Organic Geochemistry 81 (2015) 64–69

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

Investigation of the presence of an aliphatic biopolymer in cyanobacteria: Implications for kerogen formation

Patrick Biller [a,*], Andrew B. Ross [a], Stephen C. Skill [b,c]

[a] Energy Research Institute, School of Chemical and Process Engineering, University of Leeds, Leeds LS2 9JT, UK
[b] Centre for Sustainable Aquatic Research, Swansea University, Swansea SA2 8PP, UK
[c] Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, UK

ARTICLE INFO

Article history:
Received 8 October 2014
Received in revised form 21 January 2015
Accepted 31 January 2015
Available online 10 February 2015

Keywords:
Algaenan
Microalgae
Kerogen
Cyanobacteria
Chlorogloeopsis

ABSTRACT

Algaenan has been suggested to be one of the main precursors of certain kerogens. It is a non-hydrolysable and insoluble biomolecule of high molecular weight. It has been found in a limited number of microalgae species. There is considerable uncertainty about its formation and preservation, as well as its role in kerogen formation and the implications for the global C cycle. We tested whether the cyanobacterium *Chlorogloeopsis fritschii* can synthesise a biomacromolecule similar to algaenan with potential to contribute to kerogen via selective preservation. Two freshwater green microalgae, *Pseudochoricystis ellipsoidea* and *Scenedesmus obliquus*, as well as *C. fritschii*, were subjected to harsh solvent extraction and hydrolysis steps to obtain an insoluble and non-hydrolysable macromolecule. The residues from all three species were analysed using pyrolysis–gas chromatography–mass spectrometry and solid-state nuclear magnetic resonance spectroscopy. The analysis revealed that *C. fritschii* indeed contains a resistant biomacromolecule exhibiting the characteristic aliphatic structure of algaenan, similar to the algaenan residues from the two microalgae. Due to the robust nature of *Chlorogloeopsis* compared with eukaryotes, it can prevail in extreme environmental conditions such as freezing, thawing, desiccation and overheating – conditions prevalent on the primeval earth. The presence of a resistant aliphatic biopolymer in *Chlorogloeopsis* suggests that cyanobacteria could have contributed to kerogen via selective preservation.

1. Introduction

Kerogen is commonly defined as the insoluble macromolecular organic matter (OM) in sedimentary rocks (Tegelaar et al., 1989;Vandenbroucke and Largeau, 2007). Until the 1980s it was thought it was formed by random condensation and polymerisation of sugars, lipids and proteins which had been broken down by way of bacterial enzymatic activity (Tissot and Welte, 1984; Tegelaar et al., 1989; Gelin et al., 1997). Today the "selective preservation" hypothesis is also accepted, i.e. resistant biopolymers such as algaenan are selectively preserved over time due to their high resistance to microbial and chemical degradation and therefore contribute to some kerogen fractions of source rock deposits (Allard et al., 2002). Although the levels of resistant biomacromolecules represent a low amount of total biomass, their high preservation potential results in preservation and enrichment during sedimentation and catagenesis, while other biomass constituents such as polysaccharides and proteins are biodegraded. Various resistant biopolymers are found in organisms, of which algaenan from microalgae has a particularly high preservation potential (Tegelaar et al., 1989).

Algaenan is a non-hydrolysable, insoluble, resistant biomacromolecule found in some species of microalgae. Reports have demonstrated its presence in freshwater green microalgae in the Trebouxiophyceae and Chlorophyceae (Versteegh and Blokker, 2004). There are other resistant biomacromolecules found in spores and higher plants and known as sporopollenin, cutin and cutan, respectively (de Leeuw et al., 2006). However, sporopollenin contains oxygenated aromatic building blocks, while cutin is a high molecular weight biopolyester composed of various interesterified hydroxyalkanoic acids and cutan is a non-hydrolysable, polymethylenic biopolymer, distinguishing them from algaenan (Tegelaar et al., 1991; Deshmukh et al., 2005). Two pathways can lead to resistant plant macromolecules: the acetate–malonate pathway, which proceeds via lipid synthesis to algaenan from algae and cutin from plants, and the phenylpropanoid pathway which leads to sporopollenin (Versteegh and Blokker, 2004). Type 1 algal

* Corresponding author.
E-mail address: p.biller@leeds.ac.uk (P. Biller).

http://dx.doi.org/10.1016/j.orggeochem.2015.01.010
0146-6380/

kerogens in particular, characterised by high H/C and low O/C ratios, are suggested to be derived from algaenan preservation (Gelin et al., 1997). *Botryococcus braunii* has been identified as a major contributor to this type of kerogen through the preservation of its algaenan, as shown from pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) and identification of acyclic isoprenoid biomarkers (Volkman, 2014; Zhang et al., 2014).

There are no reports of the presence of algaenan in cyanobacteria and only limited reports on the occurrence of resistant biopolymers in cyanobacteria such as a *Schizothrix* sp. (Chalansonnet et al., 1988). However, Allard et al. (1997) suggested that the resistant biopolymers found by Chalansonnet et al. (1988) were melanoidin-like artefacts produced by the isolation procedure used. In our study, we tested for the occurrence of a resistant biopolymer similar to algaenan in the cyanobacterium *Chlorogloeopsis fritschii* and discuss how this could have contributed to the kerogen fraction of source rock deposits.

