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Free radical oxidation (autoxidation) of alkenones and other lipids in cells of *Emiliania huxleyi*

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Abstract

Cells of the coccolithophorid *Emiliania huxleyi* strain CS-57 grown under an atmosphere of air + 0.5% CO_2 showed oxidative damage after 10 days growth with concomitant and major changes to the lipid composition. The fatty acid profile was strongly altered and lacked appreciable amounts of the polyunsaturated fatty acids (PUFA: $C_{18:5}$, $C_{18:3}$ and $C_{22:6}$) typical of healthy cells. Oxidation products of these PUFA could not be detected, but monounsaturated fatty acids proved to be good indicators of oxidative processes. The presence (after $NaBH_4$-reduction) of a high proportion of 11-hydroxyoctadec-*cis*-9-enoic and 8-hydroxyoctadec-*cis*-9-enoic acids showed that the degradation of oleic acid involved mainly free radical oxidation processes (70–75% autoxidation and 20–25% photooxidation). We also detected large amounts of degradation products of the oxidation product 9,10-epoxyoctadecanoic acid including diols, methoxyhydrins and chlorohydrins. These oxidative effects were found in all the lipid classes examined. Products included significant amounts of chlorophyll side-chain autooxidation products *Z*- and *E*-3,7,11,15-tetramethylhexadec-3-ene-1,2-diols and *Z*-and *E*-3,7,11,15-tetramethylhexadec-2-ene-1,4-diols, while phytyldiol was present in relatively low proportions. Δ^5-3β,7-epimeric unsaturated steroidal diols arising from the autoxidation of the Δ^5 double bond of *epi*-brassicasterol and minor amounts of Δ^4-3β,6-diols were also detected. Long-chain unsaturated ketone (alkenone) content per cell was much higher in the presence of 0.5% CO_2 likely due to carbon storage under these conditions. The proportions of di- and tri-unsaturated alkenones was relatively stable throughout the growth cycle in the absence of additional CO_2, but not when grown with 0.5% CO_2. The detection of characteristic alkenone autoxidation products in cells grown under these latter conditions allowed us to attribute the significant increase in index observed to the involvement of free radical oxidation processes.

Keywords: *Emiliania huxleyi*; Free-radical oxidation processes; Alkenones; Unsaturated fatty acids; Sterols, chlorophyll phytyl side-chain; Alteration of $U^{K'}_{37}$ index

1. Introduction

Linear methyl and ethyl C_{37}–C_{40} ketones with 2, 3 or 4 double bonds having *trans* geometry, also known as alkenones, are produced by a limited number of haptophyte microalgae (Volkman et al., 1980, 1995; Marlowe et al., 1984; Conte et al., 1994; Rontani et al., 2004). *Emiliania huxleyi* appears to be the dominant source of these compounds in the open ocean (Harvey, 2000). The proportion of di- and triunsaturated C_{37} alkenones in cultured cells was found to change linearly with water temperature (Brassell et al., 1986; Prahl and Wakeham, 1987). On the basis of this finding and of the ubiquity of C_{37}–C_{40} alkenones in recent and past marine sediments, the ratio $[C_{37:2}]/([C_{37:2}]+[C_{37:3}])$, commonly referred to as $U^{K'}_{37}$ was proposed as a measure of past sea surface temperatures (SST) (Prahl and Wakeham, 1987; Prahl et al., 1989; Müller et al., 1998). This method has become a reference standard for the measurement of palaeotemperatures in palaeoceanographic studies.

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For alkenones to be useful as measures of SST in the geological record, it is essential that any effects of degradation in the water column and in sediments either do not affect the temperature signal established during their initial biosynthesis by the alga (Harvey, 2000; Grimalt et al., 2000) or, if there is a change, its extent can be reasonably estimated. The significant degradation of alkenones observed in the water column and in surface sediments (Prahl et al., 1989; Sikes et al., 1991; Freeman and Wakeham, 1992; Conte et al., 1992; Madureira et al., 1995; Hoefs et al., 1998; Harada et al., 2003; Sun et al., 2004) raises the concern that the more unsaturated components could be selectively lost or modified, thus altering the $U^{K'}_{37}$ ratio (Harvey, 2000).

Recently, the involvement of autoxidative degradative processes, which have been virtually ignored to date owing to the lack of suitable tracers, could be demonstrated in particulate matter samples collected with sediment traps in the northwestern Mediterranean Sea (Marchand et al., 2005). The autoxidative reactivity of alkenones in vitro was previously determined under an atmosphere of air in hexane containing di-*tert*-butyl nitroxide (radical initiator) and *tert*-butyl hydroperoxide (radical enhancer) at 55 °C (Rontani et al., 2006). Alkenones appeared to be more sensitive towards oxidative free radical processes than analogues of other common marine lipids and their rates of oxidation increase in proportion with their number of double bonds. As the result of this increasing reactivity with degree of unsaturation, the $U^{K'}_{37}$ ratio increased significantly (up to 0.2) during the incubation. Evidence for the autoxidation of lipids in seawater was obtained from analysis of organic matter in sediment traps deployed at the DYFAMED site in the northwestern Mediterranean Sea. High contents of specific autoxidation products of monounsaturated fatty acids and the chlorophyll phytyl side-chain were recognized in samples collected during post-bloom conditions, but not during a phytoplankton bloom event. These same samples also showed anomalously high $U^{K'}_{37}$ values (equivalent to an inferred temperature change of at least 2 °C), which is consistent with the results from the autoxidation experiments in the laboratory (Rontani et al., 2006).

In order to confirm these results, we studied the effects of autoxidative processes in cells of *E. huxleyi* in vivo. Although the occurrence of autoxidation processes was clearly demonstrated in situ during post-bloom periods (Marchand et al., 2005; Rontani et al., 2006), it is not easy to induce these processes in laboratory cultures. Indeed, the mechanism of initiation of lipid radical oxidation, which has been debated for many years, seems to be the homolytic cleavage of photochemically produced hydroperoxides in phytodetritus (Rontani et al., 2003). It is well known that metal ions play an important role in the homolysis of hydroperoxides (Pokorny, 1987; Schaich, 1992). The reaction rates with metal ions are in fact much higher than those of autocatalytic reactions. Metal ions may direct the cleavage of hydroperoxides either through alkoxyl or peroxyl radicals (Eqs. (1) and (2)). In classical culture media (such as *f*/2) the metal chelator EDTA is present in high amounts and tightly binds free catalytic metal ions and thus renders them unavailable. EDTA thus acts in the culture media as an antioxidant and strongly limits radical oxidation processes. These antioxidant properties could be strongly limited by decreasing the pH of the culture medium (Rontani, unpublished results). Indeed, at acidic pH values metal ions can be released from EDTA complexes and act as catalysts of hydroperoxide homolysis.

$$\mathrm{ROOH} + \mathrm{M}^{(n+1)+} \rightarrow \mathrm{ROO}^{\cdot} + \mathrm{H}^{+} + \mathrm{M}^{n+} \quad (1)$$

$$\mathrm{ROOH} + \mathrm{M}^{n+} \rightarrow \mathrm{RO}^{\cdot} + \mathrm{HO}^{-} + \mathrm{M}^{(n+1)+} \quad (2)$$

In order to induce autoxidative damage in cells of *E. huxleyi* strain CS-57 and to observe the effects of these processes on their lipid components, we chose to incubate this strain under an atmosphere of air + 0.5% CO_2. Indeed, we observed that these conditions induced a stress (for reasons that are not well understood) in the algae as indicated by an increase in the lag phase. We suggest that the decrease in the pH of the culture medium allowed the release of free metal ions able to catalyze homolytic cleavage of photochemically-produced hydroperoxides and thus to induce free radical oxidation processes.

