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A catalytic hydropyrolysis method for the rapid screening of microbial cultures for lipid biomarkers

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Abstract

A catalytic hydropyrolysis procedure was developed for rapidly assessing the relative abundances and variety of different biomarker lipid structures in microbial cultures by reductively converting free functionalised and polymeric lipids within whole cells into hydrocarbons. High pressure hydrogen gas and a molybdenum catalyst were used to target and cleave carbon–oxygen covalent bonds (particularly ester, alcohol, acid and ether) and the pyrolysis process was conducted in an open-system reactor configuration to minimise structural and stereochemical rearrangements in the products. A revised experimental protocol, involving a modified catalyst-loading procedure, careful use of a silica support substrate and a revised temperature program was tested and optimised for handling biomass. Partial hydrogenation of double bonds inevitably did occur although it was found that some unsaturation was preserved, particularly within branched and polycyclic hydrocarbon structures. This experimental approach aids our ability to optimally correlate fossil biomarker signals found in the sedimentary record with their lipid precursors found in extant organisms. Our technique complements more rigorous, but time-consuming, chemical approaches used for elucidating the exact chemical structures of intact functionalised lipids by providing a rapid means by which to screen microbial cultures.

1. Introduction

Biomarker lipids are commonly used as molecular tools for gaining insights into the biological origins and thermal maturity of sedimentary organic matter and for reconstructing palaeoenvironmental conditions (of redox, temperature and salinity, etc.) which prevailed in the water column during sediment deposition. Although specific biological sources have been identified for many lipids in environmental samples, the gaps in our knowledge are large. Lipid biomarker applications and

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interpretations are currently based on a few key lipid types. In practice, identification of source-specific organic compounds in extant organisms is an extremely time-consuming process involving repeated extractions with solvent mixtures, product derivatisation with chemical reagents and careful product separations using thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or solid phase microextraction (SPME) methods, prior to product identification (e.g., Bligh and Dyer, 1959; Gelpi et al., 1970; Boon et al., 1979; Volkman et al., 1980, 1981, 1988, 1989, 1998; Marlowe et al., 1984; Rohmer et al., 1984; Jones et al., 1994; Rizov and Doulis, 2001). Furthermore, it is unlikely that all lipid constituents are accessible to solvent extraction. The conventional analytical window is therefore usually limited to extractable compounds which are readily amenable to gas chromatographic (GC) or liquid chromatographic (LC) techniques. Clearly, a rapid analytical approach which can access a high proportion of the total lipid content is highly desirable.

The previous work of Love et al. (1995, 1996, 1997) pioneered the use of open-system catalytic hydropyrolysis (pyrolysis assisted by high hydrogen gas pressures, typically 15 MPa, and a molybdenum sulfide catalyst) as an analytical approach for releasing biomarker hydrocarbons from organic geomacromolecules in order to access a far higher proportion of the fossil lipid record. The hydropyrolysis (HyPy) system has the unique ability to generate high yields of biomarker hydrocarbons from complex organic matrices whilst inhibiting alteration to the structures and stereochemistries of products. The technique promotes cleavage and reductive removal of select heteroatomic bonds (particularly organic oxygen and organic sulfur cross-linkages and functional groups; including ether, sulfide, carboxyl, hydroxyl, thiols and simple thiophenic groups) in organic compound and macromolecules favouring release of hydrocarbon products. The thermal reactivity of different bond types under typical HyPy conditions has been studied in detail previously by Snape and co-workers using solid calibrants, such as silica-immobilised phenolic model compounds and synthesised polymers (Mitchell et al., 1993; Ismail et al., 1995; Brown et al., 1997; McGinn, 2002).

This study represents the first application of a modified HyPy procedure to rapidly screen algal, bacterial and archaeal cultures and convert the lipid constituents of the biomass into hydrocarbons. Hydrocarbons are convenient to analyse, being readily amenable to gas chromatography without the need for prior derivatisation. Biomarkers are typically found as hydrocarbons in ancient sedimentary organic matter and are commonly utilised for geochemical assessment. Using the HyPy approach, the structural and stereochemical features of the hydrocarbon cores of lipids in extant microorganisms can be routinely assessed and, from an organic geochemistry perspective, the products can be readily compared with the biomarker skeletons found in the geological record.

It was proposed to test the capabilities of HyPy for rapidly generating informative biomarker hydrocarbon profiles directly from a broad range of microorganisms; encompassing marine and freshwater algae, bacteria and archaea. Pyrolysis methods have previously been applied to cleave heteroatomic bonds (principally carbon–oxygen bonds, be these ether, carboxyl, hydroxyl or carbonyl) in discrete molecular lipids (Rowland, 1990; Navale, 1992, 1994; Gelin et al., 1993; Pease, 1998) and in biomacromolecules (e.g., Philp and Calvin, 1976; Berkaloff et al., 1983; Largeau et al., 1986; Derenne et al., 1988, 1991, 1992; Goth et al., 1988; Gelin et al., 1994, 1996, 1999), but these methods are not as effective at preserving molecular information in hydrocarbon products as HyPy. This is due to the long reaction times required in closed-system hydrous pyrolysis experiments (of the order of days; Rowland, 1990; Navale, 1992, 1994; Pease, 1998) or the high temperatures together with the lack of an efficient hydrogen donor medium in conventional open-system flash pyrolysis systems. Flash pyrolysis is typically performed at 600 °C, and above, and in a low-pressure inert gas atmosphere (Navale, 1992; Gelin et al., 1993, 1999; Pease, 1998). These factors result in more thermal cracking and rearrangement reactions compared with the HyPy methodology which can achieve hydrodesulfurisation and hydrodeoxygenation at relatively low temperatures (typically between 250 and 450 °C).

Chemical reagents undoubtedly offer more selectivity in covalent bond cleavage than any form of pyrolysis, including HyPy, and allow better preservation of structural and stereochemical features in products since these treatments are generally performed at near-ambient temperatures. In terms of facilitating analysis of lipids in cells, both acid and base hydrolysis reactions are routinely used for cleavage of ester bonds in glycolipids, phospholipids, acyl glycerides and wax esters to release simple carboxylic acid and alcohol constituents. More recently, chemical reagents such as boron tribromide (BBr_3), hydriodic acid (HI) and ruthenium tetroxide (RuO_4) have been used for cleavage of ether bonds in archaeal lipid molecules (de Rosa et al., 1976, 1983; Schouten et al., 1998a; Summons et al., 2002) and algaenan biomacromolecules (Gelin et al., 1996; Schouten et al., 1998b; Blokker et al., 2000). HyPy, however, does hold some advantages over chemolysis. Since it is a rapid technique (less than 1 h per experiment), and allows simultaneous cleavage of different heteroatomic covalent bond types (both intermolecular cross-links and free functional groups), the hydrocarbon products can be detected within one, readily-prepared, analytical window. Furthermore, cells need not undergo rigorous Bligh and Dyer extractions (Bligh and Dyer, 1959).

The HyPy technique has good potential as a chemotaxonomic tool by serving as a rapid screening method

to give insights into the core lipid constituents of whole or pre-extracted cells and to assess the range and relative abundance of different carbon skeletons present in any particular species. Any useful analytical approach, however, must be able to preserve the main structural and stereochemical features in lipid products. Accordingly, the purpose of this investigation was to evaluate:

(i) if the distributions of the hydrocarbon products released by hydropyrolysis from cultured cells were sufficiently distinct to act as molecular fingerprints for different classes of microorganisms;
(ii) if the detail of molecular information in hydrocarbon products, in terms of retention of biologically-inherited structural and stereochemical features, was high or whether thermal cracking and isomerisation were particularly problematic.

2. Experimental

2.1. Sample preparation

Algal cultures were grown at the Culture Collection of Algae and Protozoa (CCAP) under aseptic photoautotrophic conditions at 15 °C, or in the case of *Emiliania huxleyi* at 10 °C and at 20 °C, to late log/stationary phase. Growth conditions are documented in the Catalogue of the UK National Culture Collection (UKNCC). *Botryococcus* algaenans (races A and L) were kindly donated by Dr. Claude Largeau (ENSCP, University of Paris). These biopolymer concentrates were prepared from an early isolation procedure used prior to the development of an improved method for algaenan separation (Allard et al., 1998) and so some melanoidin-type residues may have been present in our samples. Bacteria and archaea were cultured at NASA Ames Research Centre using culture strains whose lipid compositions have been established in previous work (Summons et al., 1999). A full inventory of species which were used in this investigation is listed in Table 1.

For selected samples, freeze-dried biomass was extensively and sequentially extracted ultrasonically using dichloromethane/methanol (1:1 v/v), dichloromethane/methanol (3:1 v/v), dichloromethane, acetone and, finally, petroleum ether. For two hopanoid-producing bacteria, *Phormidium luridum* and *Microcystis aeruginosa*, exhaustive thermal extraction with chloroform/methanol (2:1 v/v) was performed in a Gerhardt Soxtherm apparatus, in addition to ultrasonic treatment, to aid the recovery of amphiphilic biohopanoid compounds. Prior to HyPy treatment, biomass was impregnated with an aqueous solution of ammonium dioxydithiomolybdate [$(NH_4)_2MoO_2S_2$], which reductively decomposes in situ under HyPy conditions above 250 °C to form a catalytically-active molybdenum sulfide (MoS_2) phase, and then freeze-dried again.