There is some uncertainty about the importance of the "selective preservation" hypothesis for kerogen formation; this stems mainly from the relatively small number of microalgal species which have been demonstrated to be algaenan producers. Additionally, the majority of these species are freshwater species, thereby introducing some uncertainty with respect to marine kerogen. Approximately 17 species from the Trebouxiophyceae and seven from the Chlorophyceae were listed by Kodner et al. (2009) to contain algaenan. The selective preservation theory and other kerogen formation pathways are of continued debate (de Leeuw, 2007; Gupta et al., 2007). The oxidative polymerisation of low molecular weight lipids is another pathway explaining the presence of the aliphatic content in kerogens (de Leeuw et al., 2006). Gupta et al. (2014) have argued in favour of the formation of geomacromolecules from lipids also contributing to the aliphatic content of kerogens due to algaenan not being as widespread ecologically and phylogenetically. In their experimental studies they obtained macromolecules with aliphatic characteristics from the high T/P pyrolysis of lipids from algae and cyanobacteria that lacked algaenan. They therefore raised the question if bacterial biomass may also have contributed to the insoluble organic fraction of ancient sediments (Gupta et al., 2014). Similarly, we would suggest that, if resistant aliphatic biopolymers are present in cyanobacteria, they could have contributed to kerogen by selective preservation.

Microfossils of the marine microorganism *Gloeocapsomorpha prisca* have been observed as a major contributor to the kerogen of Ordovician sediments (Blokker et al., 2001). Its taxonomic position and biological affinity have been debated and assigned mainly to the cyanobacteria and Chlorophyta, notably *Botryococcus braunii* as this chlorophyte produces structures morphologically similar to *G. prisca* under salt stress (Blokker et al., 2001). However, Versteegh and Blokker (2004) argue that the lack of evidence for *B. Braunii* containing the shorter chain lengths of the *n*-alkyl resorcinol building blocks, indicates it should not be attributed to *G. prisca*. Therefore, a third pathway could exist for the formation and preservation of resistant biomacromolecules (Versteegh and Blokker, 2004). Blokker et al. (2001) state that it is generally accepted that ancient microfossils are often composed of resistant biopolymers which have been selectively preserved over time; cyanobacteria could therefore be a possible source organism for microfossils such as *G. prisca*, if indeed the presence of resistant biopolymers could be shown in cyanobacteria. Foster et al. (1990) suggest *G. prisca* originates from an intertidal, marine, mat-forming benthic cyanobacterium similar to the extant cyanobacterium, *Entophysalis major*. Such properties have recently been described for *Chlorogloeopsis* (Pepe-Ranney et al., 2012).

The presence of algaenan has been suggested to have the function of protecting the alga by strengthening the cell wall and waterproofing the cell, protecting it from dehydration and attack by bacteria (Versteegh and Blokker, 2004). The main microalgae species shown to contain algaenan are *B. braunii*, *Scenedesmus* spp. and *Chlorella* spp. It has been argued that *B. braunii* is one of the main contributors of algaenan to kerogen formation (Audino et al., 2002; Volkman, 2014). Research has demonstrated that the cyanobacterium *Chlorogloeopsis* produces a high yield of oil when processed under hydrothermal liquefaction (Biller et al., 2012). This was not expected from an organism with such a low lipid content (ca. 5%), based on hydrothermal liquefaction studies with model compounds (Biller and Ross, 2011). Algaenan has been shown to contribute to oil formation during hydrothermal liquefaction (Torri et al., 2012). This led to the hypothesis that *Chlorogloeopsis* may contain an algaenan-like, resistant biomacromolecule.

Cyanobacterial cell walls differ from those of eukaryotic microalgae. They are characterised as either gram positive or gram negative using the crystal violet Gram staining method (Hoiczyk and Hansel, 2000). *Chlorogloeopsis* is gram negative, indicating a strong cell wall (Schrader et al., 1982). Its cell walls are made up of an inner, middle and outer layer surrounded by an additional sheath layer outside the cell wall (Peat and Whitton, 1967). The cell walls of gram negative bacteria generally contain an outer membrane and a thin peptidoglycan layer sandwiched between the periplasm and the outer membrane (Beveridge, 1999). Cyanobacterial cell walls have not been shown to contain algaenan and it is unlikely the same biosynthetic pathway for algaenan formation from microalgae also occurs in cyanobacteria. Nevertheless it might be possible that a similar resistant biopolymer to algaenan could be formed by some cyanobacteria via a different pathway. Cyanobacteria played a vital role in evolution to develop a photosynthetic system allowing the evolution of eukaryotic algae and plants and cyanobacteria were already widespread at the beginning of the Proterozoic (2.5×10^9 yr ago). Cyanobacterial matter contributed significantly to stromatolites and amorphous kerogens of supposed cyanobacterial origin (Chalansonnet et al., 1988). If resistant biopolymers exist in cyanobacteria, these could have contributed to these amorphous kerogens via selective preservation. *Chlorogloeopsis* has been shown to be an important contributor to the OM in living stromatolites growing in hot springs in the Yellowstone National Park (Pepe-Ranney et al., 2012). These stromatolites resemble ancient laminated forms, suggesting that *Chlorogloeopsis* could have contributed to ancient stromatolites and amorphous kerogen.

Chlorogloeopsis was originally isolated from soil particles in India (Mitra, 1950; Mitra and Pandey, 1966) and has since been found in a wide range of habitats around the world, including thermal springs (Hindák, 2008). It can differentiate into a broad range of morphological cell types in response to environmental stress, including heterocysts in fixed N depleted environments (Evans et al., 1976). It can survive a wide range of salt concentration (Hindák, 2008) and remains viable following either desiccation or repeated cycles of freeze thawing. All these properties represent a diverse suite of defence mechanisms and survival strategies that would enable *Chlorogloeopsis* to endure constantly fluctuating environmental extremes thought to have prevailed $2–3.4 \times 10^6$ yr ago.