2. Results and discussion

2.1. Oxidation of fatty acids

After growth for 10 days under an atmosphere of air + 0.5% CO_2, cells of *E. huxleyi* strain CS-57 exhibited a strongly altered fatty acid profile. Cells grown under an atmosphere of air typically contain high amounts of polyunsaturated fatty acids (PUFA) ($C_{18:5}$, $C_{18:3}$ and $C_{22:6}$) (Table 1), but these PUFA were present in lower proportions in "stressed" cells grown in the presence of additional CO_2 (Table 1). A similar trend was previously observed by Riebesell et al. (2000) during growth of *E. huxleyi* cells over a wide range of CO_2 concentrations. The possibility that this disappearance was due to a degradative effect of $NaBH_4$ treatment was examined, but we could rule this out since: (i) all the samples were treated using the same protocol and (ii) cells grown under an atmosphere of air + 0.5% CO_2 also exhibited a strongly altered fatty acid profile (relative to the cells grown under an atmosphere of air) after direct saponification (without $NaBH_4$ reduction). So, we attributed this degradation of the fatty acid profile to the involvement of photooxidative or autoxidative processes since it is well known that the rates of photooxidation (Rontani et al., 1998) and autoxidation (Frankel, 1998) of fatty acids increase with their degree of unsaturation. However, proof could not be obtained since oxidation products of these PUFA could not be detected. This is possibly due to: (i) the involvement of cross-linking reactions leading to the formation of macromolecular structures non-amenable to analysis by gas chromatography (Muizebelt and Nielsen, 1996), or (ii) the instability or volatility of these compounds.

Table 1
Fatty acid composition (fg cell^{-1}) of *E. huxleyi* strain CS-57 cells grown under an atmosphere of air or air + 0.5% of CO_2

	Air (10 days)	Air (17 days)	Air + 0.5% CO_2 (10 days)	Air + 0.5% CO_2 (17 days)
$C_{14:0}$	560 ± 56[a]	183 ± 43	535 ± 90	69 ± 17
$C_{15:0}$	61 ± 6	26 ± 7	76 ± 9	7 ± 1
$C_{16:1}$	50 ± 17	15 ± 8	63 ± 9	5 ± 3
$C_{16:0}$	308 ± 28	105 ± 35	392 ± 31	29 ± 3
$C_{17:0}$	8 ± 0	3 ± 1	13 ± 0	1 ± 0
$C_{18:5\omega3}$	140 ± 84	67 ± 7	44 ± 9	5 ± 1
$C_{18:4\omega3}$	392 ± 112	156 ± 2	196 ± 40	23 ± 2
$C_{18:2\omega6}$	25 ± 6	3 ± 2	13 ± 4	3 ± 1
$C_{18:3\omega3}$	168 ± 60	67 ± 11	80 ± 4	15 ± 1
$C_{18:1\omega9}$	224 ± 28	73 ± 8	258 ± 9	32 ± 1
$C_{18:1\omega7}$	42 ± 3	20 ± 2	71 ± 4	4 ± 1
$C_{18:0}$	56 ± 14	18 ± 1	98 ± 13	8 ± 1
$C_{22:6\omega3}$	140 ± 36	103 ± 11	40 ± 13	12 ± 2
Sum (fg cell^{-1})	2174 ± 450	839 ± 138	1879 ± 235	213 ± 34
Oleic oxidation products[b]	4.6 ± 0.1[c]	2.1 ± 1.0	14.0 ± 2.6	4.2 ± 2.3

[a] Average of duplicates.
[b] 9-Hydroxyoctadec-*trans*-10-enoic, 10-hydroxyoctadec-*trans*-8-enoic, 11-hydroxyoctadec-*trans*-9-enoic, 11-hydroxyoctadec-*cis*-9-enoic, 8-hydroxyoctadec-*trans*-9-enoic and 8-hydroxyoctadec-*cis*-9-enoic acids.
[c] Percentage relative to the parent fatty acid.

Less severe analytical procedures such as $SnCl_2$ reduction and transesterification would perhaps allow one to detect some PUFA oxidation products, but we preferred to focus on monounsaturated fatty acids which, although oxidized more slowly than PUFA, appear to be better indicators of photooxidative or autoxidative damage. Indeed, oxidation of these compounds affords a limited number of quite stable products, which can be analyzed without problem by GC/MS after $NaBH_4$-reduction and used as tracers of lipid oxidation in the marine environment (Marchand and Rontani, 2001). In contrast, most of the isomeric hydroperoxides resulting from PUFA oxidation can undergo rapid oxidation (of residual double bonds) or be decomposed to oligomeric or volatile products (for a review see Frankel, 1998) which strongly limits their suitability as efficient tracers of lipid oxidation in situ. Moreover, while it is sometimes difficult to differentiate abiotic oxidation products of PUFA (Frankel, 1998) from those resulting from lipoxygenase-mediated oxygenation (Pohnert and Boland, 2002; Pohnert, 2005), this is not the case for monounsaturated fatty acids. Indeed, enzymatic oxidation of oleic acid (for example) generally affords 9,10-epoxyoctadecanoic acid (Ruettinger and Fulco, 1981) or other very specific compounds such as: 10-hydroxyoctadec-*trans*-8-enoic (Busquets et al., 2004), 11-oxooctadec-*cis*-9-enoic (Clapp et al., 2001), 8-hydroperoxyoctadec-*cis*-9-enoic (Oliw et al., 1998) or 8-hydroxy-9,10-epoxyoctadecanoic acids (Oliw et al., 1998). In contrast, singlet oxygen-mediated photooxidation of monounsaturated fatty acids involves a direct reaction of $^1O_2$ with the carbon–carbon double bond by a concerted "ene" addition (Frimer, 1979), and leads to the formation of hydroperoxides at each carbon of the original double bond. Thus, oleic acid produces a mixture of 9- and 10-hydroperoxides with an allylic *trans*-double bond (Frankel et al., 1979). Free radical oxidation of monounsaturated fatty acids affords a group of six isomeric hydroperoxyacids. For example, oxidation of oleic acid results in the formation of 9-hydroperoxyoctadec-*trans*-10-enoic, 10-hydroperoxyoctadec-*trans*-8-enoic, 11-hydroperoxyoctadec-*trans*-9-enoic, 11-hydroperoxyoctadec-*cis*-9-enoic, 8-hydroperoxyoctadec-*trans*-9-enoic and 8-hydroperoxyoctadec-*cis*-9-enoic acids (Frankel, 1998). Autoxidative processes can be easily characterized based on the presence of *cis* allylic hydroperoxyacids, which are specific products of these degradation processes (Porter et al., 1994; Frankel, 1998).

