

Degradation of alkenones by aerobic heterotrophic bacteria: selective or not?

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Abstract

Four bacterial communities were isolated from *Emiliania huxleyi* strain TWP1 cultures before and after the algal cells had been treated with different antibiotics. Incubation of *E. huxleyi* with these bacterial communities resulted in dramatically different extents of alkenone degradation ranging from effectively none to extensive. Selective degradation of the more unsaturated alkenones was observed in experiments using the total bacterial community and one of the communities isolated from antibiotic-treated algal cells. The observed increases in U_{37}^K are equivalent to a +2°C and +3.3°C change in the inferred temperature. Our results clearly show that intense aerobic microbial degradative processes have the potential to introduce a significant ‘warm’ bias in palaeotemperature reconstruction and could explain apparent anomalies in palaeotemperatures inferred from alkenone distributions in strongly oxidizing sedimentary environments. The results show that aerobic bacteria capable of selectively degrading alkenones are not limited to particular environments such as microbial mats and can be actually associated with living *E. huxleyi* cells. The detection of epoxyketones in some cultures indicates that metabolic pathways involving attack of the terminal groups of the molecule are essentially non-selective, while those acting on alkenone double bonds are selective. The epoxyketones resulting from bacterial epoxidation of alkenone double bonds could be useful indicators of aerobic bacterial alteration of the alkenone unsaturation ratio in situ. The production of alkenols during incubation with one of the bacterial communities demonstrated for the first time that bacterial reduction of alkenones can be a potential source of these compounds in the environment. The intriguing production of small amounts of monounsaturated alkenones by one of the bacterial communities also raises the possibility of a bacterial reduction of alkenone double bonds.

1. Introduction

Alkenones are a class of unusual, very long chain mono-, di-, tri- and tetraunsaturated methyl and ethyl ketones synthesized by a limited number of haptophyte microalgae (Volkman et al., 1980a; Marlowe et al., 1984; Conte et al., 1994; Volkman et al., 1995). In the open ocean, *Emiliania huxleyi* appears to be the dominant source of the C₃₇–C₃₉ alkenones (Harvey, 2000), with additional contributions from *Gephyrocapsa oceanica* and perhaps other species (Conte et al., 1995; Volkman et al., 1995). Other haptophytes synthesizing alkenones include *Isochrysis* spp. and *Chrysotila lamellosa* (Marlowe, 1984; Marlowe et al., 1984; Rontani et al., 2004) which occur mainly in coastal waters. Alkenones are used as a palaeoceanographic proxy for reconstruction of sea surface temperatures (SSTs; e.g. Brassell et al., 1986; Prah1 and Wakeham 1987; Eglinton et al., 1992), partial pressure of CO₂ (Jasper and Hayes 1990; Jasper et al., 1994) and now, potentially, even sea surface salinity (Engelbrecht and Sachs, 2005; Schouten et al., 2006).

The proportion of di- to triunsaturated C₃₇ alkenones in cultured cells increases with increasing water temperature (Brassell et al., 1986; Prah1 and Wakeham, 1987). On the basis of this finding and the ubiquity of C₃₇–C₄₀ alkenones in recent and ancient marine sediments, the ratio $[C_{37:2}] / ([C_{37:2}] + [C_{37:3}])$, commonly referred to as $U_{37}^{K'}$, was proposed as a measure of SST (Prah1 and Wakeham, 1987; Prah1 et al., 1988; Müller et al., 1998). This method has become a reference standard for assessment of SST in palaeoceanographic studies.

An underlying assumption in the use of alkenones as a palaeotemperature proxy is that the temperature signal established during their initial biosynthesis by the alga (Harvey, 2000; Grimalt et al., 2000) is not affected by diagenetic processes or, if there is a change, its extent can be objectively evaluated. However, many studies have now documented significant

degradation of alkenones in the water column and 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; Prahl et al., 2001; Harada et al., 2003; Sun et al., 2004). If the more unsaturated components are selectively lost or modified, the process will alter the $U_{37}^{K'}$ ratio (Harvey, 2000) and so compromise its use as a reliable, absolute measure of SST.

Despite the widespread use of alkenones for palaeothermometry, comparatively few studies have investigated the effects of bacterial degradation on differential degradation of alkenones. Teece et al. (1998) conducted studies on the microbial degradation of *Emiliania huxleyi* cells under oxic and anoxic conditions in order to try to understand early diagenetic processes. Although they observed an extensive degradation of the C₃₇ methyl alkenone under all the conditions examined, with up to 85% degraded under oxic conditions, $U_{37}^{K'}$ values remained constant. Based on these results, it was concluded by several authors that the $U_{37}^{K'}$ index is unaffected by oxic biodegradation processes.

Recently, Rontani et al. (2005a) isolated bacteria from microbial mats very rich in alkenones (Rontani and Volkman, 2005). These bacteria were able to degrade alkenones efficiently under aerobic conditions and the authors observed a variable selectivity during the microbial attack on C₃₇ alkenones, resulting in variations in the $U_{37}^{K'}$ index ranging from 0 to + 0.10 that corresponds to an inferred temperature difference of 0 to +3°C. This variability could be attributed to the heterogeneity of the inoculum (microbial mats) and to the very large diversity of the aerobic heterotrophic bacteria present that could attack the alkenone molecules via different pathways. Thus, it would now appear unwise to generalize the results obtained from only one experiment with a bacterial monoculture (Teece et al., 1998) to the wide spectrum of aerobic bacterial communities in sediments and seawater.

During incubation of living cells of *Emiliania huxleyi* strain TWP1 in the dark, we observed a major and selective degradation of alkenones. This observation parallels findings

obtained in prolonged darkness with living cultures of *E. huxleyi* strain CCMP1742 (Prahl et al., 2003). During our incubation, we also noted a strong increase in the proportion of *cis*-vaccenic (18:1n-7), branched pentadecanoic and 11-methyloctadec-12-enoic fatty acids, which are generally considered as typical bacterial biomarkers (Volkman et al., 1980b; Zegouagh et al., 2000; Rontani et al., 2005b). This strong change in fatty acid profile led us to suspect that the selective degradation of alkenones during the incubation was induced by the aerobic bacteria actually associated with *E. huxleyi* cells. In order to check this hypothesis, we studied the degradation of alkenones by different aerobic bacterial communities isolated from cells of *E. huxleyi* strain TWP1 before and after different treatments of the algal cells with antibiotics.

2. Experimental

2.1. Substrate preparation for degradation studies

Emiliana huxleyi strain TWP1 obtained from the Caen (France) Algalbank were used as substrate for alkenone degradation. A 100 ml starter culture was transferred to flasks containing 1500 ml of f/2 medium and grown (10 d) at 20°C, using 116 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cool white fluorescent (Osram, fluora) light under a 12 h light:12 h dark regime. The cells were harvested using a Beckman J2-21 centrifuge at 5000 rpm for 25 min, concentrated at 15000 rpm using a Beckman GS-15R centrifuge (U.S.A.) for 15 min to remove excess water, freeze dried using a Virtis Benchtop 2K lyophilizer, homogenized using a mortar and pestle and stored at -20°C until use.

2.2. Tests for sterilization

Lyophilized *E. huxleyi* cells (10 mg x 6) were weighed in 5 ml screw cap Pyrex vials with Teflon (TFPE) lining. Two of the six vials were kept separately as non-sterilized

controls. The remaining four were kept in an air tight bottle and autoclaved at 120°C (20 min). The vials were securely tightened to exclude moisture. Lipid profiles of two of these sterilized vials were compared to those of the two non-sterilized controls to check for any changes that might have resulted from the sterilization procedure. The remaining two vials with sterilized *E. huxleyi* were then tested to verify the success of the sterilization by inoculating the contents of the sterilized vials in the bacterial and f/2 media, and incubating the flask at 20°C.

2.3. Bacterial community enrichment

A total aerobic bacterial community was enriched from *E. huxleyi* grown at 20°C (labelled TAB – total aerobic bacteria). At the Caen Algalbank, cultures of the strain were treated with various antibiotics which eliminated specific components of the bacterial community (Table 1). Bacterial communities from these antibiotic-treated algal cells were enriched and labelled ATB1, ATB2 and ATB3 (antibiotic-treated bacteria; Table 1). From these various antibiotic non-treated and pre-treated *E. huxleyi* cells, bacterial communities were enriched by successive transfers in bacterial growth medium composed of synthetic sea water (SSW) (Baumann and Baumann, 1981) supplemented with FeSO₄ (0.1 mM) and K₂HPO₄ (0.33 mM) in the presence of NaAc as carbon source (1g l⁻¹) under darkness. Cultures were incubated at 20°C under aerobic conditions in 250 ml Erlenmeyer flasks and agitated on a reciprocal shaker. Subsequently, we carried out two more transfers on the acetate medium to ensure exclusion of all the *E. huxleyi* cells.

2.4. Bacterial incubation

Sterilized, freeze dried cells of *E. huxleyi* were used as substrate for alkenone degradation. Each of the bacterial communities was incubated (in duplicate) at 20°C with 10

mg of cells, in sterile 50 ml of SSW, with continuous shaking. In order to monitor the degradation of alkenones by the bacterial communities, two sterile controls were prepared in the same way as the bacterial incubation flasks but without adding the bacterial inoculum prior to incubation.