2.2. Optimisation of HyPy experimental variables for biomass applications

Initial HyPy experiments for generating biomarker skeletons from hopane-producing bacteria produced disappointing results, with appreciable isomerisation and alkyl side-chain cracking of the hopanoid skeleton (Bishop et al., 1998). Biomass is much more prone to softening, and retrogressive transformations such as cross-linking, during sample heating as compared with relatively-inert geomacromolecular substances such as kerogens. Accordingly, the experimental HyPy protocol required a revised catalyst-loading procedure, support substrates and a modified temperature program.

Optimum HyPy conditions involved use of large quantities of sulfided Mo catalyst, typically using a loading of 3% w/w of Mo to dry biomass, adsorbed onto samples to aid low temperature reductive cleavage of oxygen functionalities and a temperature program which minimised heat-up time prior to bond-cleavage temperatures but which proceeded slowly through the main pyrolysis temperature window (from 260 to 450 °C under HyPy conditions). Coarse-grained silica (30–70 mesh), typically using no more than 300 mg, was added to form a short adsorbent plug in the reactor tube on which the sample was supported. This prevented biomass from softening and from being swept from the hot zone of the reactor prior to undergoing pyrolysis reactions.

In summary, catalyst-loaded samples (10–50 mg of organic matter per run) were heated in a stainless steel (316 grade) reactor tube from ambient temperature to 260 °C at 300 °C min^{-1} then to 500 °C at 8 °C min^{-1}. Typical run times were ca. 35 min. A constant hydrogen flow of 6 dm^3 min^{-1}, measured at ambient temperature and pressure, through the reactor bed ensured that the residence times of volatiles were extremely short, of the order of a few seconds. Total tar hydropyrolysates were collected in a stainless steel trap cooled with dry-ice and recovered in dichloromethane (DCM). Since this work was undertaken, a new trap design, incorporating a silica gel adsorbent, has been constructed and employed to allow more efficient trapping of mg quantities of hydropyrolysates (Meredith et al., 2004).

2.3. Product fractionation

The hydropyrolysis tar products were first treated by addition of pre-extracted and activated copper turnings to remove traces of elemental sulfur derived from thermal disproportionation of the catalyst. They were then separated by silica gel adsorption chromatography

Table 1
List of cultures used in hydropyrolysis experiments and their characteristic hydrocarbon products

Taxa	Strain number	Taxonomic class	Extracted/unextracted	Aliphatic hydrocarbon yield[a] (% dry biomass)	Characteristic dominant hydrocarbon products in GC–FID from HyPy
Marine microalgae					
Phaeodactylum tricornutum	CCAP 1052/1A	Bacillariophyceae	Unextracted	4.8	nC_{16}–nC_{24} (even over odd predominance); phytane/enes; C_{28} sterane/ene
Emiliania huxleyi (10 °C)	CCAP 920/2	Prymnesiophyceae	Unextracted	6.3	nC_{16}, nC_{18}, nC_{22},nC_{31}, nC_{33}, nC_{34}, nC_{36}, nC_{37} and nC_{38}alkanes/enes, phytane/enes
Emiliania huxleyi (20 °C)	CCAP 920/2	Prymesiophyceae	Unextracted	6.8	nC_{16}, nC_{18}, nC_{22}, nC_{31}, nC_{33}, nC_{34}, nC_{36}, nC_{37} and nC_{38} alkanes/enes; phytane/enes
Odontella aurita	CCAP 1054/1	Bacillariophyceae	Unextracted	5.2	nC_{16}, nC_{18}, nC_{26} alkanes/enes; phytane/enes; C_{28} and C_{29} steranes/enes
Freshwater microalgae					
Scenedesmus quadricauda	CCAP 276/21	Chlorophyceae	Unextracted	4.7	nC_{16}, nC_{18} alkanes/enes; nC_{26}, nC_{28}, nC_{30} alkanes/alkenes; phytane/enes
Scenedesmus quadricauda	CCAP 276/21	Chlorophyceae	Extracted	2.3	nC_{13}–nC_{36} alkanes/alkenes (maximum C_{30}) with even/odd predominance; phytane/enes
Cryptomonas sp.	CCAP 979/26	Cryptophyceae	Extracted	0.9	Phytane/enes; traces amounts of alkanes and C_{27}–C_{29} steranes
Ceratium hirundinella	CCAP 1110/4	Dinophyceae	Extracted	0.9	nC_{16}–nC_{24} alkanes (even over odd predominance); phytane/enes; C_{27} steranes/enes
Stephanodiscus hantzchii	CCAP 1079/4	Bacillariophyceae	Extracted	0.7	nC_{16}, nC_{24} alkanes (even over odd predominance); phytane/enes
Tribonema aequale	CCAP 880/1	Xanthophyceae	Extracted	0.8	nC_{16}, nC_{18}, nC_{22}, nC_{24}, nC_{26} alkanes; phyyane/enes; C_{27} and C_{29} steranes/enes
Algaenans					
Botryococcus braunii "A"	Paris	Chlorophyceae	Extracted	46	nC_{11}–nC_{38} alkane/alkenes (bimodal: maximum C_{29}, sub-max C_{20})
Botryococcus braunii "*L*"	Paris	Chlorophyceae	Extracted	56	C_{16}, $C_{18(max)}$, C_{19}, C_{20}, and C_{40} isoprenoid alkanes/enes; nC_{14}–nC_{35} alkanes (maximum C_{19})

Bacteria					
Microcystis aeruginosa	CCAP 1450/4	Cyanophyceae	Extracted	0.8	nC$_{14}$–nC$_{18}$ alkanes/enes; phytenes; hopanes/enes (maximum C$_{35}$), hopanes/enes
Phormidium luridum	UTEX 426	Cyanophyceae	Unextracted	3.8	C$_{14}$–C$_{20}$ normal and branched (monomethyl-, dimethyl-) alkanes; hopanes/enes
Phormidium luridum	UTEX 426	Cyanophyceae	Extracted	0.7	nC$_{14}$–nC$_{20}$ alkanes/enes; phytenes, hopanes/enes (maximum C$_{35}$)
Phormidium sp. "RCO"	Mat	Cyanophyceae	Unextracted	5.0	nC$_{14}$–nC$_{18}$ alkanes/enes; phytane and phytenes
Phormidium sp. "OLV"	Mat	Cyanophyceae	Unextracted	5.2	C$_{14}$–C$_{18}$ normal and branched (monomethyl-, dimethyl-) alkanes; hopanes/enes
Methylococcus capsulatus	ATCC 33009	Methylococcaceae	Unextracted	1.9	nC$_{13}$–nC$_{16}$ alkanes/enes; 3-Me hopanes, hopanes/enes
Chloroflexus aurantiacus	Unknown	Chloroflexaceae	Unextracted	2.7	nC$_{13}$–nC$_{20}$ alkanes/complex series of alkenes; nC$_{31}$ alkanes/ alkadienes/alkatrienes
Chlorobium tepidum	Unknown	Chlorobiaceae	Unextracted	4.0	nC$_{16}$, nC$_{18}$ and nC$_{31}$ alkanes/enes; phytane/enes; isorenieratene-derived complex PAH
Chlorobium thiosulfatophilum	Unknown	Chlorobiaceae	Unextracted	4.1	nC$_{16}$, nC$_{18}$ and nC$_{31}$ alkanes/enes; phytane/enes; isorenieratene-derived complex PAH
Prochlorothrix hollandica	CCAP 1490/11	Prochlorophycea	Unextracted	2.9	C$_{14}$–C$_{18}$ normal and branched alkanes/enes; phytenes; hopanes and 2-Me hopanes
Archaeon					
Halobacterium saccharovorum	ATCC 29252	Halobacteria	Unextracted	1.5	Phytane/enes and pristane/enes (from phytanyl glyceryl ethers); nC$_{16}$ and nC$_{18}$ alkane; small amounts of C$_{25}$ and C$_{30}$ isoprenoid alkanes

For CCAP algal cultures, all were grown to late log/stationary phase.

[a] Percentage weight of initial dry biomass recovered as C_{14+} aliphatic hydrocarbons in the dry-ice-cooled HyPy product trap (from mass of aliphatics recorded after evaporation of DCM solvent). Paris = supplied by Claude Largeau (Ecole Nationale Supérieure de Chimie de Paris, France). Mat = cultured at NASA Ames Research Center after isolation from microbial mats from Yellowstone National Park.

in short glass Pasteur pipette columns into aliphatics, "aromatics" and polars (or N, S, O compounds) by elution with *n*-hexane, *n*-hexane–dichloromethane (3:1 v/v) and dichloromethane–methanol (3:1 v/v), respectively. Aliphatic hydrocarbon fractions were characterised using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

2.4. Gas chromatography

GC was performed with a Carlo Erba 5160 HRGC instrument equipped with a flame ionisation detector (FID). Aliphatic hydrocarbon products were analysed on a fused silica capillary column (HP-1; 100% methylsilicone; dimensions, 30 m × 0.25 mm id, 0.25 μm film thickness) using on-column injection. The GC oven temperature program was 50 °C held for 2 min, then 4 °C min^{-1} to 300 °C and held at the final temperature for 35 min. The carrier gas used was hydrogen.

2.5. Gas chromatography–mass spectrometry

Mass spectral characterisation of aliphatic hydrocarbon products from HyPy was carried out using gas chromatography–mass spectrometry (GC–MS) on a Hewlett-Packard 5890 II GC with split/splitless injector interfaced to a Hewlett-Packard 5972 quadrupole mass selective detector (electron input energy 70 eV, filament current 220 μA, source temperature 160 °C, multiplier voltage 1600 V, interface temperature 300 °C). Data acquisition was controlled by a HP Vectra 486 Chemstation computer in full scan mode (50–550 Da s^{-1}) or selected ion mode (30 ions, 35 ms dwell time). The sample (1 μl) in *n*-hexane was injected by an HP7673 autosampler and the split opened after 1 min. (20R)-5α,14α,17α-[2,2,4,4-d4]-cholestane was added to selected aliphatic product fractions to serve as an internal standard.