Large amounts of photo- and autoxidation products of oleic acid could be detected in cells of *E. huxleyi* grown under an atmosphere of air + 0.5% CO_2 for 10 days (Table 1). The presence (after $NaBH_4$-reduction) of a high proportion of 11-hydroxyoctadec-*cis*-9-enoic and 8-hydroxyoctadec-*cis*-9-enoic acids (Fig. 1) showed that the degradation of oleic acid involved mainly free radical oxidation processes (70–75% autoxidation and 20–25% photooxidation; estimates calculated from the equations given in Marchand and Rontani, 2001).

We also detected large amounts of degradation products of 9,10-epoxyoctadecanoic acid (Fig. 2). These compounds (diols, methoxyhydrins and chlorohydrins) were produced during the treatment. Indeed, epoxyacids undergo alcoholysis and hydrolysis during alkaline hydrolysis and are converted to chlorohydrins and 9,10-dihydroxyacids during acidification (Marchand and Rontani, 2001). It is well known that some bacteria may convert Δ^9-unsaturated fatty acids to 9,10-epoxyacids (Ruettinger and Fulco, 1981); these bacteria can further hydrolyse these epoxides to yield 9,10-dihydroxyacids (Michaels et al., 1980). Enzymatic epoxidation of *cis*-Δ^9-unsaturated fatty acids is highly stereospecific and affords only *cis*-epoxides (Croteau and Kolattukudy, 1975). The detection of two *erythro* and *threo* pairs of diastereomeric 9,10-dihydroxyacids (Fig. 2) allowed us to rule out enzymatic epoxidation. Epoxyacids

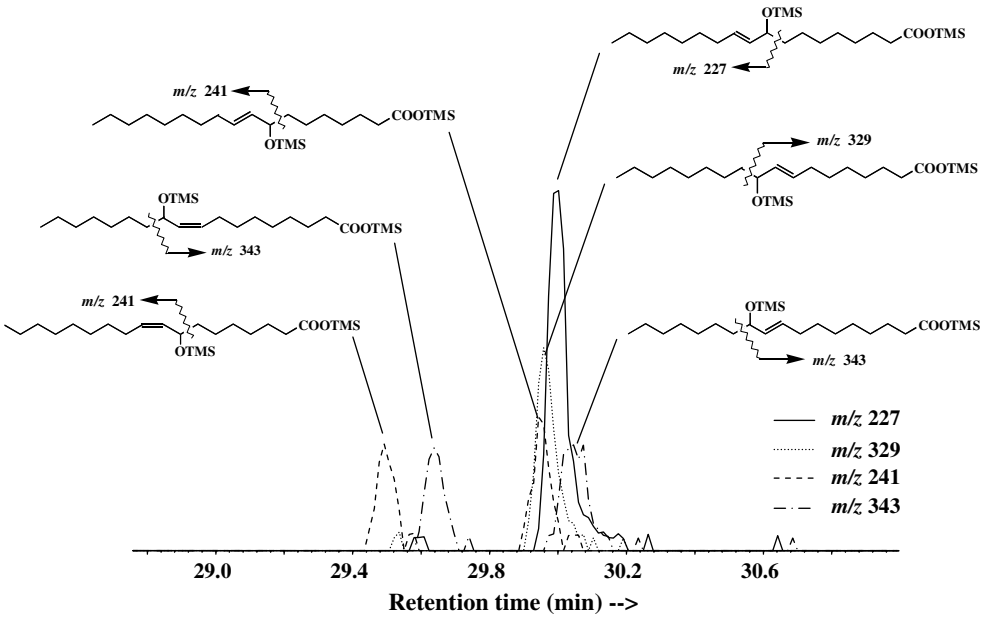

Fig. 1. Partial mass fragmentograms of *m*/*z* 227, 329, 241 and 343 revealing the presence of oxidation products of oleic acid in the saponified fraction of *E. huxleyi* strain CS-57 grown under an atmosphere of air + 0.5% CO_2 for 10 days.

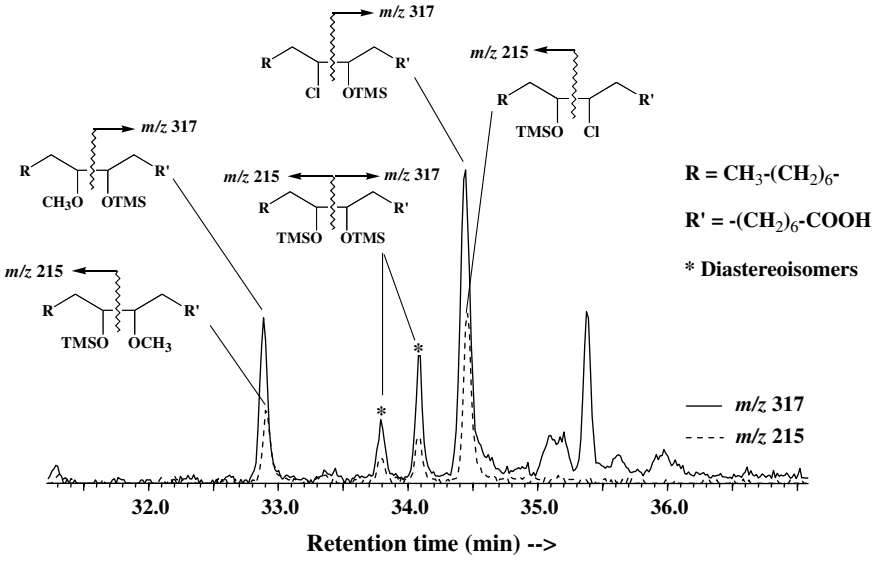

Fig. 2. Partial mass fragmentograms of *m*/*z* 317 and 215 revealing the presence of degradation products of 9,10-epoxyoctadecanoic acid in the saponified fraction of *E. huxleyi* strain CS-57 grown under an atmosphere of air + 0.5% CO_2 for 10 days.

can also be formed by cyclization of alkoxyl radicals derived from allylic hydroperoxides (Frankel, 1998). However, in this case, other epoxyacid isomers (e.g. 8,9-epoxyoctadecanoic and 10,11-epoxyoctadecanoic acids) should be formed, but we failed to detect these compounds in *E. huxleyi* cells. The formation of 9,10-epoxyoctadecanoic acid was thus suggested to be due to hydroperoxide-induced autoxidation of oleic acid (Marchand and Rontani, 2001).

After this period of stress, *E. huxleyi* cells appeared to acclimatize to the presence of additional CO_2 and we obtained a cell concentration approximately seven fold higher than under an atmosphere of air (Table 2).