2.5. Denaturing gradient gel electrophoresis (DGGE)

Cell lysis and DNA extraction were performed as described by Zhou et al. (1996). The polymerase chain reaction (PCR) conditions for 16S rRNA genes, including the hot start and a touchdown for primer annealing, were similar to those used by Muyzer et al. (1993). PCRs were carried out in a 25 μ l reaction mixture (20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂) containing 0.25 mM of each deoxyribonucleoside triphosphate, 2 μ M of each primer (Table 1), 0.5 units of *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) and 2 μ l of diluted DNA. DGGE was performed using a D-code Universal Mutation Detection System (Bio-Rad Laboratories Inc). Samples containing approximately equal amounts of PCR products (40 ng) were loaded onto 1 mm-thick, 6 % (wt/vol) polyacrylamide gels with a denaturation gradient from 30 to 50% for 16S rRNA genes (100% of denaturation corresponds to 7 M urea and 40% formamide). Electrophoresis was run at 60°C for 5.5 h at 150 V in 1x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM ethylene diamine tetraacetic acid (EDTA)). Following electrophoresis, the gels were incubated for 30 min in 1x TAE buffer containing ethidium bromide (0.5 μ g ml⁻¹) and photographed on a UV transilluminator (GelDoc 2000, gel documentation system, Bio-Rad).

2.6. Lipid extraction

After 20 d incubation, the flask contents were extracted with CHCl₃/CH₃OH/H₂O (1:2:0.8, v/v/v) using ultrasonication. Lipid recovery was tested by adding an internal standard (C₃₆ *n*-alkane). To initiate phase separation after ultrasonication, CHCl₃ and purified water

were added to the combined extracts to give a final volume ratio for $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ of 1:1:0.9 (v/v/v). The upper aqueous phase was subsequently extracted with CHCl_3 (x2) and the combined CHCl_3 extracts were dried over anhydrous Na_2SO_4 , filtered and evaporated to dryness under vacuum.

2.7. Alkaline hydrolysis

Water (25 ml), CH_3OH (25 ml) and KOH (2.8 g) were added to the organic residue obtained after ultrasonication (containing total solvent extractable lipids) and the mixture was directly saponified by refluxing (80°C) for 2 h. After cooling, the contents of the flask were acidified with HCl (to pH 1) and extracted with CH_2Cl_2 (x3). The combined extracts were dried over anhydrous Na_2SO_4 , filtered and evaporated to dryness under vacuum.

2.8. Alkenone reduction

Total lipid extracts were reduced (20 min) in $\text{Et}_2\text{O}/\text{CH}_3\text{OH}$ (3:1, v/v, 5 ml) using excess NaBH_4 or NaBD_4 (10 mg/mg extract). After reduction, a saturated solution of NH_4Cl (10 ml) was added cautiously to destroy excess reagent, the pH was adjusted to 1 with dilute HCl (2 N) and the mixture was shaken and extracted with hexane: CHCl_3 (4:1, v/v; x3). The combined extracts were dried as described above and evaporated to dryness under a stream of N_2 .

2.9. Hydrogenation

Some extracts were hydrogenated (under an atmosphere of H_2) in CH_3OH with Pd/ CaCO_3 (5% Pd, 10-20 mg/mg extract, Aldrich) as a catalyst for 12 h with magnetic stirring. After hydrogenation, the catalyst was removed by filtration and the filtrate was concentrated using rotary evaporation.

2.10. *OsO₄ derivatization*

OsO₄ (~2 mg per mg extract) and an anhydrous pyridine-dioxane solvent mixture (1:8, 5 mL) were added to each total lipid fraction. The resultant solution was homogenized by swirling and incubated at room temperature (1 h). A 16% (w/v) suspension of Na₂SO₃ in H₂O/CH₃OH (8.5:2.5, v/v) was added (6 mL) and the mixture incubated at room temperature (1.5 h). The solution was subsequently acidified (to pH 3) by dropwise addition of concentrated HCl and extracted with hexane-CHCl₃ (4:1 v/v, 5 mL each; x3). The combined extracts were dried over anhydrous Na₂SO₄, filtered and concentrated by rotary evaporation.

2.11. *Derivatization*

After solvent evaporation, residues were taken up in 400 µL of a mixture of pyridine and pure *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco) (3:1, v/v) and silylated for 1 h at 50°C. After evaporation to dryness under a stream of N₂, the derivatized residues were taken up in a mixture of ethyl acetate and BSTFA (to avoid desilylation of fatty acids) for analysis using gas chromatography-mass spectrometry (GC-MS).

2.12. *GC-MS*

GC-MS was carried out under electron ionization (EI) conditions with an Agilent 6890 gas chromatograph connected to an Agilent 5973 Inert mass spectrometer. The following conditions were employed: 30 m x 0.25 mm (i.d.) fused silica capillary column coated with SOLGEL-1 (SGE; 0.25 µm film thickness); oven temperature programmed from 70 to 130°C at 20°C min⁻¹, from 130 to 250°C at 5°C min⁻¹ and from 250 to 300°C at 3°C min⁻¹; carrier gas (He) maintained at 0.69 bar until the end of the temperature program and then programmed from 0.69 bar to 1.49 bar at 0.04 bar min⁻¹; injector (on column with retention gap) temperature, 50°C; electron energy, 70 eV; source temperature, 190°C; cycle time, 1.99

cycles s^{-1} , scan range, 50-800 Th. Quantification of alkenones or alkenols (obtained after $NaBH_4$ reduction) involved calibration with external standards.

3. Results and discussion

3.1. Sterilization tests

Sterilized *E. huxleyi* did not grow in f/2 medium, showing that the cells were indeed dead. Inoculation with bacterial medium also showed no growth of bacteria even after long incubation times. Hence, complete sterilization of *E. huxleyi* cells was achieved. Comparison also showed the relative concentrations of di- and triunsaturated C_{37} alkenones in sterilized cells were not different from those in non-sterilized control cells. Hence, sterilization was achieved without changing the cellular alkenone profile.

3.2. Degradation of *E. huxleyi* by different bacterial communities

Sterile controls carried out in parallel showed no significant change in the lipid profiles (relative to initial cells) over the course of the experiments. This demonstrates that the changes in the composition of these lipids observed in our experiments were indeed microbially mediated. After incubation for 20 d, we observed a varying degree of aerobic biodegradation of lipids with the four bacterial communities (Table 2). Fatty acids were strongly degraded in all cases, while the phytol side chain of chlorophyll and especially the main algal sterol, *epi*-brassicasterol, appeared to be more recalcitrant towards bacterial degradation.

Though alkenones are generally considered to be much more stable towards degradation than most common phytoplanktonic lipids (Harvey, 2000), we obtained (for three of the four communities) higher biodegradation rates for alkenones (Table 3) than for *epi*-brassicasterol (Table 2). These results are in good agreement with observations made during a study of the stability of alkenones in senescing cells of *E. huxleyi* (Rontani et al., 1997) and

more recently during a study of aerobic biodegradation of *E. huxleyi* by aerobic bacterial communities isolated from microbial mats (Rontani et al., 2005a). Only minor degradation of alkenones was observed with the ATB1 community, while incubation with the ATB2 community resulted in major degradation (Table 3). However, in both cases the degradation of di- and triunsaturated alkenones appeared to be non-selective. In contrast, incubation with the TAB and ATB3 communities resulted in major and *selective* alkenone degradation (Table 3). Based on an established calibration equation (Prahl et al., 1988), we observed an increase in $U_{37}^{K'}$ (relative to control) after 20 d incubation with the TAB and ATB3 communities (Table 3), equivalent to an inferred increase in growth temperature of 2°C and 3.3°C, respectively.

These results clearly show that the various antibiotic treatments of *E. huxleyi* significantly changed the composition of the bacterial communities associated with the cells. The changes in bacterial community could be visualized using DGGE analysis (Fig. 1). The DGGE pattern of the TAB community showed a complex structure illustrated by a smear containing several major bands (lane 2). The structure of the bacterial communities associated with *E. huxleyi* maintained in the presence of different antibiotics showed drastic changes. In the case of ATB1 (lane 3) or ATB2 (lane 4) communities the DGGE pattern was dominated by only one band (**1**), that seemed to be the same for both treatments. In contrast, the DGGE pattern of the ATB3 community (lane 5) exhibited three major bands (**1-3**), one showing the same electrophoretic mobility as band **1** of ATB1 or ATB2 and another (band **3**) corresponding to one of the major bands of TAB.

The very distinct results obtained with the four communities strongly suggest the existence of at least two functional classes of aerobic bacteria capable of degrading alkenones, i.e. those able to degrade them either non-selectively or selectively. It is likely that these

differences in degradative outcome are strongly dependent on the particular metabolic pathways used by the two groups.

3.3. *Metabolic products of alkenone degradation*

Analyses of the total lipid extracts obtained after incubation of *E. huxleyi* with the ATB3 community showed the production of small amounts of methyl and ethyl alkenols (ranging from 4.2 to 2.0% of the corresponding alkenone), which were lacking in the sterile controls (Fig. 2). To our knowledge, this is the first observation of a probable bacterial production of alkenols from the corresponding alkenones. Alkenols were previously detected in trace amounts in several haptophytes (Rontani et al., 2001) and in microbial mats (Rontani and Volkman, 2005). Their source in microbial mats was thought to be either from bacterial reduction of alkenones or direct input by haptophytes. The present observation supports the first assumption. This reductive pathway probably results from non-specific dehydrogenase-hydrogenase activity (Platen and Schink, 1989), which is not necessarily specifically related to alkenone degradation.