To investigate the hypothesis that *C. fritschii*, a *cyanobacterium*, contains an aliphatic biopolymer similar to algaenan, it and two microalgae, *Pseudochoricystis ellipsoidea* and *Scenedesmus obliquus*, were investigated for the presence of insoluble, non-hydrolysable residues, which were compared using a range of analytical methods.

2. Material and methods

2.1. Algaenan isolation

S. obliquus (Chlorophyceae) was obtained from the University of Bath, UK and grown outdoors in a raceway bond. *P. ellipsoidea* (Trebouxiophyceae) was sourced from the Denso Corporation (Japan)

and grown in photobioreactors; *C. fritschii* (1411–1a) was obtained from the culture collection of algae at the University of Göttingen (SAG) and cultivated in a 16,000 l tubular photobioreactor (Skill, 2010) in BG11 growth medium at 45–55 °C utilising hot flue gases (90–100 °C) derived from a combined cycle gas power station as carbon source (3.5% CO_2). *Chlorogloeopsis* biomass was harvested by auto-flocculation and passive settling to 14 wt% solids (Balasundaram et al., 2012). The biomass 'cake' was frozen and stored at −20 °C.

Algaenan was isolated using the method described by Allard et al. (1998) and adapted by Salmon et al. (2009). The effect of different isolation procedures was recently compared by Obeid et al. (2014). The procedure developed by Allard et al. (1998) was chosen as it avoids the formation of melanoidin-like polymer artefacts. About 15 g freeze dried material were solvent extracted in 150 ml $CHCl_3$/CH_3OH (2:1% v/v) in an ultrasonic water bath at 40 °C for 60 min. After centrifuging, the supernatant was recovered and the solvent removed by evaporation. The precipitate was subjected (2×) to extraction with 150 ml hexane under the same conditions. The residue was water washed (2×) and hydrolysed (2×) with 3 M trifluoroacetic acid (TFA) for 3 h under reflux at 100 °C and water washed between and after each hydrolysis step. A 4 M TFA hydrolysis step was also performed for 18 h, with subsequent water washing, before a final 6 M TFA 18 h hydrolysis step followed by water washing. Saponification was then performed on the residue with KOH (5 wt%) in CH_3OH/H_2O (88/12% v/v) at 80 °C for 1 h. The resulting residue was washed with distilled water (3×) before lyophilisation.

2.2. Py–GC–MS

Samples of freeze-dried residue and microalgae were analysed using Py–GC–MS with a CDS 5000 series pyrolyser (CDS Analytical Inc., Oxford, PA, USA) connected to a Shimadzu 2010 GC–MS instrument (Shimadzu Corporation, Kyoto, Japan). Approximately 0.2 mg of weighed sample were added to a pre-weighed quartz tube (25 mm × 0.5 mm i.d.) with quartz wool at one end. The other end of the tube was then filled with a small quartz wool plug to keep the sample in place. The tube was reweighed before pyrolysis. Pyrolysis was performed at a 600 °C with a ramp rate of 20 °C/ms with a hold time of 20 s. The products were trapped on a TENAX adsorbent trap before being desorbed at 300 °C onto a heated transfer line held at 300 °C. A purge flow (20 ml/min) of He was used to remove O_2 from the sample prior to pyrolysis. The heated transfer line (280 °C) was connected to a split/splitless injector (split ratio 20:1) in the GC inlet port (280 °C). The pyrolysis products were separated on an Rtx 1701 column (60 m × 0.25 mm i.d., 0.25 μm film thickness) using a temperature programme of 40 °C (2 min) to 280 °C (held 15 min) at 6 °C/min and a constant column head pressure of 2.07 bar. After pyrolysis the tube was reweighed to determine the amount of sample pyrolysed. This allowed calculation of normalised peaks as an area per mg sample pyrolysed. The ion source was at 230 °C and the interface at 280 °C, with a scan rate of 1 s over a range of m/z 50–550. Peaks were assigned using the NIST mass spectral database.

2.3. Solid state NMR

Samples of residue and algae were also analysed at the EPSRC solid state NMR facility at the University of Durham, UK. Solid-state ^{13}C spectra were recorded at 100.56 MHz using a Varian VNMRS spectrometer and a 4 mm (rotor o.d.) magic angle spinning probe. They were obtained using cross polarisation with a 2 s recycle delay, 3 ms contact time, at ambient probe temperature (ca. 25 °C) and at a sample spin rate of 10 kHz. Between 1000 and 1600 scans were accumulated. Spectral referencing was with respect to an external sample of tetramethylsilane (carried out by setting the high frequency signal from adamantane to 38.5 ppm).

2.4. Biochemical composition of microalgae

The composition of the microalgae strains was determined using traditional techniques; for protein analysis, a modified Lowry method was used (Waterborge, 2002). Total carbohydrate content was determined via a sulfuric acid hydrolysis method (Gerchakov and Hatcher, 1972). Lipid extraction was performed using the Folch method, employing 2:1 $CH_3OH/CHCl_3$ extraction at 35 °C, using an ultrasonic bath for 45 min in duplicate. The microalgae samples were analysed using thermo gravimetric analysis (TGA) for ash and moisture content with a TA Instruments IR5000Q TGA in air. The TGA oven temperature was increased to 105 °C (held 15 min), subsequently ramped to 550 °C (held 80 min) to determine the moisture and ash content respectively.