GC separation was performed on a fused silica capillary column (HP-5; 25 m × 0.25 mm id, 0.25 μm film thickness) using helium as the carrier gas (flow 1 ml min^{-1}, pressure of 50 kPa, split at 10 ml min^{-1}). The temperature was programmed from 40 to 175 °C at 10 °C min^{-1}, 175–225 °C at 6 °C min^{-1} and then at 225–300 °C at 4 °C min^{-1} and held at final temperature for 20 min. Compound identification was based on relative retention times and comparison of mass spectra with those reported in the literature.

3. Results and discussion

3.1. How distinctive are hydrocarbon profiles for individual microorganisms?

A highly distinctive distribution of hydrocarbon products was generated for each of the species subjected to HyPy, broadly in agreement with the expected lipid compositions as previously reported in the literature for these, or related, species. A summary of the principal characteristic hydrocarbons produced for each of the species investigated is given in Table 1.

The total ion chromatograms shown in Fig. 1 illustrate the ranges of HyPy hydrocarbon products for four of the microbial cultures tested. *Prochlorothrix hollandica* yielded predominantly *n*-C_{16} and *n*-C_{18} acyclic hydrocarbon chains (from C_{16} and C_{18} fatty acids), phytenes (from free phytol and from phytyl chains of chlorophylls *a* and *b*), C_{29} and C_{30} hopenes (from diploptene/diplopterol) and 17β,21β(H)-extended hopanes maximising at C_{35} (along with the 2-Me analogues) from bacteriohopanepolyols (Volkman et al., 1988; Simonin et al., 1996; Summons et al., 1999). No steroids were detectable. *Scenedesmus quadricauda*, a freshwater green alga, also produced C_{16} and C_{18} alkanes and phytane/enes as the dominant aliphatic products although significant amounts of higher molecular weight *n*-hydrocarbons were also generated (mostly as *n*-alkanes, but different *n*-alkene homologies were also found). The most abundant long-chain linear hydrocarbons (C_{26}, C_{28}, C_{30} and C_{32}) are derived from aliphatic biomacromolecules (algaenans) previously shown to occur in the outer cell walls of *Scenedesmus communis* (Derenne et al., 1991). The straight-chain hydrocarbon distributions obtained for unextracted and extracted cells of *S. quadricauda* are discussed in detail in Section 3.4 in the context of algaenan chemical structure. C_{28} and C_{29} steroid products were only present in low abundance relative to the straight-chain hydrocarbons and are difficult to discern in the TIC trace although GC–MS analysis confirmed their presence (see Section 3.5).

The archaeon, *Halobacterium saccharovorum*, generated predominantly regular isoprenoids ($^{i}C_{18,\ 19,\ 20,\ 25\ and\ 30}$) with phytane/enes ($^{i}C_{20}$) as the main products. Phytane and phytenes are derived from cleavage of ether bonds within archaeol, 2,3-di-*O*-phytanyl-*sn*-glycerol, and related phosphoglycolipids and sulfated glycolipids (Lanzotti et al., 1988; Tindall, 1990), while the lower relative amounts of shorter-chain isoprenoid compounds arise from partial thermal cracking of the phytanyl chain. The low amounts of $^{i}C_{25}$ and $^{i}C_{30}$ compounds detected are most probably derived from an archaeol-type lipid containing these longer isoprenoid chains ether-linked to glycerol. Smaller amounts of *n*-C_{16} and *n*-C_{18} were also detectable and most probably derived from fatty acids of the same carbon number (see Section 3.2). No hopanoid or steroid products were found in this hydropyrolysate, as expected.

E. huxleyi is known to biosynthesise a variety of characteristic straight-chain aliphatic lipids including *n*-C_{36}–C_{39} alkenones and alkenoates with varying degrees of unsaturation (Volkman et al., 1980, 1981, 1998; Marlowe et al., 1984). There are also *n*-C_{31}, *n*-

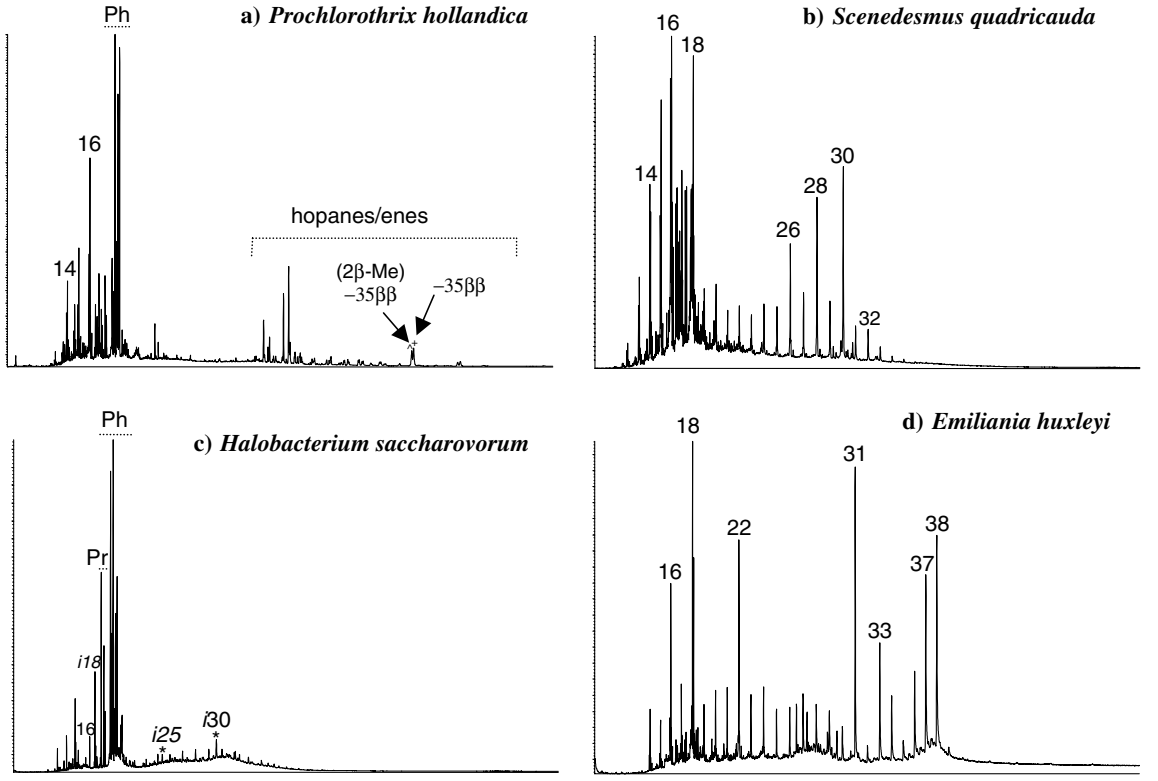

Fig. 1. Total ion chromatograms (TICs) showing the aliphatic hydrocarbon product distributions generated from HyPy of unextracted cells of (a) *P. hollandica*, (b) *S. quadricauda*, (c) *H. saccharovorum*, and (d) *E. huxleyi* (grown at 10 °C). (Numbers refer to carbon chain lengths of *n*-alkane products; Ph = phytane + phytenes; Pr = pristane + pristenes; *i*18, *i*25 and *i*30 = C_{18}, C_{25} and C_{30} regular isoprenoids.)

C_{33}, *n*-C_{37} and *n*-C_{38} alkenes (Conte et al., 1995), *n*-C_{31} and *n*-C_{33} alkadienes (Rieley et al., 1998), as well as saturated *n*-C_{16} and polyunsaturated *n*-C_{18} and *n*-C_{22} monocarboxylic fatty acids (Bell and Pond, 1996; Pond and Harris, 1996). Most published lipid analyses have been performed on coccolith-forming strains of *E. huxleyi.* Our investigation used a strain which did not form coccoliths but the principal lipid compounds noted previously should be common constituents of both. The dominant aliphatic products were, in fact, C_{16}, C_{18}, C_{22}, C_{31}, C_{33}, C_{34}, C_{36}, C_{37} and C_{38} *n*-alkanes, with the homologous series extending up to *n*-C_{39} (Fig. 1), and this carbon chain length pattern is compatible with what one expects to be generated from HyPy based on the reported free lipid biomarker composition of *E. huxleyi*. Alkenes and polyenes of similar carbon number were also generated but their distributions, which probably result from double bond migrations, are too complex to discuss in detail here. The same characteristic hydrocarbons were generated for *E. huxleyi* cultures grown at both 10 and 20 °C, although the higher molecular weight hydrocarbons (*n*-C_{31+}) were relatively more abundant in comparison to C_{16}, C_{18} and C_{22} hydrocarbons in the lower temperature culture products.

Due to significant hydrogenation of mid-chain unsaturations during HyPy, it is impossible to gauge whether the relative abundances of tri- to di-unsaturated C_{37} and C_{38} hydrocarbons products varied with growth temperature for the two culture temperatures used (10 and 20 °C). The ratio of tri- to di-unsaturation in C_{37} alkenones forms the basis of the $U^{K'}_{37}$ parameter used to estimate surface sea temperature (Brassell et al., 1986; Prahl and Wakeham, 1987; Conte et al., 1994) but it appears that HyPy cannot be used as a convenient route to provide an analogous measurement for any alkenes generated. C_{28} steroids comprising 24-methylcholestane and related sterenes and diasterenes were important products as discussed in Section 3.5. The main point to note from the *E. huxleyi* analysis is that the principal and characteristic alkyl chain lengths are obvious from the TIC trace of total aliphatic hydrocarbons produced from HyPy (Fig. 1), even for the longest alkyl chain products expected (*n*-C_{37}–*n*-C_{39}). This suggests that thermal cracking is not a major deficiency when using these HyPy conditions.