NaBH₄ reduction of total lipid extracts obtained after incubation with the ATB3 community also showed the presence of three peaks eluting close to the resulting C₃₉ alkenols (Fig. 3). These compounds, absent from reduced extracts from the sterile controls, clearly resulted from bacterial degradation of the alkenones. It is interesting to note that they were also present (but in lesser amount) in the reduced extracts obtained after incubation with the TAB community. On the basis of their EI mass spectra (Fig. 4), we assigned them as isomeric diols and attribute their formation to the partial NaBH₄ reduction of the corresponding keto epoxides produced after bacterial oxidation of alkenone double bonds. To test this hypothesis, the total lipid extracts were reduced with NaBD₄ instead of NaBH₄. EI MS data provided

unambiguous validation of the hypothesis as shown, for example, by results obtained for peak 1 (Fig. 5).

Attack on double bonds by peroxy radicals (autoxidation) can also result in epoxide production (Fossey et al., 1995). When the double bonds have allylic hydrogens there is competition between addition of the peroxy radical to the double bond (epoxide formation) and allylic hydrogen abstraction (formation of allylic hydroperoxides). We previously demonstrated that free radical oxidation of alkenones in solvent (Rontani et al., 2006a) and *E. huxleyi* (Rontani et al., 2007) mainly involves allylic hydrogen abstraction. On the basis of these results, the epoxidation of alkenone double bonds observed after bacterial incubation was attributed to bacterial action rather than abiotic chemical processes since such compounds were lacking in sterile controls. Moreover, in NaBH₄-reduced lipid extracts obtained after incubation with the ATB3 community, we also detected large amounts of 11-hydroxyoctadecanoic and 12-hydroxyoctadecanoic acids (Fig. 6B), which were present only in trace amounts in the controls (Fig. 6A). Reduction with NaBD₄ instead of NaBH₄ demonstrated unambiguously that these compounds resulted from the reduction of 11,12-epoxyoctadecanoic acid. The lack of hydroxyacids resulting from the reduction of the 12,13-epoxy-11-methyloctadecanoic acid, even though the 11-methyloctadec-12-enoic acid was present in similar proportion to *cis*-vaccenic acid, clearly demonstrates the specificity of epoxidation and allowed us to attribute the formation of epoxyalkenones unambiguously to enzymatic epoxidation processes.

The selectivity during alkenone biodegradation by the ATB3 and TAB communities via enzymatic attack of double bonds must be due to specific components of these communities. Indeed, these bacteria appear capable of oxidizing all the alkenone double bonds (Fig. 4), but with some selectivity (22, 32 and 46% oxidation of the ω 15, ω 22 and ω 29

double bonds, respectively). These estimates are based on the abundances of fragment ions resulting from α -cleavage of the TMS ether groups of the fully hydrogenated diols measured at 20 eV (in order to avoid subsequent selective cleavage of these fragment ions) and taking into account the proportion of initial alkenones. The higher reactivity of the ω 29 double bond towards enzymatic epoxidation processes is in good agreement with the observed preferential degradation of triunsaturated alkenones. The presence of a high proportion of bacteria able to attack alkenone double bonds via such selective processes could thus induce a non-negligible increase in calculated values of the alkenone unsaturation ratio and thereby lead to warmer inferred growth temperatures.

Epoxidation of double bonds by aerobic bacteria is a well known process induced by cytochrome P-450-dependent monooxygenases. These enzymes can produce epoxides from a broad range of lipophilic substrates such as *n*-alkenes (Klug and Markovetz, 1968; Hartmans et al., 1989a; Soltani et al., 2004), terpenes (Duetz et al., 2003), unsaturated fatty acids (for a review see Ratledge, 1994) and styrene (Hartmans et al., 1989b). Enzymes from a large number of classes, including dehydrogenases, lyases, carboxylases, glutathione S-transferases, isomerases and hydrolases, are involved in the microbial degradation of epoxides (van der Werf et al., 1998). Among these, epoxide hydrolases, which catalyze the addition of water to epoxides to form the corresponding diol, have been extensively studied and seem to be widely distributed in bacteria (e.g. Michaels et al., 1980; van der Werf et al., 1998; Johansson et al., 2005). Thus, we propose the pathways in Fig. 7 for the bacterial degradation of alkenones via an initial double bond epoxidation. The processes involve hydrolysis of the epoxide to the corresponding diol and subsequent oxidative cleavage. The resulting ketoacid and acid fragments are then totally assimilated by way of classical β -oxidation.

In total lipid extracts obtained after incubation with the ATB3 community, we also detected small amounts of monounsaturated methyl C₃₇ and ethyl C₃₈ alkenones (detected as alkenol derivatives, see Fig. 3). Small amounts of monounsaturated alkenones were previously identified in several haptophytes (Rontani et al., 2001) and in Black Sea Unit II samples (Xu et al., 2001; Rontani et al., 2006b). While it is conceivable that these intriguing compounds could be produced by *E. huxleyi* strain TWP1 before sterilization, they were lacking in all the other lipid extracts analyzed (sterile controls and other bacterial incubations) so their formation would seem linked to a bacterial activity specific to the ATB3 community. These compounds could be formed by a partial bacterial hydrogenation of diunsaturated alkenones. GC-MS of these extracts after OsO₄ treatment and subsequent silylation allowed us to confirm this hypothesis. Indeed, while the monounsaturated methyl C₃₇ and ethyl C₃₈ alkenones previously described (Rontani et al., 2001) possessed a double bond in the ω₁₇ position (which does not correspond to that observed in “classical” alkenones), each GC peak for monounsaturated alkenols observed after incubation with the ATB3 community appeared to be composed of two ω₁₅ and ω₂₂ double bond positional isomers (Fig. 8) resulting from the reduction of one double bond of the diunsaturated alkenones. The saturation of fatty acids (biohydrogenation) by mixed cultures of rumen bacteria has long been recognized (for a review see Hammond, 1988), but the processes are generally considered to be restricted to anaerobic bacteria. However, Pereira et al. (2002) and Koritala et al. (1987) observed that bacteria and yeasts, respectively, could convert 18:3(n-3) acid to 18:2(n-6) and 18:1(n-9) acids under aerobic conditions. Although the proportions of monounsaturated alkenones produced in our experiments were low (2-3% of the corresponding diunsaturated alkenone), the existence of alkenone bacterial hydrogenation processes could constitute a problem for palaeotemperature reconstruction if this process proves widespread and quantitatively significant in nature.

3.4. Selectivity of aerobic bacterial processes towards alkenones

Aerobic bacterial metabolism of unsaturated aliphatic ketones such as alkenones may be initiated either by the same mechanisms employed in alkanone metabolism or via attack at the double bonds. Three main patterns of initial attack of an aliphatic methyl ketone have been recognized (Fig. 9): (i) terminal methyl oxidation (Ratledge, 1978) (Pathway A), (ii) oxidation of the keto terminal methyl and decarboxylation of the resulting α -ketoacid (Gillan et al., 1983) (Pathway B) and (iii) enzymatic oxidation of the keto group to an ester, analogous to Baeyer-Villiger oxidation with peracids (Britton et al., 1974) followed by hydrolysis of the ester to a primary alcohol and acetic acid (Pathway C). The different compounds formed in each pathway are then assimilated by way of β -oxidation. The previous detection of C_{35:2}, C_{35:3} and C_{35:4} alken-1-ols in alkenone-rich Camargue microbial mats (Rontani and Volkman, 2005) provides support for the degradation of alkenones *via* a bacterially mediated Baeyer-Villiger sequence (Pathway C) in the natural environment. The positions of the double bonds in the alkyl chain do not constitute a metabolic blockage for β -oxidation owing to the occurrence of 2,3-enoyl-CoA isomerases in bacteria (Ratledge, 1994). Consequently, the presence or absence of an additional double bond in the chain of alkenones has no significant effect on degradation rates. Bacteria degrading alkenones non-selectively, as in the case of the ATB1 and ATB2 communities and in the experiments of Teece et al. (1998), probably metabolized them via one of the three proposed pathways (Fig. 9).

In contrast, bacteria able to degrade alkenones selectively, present in TAB and ATB3 communities, and probably in the inocula from microbial mats previously used by Rontani et al. (2005a), likely do so by attack at the alkenone double bonds. This appears to involve the formation of epoxides (pathway D in Fig. 10) in the case of ATB3 and TAB communities, but the involvement of other processes such as direct oxidation of double bonds by dioxygenases

(pathway E in Fig. 10) or addition of water by hydratases (Seo et al., 1981) followed by dehydrogenation and carboxylation (pathway F in Fig. 10) cannot be excluded. In the case of bacteria using one of the pathways (D, E or F) to degrade alkenones, the presence of an additional double bond could significantly increase the degradation rates and thereby control selectivity.

We note that hydratases can also act under anaerobic conditions (Schink, 1985; Rontani et al., 2002) and thus induce selectivity during the degradation of alkenones by anaerobes. However, alkenones appeared to be degraded non-selectively under methanogenic, sulfate reducing and denitrifying conditions (Teece et al., 1998; Rontani et al., 2005a). Although the existence of anaerobes able to hydrate alkenone double bonds cannot be totally excluded, we speculate that anaerobic degradation of alkenones mainly involves attack at the carbonyl group and consequently occurs non-selectively.

3.5. Biogeochemical implications

Our results confirm the previous observations of Rontani et al. (2005a) and show that aerobic bacteria capable of degrading alkenones selectively are not limited to particular environments such as microbial mats and can be actually associated with *E. huxleyi* cells.