3. Results and discussion

3.1. Algaenan isolation

All three species were investigated for their composition; ash and moisture contents were determined using thermogravimetric analysis. The algaenan content was determined by successive lipid extractions, water washing and hydrolysis steps as described in Section 2.1. The final residue after purification was dried and weighed, with the values expressed as a mass fraction of the original material. The residue fraction and its composition are presented in Table 1. The residue content varied from 0.7 wt% for *Pseudochoricystis*, to 2.7 wt% for *Chlorogloeopsis* and 5.5 wt% for *Scenedesmus*. The amount for *Pseudochoricystis* and *Chlorogloeopsis* is similar to the algaenan level (1–2%) reported for *Nannochloropsis* spp. and *Chlorella spaerckii* (Gelin et al., 1997). The amount for *Scenedesmus* (5.5 wt%) is higher than the level (2.6%) reported for *Scenedesmus communis* (CCAP 276/4b; Allard et al., 1998), but similar to the amount (4%) reported by Blokker et al. (1998). For *B. braunii*, Allard et al. (1998) report an algaenan content as high as 11 wt%, so the levels of algaenan here broadly agree with previous studies (Blokker et al., 1998). Due to the harsh and repeated hydrolysis conditions employed during purification of the residue, the results do indeed reveal that there is a non-hydrolysable organic biopolymer in the cyanobacterium. This is the first time such a non-hydrolysable, insoluble biomacromolecule has been reported in a cyanobacterium. *Scenedesmus* has been shown to produce algaenan, so the likelihood was high that our strain indeed contained algaenan (Kodner et al., 2009).

Pseudochoricystis has not been investigated previously but as a green alga from the Trebouxiophyceae, where algaenan is most prevalent, it is likely that the residue was algaenan. As cyanobacteria have not previously been shown to contain algaenan or similar resistant biopolymer, further analysis was required to determine if the insoluble, non-hydrolysable residue was indeed a biopolymer with similar structure to algaenan. This is addressed below.

3.2. Py–GC–MS

The residues were analysed using Py–GC–MS at 600 °C and the product chromatograms are plotted in Fig. 1a–c. Fig. 1a shows the residue from *Pseudochoricystis*; among the 50 largest peaks assigned using the NIST MS database, only one non-hydrocarbon could be assigned. The hydrocarbons consist of alkane/alkene pairs from C_8–C_{29}, denoted as triangles and circles respectively. The notable non-hydrocarbon, 3,7,11-trimethyldodecanol (diamond in

Table 1
Biochemical analysis and residue weight fraction from algaenan isolation method.

	Ash	Moisture	Protein	Carbohydrate	Lipid	Residue
	Wt%		Wt% (dry ash free)			
Pseudochoricystis	0.7	2.5	20.0	35.6	34.3	0.7
Chlorogloeopsis	4.6	5	41.8	37.8	8.2	2.7
Scenedesmus	7.1	5	36.1	28.3	17.0	5.5

Fig. 1), is the only oxygenated species, possibly originating from the cleavage of algaenan ester bonds. It has been shown by Obeid et al. (2014) that pyrolysates of algaenan can include fatty acids from cleavage of algaenan ester bonds but we did not find any. The most abundant alkane/alkene pair was C_{27}. The overall profile of pyrolysate hydrocarbons is very similar to published Py–GC chromatograms of algaenan from microalgae (Gelin et al., 1997; Kodner et al., 2009). Fig. 1b depicts the pyrolysis chromatogram from the *Chlorogloeopsis* residue, with some distinct differences; the chromatogram shows a toluene peak at 7.5 min as well as five pyrrole peaks, together 6.5% of the entire chromatogram area. These could potentially represent impurities in the residue from protein derived material. We have previously shown that these are typical pyrolysates from protein (Biller and Ross, 2014). Since there are no N containing peaks in the other pyrograms, which were derived using the exact same isolation procedure, it is unlikely that the *Chlorogloeopsis* residue still contained protein impurities. Therefore, the differences between the pyrograms for the two microalgae and the cyanobacterium are probably due to the different structural composition of the isolated residue.

The *Chlorogloeopsis* chromatogram also contains four alcohol peaks, including 3,7,11-trimethyldodecanol, also found for *Pseudochoricystis*. The alcohols (inverted triangles) are not attributed to impurities in the residue, as algaenan for example also contains