2.2. Oxidation of chlorophyll phytyl side-chain

Photo-oxygenation of the olefinic C-2 of the chlorophyll phytyl side-chain involves selective hydrogen abstraction by singlet oxygen on the allylic methyl group *Z*-oriented to the carbinol group (Rontani et al., 1994). This strong regioselectivity, which is in accord with the results obtained by Schulte-Elte et al. (1979) in the case of 3-methyl-3-alkyl-substituted allylic alcohols, suggests that 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol) (Fig. 3) can be used as a specific tracer of chlorophyll photodegradation processes (Rontani et al., 1994; Cuny and Rontani, 1999).

Table 2
Alkenone concentrations, $U^{K'}_{37}$ and cell count values measured in cells of *E. huxleyi* strain CS-57 grown under atmospheres of air and air + 0.5% CO_2

	Air growth for 10 days	Air growth for 17 days	Air + 0.5% CO_2 growth for 10 days	Air + 0.5% CO_2 growth for 17 days
$U^{K'}_{37}$	0.54 ± 0.01	0.54 ± 0.01	0.65 ± 0.04	0.50 ± 0.02
[Alkenones] (ng cell^{-1})	2.29	0.68	6.58	0.42
Cell counts (cells ml^{-1})	225,000	1,250,000	140,000	8,650,000

Recently, the production of *Z*- and *E*-3,7,11,15-tetramethylhexadec-3-ene-1,2-diols and *Z*- and *E*-3,7,11,15-tetramethylhexadec-2-ene-1,4-diols was observed during autoxidation of the chlorophyll phytyl side-chain (Rontani and Aubert, 2005) (Fig. 3). These new compounds were proposed as specific indicators of radical-mediated oxidation processes.

Analyses of the lipid extracts obtained from *E. huxleyi* cells grown in the presence of additional CO_2 for 10 days showed the presence of significant amounts of *Z*- and *E*-3,7,11,15-tetramethylhexadec-3-ene-1,2-diols and *Z*- and *E*-3,7,11,15-tetramethylhexadec-2-ene-1,4-diols (Fig. 4), while phytyldiol was present in relatively low proportions. These results confirmed that autoxidative processes play an important role during the degradation of the lipid components in stressed cells of *E. huxleyi*. In contrast, the lack of oxidation products of the chlorophyll phytyl side-chain in cells grown in the presence of additional CO_2 for 17 days, and under an atmosphere of air attested to their relatively good physiological state.

2.3. Oxidation of sterols

Singlet-oxygen-mediated photooxidation of sterols produces mainly Δ^6-5α-hydroperoxides and to a lesser extent Δ^4-6α/6β-hydroperoxides (Nickon and Bagli, 1961; Kulig and Smith, 1973), whereas autoxidative processes give Δ^5-7α/7β-hydroperoxides (Smith, 1981). Δ^6-5α-Hydroperoxysterols are relatively unstable and may undergo allylic rearrangement to Δ^5-7-hydroperoxysterols (Smith, 1981) (Fig. 5). The characterization of Δ^6-3,5-dihydroxysterols (after $NaBH_4$ reduction of the corresponding hydroperoxides) by GC-MS presents some difficulties. Indeed, derivatization of this diol with pyridine/BSTFA results in the silylation of only the hydroxyl group at position 3 and the resulting monosilylated derivative undergoes quantita-

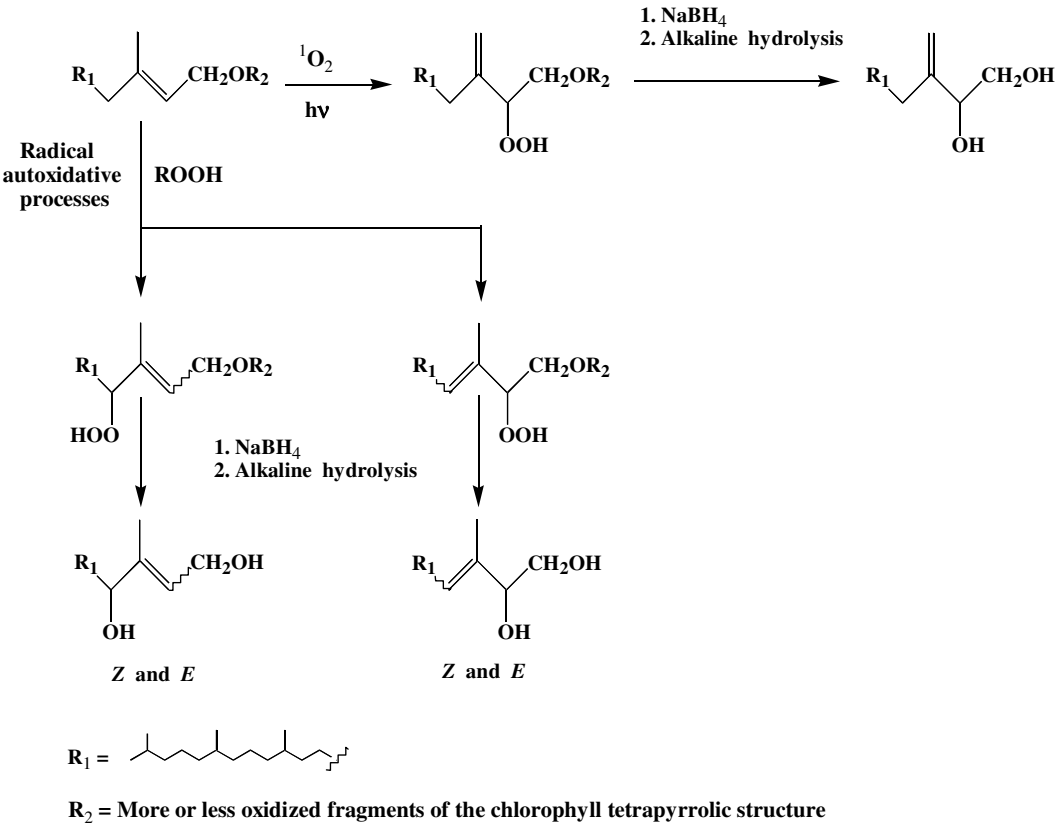

Fig. 3. Formation of phytyldiol, 3,7,11,15-tetramethylhexadec-3-ene-1,2-diols and 3,7,11,15-tetramethylhexadec-2-ene-1,4-diols from the chlorophyll phytyl side-chain.