Intense aerobic microbial degradative processes have the potential to introduce a bias in palaeotemperature reconstruction, so this factor should be considered when sediments in which there is clear evidence of substantial aerobic microbial degradation of the deposited organic matter are examined. Conceivably, the involvement of selective aerobic microbial degradative processes could explain the increasing mismatch between $U_{37}^{K'}$ values measured in annually averaged sediment trap materials and surface sediment documented by Prahl et al. (1993) along an offshore transect at $\sim 42^\circ\text{N}$ in the northeast Pacific Ocean. Indeed, burial efficiency at the most offshore, open ocean site, where the mismatch was greatest (0.335 vs.

0.447), was very low (~1%) because of high oxygen exposure. Our results are also in good agreement with the observations of Conte et al. (2006). From an extensive compilation of several $U_{37}^{K'}$ measurements (n = 629), these authors observed that $U_{37}^{K'}$ values in surface sediments were systematically higher than the surface water production temperature. They concluded that the deviations could be attributed to seasonality in production and/or thermocline production as well as differential degradation of C_{37:3} and C_{37:2} alkenones.

The epoxy ketones resulting from bacterial epoxidation of alkenone double bonds may prove useful as indicators of in situ aerobic bacterial alteration of the alkenone unsaturation ratio. Their detection is much easier after NaBH₄ reduction to the corresponding diols and subsequent silylation. This treatment also allows for better quantification of alkenones in the form of silylated alkenols (Rontani et al., 2001).

4. Conclusions

Although various studies have inferred biodegradation of alkenones in different environments, very few have yet examined the details of the biodegradative processes (Teece et al., 1998; Rontani et al., 2005a). Four bacterial communities isolated from *E. huxleyi* strain TWP1 cultures before and after different antibiotic treatments were used in the present work to study the biodegradation of alkenones under laboratory-controlled conditions. The communities degraded alkenones to varying degrees, ranging from effectively none to extensive. We observed not only an extensive degradation of alkenones but also an important selectivity during the degradation of the different alkenones by some of the bacterial communities used. Observed increases in $U_{37}^{K'}$ values are equal in these cases to a +2°C and +3.3°C change in the inferred temperature when interpreted using a standard calibration equation (Prahl et al., 1988). The differences are sufficiently large to cause concern about data interpretations of palaeotemperature reconstructed from the alkenone unsaturation index,

because some reconstructed temperature differences between the last glacial and interglacial period are only 1–3°C in magnitude.

The detection of epoxy ketones in some cultures indicates that metabolic pathways involving attack on the terminal groups of the molecule are essentially non-selective, while those acting on alkenone double bonds are selective. Some other interesting metabolic products of the alkenone degradation were observed during incubation. The production of alkenols with the ATB3 community demonstrated for the first time that bacterial reduction of alkenones can be a potential source of these compounds in the environment. The intriguing production by this community of small amounts of monounsaturated alkenones also raises the possibility of a bacterially mediated hydrogenation of alkenone double bonds, a biodegradation process worthy of further investigation.

Finally, our results demonstrate that bacterial degradation can play a role in the selective degradation of alkenones, a phenomenon observed during incubation of live *E. huxleyi* cells under prolonged darkness conditions (Prahl et al., 2003). Although selective metabolic consumption of alkenones by the alga (as energy reserves) is supported by that data set, it now seems likely that growth of bacteria associated with living *E. huxleyi* cells, perhaps induced by cessation in the dark of antibiotic production by the alga (F. Van Wambeke, personal communication) may also have contributed to some extent to the observed selective loss of alkenones in these non-axenic experiments.

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FIGURE CAPTIONS

Fig. 1. Negative images of DGGE profiles of the 16S rDNA fragments obtained with primers specific for the domain bacteria and template DNA extracted from TAB, ATB1, ATB2 and ATB3 communities. Markers correspond to a mixture of PCR products amplified from *Clostridium perfringens*, *Marinobacter hydrocarbonoclasticus* sp. cab and *Micrococcus luteus*.

Fig. 2. Partial total ion chromatogram of total lipid extracts obtained after incubation of *E. huxleyi* strain TWP1 in sterile medium (A) and with the ATB3 community (B) for 20 days. The insert shows m/z 117 and 131 mass chromatograms of the lipid extract obtained after incubation with the ATB3 community.

Fig. 3. Partial total ion chromatogram of silylated NaBH₄-reduced total lipid extracts showing the presence of monounsaturated species and isomeric diols after incubation of *E. huxleyi* strain TWP1 with the ATB3 community for 20 days.

Fig. 4. EI mass spectra of peaks 1 (A), 2 (B) and 3 (C); see insert in Fig. 3.

Fig. 5. EI mass spectrum of peak 1 in Fig. 3 obtained after reduction with NaBD₄.

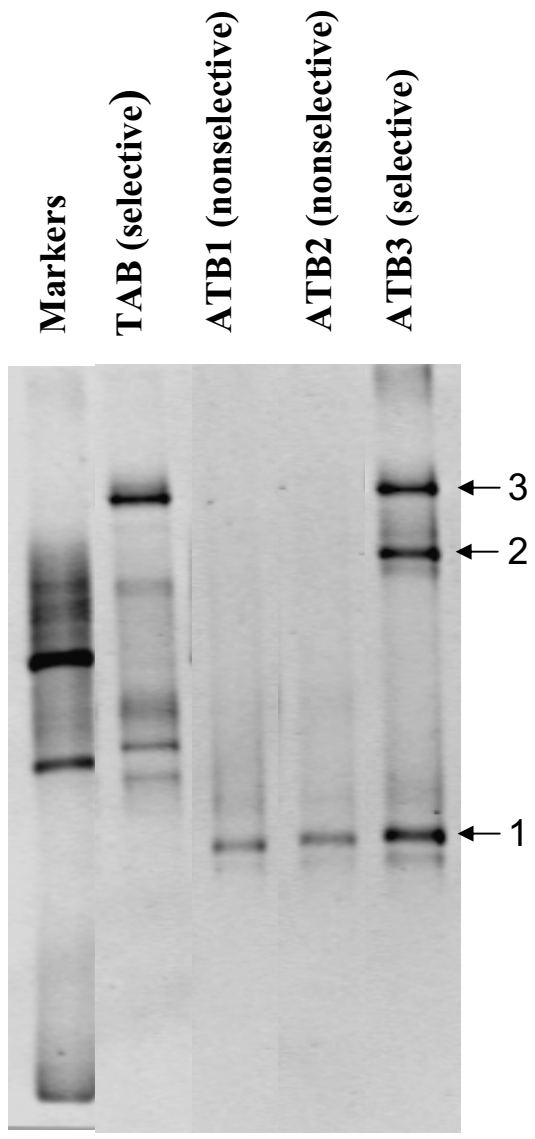
Fig. 6. Partial m/z 345 and 359 chromatograms showing amounts of 11-hydroxyoctadecanoic and 12-hydroxyoctadecanoic acids after incubation of *E. huxleyi* strain TWP1 in sterile medium (A) and with the ATB3 community (B) for 20 days.

Fig. 7. Proposed pathways for biodegradation of the C_{37:3} alkenone involving initial epoxidation of ω 29 double bond.

Fig. 8. Partial m/z 117, 299, 397, 297, 395, 131, 311 and 409 chromatograms of silylated triols derived from OsO_4 treatment of (A) $\text{MeC}_{37:1}$ and (B) $\text{EtC}_{38:1}$ alkenols showing the initial presence of two double bond positional isomers.

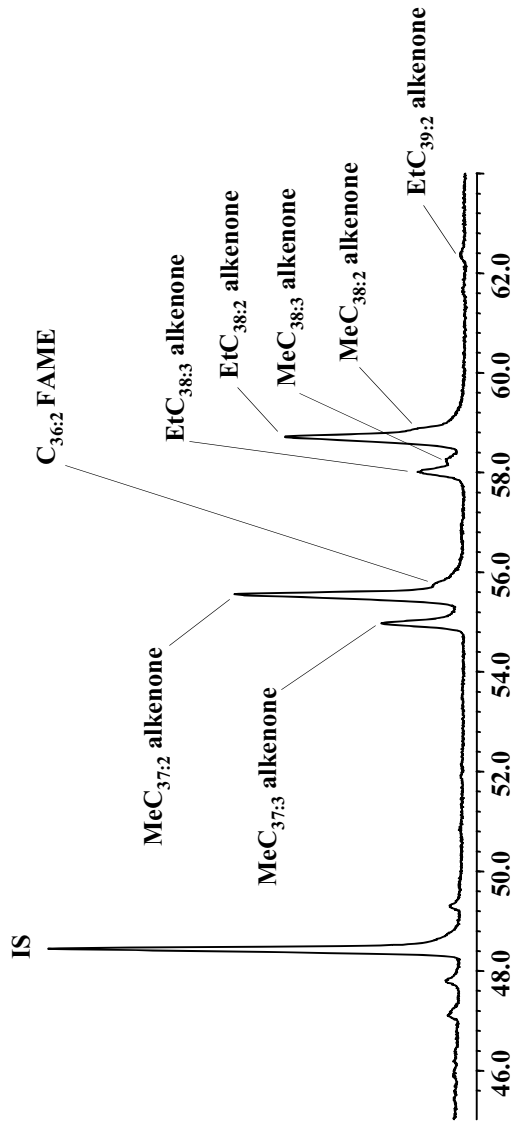
Fig. 9. Metabolic pathways involved during aerobic bacterial degradation of methyl alkenones: attack on terminal methyl and keto groups.

