) in phytoplankton can contribute to carbon isotope fractionation and therefore cell dimensions and growth rates should be considered if stable carbon isotope compositions of organic material are to be used in determining, e.g. palaeo-[CO_2(aq)] levels (Popp et al., 1998). It has also been shown that variations in carbon and hydrogen isotopes of individual compounds within the same and different organisms can occur, and this may result from different and competing biosynthetic pathways, isotopic enrichment in biosynthetic precursors or substrates involved in biosynthesis (e.g. NADPH) (Sessions et al., 1999; Rowland et al., 2001b). Clearly then,

changes in distributions of individual lipids may cause variations in measured isotopic fractionation. The fact that changes in distributions may also be associated with cell dimensions as shown here suggests that the measurement of isotopic fractionation of individual lipids as a function of cell size or dimensions could be highly informative. Such an investigation is currently an area of research in our laboratory.

3. Experimental

3.1. Isolation and culturing of R. setigera

3.1.1. Experiment 1: RS-1

R. setigera was isolated from Le Croisic, France (8/8/2000) using a plankton net (75 μm). In the laboratory, single cells (ca. 20 μm width) were isolated under the microscope and grown in 250 ml Erlenmeyer flasks containing 150 ml F/2 Guillard medium under controlled conditions (14 °C, 100 μmol photons cm^{-2} s^{-1}, 14/10 h light/dark cycle). In order to ensure complete equilibration with the culture conditions, cells were replicated several times over a period of 1 month. After this equilibration period, 150 ml of new F/2 medium was inoculated with a concentration of 100 cell ml^{-1} (cycle 1). At the end of the exponential growing phase (11 days), cells were harvested by filtration, with a subsample being used to inoculate a second flask (cycle 2) with the same cell concentration as per cycle 1. This procedure of culturing, harvesting and further inoculation was repeated a further four times to yield a total of six cycles.

For each cycle, cell counting was performed using light microscopy and cell volumes were determined with a Beckman Coulter-Counter.

3.1.2. Experiment 2:RS-2

R. setigera (R.s. 99) was isolated from Etel, France (25/03/1999) and cultured using the same general method as described for Experiment 1 with the following exceptions : the cell concentration at the beginning of each cycle was 500 cell ml^{-1} and cells were harvested at the end of each exponential growth phase (ca. 7 days). In addition, single cells were isolated from R.s. 99 which had equilibrated in the laboratory for more than