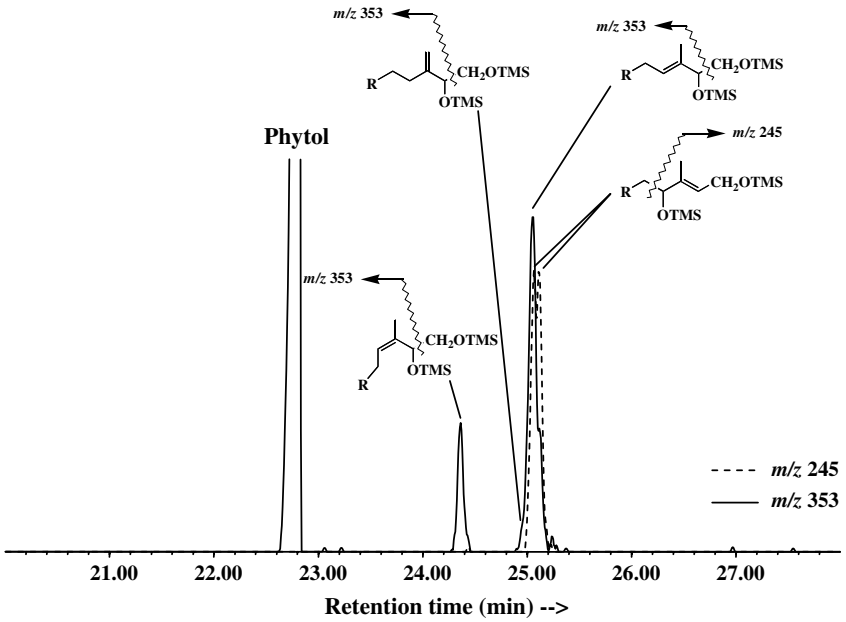

Fig. 4. Partial mass fragmentograms of *m/z* 353 and 245 revealing the presence of oxidation products of chlorophyll phytyl side-chain in the unsaponified fraction of *E. huxleyi* strain CS-57 grown under an atmosphere of air + 0.5% CO_2 for 10 days.

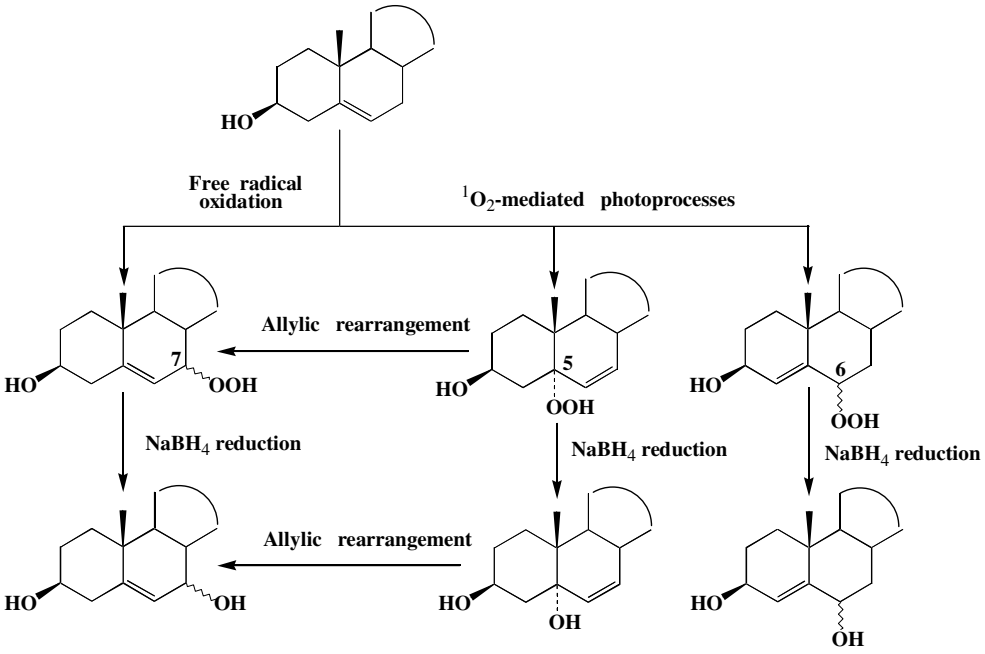

Fig. 5. Autoxidation and photooxidation of Δ^5-sterols.

tive allylic rearrangement to Δ^5-3,7-dihydroxysterols during GC analysis (Rontani and Marchand, 2000).

GC-EIMS analyses allowed detection of significant amounts of Δ^5-3β,7-epimeric unsaturated steroidal diols arising from the oxidation of the Δ^5 double bond of *epi*-brassicasterol in *E. huxleyi* cells grown in the presence of additional CO_2 for 10 days. Δ^4-3β,6-Diols were present in very low amounts. Δ^5-3β,7-Diols may arise from radical oxidation of the corresponding sterols or from allylic rearrangement of photochemically produced Δ^6-3β,5α-diols (Fig. 5). On the basis of the very low value of the ratio Δ^4-3β,6-diols/Δ^5-3β,7-diols (0.1), which was much lower than the values of the ratio Δ^4-6-hydroperoxides/Δ^6-5-hydroperoxides measured in photodegraded phytoplanktonic cells (0.34; Rontani et al., 1997), it could be estimated that the major part of the Δ^5-3β,7-diols detected arose from autoxidative processes. These diols were present only in trace amounts in cells grown in the presence of additional CO_2 for 17 days and in those cells grown under an atmosphere of air.

2.4. Oxidation of alkenones and derivatives

After 10 days of growth, alkenone content per cell was much higher in the presence of additional CO_2 (Table 2). This observation is in good agreement with the results previously obtained by Riebesell et al. (2000), who observed a strong increase in alkenone contents per cell with increasing dissolved CO_2 concentrations. These authors attributed this result to storage of excess photosynthate as storage lipids, which is one of the functions of alkenones. This hypothesis was validated recently by Eltgroth et al. (2005), who showed that *E. huxleyi* packaged alkenones into cytoplasmic vesicles. After this phase of lipid storage, alkenones must have been consumed since the content per cell observed after growth for 17 days in the presence of additional CO_2 was quite similar to that of cells grown under air (Table 2).

Cells of *E. huxleyi* grown under an atmosphere of air at 20 °C exhibited stable $U^{K'}_{37}$ values (0.54 ± 0.01) throughout the growth cycle (Table 2, Fig. 6a). In contrast, after growth in the presence of additional CO_2 for 10 days, $U^{K'}_{37}$ values were significantly higher (ranging from 0.61 to 0.69) (Table 2, Fig. 6b). These surprisingly high values cannot simply be attributed to the presence of additional CO_2 since it was previously demonstrated that increases in CO_2 concentration have no systematic effect on $U^{K'}_{37}$ values (Riebesell et al., 2000). Moreover, after acclimatization of the cells to the CO_2 concentration (after growth for 17 days), $U^{K'}_{37}$ values recovered to approximate those observed in cells grown under an atmosphere of air (Table 2). An increase in $U^{K'}_{37}$ value seems to be related to the "stressed state" of the cells. On the basis of the large amounts of autoxidation products of other lipids detected in the cells