Fig. 10. Metabolic pathways involved during aerobic bacterial degradation of alkenones: attack on double bonds.



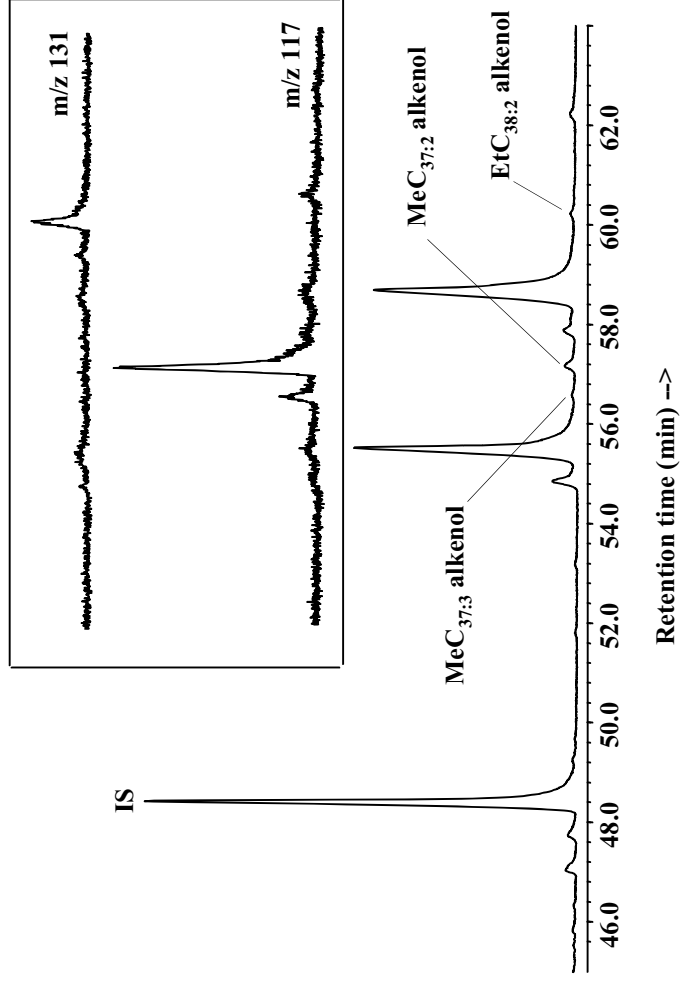
A

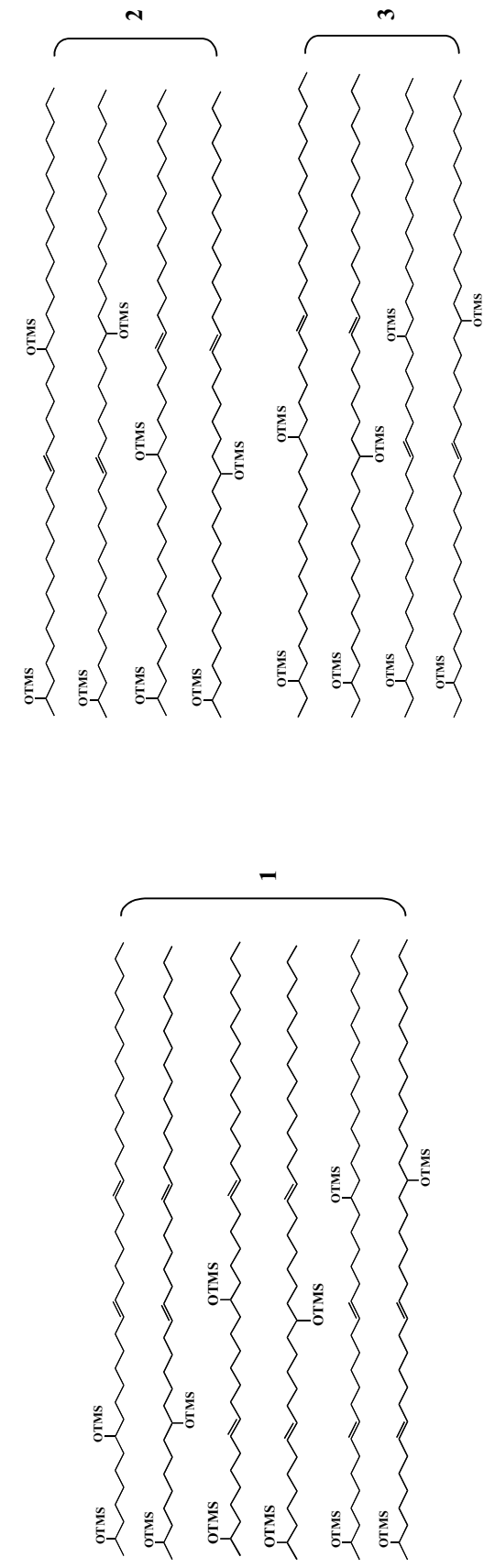
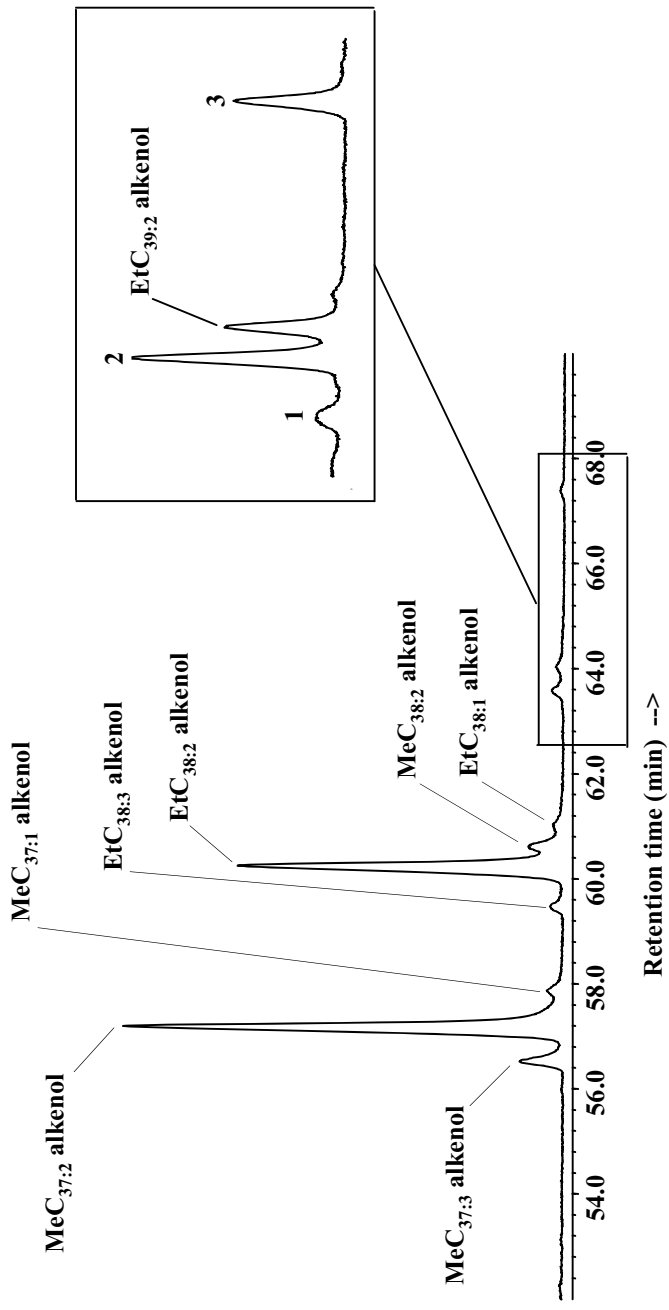
Sterile control
(20 days)