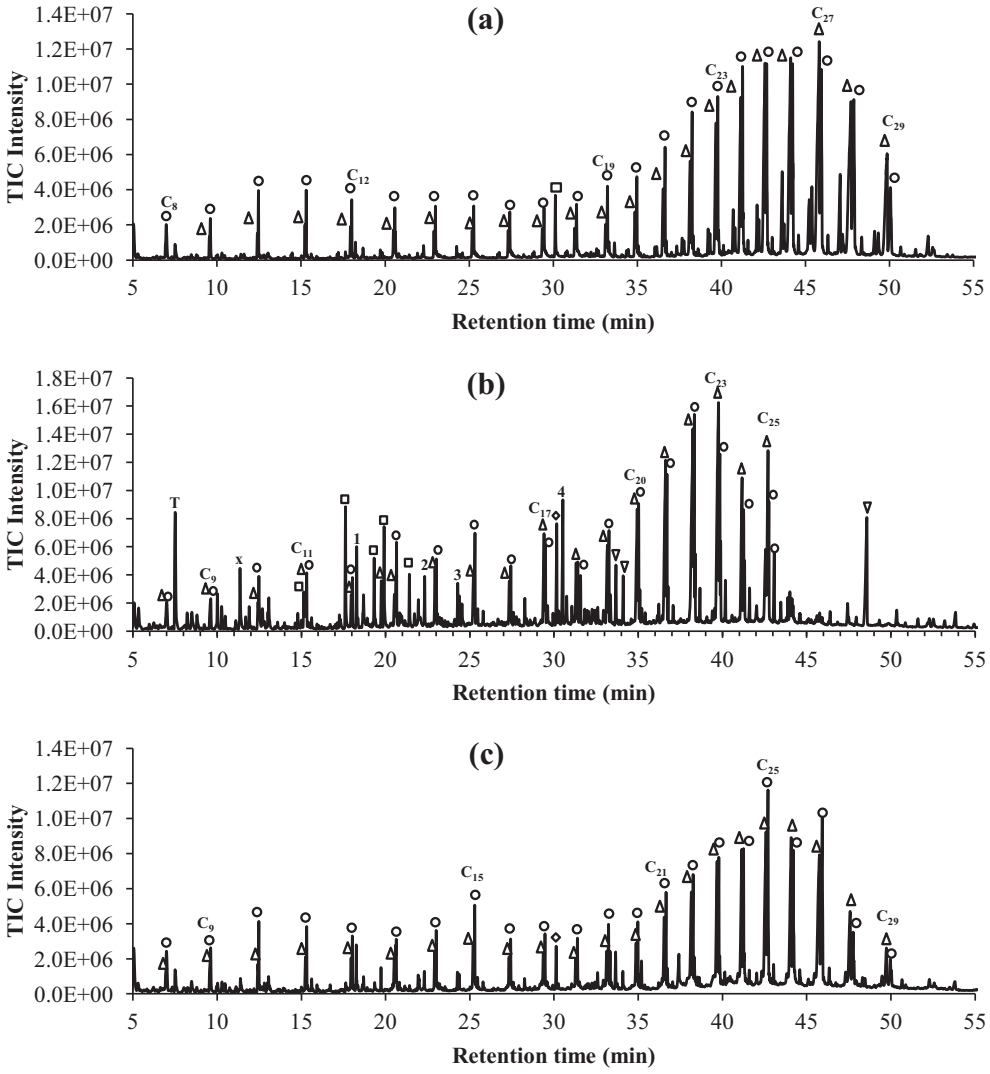

Fig. 1. Total ion Py–GC–MS chromatograms of (a) *Pseudochoricystis*, (b) *Chlorogloeopsis* and (c) *Scenedesmus* at 600 °C. Triangles, *n*-alkanes; circles, *n*-alkenes; diamond, 3,7,11-trimethyl dodecanol; T, toluene; x, styrene; 1, 2,6-dimethylundecane; 2, 2,6,10,15-tetramethylhexadecane; 3, 2,6,10,15-tetramethylheptadecane; 4,4-methylpentadecane; inverted triangles, alcohols.

oxygen, so these peaks are likely pyrolysis products of the aliphatic macromolecule. Nonetheless, the majority of compounds from *Chlorogloeopsis* are alkane/alkene pairs, together 87.5% of the chromatogram peak areas. The most abundant hydrocarbons were in the range C_{21}–C_{25}, shorter than from *Pseudochoricystis*. The differences between *Pseudochoricystis* and *Chlorogloeopsis* are most likely due to the inherently different structure of the isolated aliphatic biopolymer.

Fig. 1c shows the pyrolysis products from the *Scenedesmus* residue. It can be seen that the chromatogram is very similar to *Pseudochoricystis*, with some slight shifts to different chain length hydrocarbons. The most abundant alkane/alkene pair is at C_{25}. All three samples had a similar concentration of C_8–C_{14} hydrocarbons. However, the *Chlorogloeopsis* pyrogram reveals some additional branched alkanes, assigned as 2,6-dimethylundecane, 2,6,10,15-tetramethylhexadecane, 2,6,10,15-tetramethylheptadecane and 4-methylpentadecane, indicated with the numbers 1–4 in Fig. 1b. The exact position of methyls are difficult to determine via MS, so their actual positions could differ. The longest alkane/alkene pair chain length for all three samples is C_{29}, the most abundant being C_{27} for *Pseudochoricystis*, and C_{23} and C_{25} for *Chlorogloeopsis* and *Scenedesmus* respectively. While *Pseudochoricystis* and *Scenedesmus* show a gradual decrease in the most abundant chain lengths to the longest at C_{29}, *Chlorogloeopsis* appears to lack the C_{27} and C_{28} alkane/alkene pairs. Other reports have shown slightly different chromatograms for algaenan, especially concerning maximum chain length and most abundant chain length (Gelin et al., 1997; Kodner et al., 2009). Kodner et al. (2009) show an example of pyrolysis products from algaenan of four *Scenedesmus* species, where the dominant chain length varied but the characteristic aliphatic nature was apparent.

Overall, the microalgae pyrolysis chromatograms show clear evidence for the presence of algaenan, based on comparison with the literature, due to the presence of the characteristic alkane/alkane peaks, similar to those observed in the products from pyrolysis of kerogen (Horsfield, 1989). The pyrolysate of the *Chlorogloeopsis* residue reveals a very similar structure, with a slight shift to shorter chain length hydrocarbons and additional compounds. Nevertheless, it can be concluded from the results that the cyanobacterium contains an aliphatic biopolymer very similar to algaenan and which could therefore have contributed to the aliphatic content in kerogen via the selective preservation pathway.

