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Geochemical characterization of the hydrous pyrolysis products from a recent cyanobacteria-dominated microbial mat

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ABSTRACT

Hydrous pyrolysis experiments were performed on a recent microbial mat sample from Lagoa Vermelha, Brazil, to determine whether crude oil can be generated and expelled during artificial maturation of the Organic Matter (OM). The experiments were conducted at 280ºC, 330ºC and 350ºC during 20h. Two types of liquid pyrolysis products, assigned as free oil and bitumen, were isolated and analyzed. Free oil represents free organic phase released by hydrous pyrolysis, whereas bitumen was obtained by extraction from the solid pyrolysis residue with dichloromethane. Changes in the OM maturity were determined using Rock-Eval parameters and biomarker maturity ratios of original sample and pyrolysis products. Biomarker compositions of original sample extract and liquid pyrolysates were used for determination of dominant bacterial source. The yields of free oil and bitumen showed that a microbial mat OM has a high liquid hydrocarbons generation potential. Rock-Eval maturity parameters, biopolymer and biomarker compositions indicate a significant increase of the OM maturity during hydrous pyrolysis. At 280ºC the release of free, adsorbed and occluded compounds was observed; however, without a cracking of the OM. At 330ºC the generation of bitumen and free oil is mostly related to the OM cracking. The highest yield of free oil was recorded at this temperature. Distribution of biomarkers in the extract of original sample and liquid pyrolysates confirms cyanobacteria-dominated microbial mats, whereas the identification of long chain *n*-alkane series, with maximum at C_{26}, and prominent C_{30} hop-17(21)-ene additionally suggest the presence of sulfate reducing bacteria.

KEYWORDS | **Microbial mat. Hydrous pyrolysis. Hydrocarbon potential. Biomarkers. Lagoa Vermelha.**

INTRODUCTION

Microbial mats are multi-layered microbial communities spatially organized as a result of physicochemical gradients (Sánchez *et al*., 2006). They typically have steep gradients of oxygen and sulfide, and characteristically are dominated by only a few functional groups of microbes: cyanobacteria, colorless sulfur bacteria, purple sulfur bacteria and sulfate-reducing bacteria (van Gemerden, 1993). These communities are developed mostly in the environmental interfaces of water and solid substrates, forming a laminated multilayer of biofilms and largely altering the environmental microgradients in this interface as a result of their own communal metabolism (Rontani and Volkman, 2005). They usually develop in heat- and/or salinity-stressed habitats, where the activity of eukaryotic macro- and microorganisms is highly restricted. Thus, the lower diversity of species of

these ecosystems facilitates the assignment of sources of Organic Matter (OM) and the recognition of early diagenetic processes, which provides reference data for biogeochemical modeling (Grimalt *et al.*, 1992).

The mats in evaporitic environments constitute stratified benthic communities composed of slime-rich cohesive layers that contain dense populations of microorganisms, where cyanobacteria are dominant in the upper levels, whereas anoxygenic phototrophic and chemoautotrophic bacteria may bloom at the greater depths (Grimalt *et al.*, 1992).

In last decade the significance of bacterial diversity of a cyanobacterial mat in degradation of petroleum compounds has proved a promising tool for bioremediation after serious oil spills (Abed *et al.*, 2006; Gallego *et al.*, 2006, 2007; Al-Thani and Potts, 2012). Since the spills from the commercial trafficking of oil often accumulate in coastal regions where cyanobacterial mats are prevalent, they have play an important role in the pollutant degradation via their own activity and by establishing oxygen gradients and/or supplying nutrients for heterotrophic bacteria (Al-Hasan *et al.*, 1998; Cohen, 2002). Moreover it was shown that novel halotolerant and thermotolerant microorganisms could grow or even become dominant within cyanobacterial microbial mats after massive oil spills. These bacteria are capable for degradation of oil pollutants under extreme conditions of temperature and salinity (Abed *et al.*, 2006).

As well documented in the literature, petroleum is derived from the geothermal maturation of kerogen, which is a heterogeneous, polymeric substance formed from a biomass consisting of variable proportions of the remains of algae, higher plants and bacteria (Tissot and Welte, 1984). The contributions of algae and higher plants is well documented and there is substantial evidence for a contribution from bacteria, based on kerogen degradation experiments (*e.g.* Chappe *et al.*, 1979, 1982) and the widespread occurrence of extended hopanes in crude oils (Peters *et al.*, 2005; Nytoft, 2011). Consequently, a study of microbial mats by hydrous pyrolysis may provide reference data for the understanding of the transformation of the OM during sedimentation processes.