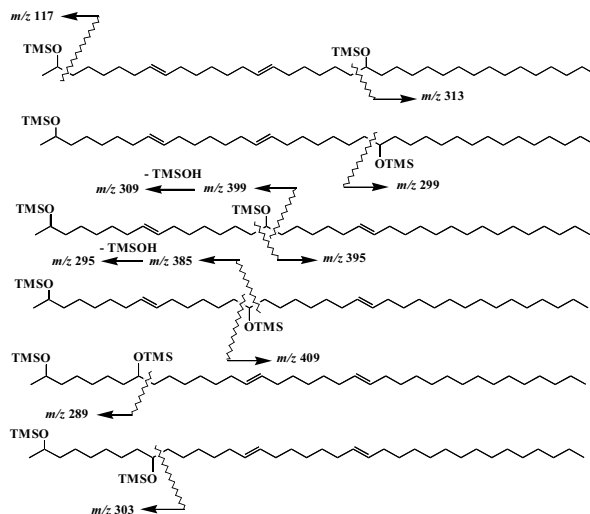
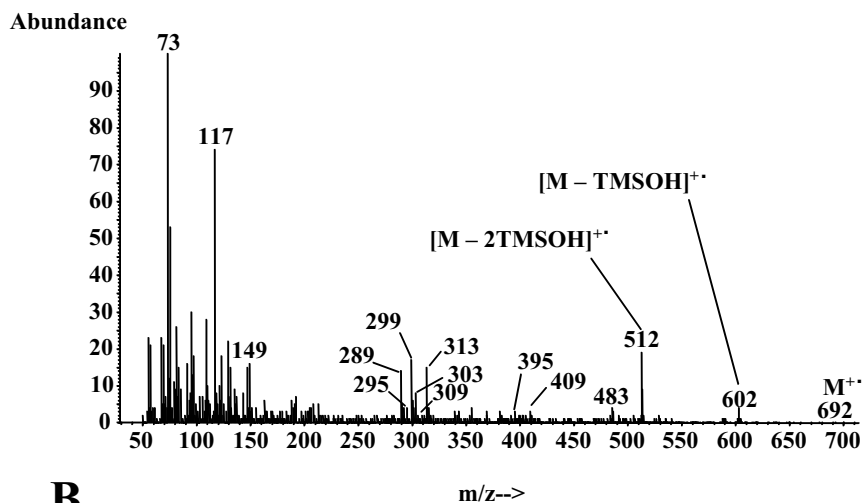
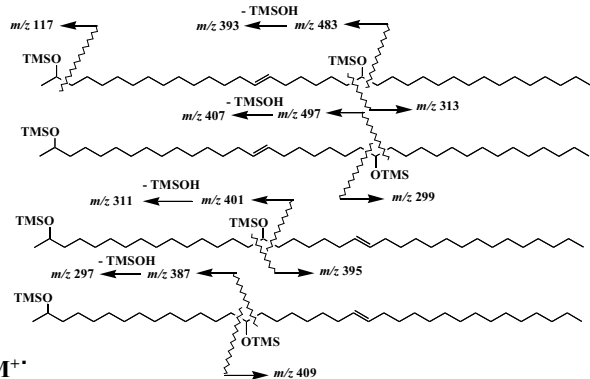
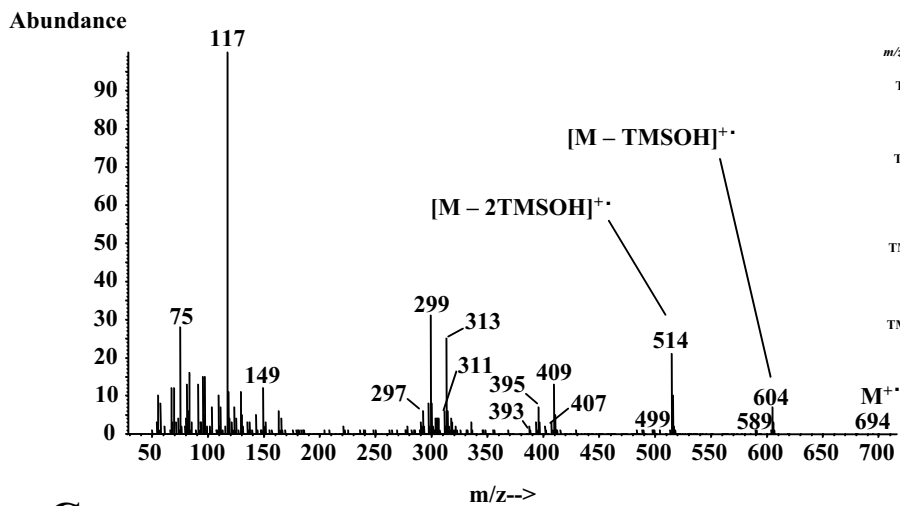
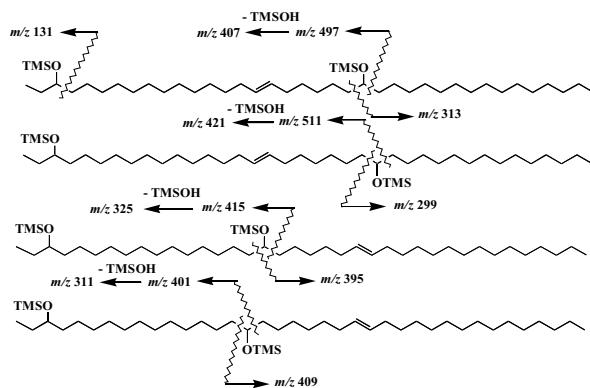
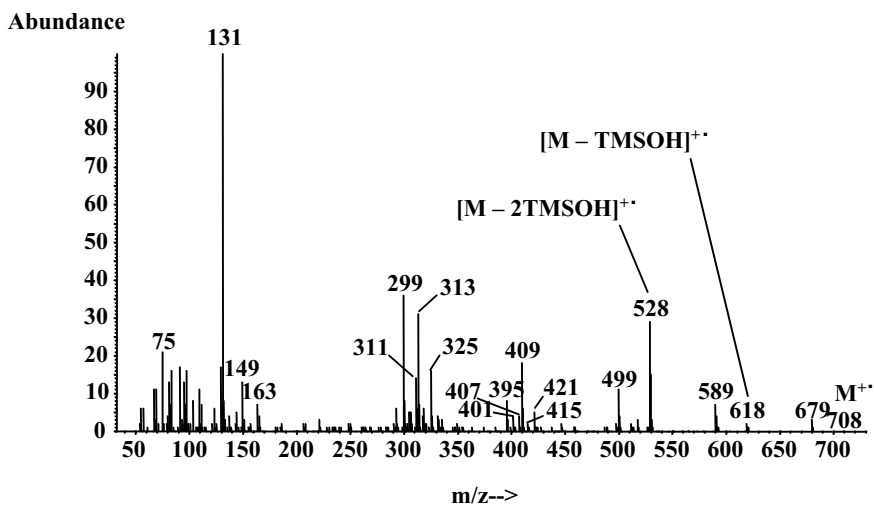


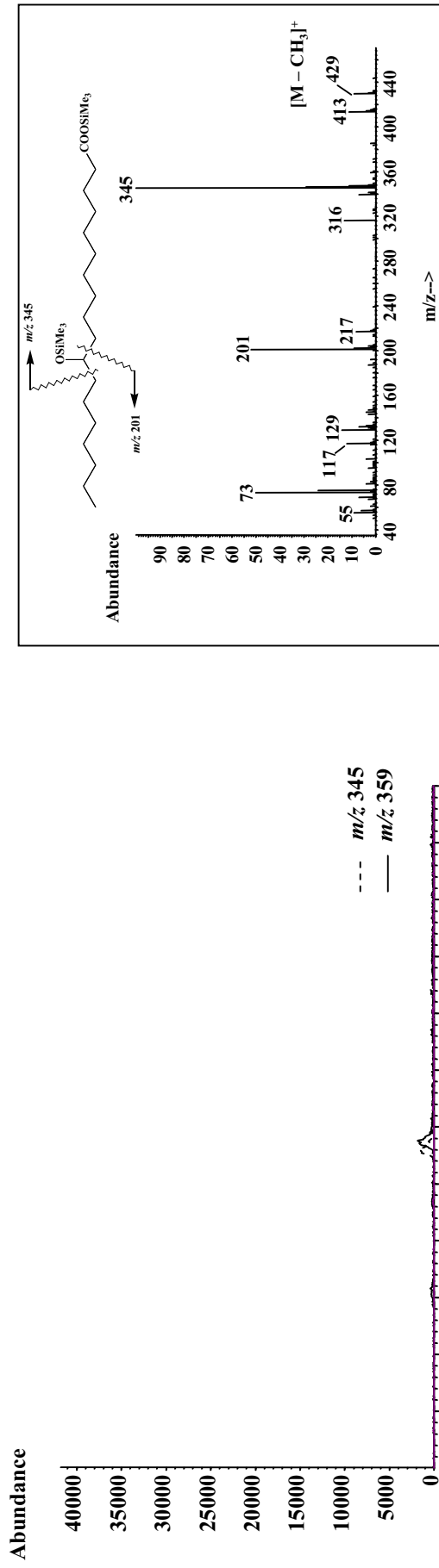
B

ATB3 Community
(20 days)