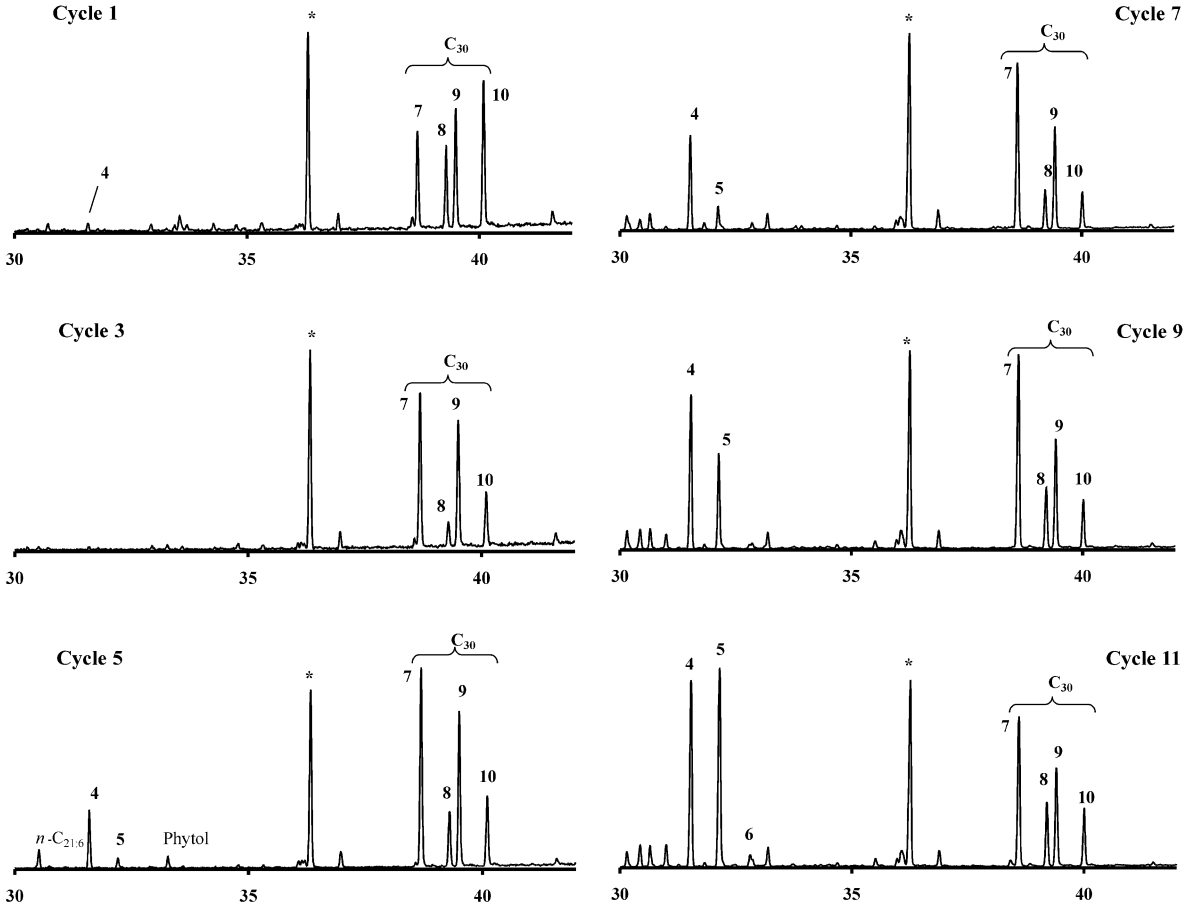

Fig. 5. Partial TIC chromatograms corresponding to alternate cycles of a culture of *R. setigera* (RS-2). The peak marked * is due to the internal standard. The peak numbers refer to the C_{25} and C_{30} structure numbers in Figs. 1 and 2.

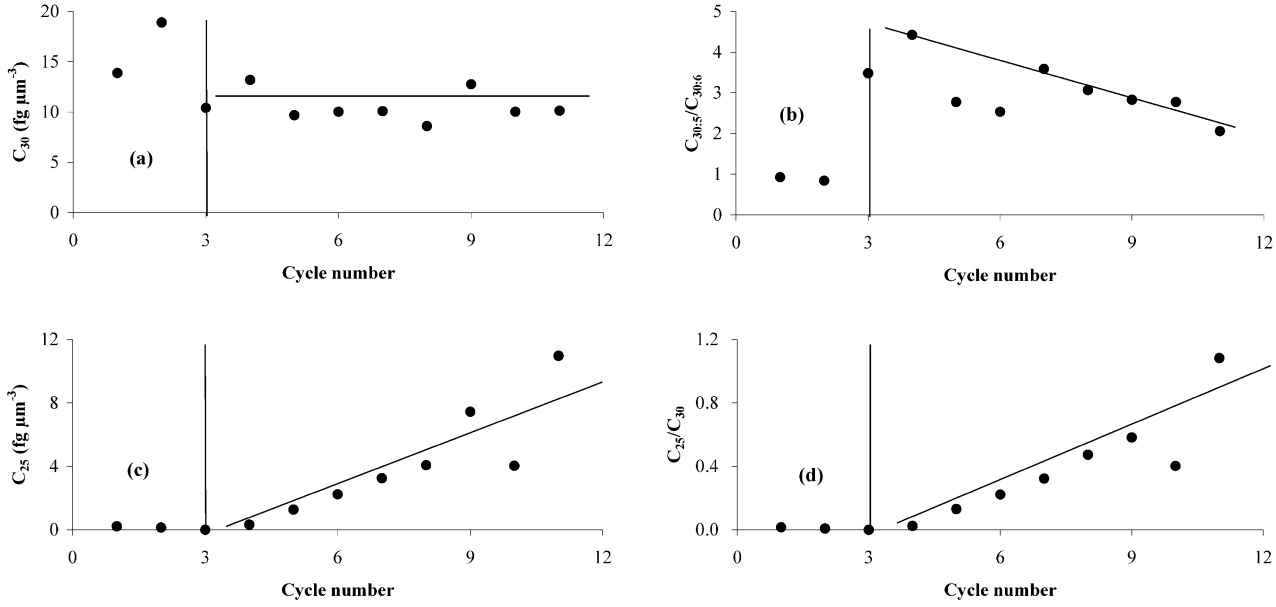

Fig. 6. Concentrations and ratios of C_{25} and C_{30} HBI alkenes as a function of cycle number for *R. setigera* RS-2. The vertical line at cycle 3 denotes the onset of auxosporulation. The horizontal and diagonal lines are trend lines only.

one year. As a result, all of the cells were extremely small (ca. 4 μm width) and believed to be close to the critical size for auxospore formation and the onset of auxosporulation.

3.2. Analysis of C_{25} and C_{30} HBIs by GC–MS

Analysis of HBI alkenes was as previously described (Wraige et al., 1997, 1999; Belt et al., 2000a,b). Briefly, cells were filtered at the end of each exponential growth phase and extracted with hexane following addition of an internal standard (7-hexylnonadecane; 1.1 μg filter^{-1}). Extracts were saponified (5% (w/w) KOH/80% MeOH/20% H_2O) and then re-extracted into hexane, dried and derivatised (BSTFA) to give a non-saponifiable lipid (NSL) fraction. GC–MS analysis of these NSL fractions was performed with a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard Mass Selective Detector (5970 series) fitted with a 12 m (0.2 mm i.d.) fused silica column (HP-1 stationary phase). Auto splitless injection and He 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. MS operating conditions were: ion source temperature 250 °C and 70 eV ionisation energy. Spectra (50–550 Da) were collected using Chemstation software. Identification of individual compounds was achieved by comparison of retention indices and mass spectra with authentic standards. Systematic errors linked to variations in HBI concentrations from duplicate or replicate determinations are typically 10–15% while random errors associated with, e.g. isomer ratios are less than 5%.

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