3.2. Comparison of alkyl chain lengths of lipids in algae and prokaryotes

A rapid and convenient way to assess the total distribution of the polymethylenic and branched alkyl contents of microorganisms is to isolate a total aliphatic hydrocarbon fraction produced from HyPy and then monitor *m/z* 85 ion chromatograms from routine GC–MS analyses (Figs. 2 and 3). In general, it can be seen that bacteria and archaea biosynthesise a more restricted distribution of polymethylenic chain lengths which are, typically, *n*-C_{20} and shorter. In contrast, all of the algal cultures investigated here generated significant quantities of waxy linear hydrocarbons with chain lengths greater than C_{20}. The presence of long *n*-alkyl chain constituents of microalgae is not always apparent from analysis of extractable fatty acid components in algal cultures, which generally comprise C_{16} and C_{18} compounds as the dominant carbon numbers (Schouten et al., 1998c). Long-chain C_{26}, C_{28} and C_{30} ($\omega - 1$)-hydroxy fatty acids

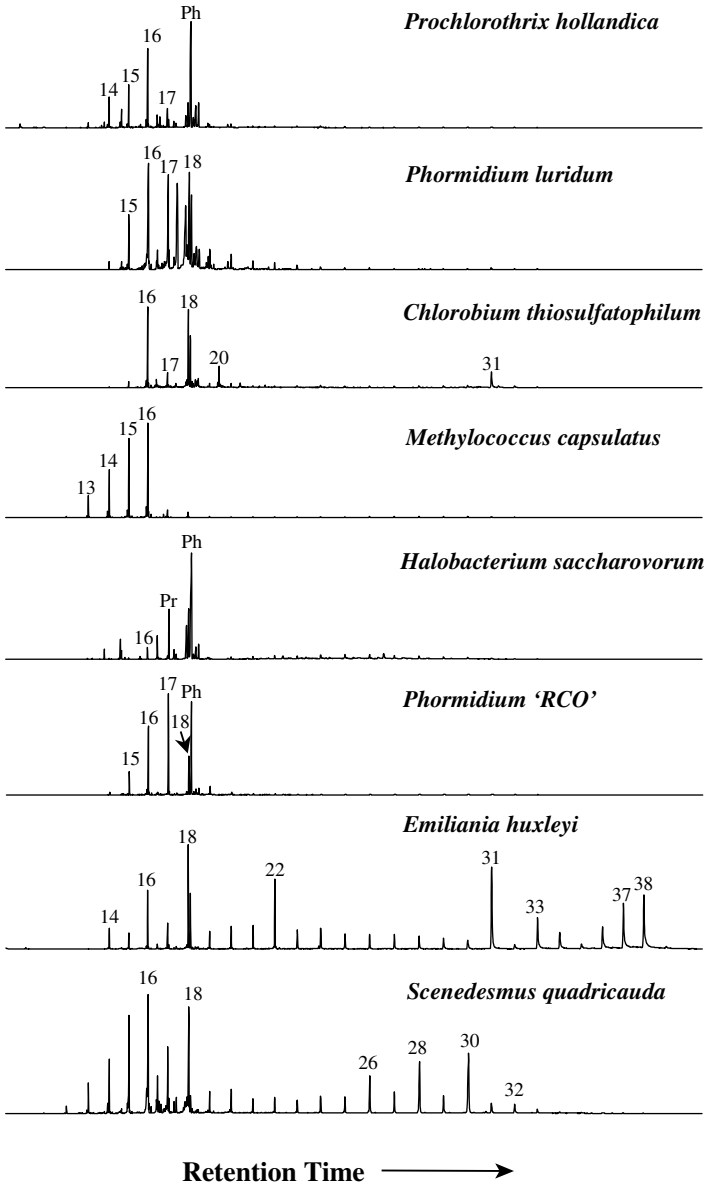

Fig. 2. *m/z* 85 ion chromatograms showing the full distribution of *n*-alkanes and isoprenoids generated from HyPy of a selection of unextracted microbial cultures. (Numbers refer to carbon chain lengths of *n*-alkane products; Ph = phytane.)

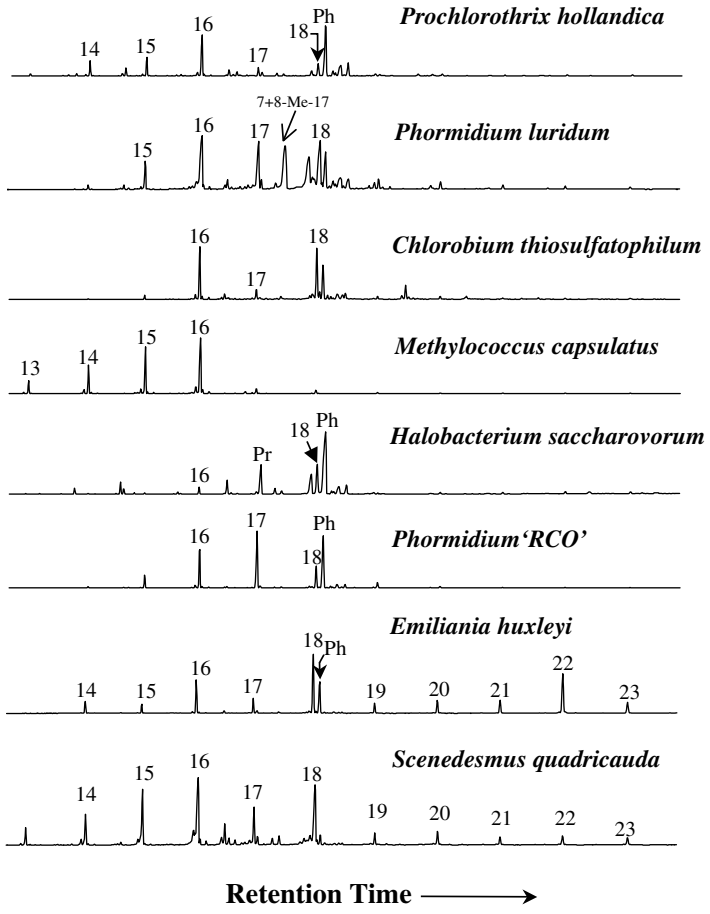

Fig. 3. Partial *m/z* 85 ion chromatogram showing the distribution of *n*-alkanes and isoprenoids in the C_{13}–C_{23} carbon number range produced from HyPy of a selection of unextracted microbial cultures. (Numbers refer to carbon chain lengths of *n*-alkane products; Ph = phytane.)

have been reported as lipid constituents of methane-utilising bacteria (Skerratt et al., 1992), and so prokaryotes appear capable of biosynthesising waxy *n*-alkyl chains. However, linear alkyl chains with 20 carbon atoms or less are generally found in prokaryotes.

Exceptions to the rule of shorter polymethylenic carbon chain lengths for prokaryotes, were found for two *Chlorobium* sp. and *Chloroflexus aurantiacus* which all generated *n*-C_{31} alkane (and alkenes) from HyPy. It has been shown previously that *Chloroflexus* sp. biosynthesise *n*-C_{31} alkyl chains, particularly in the form of $C_{31:3}$ alkatriene (Shiea et al., 1991; van der Meer et al., 2000), although it has not previously been reported, to our knowledge, that *Chlorobium* sp. biosynthesise *n*-C_{31} alkyl chains. For example, no lipids containing *n*-C_{31} alkyl chains are reported by van der Meer et al. (1998) from *Chlorobium* sp. cultures. It should also be noted that the archaeon investigated here, *H. saccharovorum*, generated significant amounts of linear C_{16} and C_{18} alkanes from HyPy as well as isoprenoidal hydrocarbons (Fig. 3). C_{16} and C_{18} linear fatty acids have previously been detected as bound molecular constituents esterified to proteins in the red membrane of the archaea *Halobacterium cutirubrum* and *Methanobacterium thermoautotrophicum* (Pugh and Kates, 1994).