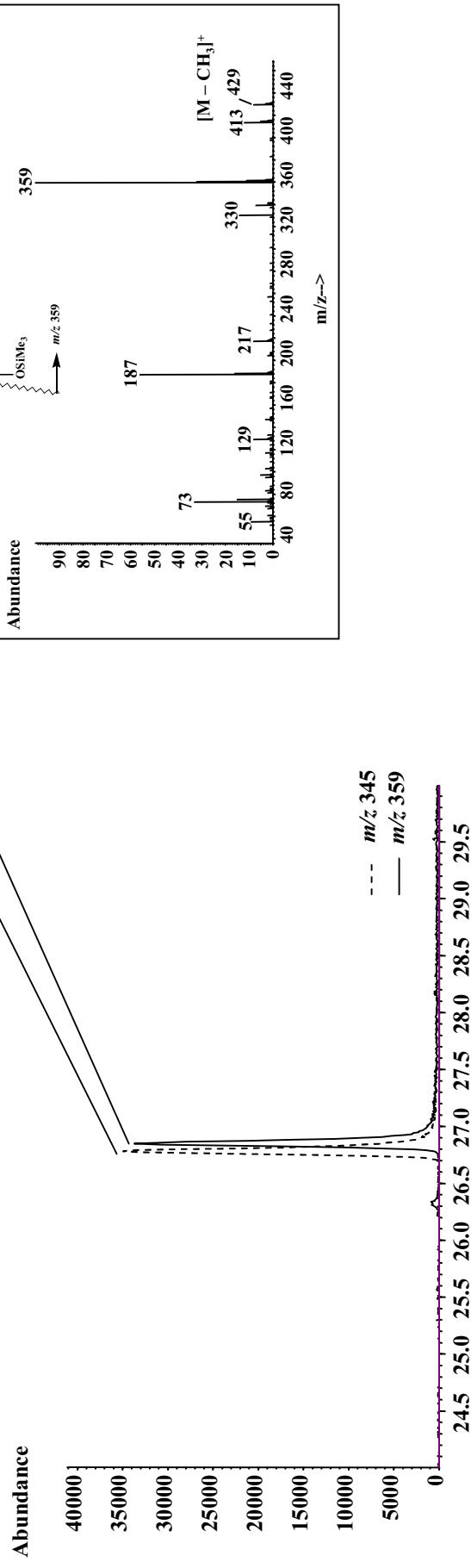




A**B****C**

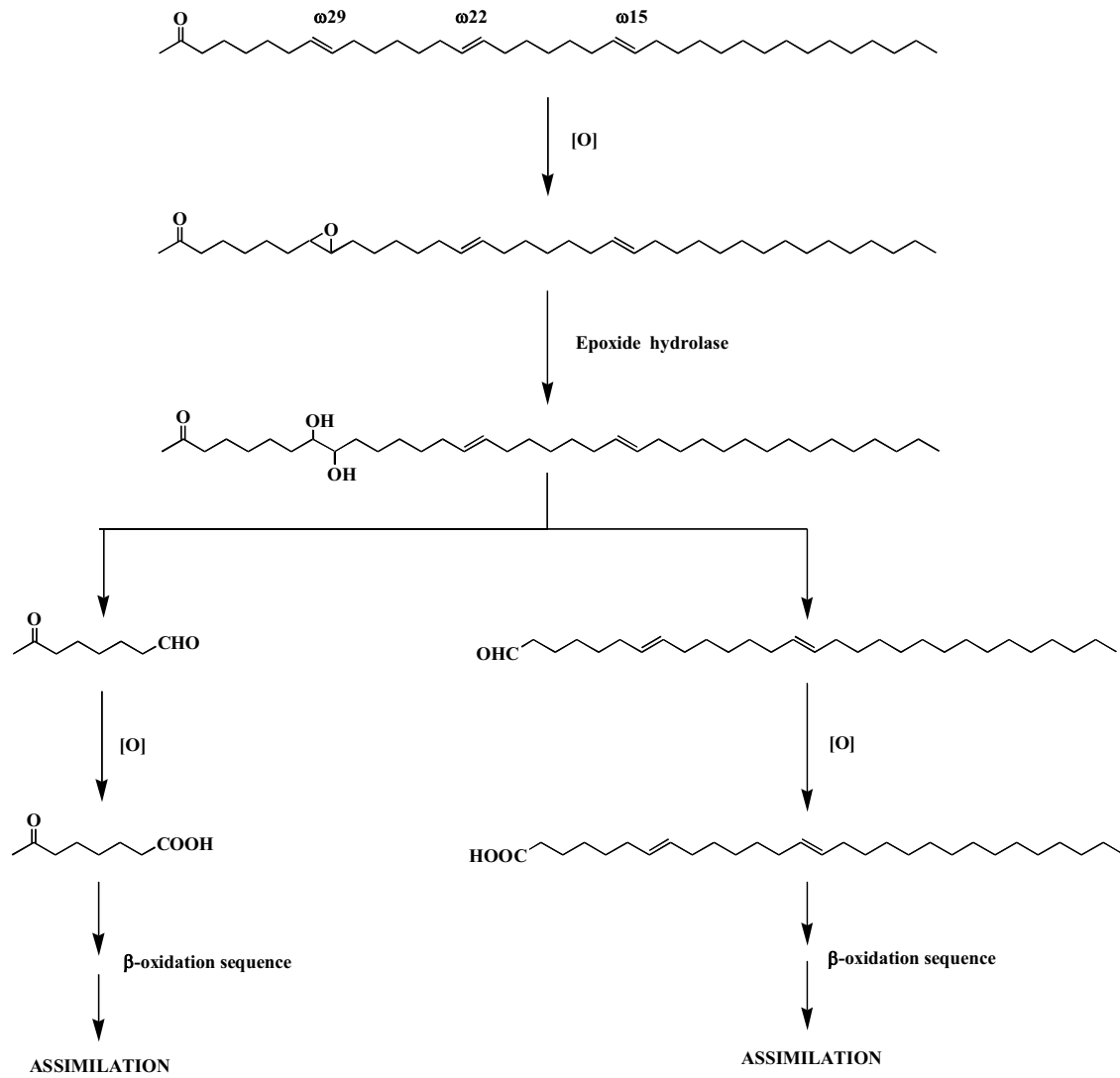
A

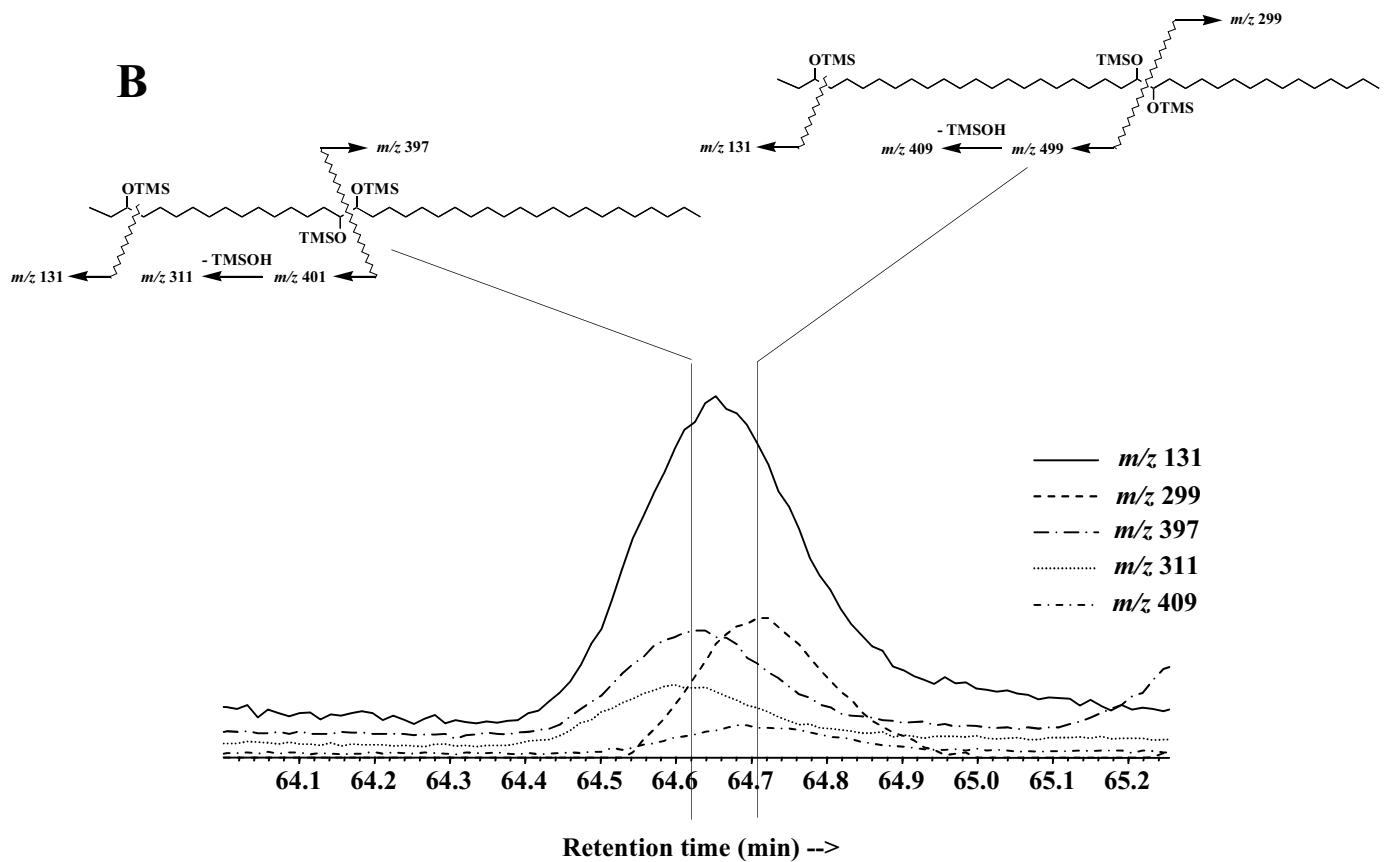
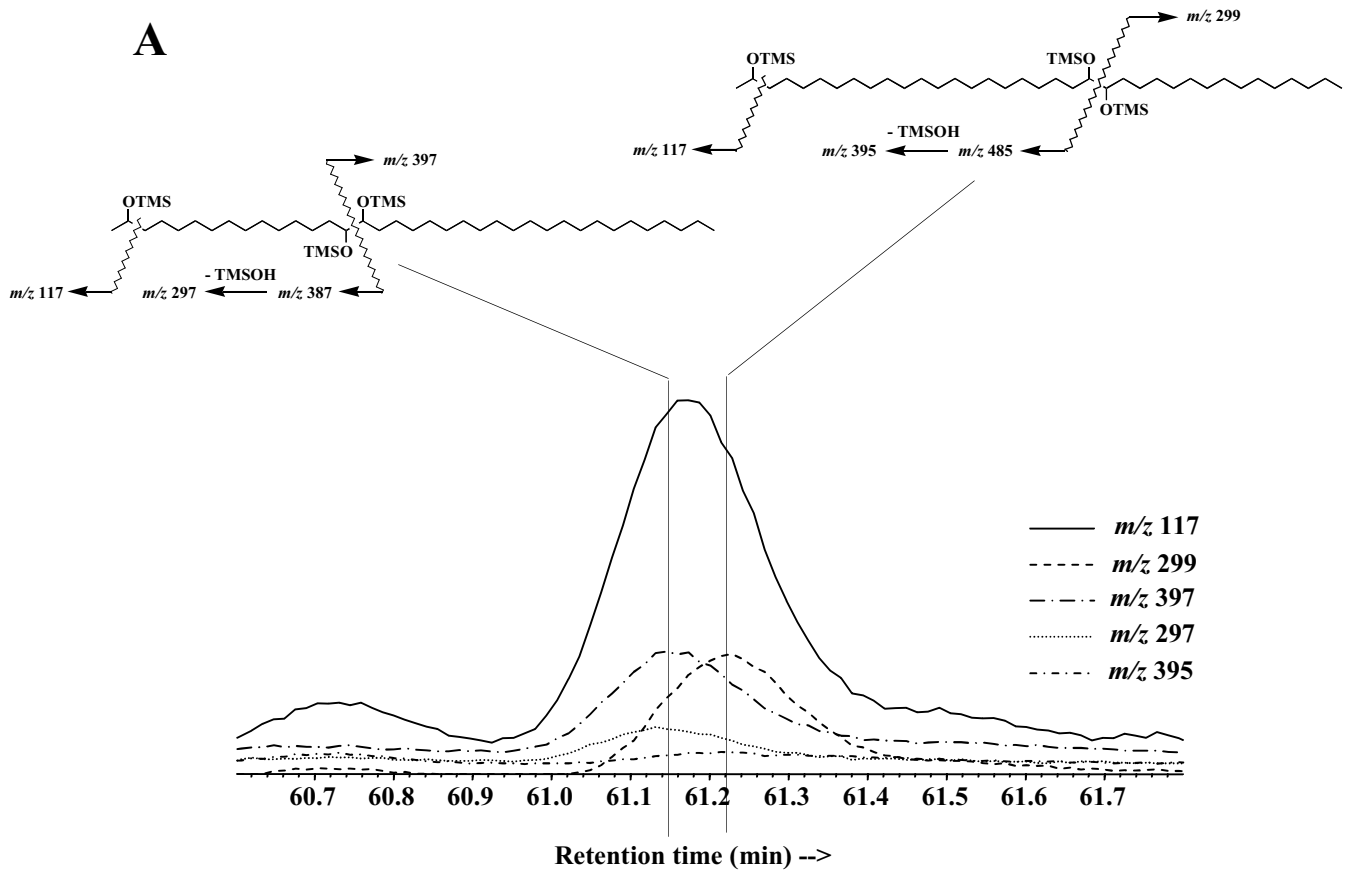
Retention time (min) -->