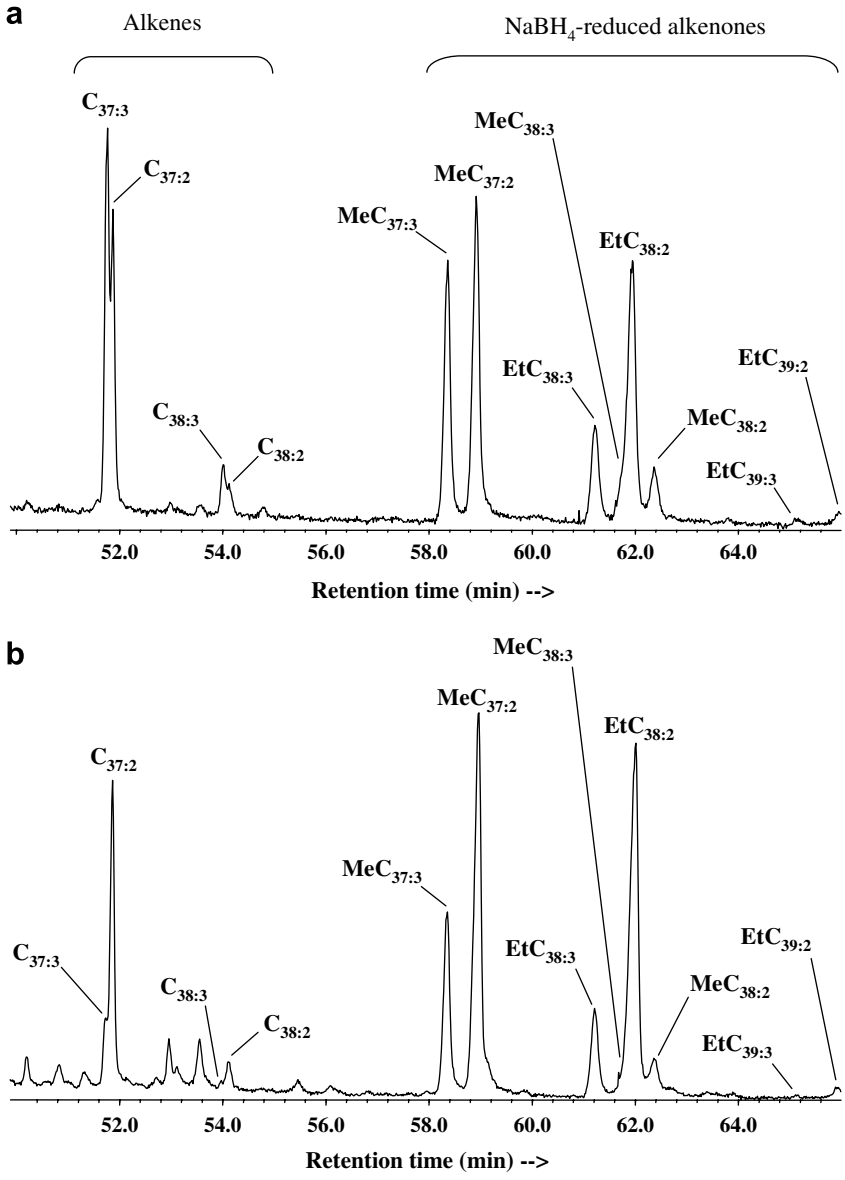

Fig. 6. Partial total ion current chromatogram of the silylated $NaBH_4$-reduced total lipid fraction of *E. huxleyi* strain CS-57 grown under an atmosphere of air for 10 days (a) and under an atmosphere of air + 0.5% CO_2 for 10 days (b).

at this time (see above), this increase is most likely due to the selective radical-induced oxidation of alkenones.

Autoxidation of alkenones mainly involves allylic hydrogen abstraction and subsequent oxidation of the allylic radical thus formed affording isomeric allylic hydroperoxides. Oxidation of each double bond in the alkenones and subsequent $NaBH_4$ reduction affords four positionally isomeric alkenediols (Rontani et al., 2006). In order to demonstrate the involvement of such degradative processes in *E. huxleyi* cells, we prepared standard autoxidation products from purified alkenones as previously described (Rontani et al., 2006) and we reduced (with $NaBH_4$) the resultant hydroperoxyketones to the corresponding alkenediols. Subsequent comparison of retention times and mass spectra with these standard compounds allowed us to demonstrate unambiguously the presence of alkenone autoxidation products in *E. huxleyi* cells grown in the presence of additional CO_2 for 10 days (Fig. 7). These results show that in cells of *E. huxleyi*, oxidation due to free radical processes can act not only on unsaturated fatty acids, sterols and the chlorophyll phytyl side-chain but also on alkenones inducing a significant increase in $U^{K'}_{37}$ values (ranging from 0.07 to 0.15).

By plotting the variation in $U^{K'}_{37}$ values observed according to the percentage of specific *cis* (i.e. *Z*)-autoxidation products of oleic acid (relative to the residual parent acid) measured, we obtained a relatively good polynomial correlation ($U^{K'}_{37} = 0.0083 \times [\%Cis]^2 + 0.0055 \times [\%Cis] + 0.533$; $r^2 = 0.97$) (Fig. 8). This result confirms the existence of a link between the increase in $U^{K'}_{37}$ values and the autoxidative degradation state of the cells. This proxy could be useful to correct biases induced by free radical-mediated oxidation processes during alkenone-based temperature estimations. Unfortunately, it would be difficult to use this proxy for natural samples given the many sources of fatty acids and sterols present in aquatic environments and uncertainties caused by the presence of both fresh and degraded organic matter. This proxy might work to some extent where *E. huxleyi* is the major source of lipids.

We would also expect C_{37} and C_{38} alkadienes and trienes, which are structurally related to methyl C_{37} and C_{38}