A common feature of all the straight-chain hydrocarbon distributions produced from HyPy of cultures is the presence of a significant even-over-odd carbon number predominance (EOP) of shorter carbon chain lengths ($< C_{22}$). The distinct EOP reflects the fact that the molecular precursors of these hydrocarbon products are generally either free or bound carboxylic acids and alcohols possessing a similar chain-length predominance. C_{16} and C_{18} are generally the most abundant short chain fatty acids in extant organisms (with various degrees of unsaturation) and the presence of C_{16} and C_{18} acyclic hydrocarbons as common and dominant

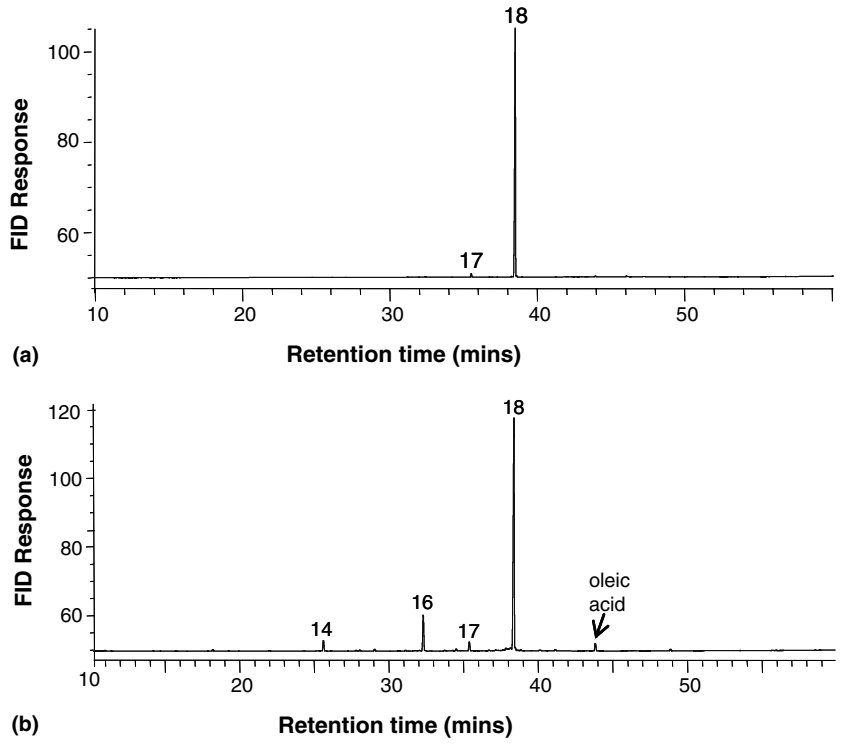

Fig. 4. GC–FID traces showing the total product distributions obtained from HyPy of fatty acid model compounds, (a) stearic acid and (b) oleic acid, which had been pre-adsorbed on silica gel. (Numbers refer to carbon chain lengths of *n*-alkane products.) Taken from Craig et al., 2004 (reproduced with permission).

constituents in HyPy products indicates that hydropyrolysis reductively converts acyclic acid and alcohol lipids to alkane chains of similar length. So, complete reduction of fatty acids to even-carbon-numbered hydrocarbons is favoured over decarboxylation, although the latter reaction does occur to some degree. To illustrate this, stearic acid (*n*-octadecanoic acid, a saturated *n*-C_{18} carboxylic acid) and oleic acid (*cis*-9-octadecenoic acid, an unsaturated *n*-C_{18} carboxylic acid) were separately pre-adsorbed on a silica gel support, loaded with catalyst precursor, and subjected to catalytic HyPy treatment (Craig et al., 2004). For stearic acid, *n*-C_{18} alkane was by far the dominant product (98% of the total GC–FID signal) and only a small amount of *n*-C_{17} alkane and lower carbon numbered compounds were generated. With the unsaturated acid, oleic acid, thermal cracking of C–C bonds via a β-cleavage process is slightly more problematic but *n*-C_{18} alkane was still by far the main product generated (79% of the total GC–FID signal). Catalytic HyPy is thus fairly selective at cleaving heteroatomic linkages (here carbon–oxygen bonds are targeted, but also carbon–sulphur linkages in geopolymers) and produces much less cracking of C–C bonds in alkyl chains in comparison with conventional flash pyrolysis methods.

3.3. Assessing molecular information content in HyPy-generated hydrocarbon profiles

The preservation of important molecular information, in terms of structural and stereochemical detail, in the polycyclic biomarker HyPy products is illustrated in Fig. 5. Biohopanoids, in particular bacteriohopanepolyols (BHP), pose an excellent test of the capability of the optimised HyPy method to preserve structural detail since: (i) these structures are particularly susceptible to undergoing configurational isomerisation at C-17, C-21 and C-22 even when only a low level of thermal stress is applied; (ii) selective cleavage of four or more oxygen functionalities is required to convert the poly-functionalised BHP precursors to the corresponding hydrocarbon (Fig. 5).

It can be seen from the *m/z* 191 chromatogram displayed in Fig. 5 that the hopanoid-producing cyanobacterium *P. luridum* has (22R)-17β,21β(H) pentakishomohopane (C_{35} homohopane), the basic carbon skeleton of all BHP precursors, as 45% of the total extended hopane products. The presence of lower amounts of C_{31}–C_{34} homohopanes, and detectable amounts of 17β,21α(H)- and 17α, 21β(H)-isomers indicated that some thermal cracking and rearrangement seems inevi-

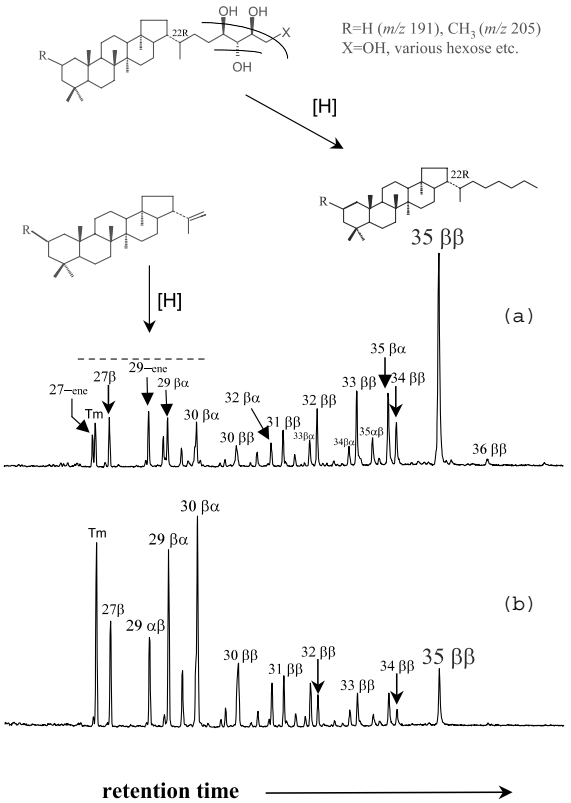

Fig. 5. *m/z* 191 ion chromatograms showing the distributions of hopanes produced from HyPy of *P. luridum* (cyanobacterium) whole cells using (a) the optimised HyPy procedure with a Mo catalyst, and (b) HyPy without a Mo catalyst.

table. This may be because the C–C covalent bonds in the hopanoid extended side-chain are weakened due to the presence of adjacent hydroxyl groups (Rohmer et al., 1984). Even for these shorter chain hopanes though, the biological (22R)-17β,21β(H) configuration of BHP was retained in ca. 80% of extended hopane products released by HyPy. In the absence of molybdenum catalyst, the hopane profiles (Fig. 5) indicated a much higher degree of thermal cracking and isomerisation. The presence of a catalytically-active molybdenum sulfide phase (MoS_2) during HyPy is beneficial since it results in more selective cleavage of oxygen functionalities at lower temperatures in the HyPy heating cycle. To date, no other pyrolysis method has demonstrated this level of preservation of structural and stereochemical features for hopanes and, therefore, HyPy can be regarded as a relatively "mild" technique.

3.4. Searching for evidence for insoluble aliphatic biomacromolecules

The only strong evidence that was obtained from HyPy-generated hydrocarbon signals for the presence of aliphatic biomacromolecules was for the freshwater green alga *S. quadricauda*. The closely-related species *S. communis* has already been shown to contain algaenans in the outer cell walls (*S. quadricauda* used by Derenne et al., 1991 was subsequently reclassified as *S. communis*). HyPy of this species produced a bimodal series of predominantly *n*-alkanes with smaller amounts of *n*-alkenes up to at least *n*-C_{36} (maximum at *n*-C_{16} and sub-maximum *n*-C_{30} for unextracted cells, see Fig. 3(a)), with a distinctive even-over-odd (EOP) carbon number predominance. A significant yield of aliphatic hydrocarbons (amounting to 2.3% w/w of initial dry cells) was generated even after the cells had been thoroughly extracted with solvents (see Fig. 6). By comparison, the pre-extracted cells of the other algal species generated lower amounts of aliphatic hydrocarbons (<1% w/w of extracted cell weight). The molecular profiles produced from these were not just dominated by series of linear alkanes and alkenes but contained straight-chain, isoprenoidal and steroidal hydrocarbons (Table 1) which had evaded solvent extraction.

For pre-extracted cells of *S. quadricauda*, the dominant aliphatic constituents were straight-chain hydrocarbons (predominantly *n*-alkanes but also *n*-alkenes at least up to *n*-C_{36}) although the molecular profile generated for extracted cells were relatively enriched in waxy *n*-C_{24} to *n*-C_{36} hydrocarbons compared with the lower

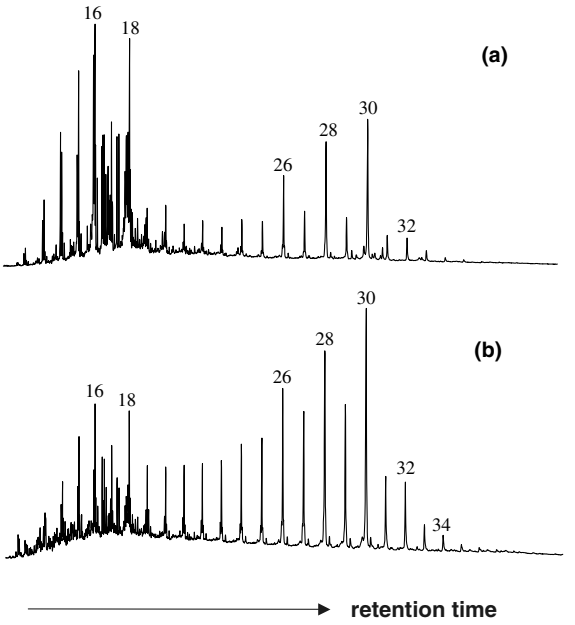

Fig. 6. Total ion chromatograms (TICs) showing the distributions of aliphatic hydrocarbons generated from HyPy of (a) unextracted, and (b) extracted cells of the freshwater chlorophyte *S. quadricauda*. (Numbers refer to carbon chain lengths of *n*-alkane products.)

molecular weight envelope (n-C_{14} to n-C_{18}). This is not surprising since n-C_{14}, n-C_{16} and n-C_{18} acids are the principal alkyl chain lengths of readily-extractable fatty acid constituents of *S. quadricauda* (Tadesse et al., 2003) and so should be less abundant in cell residue products. The extremely low amounts of steroidal hydrocarbons (steranes, sterenes and diasterenes) detected in the extracted cells in comparison with unextracted cells showed that solvent extractions had been efficient. Unlike a previous pyrolysis investigation into the structure of *Scenedesmus* sp. algaenans (Derenne et al., 1991), n-alkylcyclohexane and alkylbenzene compounds were virtually absent in the hydropyrolysates produced from this species (i.e., below detection limits), indicating that these compounds are likely produced as a consequence of the pyrolysis method used through cyclisation and subsequent dehydrogenation of n-alkyl radical intermediates.