Hydrous pyrolysis is a laboratory technique developed to simulate the natural maturation process, by heating organic rich sedimentary rock in a closed reactor in the presence of liquid water at subcritical temperatures (Lewan *et al.*, 1979; Lewan, 1983). Previous studies have shown that reactions involving biomarkers during hydrous pyrolysis experiments mimic those occurring during natural maturation (*e.g.* Eglinton and Douglas, 1988; Koopmans *et al.*, 1996; Lewan, 1997; Ruble *et al.*, 2001; Elie and Mazurek, 2008). Therefore, the main goal of this research was to determine whether any crude oil is generated and expelled by artificial maturation of a recent microbial mat. Changes in the OM maturity were determined using Rock-Eval parameters and biomarker maturity ratios of original sample and pyrolysis products. Moreover, biomarker compositions of original sample extract and liquid pyrolysates, along with macroscopical characterization of the sample, were used for determination of the dominant bacterial source.

STUDY AREA

As a result of a particular semi-arid microclimate, within an otherwise tropical environment, and the influence of an adjacent marine upwelling zone, systems of Holocene shallow hypersaline and carbonaceous lagoons are found in the southeast coast of the state of Rio de Janeiro, Brazil. The Lagoa Vermelha (22º55'39"-22º56'06"S; 42º21'29"-42º24'13"W) is approximately 4.5km long and 250 to 850m wide, covering an area of 1.90km^2 with a mean water depth of 2.0m (Fig. 1). It is located between two parallel dune systems, a younger (Holocene) which separates it from the Atlantic Ocean and an older (Pleistocene) which separates it from a much larger lagoon (Araruama system).

There is no surface drainage in the Lagoa Vermelha environment. Therefore, water column depths ultimately depend on weather conditions (dry or rainy season), as well as on the sea conditions promoting seepage, *e.g.* spring tides and local storms, which can considerably increase the lagoon's water volume.

Due to the elongated geometry and flat bottom topography of the lagoon, the water balance is strongly influenced by small fluctuations in the water cycle, like variation in the evaporation/precipitation ratio, which result in changes in the lagoon surface area and in the water chemistry (van Lith *et al.*, 2003).

Sample description

A polygonal microbial mat (Fig. 2A) was found at a temporary pond in a high meso-littoral region of Lagoa Vermelha. External morphology showed traditional features like upturned crack margins producing saucer-shaped polygons, approximately 15cm wide and 1.5cm thick. The mat had a flat dark-green pigmented surface and internally was subdivided into different colored layers (Fig. 2B). It was composed of a green layer at the top (3mm), followed by a thin red layer (4-5mm) and a thicker brown layer at the bottom (6-15mm). Irregular and thin carbonate laminations were mainly observed in the brown layer.

The polygonal mat harbored a variety of morphologically recognizable filamentous and coccoid cyanobacterial taxa,