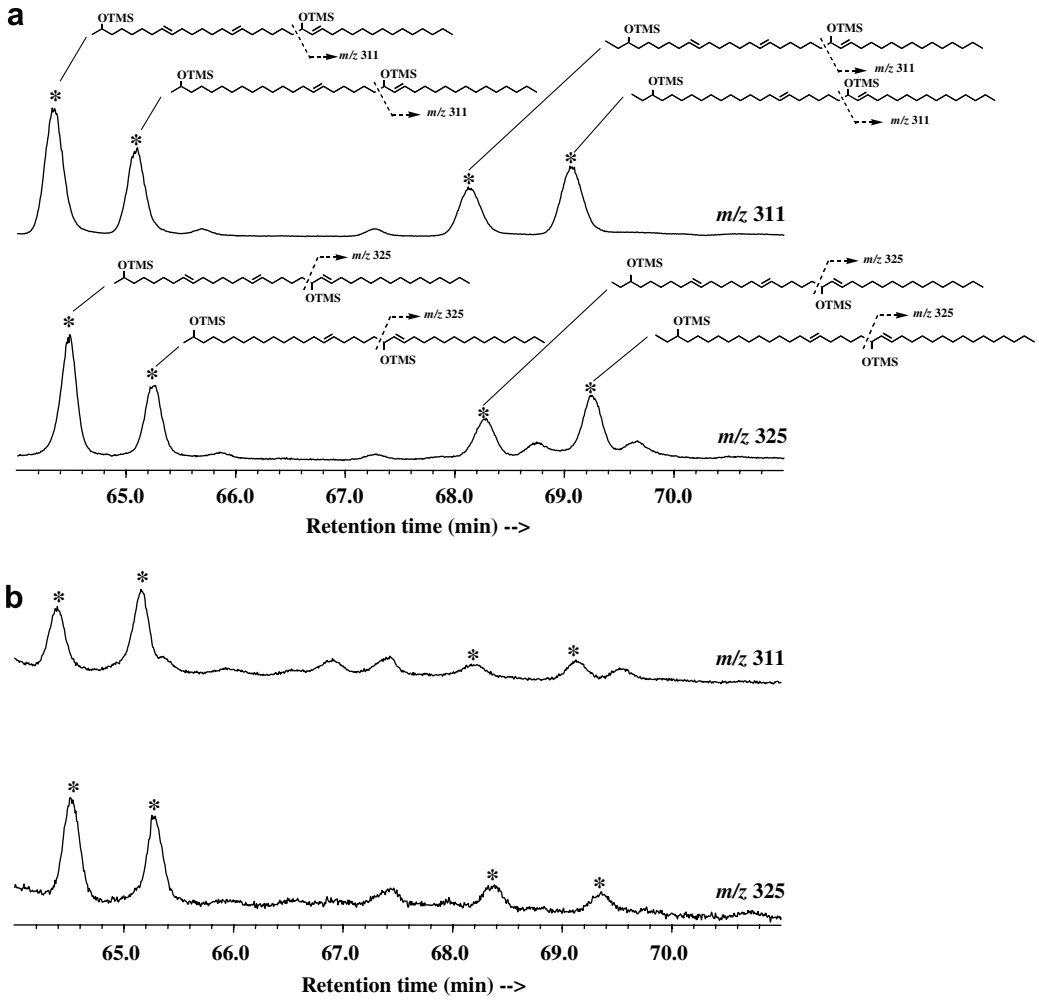

Fig. 7. Partial mass fragmentograms of *m/z* 311 and 325 revealing the presence of silylated C_{37} and C_{38} alkenediols after $NaBH_4$-reduction and silylation of standard autoxidized alkenones (a) and of the total lipid fraction of *E. huxleyi* strain CS-57 grown under an atmosphere of air + 0.5% CO_2 for 10 days (b).

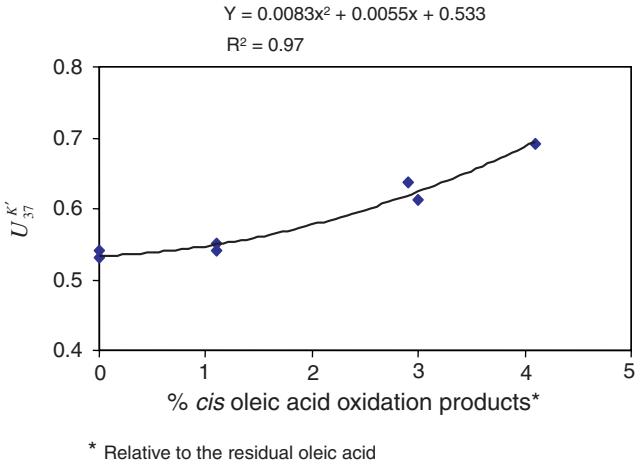

Fig. 8. $U^{K'}_{37}$ values plotted against the percentage of *cis* oxidation products of oleic acid indicating that variations in $U^{K'}_{37}$ values are likely due to alkenone oxidation.

alkenones, to be strongly affected by autoxidative reactions. Unfortunately, it was not possible to estimate the actual impact of these degradative processes on these compounds since the proportions of di- and triunsaturated alkenes changed (for unknown reasons) in "unstressed" cells during growth.

2.5. Comparison to observations of autoxidation in the natural environment

Anomalously high $U^{K'}_{37}$ values (equivalent to an inferred temperature change of at least 2 °C) were previously detected in particulate matter samples collected during post-bloom periods at the DYFAMED Station (Ligurian Sea) and attributed to the involvement of autoxidative processes due to the presence of oxidation products of monounsaturated fatty acids and the chlorophyll phytyl side-chain (Rontani et al., 2006). Our new results provide strong support for this hypothesis.

Free radical oxidation processes, which can act throughout the water column and in the oxic zone of the sediments, could explain the selective degradation of alkenones previously observed in some aerobic sediments (Hoefs et al., 1998; Gong and Hollander, 1999; Prahl et al., 2003) and suspended particles (Sawada et al., 1998). Autoxidation is also consistent with the fact that $U^{K'}_{37}$ values recorded in sediments are often higher than those in the particles settling through the overlying water column (Prahl et al., 1993; Ternois et al., 1996; Cacho et al., 1999).

Alkenediols resulting from $NaBH_4$-reduction of autoxidation products of alkenones would be very useful indicators of autoxidative alteration of $U^{K'}_{37}$ values but, unfortunately, they do not accumulate (Rontani et al., 2006). Indeed, due to the presence of additional reactive double bonds, hydroperoxyalkenones may undergo subsequent oxidation reactions affording, after $NaBH_4$ reduction, alkene-triols, tetraols or pentaols according to the degree of unsaturation of the starting alkenone. Owing to their very high molecular weight and polarity, these polyols display poor chromatographic characteristics and thus cannot be used as effective tracers of alkenone autoxidation in natural samples. It may be that future studies might identify new markers (e.g. from chain-cleavage of oxidation products). As an interim solution, we propose that researchers check for the presence of the specific autoxidation products of oleic acid and chlorophyll phytyl side-chain above described in order to identify cases where autoxidation of organic matter has been significant. In these cases, an overestimation of $U^{K'}_{37}$ values would be expected.

3. Conclusions

By culturing the strain *E. huxleyi* strain CS-57 under an atmosphere of air + 0.5% CO_2, we could induce autoxidative degradative processes in this alga and study the effects of these processes on its main lipid components. Free radical-mediated processes acted intensively on monounsaturated fatty acids, sterols and the chlorophyll phytyl side-chain affording different oxidation products. Some of these compounds (11-hydroxyoctadec-*cis*-9-enoic and 8-hydroxyoctadec-*cis*-9-enoic acids, *Z*- and *E*-3,7,11,15-tetramethylhexadec-3-ene-1,2-diols and 3,7,11,15-tetramethylhexadec-2-ene-1,4-diols) appeared to be sufficiently specific to be proposed as tracers of these degradative processes. This autoxidative damage in the cells of *E. huxleyi* is comparable to that previously observed in situ in some particulate matter samples from the Mediterranean (Marchand et al., 2005; Rontani et al., 2006). Growth of the alga in the presence of additional CO_2 as an autoxidative stress allowed us to simulate these natural autoxidative processes.