Discrete high molecular weight lipids, principally linear polyesters, mono- and dicarboxylic acids up to C_{120} (Allard et al., 2002), have recently been detected as cell wall constituents of freshwater microalgae, including *S. communis*. Based on these findings, the authors proposed that algaenans are more likely composed of such high molecular weight lipid species rather than comprising a network of C_{26}–C_{34} even-carbon-numbered aliphatic chains cross-linked by strong covalent bonds such as ether bridges (Derenne et al., 1991; Gelin et al., 1996; Blokker et al., 1998, 2000; Schouten et al., 1998b). The presence of significant C_{20}–C_{25} n-alkanes and n-alkenes in the hydropyrolysates (as well as the C_{26}–C_{34} straight-chain hydrocarbons) and a reduced EOP of n-alkyl chain lengths for extracted cell products compared with unextracted cells, however, suggests a reasonable degree of covalent cross-linking (possibly involving C–C bonds as well as C–O) at non-terminal positions in n-alkyl chains within the cell residue. These stable cross-links within the 3-D architecture of the biomacromolecules are likely cleavage sites during HyPy conversion, resulting in significant release of smaller chain length products (such as C_{20}–C_{25}) from a significant proportion of the bound C_{26}–C_{34} linear chains. If algaenans were composed predominantly of discrete long-chain mono- and dicarboxylic acids and esters (Allard et al., 2002) rather than a biopolymer, then one would expect a much narrower distribution of dominant carbon chain-lengths to be produced from HyPy of *S. quadricauda* cell material than is observed here. Tetramethylammonium hydroxide (TMAH) thermochemolysis of algaenans prepared from the freshwater algae *Chlorella emersonii*, *Tetraedron minimum* and *S. communis* (Allard et al., 2002) also produced a wide carbon number distribution (from C_8 to C_{32}) of methyl esters of straight-chain carboxylic acids, α,ω-dicarboxylic acids, ω-methyl ether acids and other unidentified compounds suggesting some degree of three-dimensional cross-linking in the algaenan structures.

As has been demonstrated previously (Fig. 4), HyPy is a mild pyrolysis technique that can cleave oxygen functionalities in lipid structures without significant reduction in alkyl chain lengths. Our results are, therefore, more consistent with the established 3-D macromolecular models of algaenan structure for this freshwater chlorophyte than with the algaenan composition proposed by Allard et al. (2002). It appears that a significant portion of the aliphatic cell wall material may consist of highly cross-linked biomacromolecules, as well as the discrete linear polyesters and dicarboxylic acids up to C_{120} observed by Allard et al. (2002). Unfortunately, our analytical window was restricted to n-C_{40} compounds and below, since only conventional gas chromatography columns were employed.

The chemical structures of isolated algaenans from the freshwater green alga, *Botryococcus braunii*, races A and L, have been examined in a number of previous studies using both chemical degradation and pyrolysis techniques (Berkaloff et al., 1983; Largeau et al., 1986; Derenne et al., 1989; Gelin et al., 1994; Behar et al., 1995; Schouten et al., 1998b; Berthéas et al., 1999; Blokker et al., 2000). Accordingly, algaenans prepared from both races were subjected to HyPy treatment. The *B. braunii* L-race algaenan used in this study produced significant amounts of both isoprenoids and linear alkyl chains up to C_{40} (Fig. 7). This product pattern was initially interpreted as being a consequence of the algaenans being from mixed race cultures (races L and A; Behar et al., 1995). More recent work has shown that the algaenan of *B. braunii* L-race is also predominantly polymethylenic in structure and that the isoprenoidal components in pyrolysates are derived from cleavage of free lycopenediol ($^{i}C_{40}$ compound) which was incompletely hydrolysed and only partially removed during the algaenan isolation process (Berthéas et al., 1999). The algaenans used in this study were prepared using the early isolation procedure (Allard et al., 1998) and, therefore, the results record this through the presence of lycopane, lycopa-enes and C_{40} monoaromatic derivatives in the pyrolysates. For the *B. braunii* A-race algaenan, the enhanced levels of odd-carbon-numbered C_{25}–C_{31} straight-chain hydrocarbons generated from HyPy in comparison with the L-race, probably arises from incomplete removal of free C_{25}–C_{31} n-alkadiene and trienes (Metzger et al., 1985) during the algaenan isolation procedure. The algaenans in *B. braunii* are now thought to result from the polycondensation, via aldolisation, of linear C_{32} α,ω-dialdehyde monomers (Berthéas et al., 1999).

The overall distributions of total products obtained by HyPy for both *Botryococcus* strains (Fig. 7) appeared less complex than those published previously from chemolysis or analytical pyrolysis regimes (Berkaloff et al., 1983; Largeau et al., 1986; Derenne et al., 1988; Gelin et al., 1994; Behar et al., 1995; Schouten et al., 1998b; Berthéas et al., 1999; Blokker et al., 2000) and

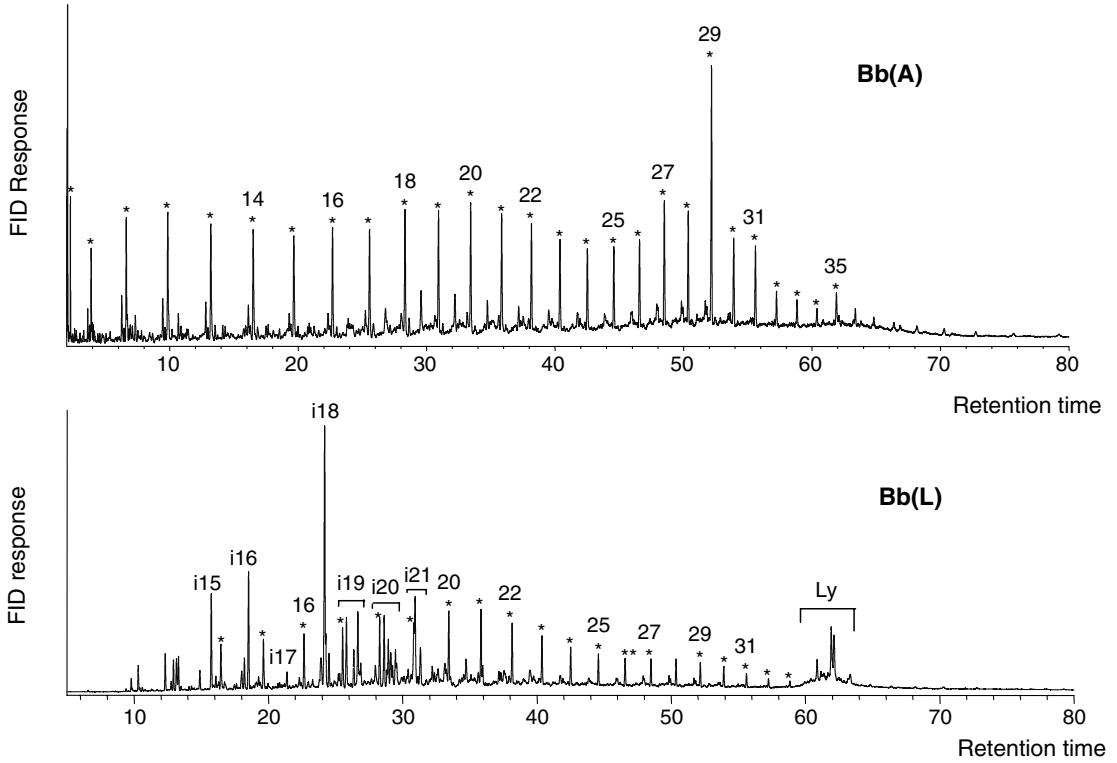

Fig. 7. GC–FID profiles of total products generated from HyPy of algaenans of *B. braunii* races A [Bb(A)] and L [Bb(L)]. (Numbers refer to carbon chain lengths of *n*-alkane products; *i* denotes isoprenoids; Ly = lycopane, lycopa-enes and monoaromatic $^{i}C_{40}$.)

the hydrocarbons extended up to higher carbon numbers (up to *n*-C_{40}) for *B. braunii* A-race algaenan. This is principally due to efficient reductive cleavage and removal of alcohol, acid, ester and ether bonds during HyPy treatment resulting in a lack of oxygenated-products. Partial hydrogenation of mid-chain unsaturations reduced the total number of alkene isomers and produced a more restricted range of structural isomers in comparison with other pyrolysis studies. The overall yields of recovered aliphatic hydrocarbons produced from HyPy of both algaenans (46% w/w for A-race and 56% w/w for L-race, Table 1) were slightly higher than those reported previously from open-system pyrolysis performed at 400 °C using a helium sweep gas flow (32% w/w for A race, Largeau et al., 1986; 52% w/w for L-race, Berthéas et al., 1999). The steady supply of chemisorbed hydrogen available in the HyPy system stabilises reactive molecular intermediates produced from covalent bond cleavage (radicals and ionic species) and helps inhibit cyclisation and aromatisation reactions. This is why yields of aliphatic hydrocarbons from HyPy are generally higher than for pyrolysis performed in inert gas atmospheres (Love et al., 1995). The increase in pyrolysate yields between HyPy and low pressure inert gas systems becomes more pronounced with increasing aromaticity of the organic substrate being pyrolysed, since the availability of hydrogen within the sample controls the overall pyrolysate yields if no external hydrogen supply is added (Rocha et al., 1997).