3.3. Solid state ^{13}C NMR

The spectra of the residues are plotted in Fig. 2a–c. The initial biomass shows large and broad shifts in the areas around 30 ppm, 75 ppm, 105 ppm and 174 ppm. The shifts at 74 and 105 ppm are attributed to the carbohydrate fraction of the biomass (Zang et al., 2001). Carboxyl and amide carbons, which encompass the protein fraction, are represented by the chemical shift at 174 ppm. Paraffinic carbon shifts are those in the region of 30 ppm and olefinic carbons at 129 ppm (Zelibor et al., 1988).

All three initial biomass samples are essentially quite similar (Fig. 2a–c), with variation in the main peak abundances due to the different biochemical composition presented in Table 1. The dominant shift is in the paraffinic carbon area at 30 ppm. Two distinct peaks are recognisable at 30 and 33 ppm, which represent CH_2– and –CH-moieties, respectively (Salmon et al., 2009). These are therefore attributed to the aliphatic nature of algaenan, representing the alkane/-ene pairs observed from Py–GC–MS analysis. The *Pseudochoricystis* algaenan spectrum (Fig. 2a), shows that the residue is almost pure, with only a very small peak in the carbohydrate region (74 ppm). This spectrum is in agreement with published spectra of algaenan from other microalgae (Derenne et al., 1991; Gelin et al., 1996; Blokker et al., 1998). The spectrum of the *Chlorogloeopsis* residue (Fig. 2b) does not appear to be as pure as the *Pseudochoricystis* residue. There are peaks in the 174 ppm and 129 ppm regions and shoulders in the paraffinic carbon range around 30 ppm. This supports the observation from Py–GC–MS analysis that the residue is different from the *Pseudochoricystis* sample or that there are impurities present. The *Scenedesmus* sample exhibits less of a shoulder in the paraffinic carbon range and only has very small peaks around 75, 105 and 129 ppm. The algaenan residues from *Pseudochoricystis* and *Scenedesmus* are virtually identical to the algaenan NMR data published previously and the *Chlorogloeopsis* sample shows the dominant characteristics of an algaenan spectrum. This leads to the conclusion that the two microalgae residue samples are algaenan, while the isolation procedure from *Chlorogloeopsis* led to an aliphatic residue very similar to algaenan. We believe the data demonstrate to a reasonable level of certainty that the cyanobacterium *Chlorogloeopsis* indeed contains an aliphatic biopolymer similar to algaenan which could have contributed to source rock deposits and kerogen.

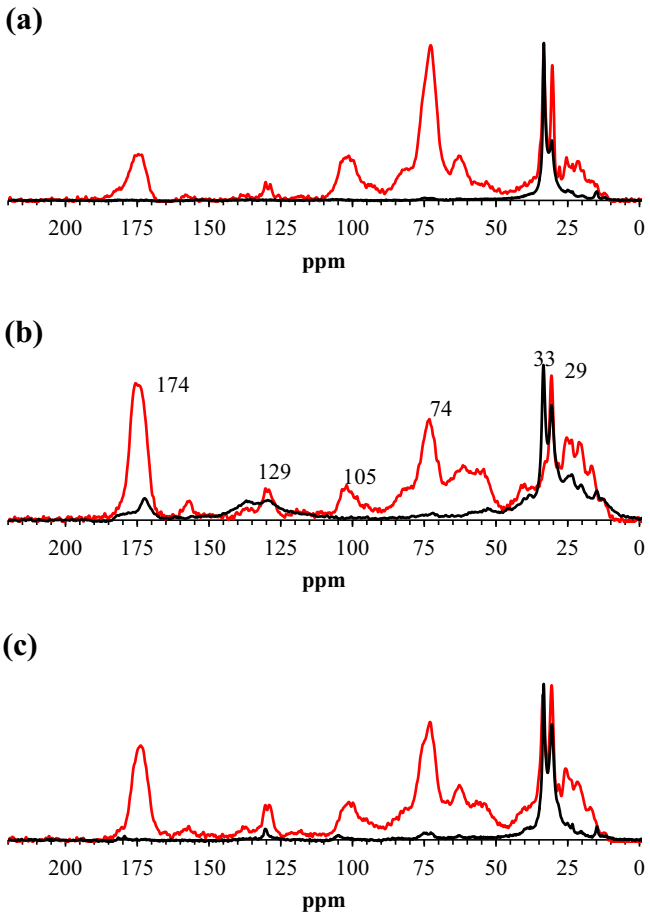

Fig. 2. ^{13}C NMR spectra of (a) *Pseudochoricystis*, (b) *Chlorogloeopsis* and (c) *Scenedesmus*. Red lines, initial biomass, black lines, aliphatic biopolymer (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

4. Conclusions

The methodology employed to isolate algaenan, widely regarded as the most conclusive proof of the presence of algaenan in microalgae, allowed positive identification of algaenan in both microalgae species and the presence of a resistant biopolymer in the cyanobacterium *Chlorogloeopsis*. Py–GC–MS confirmed its char-

acteristic aliphatic nature. Diagnostic alkane/alkene pairs were found in the residues of all three species; dominant carbon chain length varied but is consistent with literature data. Py–GC–MS also revealed that the *Chlorogloeopsis* residue contained additional functional groups compared with algaenan. However, the chromatogram and the NMR data emphasised the highly aliphatic nature of the *Chlorogloeopsis* residue. A combination of the analyses of the residues and the isolation procedure prove that *Chlorogloeopsis* contains a resistant aliphatic biomacromolecule with high similarity to the algaenan. Its presence suggests that cyanobacteria like *Chlorogloeopsis* could have contributed to the aliphatic content of kerogens via selective preservation.