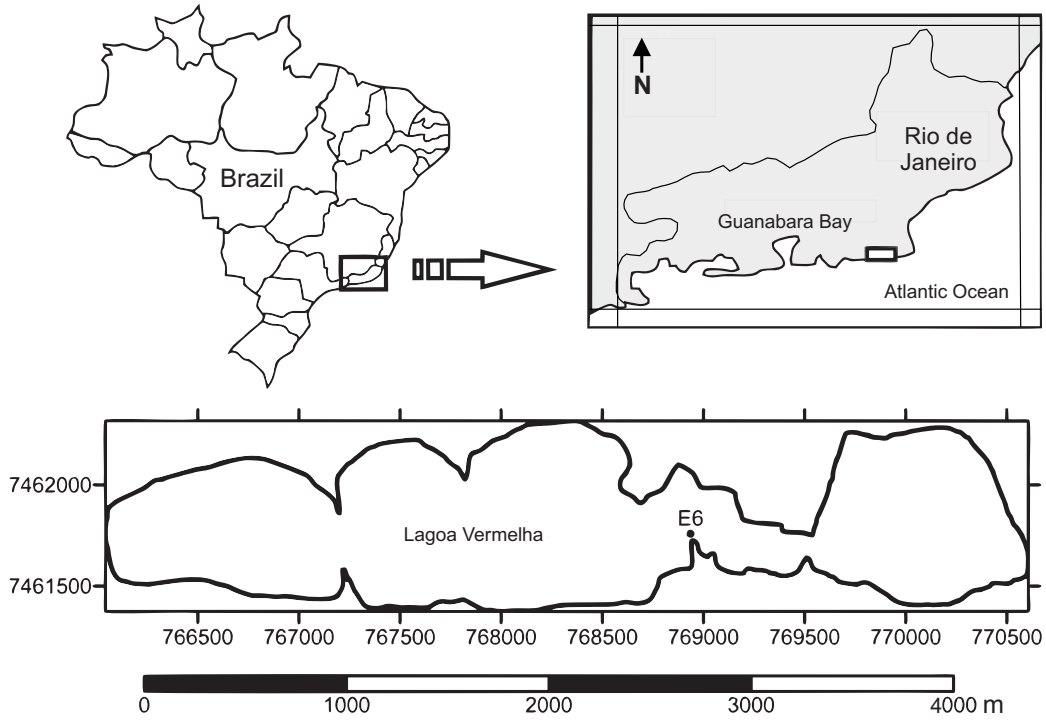

FIGURE 1. Location of Lagoa Vermelha on the southeastern coast of the state of Rio de Janeiro, Brazil (sampling point, E6 is also depicted).

which include: *Jaaginema*, *Phormidium*, *Microcoleus*, *Schizothrix*, *Spirulina*, *Aphanothece*, *Aphanocapsa*, *Chroococcus*, *Gloeocapsopsis*, *Gomphosphaeria*, *Synechococcus* and *Johannesbaptistia*. The predominant cyanobacterial morphotype was the filamentous *Microcoleus chthonoplastes*.

The particulate OM encountered in this sample is predominantly amorphous. Around 97% of the OM is composed of bacterial amorphous OM, while 2% corresponds to the palynomorph group and 1% to the phytoclast group.

MATERIAL AND METHODS

Sampling

A microbial mat sample was collected in the intertidal zone of Lagoa Vermelha at E6 station (Fig. 1) in April 2012. The mat morphotype was determined according to its geometry, texture and color at the sampling site. The sample was collected using a metal spatula and placed into aluminum container, which was refrigerated during transport.

Biopolymer analysis

Biopolymer analyses were performed on the fresh sample and residues from hydrous pyrolysis experiments. Protein analyses were carried out after the extraction with sodium hydroxide (NaOH 0.2mol/dm^3) according to the procedure of Hartree (1972), modified by Rice (1982). Carbohydrates were analyzed following the Dubois method (Dubois *et al.* 1956), modified by Gerchacov and Hacher (1972). Lipids were isolated using the method of Marsh and Wenstein (1966).

Cyanobacterial identification

Slides from mat were observed microscopically (Axiovision Imager. A1 Zeiss), about ten, to ensure a good overall representation of resident morphotypes.

Cyanobacterial identification was carried out in accordance with traditional morphological features as described by Komárek and Anagnostidis (1999, 2005).

Determination of total organic carbon and Rock-Eval pyrolysis

Crushed samples were pulverized and sifted to obtain an 80-mesh grain size for the determination of Total Organic Carbon (TOC) and Rock-Eval pyrolysis. TOC contents (weight %) was determined by combustion in a Leco SC-144 carbon analyzer with an infrared detector, after removal of carbonates by acidification (HCl).

The Rock-Eval pyrolysis, developed by Espitalié *et al.* (1977), was carried out in a Rock-Eval 6 equipment by

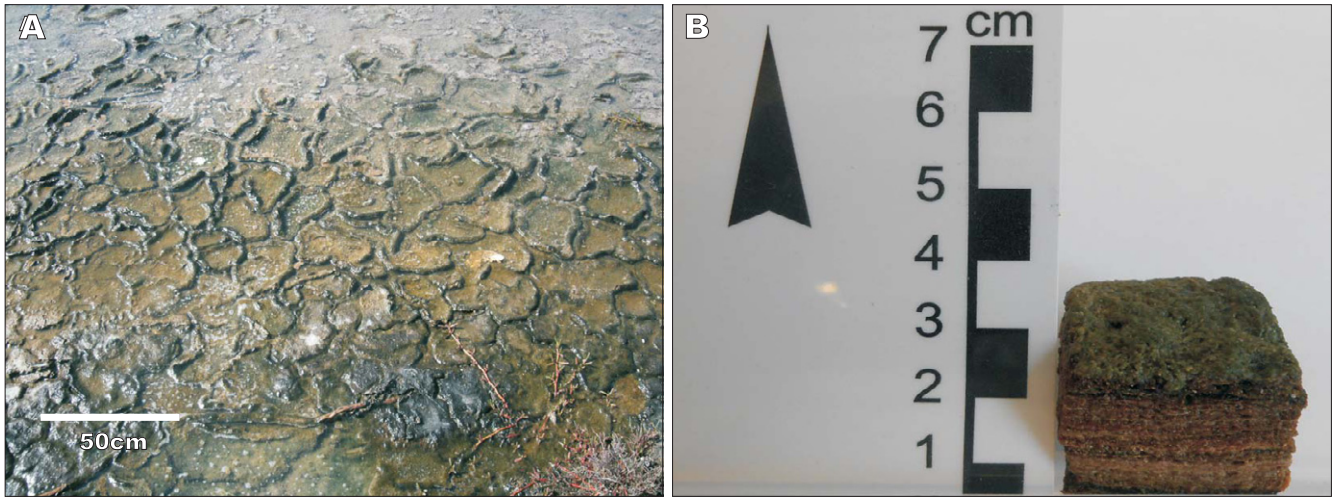

FIGURE 2. Polygonal mat from Lagoa Vermelha at location E6 (See Fig. 1). A) View of microbial mat at sampling site and B) internal stratification of microbial mat selected for this study.