The signal intensities of *n*-alkylcyclohexanes, alkylbenzenes and other aromatised products were generally lower in abundance relative to the main aliphatic hydrocarbon products in *Botryococcus* hydropyrolysates compared with those of previous pyrolysis studies. This suggests that cyclic molecules are not native to this biopolymer but are a result of thermal rearrangement reactions occurring during conventional open-system pyrolysis performed in inert gas atmospheres. A similar interpretation of the origins of cyclic compounds in *Botryococcus* algaenan pyrolysis products has been expressed previously (Gelin et al., 1994). The fact that *n*-alkylcyclohexane acids and alkylbenzene acids were not reported as significant products from ruthenium tetroxide degradation of *Botryococcus* algaenan (Blokker et al., 2000) provides additional compelling evidence that any alkylcyclohexane and alkylbenzene species produced from *Botryococcus* biomass were formed predominantly as pyrolysis artefacts.

In general, since the sample capacity (>1 g sample) of the apparatus is large compared to other open-system analytical pyrolysis methods, no strong acid or base concentration steps need routinely be performed prior to hydropyrolysis. This reduces the risk of forming melanoidin-like polymers and other structural artefacts during the algaenan isolation (Allard et al., 1998). If present in significant amounts, aliphatic biopolymers in microalgae might be evident as high MW alkanes in HyPy products of solvent-extracted biomass.

3.5. Determination of sterane carbon number patterns in microalgae

Ternary diagrams constructed from the relative proportions of C_{27}–C_{29} steroid constituents, often based on the relative peak areas of 20(R)-5α,14α,17α(H) steranes in *m/z* 217 ion chromatograms, are often used in petroleum geochemistry to characterise petroleum source rocks and oils and to facilitate correlation studies beneficial for aiding petroleum exploration (Huang and Meinschein, 1979). The relative proportions of the different carbon numbers of sterane biomarkers preserved in ancient sediments strongly reflects the combined initial eukaryotic input from different classes of algae, higher plants and fungi. Steroid diagenesis is a very complex process however, involving reductive transformation of sterol and stanol precursors via a number of sterene and diasterene intermediates before the fully saturated sterane and diasterane hydrocarbons can be formed (Mackenzie et al., 1982; Mackenzie, 1984). Thus, both unsaturated and saturated steroid hydrocarbon products are expected to be released from catalytic HyPy treatment of algal cells unless complete hydrogenation of alkene bonds takes place.

Major steroidal products identified in hydropyrolysates of algal cultures included sterenes, steradienes and diasterenes, as well as fully saturated steranes. The isomer distributions showed 'immature' patterns as anticipated, with (20R)-5α, 14α,17α(H) and (20R)-5β,14α,17α(H) being the dominant isomers (Fig. 8). From GC–FID analyses, $\Delta^{13(17)}$ diasterenes and steranes were generally found to be the most abundant steroid products. $\Delta^{13(17)}$ Diasterenes, which give a characteristic *m/z* 257 fragment ion, were generated as metastable products from rearrangement of sterenes and steradienes. These were, in turn, formed from elimination of the hydroxyl group at C-3 and subsequent mobilisation of double bonds (Peakman and Maxwell, 1988; de Leeuw et al., 1989). Diasterene formation involves both methyl group and double bond migrations, but the tetracyclic backbone remains unchanged. Diasterenes are not readily hydrogenated to diasteranes under HyPy conditions due to the hindered unsaturation in the tetracyclic nucleus and this explains the virtual absence of the latter compounds in these hydropyrolysates. In

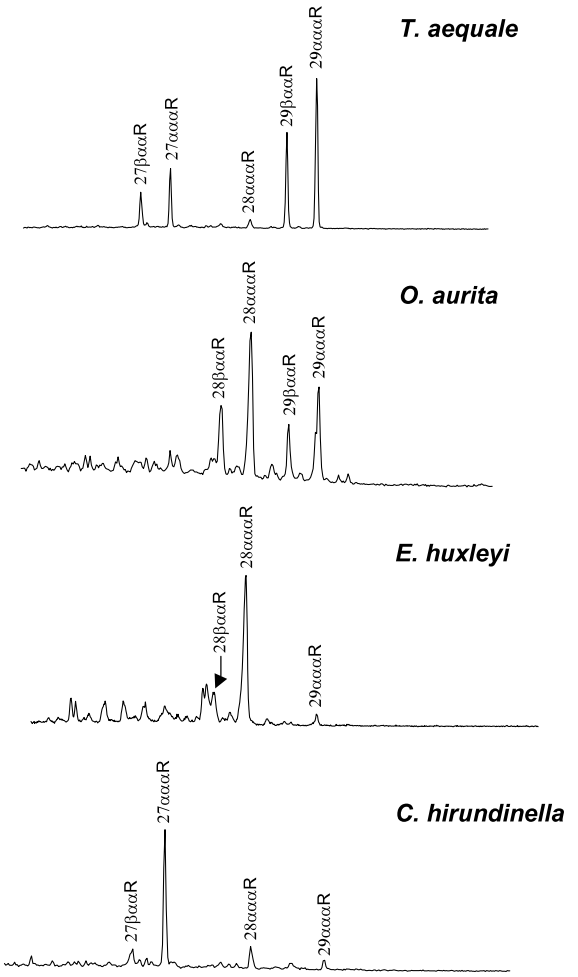

Fig. 8. *m/z* 217 ion chromatograms showing the sterane profiles generated from HyPy of four different species of microalgae *T. aequale*, *O. aurita*, *E. huxleyi* (10 °C culture) and *C. hirundinella*. Note that the sterane profiles shown for *T. aequale* and *C. hirundella* were obtained for pre-extracted cells (Table 1).

theory, steroid carbon number distributions can be gauged from both sterene and diasterene distributions, but co-elution of unsaturated compounds of different carbon number complicates the situation. For this reason, the carbon number patterns of steroid components were gauged in this particular study from the distribution of sterane biomarkers as observed in *m/z* 217 ion chromatograms.

The relative proportions of C_{27}–C_{29} steranes released by HyPy (Fig. 8) were in good agreement with published information about the steroid content of these algae (Mercer et al., 1974; Volkman, 1986, 2003; Barrett et al., 1995; Volkman et al., 1998; Gatenby et al., 2003). A selection of sterane profiles is shown for four algal species in Fig. 8. *Tribonema aequale*, a freshwater

xanthophyte, yielded 20(R)-5α,14α,17α(H) and 20(R)-5β,14α,17α(H) isomers of cholestane and 24-ethylcholestane (C_{27} and C_{29} steranes) as the dominant sterane products from HyPy (Fig. 8), arising predominantly from reduction of cholesterol and 24-ethylcholesterol precursors (Mercer et al., 1974). Only trace amounts of C_{28} products (<5% of *m/z* 217 response for total C_{27}–C_{29} steranes) were formed. In contrast, the hydropyrolysates produced from the unextracted marine diatoms, *Odontella aurita* (Fig. 8) and *Phaeodactylum tricornutum* (not shown), were relatively enriched in C_{28} products with both 5α(H) and 5 β(H) isomers present. C_{28} sterols are often the most abundant steroidal components of diatoms (Barrett et al., 1995). No C_{26} steroids (or steroids of lower carbon number) were detected.

Diatoms are not the only class of microalgae which biosynthesise C_{28} sterols (Volkman, 1986, 2003; Volkman et al., 1998) and appreciable amounts of C_{28} steranes were observed in hydropyrolysates of *E. huxleyi* and *S. quadricauda*, although C_{29} isomers were dominant in the latter. A simple but distinct distribution of sterane products was obtained for the freshwater dinoflagellate, *Ceratium hirundinella*, characterised by an anomalously low abundance of 5β(H) sterane isomers relative to the dominant (20R)-5α,14α,17α(H) isomer of cholestane in the *m/z* 217 ion chromatogram (Fig. 8). It is known that 5α(H)-stanols are often directly biosynthesised by dinoflagellates but rarely by other microalgae (Volkman et al., 1998) and this likely explains the dominance of the 5α(H) configuration in the sterane products in this particular instance. This may have also caused the diasterene to sterane ratio to be the lowest for all algal species studied. Hydrogenation of the double bond at C-5 in Δ^5 sterols, found commonly in other algal species, produced a characteristic mixture of 5β(H) and 5α(H) isomers from HyPy, although not in an exact 1:1 ratio as the more thermodynamically stable 5α(H) isomer was formed preferentially.