--- m/z 345
— m/z 359**B**

Retention time (min) -->

--- m/z 345
— m/z 359





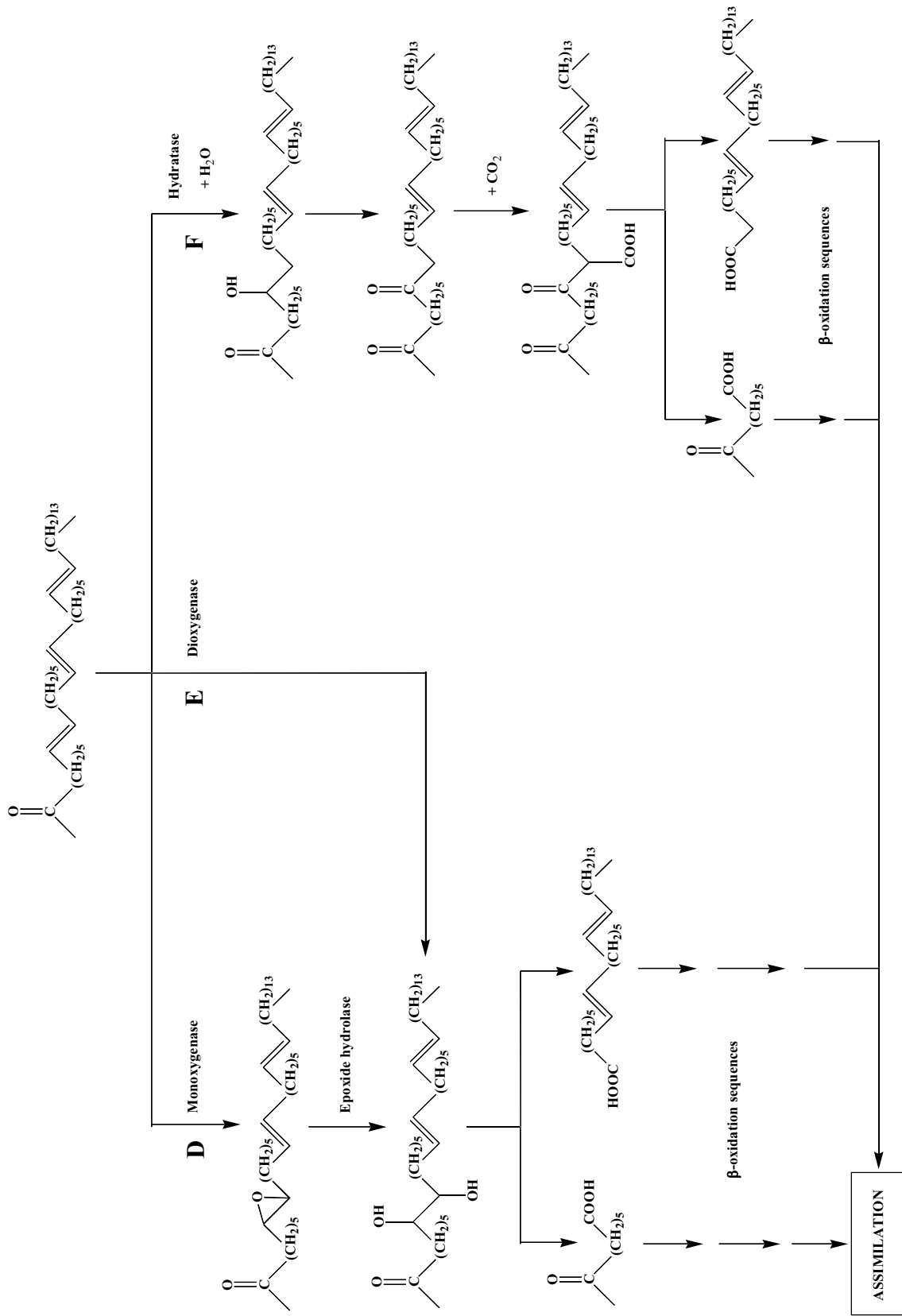


Table 1

Origin of the different bacterial communities used

Bacterial community	Origin	Ratio (bacterial number/ <i>E. hux.</i> cells) in algal cultures at time of bacterial isolation ^a
TAB	Untreated <i>E. hux.</i>	25.1
ATB1	<i>E. huxl.</i> treated (24 h) with Provasoli's concentrate ^b (Sigma)	6.6
ATB2	<i>E. hux.i</i> treated (24 h) with mixture of penicillin G, streptomycin and gentamycin	4.5
ATB3	<i>E. hux.</i> treated (24 h) with Provasoli's antibiotic concentrate ^b (Sigma) and transferred x 2 in sterile <i>f/2</i> medium.	8.4

^a Cells counted using epifluorescence technique with fluorochrome [4', 6'-diamidine-2'-phenylindole dihydrochloride (DAPI; Rontani et al., 1999].

^b Mixture of penicillin G, chloramphenicol, polymyxin B and neomycin.

Table 2

Lipid degradation (%) after incubation of sterile cells of *E. hux.* with different aerobic bacterial communities

	C _{14:0} fatty acid ^a	C _{22:6} fatty acid	Epibrassicasterol	Phytol
TAB community	91 ± 2	93 ± 2	17 ± 8	21 ± 10
ATB1 community	57 ± 6	70 ± 5	16 ± 3	30 ± 8
ATB2 community	61 ± 2	93 ± 4	8 ± 1	32 ± 9
ATB3 Community	86 ± 4	97 ± 1	- ^b	54 ± 1

^a Calculated relative to sterile controls.

^b No significant degradation.

Table 3

Alkenone degradation (%) after incubation of sterile cells of *E. hux.* with different aerobic bacterial communities

		MeC _{37:3} ^a	MeC _{37:2}	U_{37}^k	EtC _{38:3}	EtC _{38:2}	MeC _{38:3}	MeC _{38:2}
Sterile control	Duplicate 1	-	-	0.77	-	-	-	-
	Duplicate 2	-	-	0.76	-	-	-	-
TAB Community	Duplicate 1	48.6	24.6	0.83	38.0	23.3	47.7	37.7
	Duplicate 2	49.4	25.7	0.83	39.7	28.2	44.7	36.0
ATB1 community	Duplicate 1	8.0	12.0	0.76	5.1	16.3	7.8	18.9
	Duplicate 2	10.0	12.3	0.76	7.6	12.8	7.0	9.1
ATB2 Community	Duplicate 1	25.0	27.8	0.76	21.1	25.8	40.8	42.4
	Duplicate 2	45.3	45.4	0.77	37.7	37.8	48.8	50.7
ATB3 Community	Duplicate 1	67.0	23.4	0.88	72.1	21.5	68.8	36.8
	Duplicate 2	61.7	21.2	0.87	77.7	32.7	65.1	38.5

^a Calculated relative to sterile controls.