Acknowledgements

Solid-state NMR spectra were obtained at the EPSRC UK National Solid-state NMR Service at Durham. The authors would like to thank P. Mozzanega of the University of Bath for providing *Scenedesmus*. Gratitude is extended to the Denso Corporation (Japan) for provision of *Pseudochoricystis* samples. The authors would like to thank the EPSRC (EP/I014365/1), the Technology Strategy Board (CRD200094), ERDF INTERREG IVB (EnAlgae) for financial support and the Doctoral Prize Fellowship for P.B. (EP/L504993/1). Finally, we would like to extend our gratitude to N.S. Gupta and E. Salmon for constructive comments.

Associate Editor—**J.K. Volkman**

References

Allard, B., Rager, M.-N., Templier, J., 2002. Occurrence of high molecular weight lipids (C_{80}+) in the trilaminar outer cell walls of some freshwater microalgae. A reappraisal of algaenan structure. Organic Geochemistry 33, 789–801.

Allard, B., Templier, J., Largeau, C., 1997. Artifactual origin of mycobacterial bacteran. Formation of melanoidin-like artifact macromolecular material during the usual isolation process. Organic Geochemistry 26, 691–703.

Allard, B., Templier, J., Largeau, C., 1998. An improved method for the isolation of artifact-free algaenans from microalgae. Organic Geochemistry 28, 543–548.

Audino, M., Grice, K., Alexander, R., Kagi, R.I., 2002. Macrocyclic alkanes in crude oils from the algaenan of *Botryococcus braunii*. Organic Geochemistry 33, 979–984.

Balasundaram, B., Skill, S.C., Llewellyn, C.A., 2012. A low energy process for the recovery of bioproducts from cyanobacteria using a ball mill. Biochemical Engineering Journal 69, 48–56.

Beveridge, T.J., 1999. Structures of gram-negative cell walls and their derived membrane vesicles. Journal of Bacteriology 181, 4725–4733.

Biller, P., Ross, A.B., 2011. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. Bioresource Technology 102, 215–225.

Biller, P., Ross, A.B., 2014. Pyrolysis–GC–MS as a novel analysis technique to determine the biochemical composition of microalgae. Algal Research 6, 91–97.

Biller, P., Ross, A.B., Skill, S.C., Lea-Langton, A., Balasundaram, B., Hall, C., Riley, R., Llewellyn, C.A., 2012. Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process. Algal Research 1, 70–76.

Blokker, P., Schouten, S., van den Ende, H., de Leeuw, J.W., Hatcher, P.G., Sinninghe Damsté, J.S., 1998. Chemical structure of algaenans from the fresh water algae *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum*. Organic Geochemistry 29, 1453–1468.

Blokker, P., van Bergen, P., Pancost, R., Collinson, M.E., de Leeuw, J.W., Sinninghe Damsté, J.S., 2001. The chemical structure of *Gloeocapsomorpha prisca* microfossils: implications for their origin. Geochimica et Cosmochimica Acta 65, 885–900.

Chalansonnet, S., Largeau, C., Casadevall, E., Berkaloff, C., Peniguel, G., Couderc, R., 1988. Cyanobacterial resistant biopolymers. Geochemical implications of the properties of *Schizothrix* sp. resistant material. Organic Geochemistry 13, 1003–1010.

de Leeuw, J.W., 2007. On the origin of sedimentary aliphatic macromolecules: a comment on recent publications by Gupta. Organic Geochemistry 38, 1585–1587.

de Leeuw, J., Versteegh, G.M., van Bergen, P., 2006. Biomacromolecules of algae and plants and their fossil analogues. In: Rozema, J., Aerts, R., Cornelissen, H. (Eds.), Plants and Climate Change. Springer, Netherlands, pp. 209–233.

Derenne, S., Largeau, C., Casadevall, E., Berkaloff, C., Rousseau, B., 1991. Chemical evidence of kerogen formation in source rocks and oil shales via selective preservation of thin resistant outer walls of microalgae: origin of ultralaminae. Geochimica et Cosmochimica Acta 55, 1041–1050.

Deshmukh, A.P., Simpson, A.J., Hadad, C.M., Hatcher, P.G., 2005. Insights into the structure of cutin and cutan from *Agave americana* leaf cuticle using HRMAS NMR spectroscopy. Organic Geochemistry 36, 1072–1085.

Evans, E.H., Foulds, I., Carr, N., 1976. Environmental conditions and morphological variation in the blue-green alga *Chlorogloea fritschii*. Journal of General Microbiology 92, 147–155.

Foster, C.B., Wicander, R., Reed, J.D., 1990. *Gloeocapsomorpha prisca* Zalessky, 1917: a new study part II: Origin of Kukersite, a new interpretation. Geobios 23, 133–140.

Gelin, F., Boogers, I., Noordeloos, A.A.M., Sinninghe Damsté, J.S., Hatcher, P.G., de Leeuw, J.W., 1996. Novel, resistant microalgal polyethers: an important sink of organic carbon in the marine environment? Geochimica et Cosmochimica Acta 60, 1275–1280.

Gelin, F., Boogers, I., Noordeloos, A.A.M., Sinninghe Damsté, J.S., Riegman, R., de Leeuw, J.W., 1997. Resistant biomacromolecules in marine microalgae of the classes Eustigmatophyceae and Chlorophyceae: geochemical implications. Organic Geochemistry 26, 659–675.