The sterane profiles, and absence of C_{26} or lower products, for the different algal cultures, provided good evidence that alkyl side-chain shortening is minimal and does not significantly compromise the sterane carbon number patterns. The similarity in carbon number profiles found for steranes and diasterene compounds, as illustrated for the xanthophyte *T. aequale* (Fig. 9), is a good internal check that no significant carbon number bias accompanies the hydrogenation of steroid precursors. It should be noted that the extent of epimerisation at C-20 (20R into 20S form) was well advanced in diasterene products, but not at all in steranes (Fig. 9). This is attributed to the double bond migration process required for the formation of diasterenes resulting in hydrogen shuttling and exchange (via dehydrogenation and subsequent non-stereospecific hydrogenation) at carbon sites throughout the tetracyclic steroid backbone (Peakman and Maxwell, 1988; de Leeuw et al., 1989). Removal

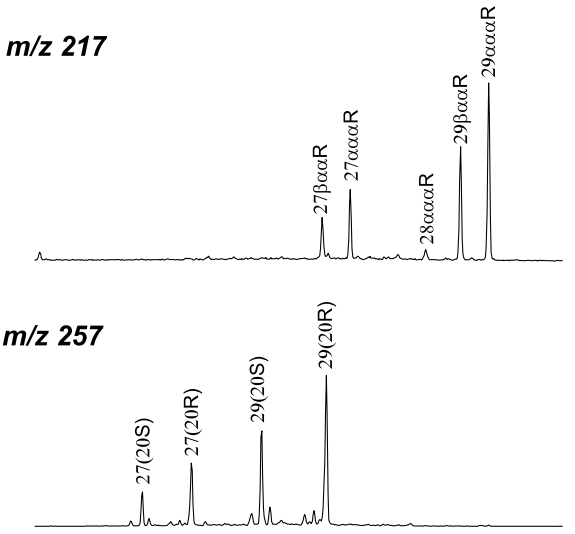

Fig. 9. Partial ion chromatograms showing the distribution of sterane (*m/z 217*) and diasterene (*m/z 257*) products generated from pre-extracted cells of *T. aequale*.

and exchange of the C-20 hydrogen must then occur during the formation of $\Delta^{13(17)}$ diasterenes. In contrast for sterane products, rapid hydrogenation of any unsaturation in the tetracyclic core means that double bond migration and hydrogen shuttling/exchange are terminated before hydrogen atoms in the D-ring and alkyl side-chain can become involved.

3.6. Generation of biomarker hydrocarbons from pre-extracted biomass

Significant quantities of biomarker hydrocarbons were still produced from HyPy treatment of microbial cells that had been carefully pre-extracted with solvents. For example, *P. luridum* cells were repeatedly extracted, both ultrasonically and with boiling solvent, prior to HyPy and yet appreciable quantities of acyclic and cyclic biomarker hydrocarbons were generated. Addition of a deuterated standard [(20R)-5α,14α,17α-[2,2,4,4-d4]-cholestane] to HyPy products allowed us to estimate the residual hopane content at ca. 32 $\mu g\,g^{-1}$ dry wt., in comparison to ca. 205 $\mu g\,g^{-1}$ dry wt. of hopanes generated from untreated cells. Differences in hopane carbon number distribution and in the ratio of 2-methyl- to desmethylhopanes produced from extracted biomass compared to unextracted cells were observed. While it is tempting to infer that the additional hydrocarbons released from extracted cells represent products derived from cleavage of lipids covalently-bound into macromolecular structural components, the compositional differences indicated they were most probably derived from lipids associated with

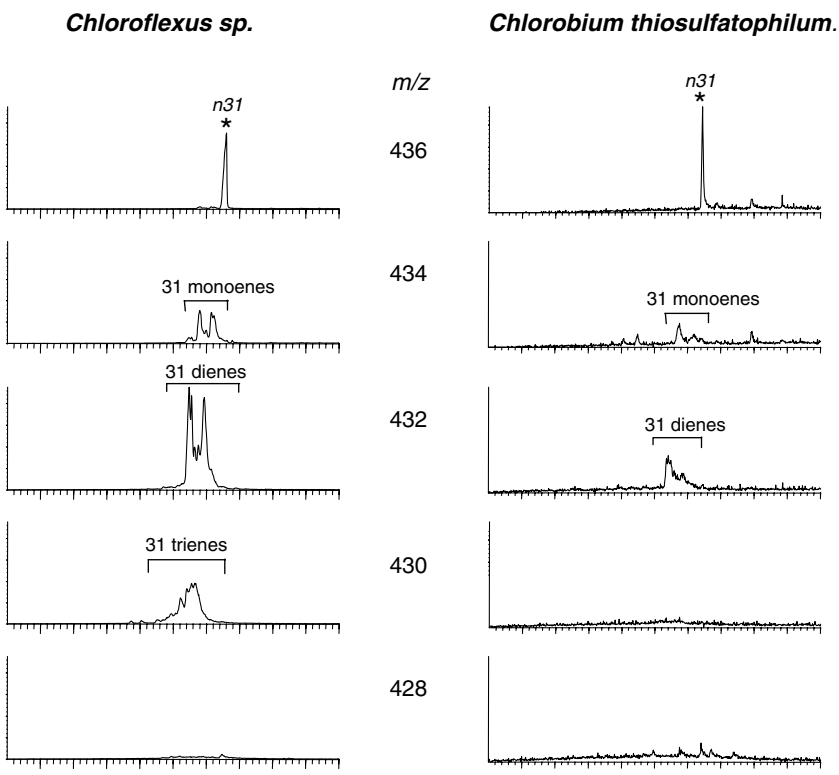

Fig. 10. Distribution of n-C_{31} alkanes and alkenes generated from HyPy of *C. aurantiacus* and *Chlorobium thiosulfatophilum*, using the relative abundances of molecular ions detected by GC–MS.

specific membranes that were not amenable to solvent treatment. An exception is for organisms containing aliphatic biomacromolecules as major structural components of cell walls (see Section 3.4). Regardless of the biological function and spatial location within cells of these unextractable compounds, the important result is that HyPy treatment extends the analytical window such that the hydrocarbon skeletons of these compounds become detectable.

3.7. Loss of molecular information through defunctionalisation and hydrogenation and the potential role of HyPy in lipid chemotaxonomy

The modified HyPy method is a useful technique for cleaving chemical bonds in complex lipids and biopolymers and generating tractable hydrocarbon products. The loss of oxygen functionalities and partial hydrogenation of alkenes, however, means that it may be difficult to predict which particular functionalised lipid precursor(s) species have generated which hydrocarbon products. As a general rule, we found that sites of unsaturation were generally better preserved within highly branched (for example, in phytenes and phytadienes) and polycyclic hydrocarbon structures (hopenes, sterenes and diasterenes) in comparison with linear alkyl chains. It was still possible to detect remnant unsaturation in linear alkyl chains, which may prove useful for determining the degrees of unsaturation in lipid precursors. For example, Fig. 10 shows that by monitoring the relative abundance of molecular ions for n-C_{31} hydrocarbons released by HyPy from *C. aurantiacus*, then levels of unsaturation in the alkyl chain up to triunsaturation are predicted for the n-C_{31} lipid precursors. This is in accord with published results since n-C_{31} alkatrienes are known to be biosynthesised by *Chloroflexus* sp. (Shiea et al., 1991; van der Meer et al., 2000). For *Chlorobium* sp., however, di-unsaturated lipid precursors seem more likely since the n-C_{31} alkatriene was generated in only trace amounts while n-C_{31} alkane, monoenes and dienes were more abundant. It remains to be seen whether this prediction proves to be accurate. For future culture work, it should be possible to reduce the hydrogen pressure used in HyPy from 150 bar to around 50 bar to significantly reduce the extent of hydrogenation of unsaturated sites in linear alkyl chains whilst still favouring the formation of hydrocarbon compounds over functionalised species.

Some important molecular structural information, needed for evaluating and exploiting the potential of

lipids as source-specific biomarkers and as geochemical indicators, is undoubtedly lost during HyPy treatment. We are not proposing that our HyPy method will ever replace the rigorous and time-consuming direct analyses of intact lipids in cultured organisms by traditional chemical and spectroscopic methods on components of solvent extracts. Instead, we see the method serving predominantly as a screening tool for identifying whether a particular biomarker structure is a source-specific molecular marker or whether it has a wider taxonomic distribution. The identification of an unexpected biomarker hydrocarbon in the hydropyrolysate of a particular species can signal the presence of novel precursors requiring structure elucidation.

The HyPy technique has already been demonstrated to be a highly effective analytical pyrolysis method for fragmenting recent and ancient geopolymers, including kerogen, and releasing hydrocarbons with excellent preservation of structural and stereochemical features (e.g., Love et al., 1995–1998; Bishop et al., 1998; Murray et al., 1998; Farrimond et al., 2003). We have found that biomarker profiles generated from HyPy of recent and ancient geopolymers are not affected by the fine-tuning in methodology required here to generate optimum biomarker signals from biomass. This is because the bound biomarker lipid species within geopolymers are linked within a stable macromolecular network that does not soften and rearrange like biomass when heated. Our improvements to the HyPy procedure and the generation of hydrocarbons directly from biomass facilitates comparison of extant and fossil lipids within the same analytical window.

4. Conclusions

The application of catalytic hydropyrolysis to generate characteristic hydrocarbon profiles directly from biomass is a novel approach. The modified HyPy procedure allowed us to rapidly detect the presence of lipid carbon skeletons with good retention of structural and stereochemical features. The technique was particularly effective at revealing carbon number patterns of linear, branched and polycyclic aliphatic lipids in different microbial species as well as for identifying and characterising any resistant aliphatic biopolymer constituents. The catalytic HyPy method can serve as a useful screening tool for identifying whether a particular biomarker structure has the potential to be used as a source-specific molecular marker or whether it has a wider taxonomic distribution. Unequivocal assignment of the biological origin of lipid compounds is of fundamental importance for improving our overall understanding of biomarker records in the natural environment and geological record.

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