We previously showed in laboratory experiments that alkenones were autoxidized faster than analogues of other common marine lipids such as phytyl acetate, methyl oleate and cholesteryl acetate (Rontani et al., 2006). During the present work, we could detect autoxidation products of alkenones in cells of *E. huxleyi* and so demonstrate that free radical oxidation processes may also act intensively on alkenones within the living cell. Owing to their selectivity these degradative processes may induce a significant increase in $U^{K'}_{37}$ values (ranging from 0.07 to 0.15 and corresponding to inferred temperature changes ranging from 2 to 4 °C). Our work demonstrates that autoxidation of lipids may occur in cells of *E. huxleyi* and that these processes have the potential to affect alkenone distributions leading to a warm bias in estimates of palaeotemperature derived from alkenone ratios in sediments.

4. Experimental

4.1. Chemicals

Standard oxidation products of oleic acid, chlorophyll phytyl side-chain and brassicasterol were produced accord-

ing to previously described procedures (Rontani and Marchand, 2000; Marchand and Rontani, 2001; Rontani and Aubert, 2005). Alkenones were isolated from *E. huxleyi* strain CS-57 freeze–dried cells as previously described (Rontani et al., 2006).

4.2. Algal cultures

The strain used was CS-57 *Emiliania huxleyi* (Lohmann) Hay et Mohler, isolated by R. Guillard in 1960 from an unknown location in the Sargasso Sea. This strain is maintained at the CSIRO laboratories in 50 ml glass Erlenmeyer flasks in *f*/2 medium (Jeffrey and LeRoi, 1997) at 20 °C and 80 μmol photons m^{-2} s^{-1}. A 40 ml subsample of a logarithmic phase culture was used to inoculate 4 two-litre flasks with 1600 ml *f*/2 media. Two of these flasks were aerated with 0.2 μm filtered air only and the other two flasks with 0.2 μm filtered air + 0.5% CO_2 using an Aalborg Instruments flow meter. The $+CO_2$ treatment used Industrial Grade compressed CO_2 (UN No. 1013) set to 0.5% of the air flow (actual range 0.41%–0.53%) with air and CO_2 levels monitored twice daily and adjusted if necessary. The light intensity was 100 μmol photons m^{-2} s^{-1} on a 16:8 h light:dark cycle. Cell growth was tracked on a daily basis by aseptically transferring 40 ml subsamples into sterile 25 mm diameter screw capped tubes and reading in vivo fluorescence on a Turner Designs 10AU fluorometer. The subsamples were aseptically returned to the flasks; 800 ml of each flask was aseptically harvested after 10 and 17 days when fluorescence indicated the cultures were in mid-log phase and end of log phase respectively. Cell yield at day 10 and 17 was estimated from 5 ml subsamples counted using a Neubauer hemocytometer. The harvested aliquots of each flask were split into two Beckman 500 ml centrifuge tubes and centrifuged on a Sorvall RC26 Plus centrifuge (SLA-3000 rotor) at 2000 rpm for 15 min. The concentrates were combined into a single tube and the centrifuging step repeated. The supernatant was then reduced to 45 ml and the concentrates were transferred to Corning 50 ml tubes and centrifuged on a Sorvall RT6000 benchtop unit at 2000 rpm for 12 mins. The supernatant was reduced to 10 ml and the concentrate vortexed for 10–20 s and transferred to Labconco 80 ml flasks, stored overnight at −20 °C and then freeze–dried in a Labconco Freezone 6. Microscopic examination of centrifuged and freeze dried material was undertaken on a Zeiss Axioplan microscope. Preparation methods appeared to be non-destructive as microscopic examination of freeze–dried material resuspended in sterile seawater revealed intact cells.

4.3. Chemical treatment of the cells

Oxidation products were obtained after $NaBH_4$ reduction and subsequent saponification of lyophilized cells. All manipulations were carried out with foil-covered vessels in order to exclude photochemical artifacts. This reduction of hydroperoxides with $NaBH_4$ allowed us to avoid the production of autoxidative artifacts during alkaline hydrolysis.

4.4. Reduction of hydroperoxides to alcohols

Hydroperoxides were reduced to the corresponding alcohols in methanol (25 ml) by excess $NaBH_4$ (10 mg/mg of freeze–dried cells) (30 min at room temperature). During this treatment, alkenones are also partially reduced to alkenols and the possibility of some ester cleavage cannot be excluded.

4.5. Alkaline hydrolysis

Saponification was carried out on reduced samples. After $NaBH_4$ reduction, 25 ml of water and 2.8 g of potassium hydroxide were added and the mixture was directly saponified by refluxing for 2 h. After cooling, the content of the flask was extracted three times with hexane. The combined hexane extracts were dried over anhydrous Na_2SO_4, filtered and concentrated to give the unsaponified fraction. The aqueous phase was then acidified with hydrochloric acid (pH 1) and subsequently extracted three times with dichloromethane. Treatment of the combined dichloromethane extracts as described above gave the saponified fraction. The separation of unsaponified and saponified lipids appeared to be inefficient for alkenones and alkenols (which were present in significant amounts in the saponified extracts), but this separation is needed to detect oxidation products of sterols. Indeed, some of these compounds may react with methanol when the extraction is performed after acidification.

4.6. Reduction of alkenones

After analyses of other lipids (sterols, fatty acids and isoprenoids), saponified and unsaponified extracts were combined and reduced (20 min) in diethyl ether:methanol (2:1, v/v) (5 ml) by excess $NaBH_4$ (10 mg/mg of extract) in order to achieve alkenone reduction. After reduction, 10 ml of a saturated solution of ammonium chloride was added cautiously to destroy excess $NaBH_4$, the mixture was shaken and extracted three times with hexane:chloroform (4:1, v/v). The combined extracts were dried over anhydrous Na_2SO_4, filtered and concentrated under a stream of nitrogen.

4.7. Derivatization

After evaporation of the solvents, the residues were taken up in 300 μl of a mixture of pyridine and BSTFA (Supelco) (2:1, v/v) and silylated for 1 h at 50 °C. After evaporation to dryness under nitrogen, the residues were taken up in a mixture of ethyl acetate and hexane and analyzed by gas chromatography-electron impact mass spectrometry (GC-EIMS).

4.8. Gas chromatography-electron impact mass spectrometry (GC-EIMS)

GC-EIMS analyses were carried out with a HP 5890 Series II Plus gas chromatograph connected to a HP 5972 mass spectrometer. The following operating conditions were employed: 30 m × 0.25 mm (i.d.) fused silica capillary column coated with SOLGEL-1 (SGE; film thickness, 0.25 μm). The following conditions were used: oven temperature programmed from 60 to 130 °C at 30 °C min^{-1} and then from 130 to 300 °C at 4 °C min^{-1}; carrier gas (He) maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 to 1.5 bar at 0.04 bar min^{-1}; injector (splitless) temperature, 300 °C; injector (on column with retention gap) temperature, 50 °C; electron energy, 70 eV; source temperature, 170 °C; cycle time, 1.5 s. An on column injector was used for the analysis of alkenols and sterols and a splitless injector for the analysis of the other lipids.

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