Gerchakov, S.M., Hatcher, P.G., 1972. Improved technique for analysis of carbohydrates in sediments. Limnology and Oceanography 17, 938–943.

Gupta, N.S., Briggs, D.E., Collinson, M.E., Evershed, R.P., Michels, R., Pancost, R.D., 2007. De Leeuw comment "On the origin of sedimentary aliphatic macromolecules". Organic Geochemistry 38, 1588–1591.

Gupta, N.S., Steele, A., Fogel, M., Griffin, P., Adams, M., Summons, R.E., Yang, H., Cody, G.D., 2014. Experimental formation of geomacromolecules from microbial lipids. Organic Geochemistry 67, 35–40.

Hindák, F., 2008. On *Chlorogloeopsis fritschii* (Cyanophyta/Cyanobacteria) from thermal springs in Slovakia and from a saline lake in Tunisia. Algological Studies 126, 47–64.

Hoiczyk, E., Hansel, A., 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. Journal of Bacteriology 182, 1191–1199.

Horsfield, B., 1989. Practical criteria for classifying kerogens: some observations from pyrolysis–gas chromatography. Geochimica et Cosmochimica Acta 53, 891–901.

Kodner, R.B., Summons, R.E., Knoll, A.H., 2009. Phylogenetic investigation of the aliphatic, non-hydrolyzable biopolymer algaenan, with a focus on green algae. Organic Geochemistry 40, 854–862.

Mitra, A., 1950. Two new algae from Indian soils. Annals of Botany 14, 457–464.

Mitra, A.T., Pandey, D., 1966. On a new genus of the blue-green alga *Chlorogloeopsis* with remarks on the production of heterocysts in the alga. Phykos 5, 106–114.

Obeid, W., Salmon, E., Hatcher, P.G., 2014. The effect of different isolation procedures on algaenan molecular structure in *Scenedesmus* green algae. Organic Geochemistry 76, 259–269.

Peat, A., Whitton, B., 1967. Environmental effects on the structure of the blue-green alga, *Chlorogloea fritschii*. Archiv für Mikrobiologie 57, 155–180.

Pepe-Ranney, C., Berelson, W.M., Corsetti, F.A., Treants, M., Spear, J.R., 2012. Cyanobacterial construction of hot spring siliceous stromatolites in Yellowstone National Park. Environmental Microbiology 14, 1182–1197.

Schrader, M., Drews, G., Golecki, J.R., Weckesser, J., 1982. Isolation and characterization of the sheath from the cyanobacterium *Chlorogloeopsis* PCC 6912. Journal of General Microbiology 128, 267–272.

Salmon, E., Behar, F., Lorant, F., Hatcher, P.G., Metzger, P., Marquaire, P.-M., 2009. Thermal decomposition processes in algaenan of *Botryococcus braunii* race L. Part 1: experimental data and structural evolution. Organic Geochemistry 40, 400–415.

Skill, S.C., 2010. Apparatus for treatment of fluid streams and method of conducting the same. Great Britain, Patent: WO2010086589 A3.

Tegelaar, E.W., de Leeuw, J.W., Derenne, S., Largeau, C., 1989. A reappraisal of kerogen formation. Geochimica et Cosmochimica Acta 53, 3103–3106.

Tegelaar, E.W., Kerp, H., Visscher, H., Schenck, P.A., de Leeuw, J.W., 1991. Bias of the paleobotanical record as a consequence of variations in the chemical composition of higher vascular plant cuticles. Paleobiology 17, 133–144.

Tissot, B., Welte, D.H., 1984. Petroleum Formation and Occurence, Second ed. Springer, Berlin.

Torri, C., Garcia Alba, L., Samorì, C., Fabbri, D., Brilman, D.W.F., 2012. Hydrothermal treatment (HTT) of microalgae: detailed molecular characterization of HTT oil in view of HTT mechanism elucidation. Energy & Fuels 26, 658–671.

Vandenbroucke, M., Largeau, C., 2007. Kerogen origin, evolution and structure. Organic Geochemistry 38, 719–833.

Versteegh, G.J.M., Blokker, P., 2004. Resistant macromolecules of extant and fossil microalgae. Phycological Research 52, 325–339.

Volkman, J.K., 2014. Acyclic isoprenoid biomarkers and evolution of biosynthetic pathways in green microalgae of the genus *Botryococcus*. Organic Geochemistry 75, 36–47.

Waterborge, J., 2002. The Lowery method for protein quantification. In: Walker, J.M. (Ed.), The Protein ProtocolsHandbook. Human Press, New Jersey.

Zang, X., Nguyen, R.T., Harvey, H.R., Knicker, H., Hatcher, P.G., 2001. Preservation of proteinaceous material during the degradation of the green alga *Botryococcus braunii*: a solid-state 2D ^{15}N ^{13}C NMR spectroscopy study. Geochimica et Cosmochimica Acta 65, 3299–3305.

Zelibor, J., Romankiw, L., Hatcher, P., Colwell, R., 1988. Comparative analysis of the chemical composition of mixed and pure cultures of green algae and their decomposed residues by ^{13}C nuclear magnetic resonance spectroscopy. Applied and Environmental Microbiology 54, 1051–1060.

Zhang, Z., Volkman, J.K., Greenwood, P.F., Hu, W., Qin, J., Borjigin, T., Zhai, C., Liu, W., 2014. Flash pyrolysis of kerogens from algal rich oil shales from the Eocene Huadian Formation, NE China. Organic Geochemistry 76, 167–172.