heating samples in an open pyrolysis system under non-isothermal conditions. This technique uses temperature programmed heating (from 300 to 650ºC at 25ºC/min rate) of a small amount of sample (100mg) in an inert atmosphere (N_2) in order to determine the quantity of free hydrocarbons in the sample and of those that can potentially be released during maturation. The released hydrocarbons are monitored by a Flame Ionization Detector (FID), forming the so-called S1 (thermo-vaporized free hydrocarbons) and S2 (pyrolysis products from cracking of the OM) peaks. CO and CO_2 released during pyrolysis (S3 peak) are monitored on-line, by means of an infra-red cell.

Hydrous pyrolysis

Hydrous pyrolysis experiments were performed in a stainless steel reactor (350cm^3), which was loaded with 50g of a freeze dried microbial mat sample and 130cm^3 of distilled water. These amounts of sample and water were calculated based on the approximate density of the sample and the volume of the reactor, to ensure that the sample remained submerged in a liquid water phase throughout the experiment. Then the sealed reactor was filled with 1000psi of He to check for leaks. When no leaks were detected, the system was purged three times with He in order to remove the air. The final He pressure was reduced to 100psi. An artificial maturation was achieved by isothermal heating of the sample at 280ºC, 330ºC and 350ºC during 20h. Throughout the experiments the temperature was continuously monitored at 30s intervals using Type J thermocouple. Obtained standard deviations ranged between 0.3 and 0.6ºC.

After the experiments were completed, the reactor was cooled down to room temperature overnight. It was then vented and opened to collect the liquid products and solid residue. Immiscible oil floating on the water surface, designed as free oil, was generated during hydrous pyrolysis experiments. Free oil was collected using a Pasteur pipette and transferred into a pre-weighed glass vial. Water was removed from the reactor with a volumetric pipette and the solid residue was collected with a large spatula and placed into a pre-weighed Petri dish. The recovered solid residue was dried in a fume hood until the constant weight was obtained.

After the removal of the free oil, water and the solid residue from the system, the reactor was rinsed with dichloromethane to recover any adsorbed oil film, designated as rinse oil. The rinse oil was transferred into a tared glass vial and weighed after solvent evaporation under a fume hood. Total expelled oil represents the sum of free oil and rinse oil (Lewan, 1993).

Biomarker analysis

The original sample (in further text: unheated sample) and the solid residues obtained after hydrous pyrolysis experiments (~5g) were extracted with dichloromethane/methanol (2:1, v/v) using an Accelerator Solvent Extractor (ASE). The extract from the solid residue after hydrous pyrolysis experiment is designated in this study as bitumen. Aliquots of unheated sample extract, bitumens and free oils were fractionated using benzenesulfonic acid bonding Solid Phase Extraction (SPE) columns (DSC-SCX; 500mg, 3cm^3). The fractions were eluted sequentially with dichloromethane, acetone and methanol to obtain neutral lipids, glycolipid and phospholipid fractions, respectively. The hydrocarbon fraction was isolated from neutral lipids using column chromatography (silica-gel, 70-230mesh) by elution with n-hexane.

This fraction was analyzed by gas chromatography-mass spectrometry in an Agilent Technologies instrument (from USA) comprising a 7890A model gas chromatograph equipped with a 7693 auto sampler and coupled to a triple quadrupole 7000B Mass Spectrometer (MS). Helium was the carrier gas, in constant flow mode, at 1.2cm^3/min. A DB-1 column (100% dimethylpolysiloxane, 30m-long, with 0.25mm inner diameter and 0.25µm film thickness) was used. The column was heated from 40ºC (1min, hold) to 140ºC at a rate of 20ºC/min and then to 280ºC at 2ºC/min. The final temperature of 280°C was maintained for an additional 30 minutes. The injector and transfer line temperatures were 280ºC. The MS was operated under the following conditions: the ion source temperature was 280ºC, the interface temperature was 300ºC and the quadrupole temperature was 150ºC. Electron impact ionization (70eV) was used and full scan spectra were obtained by scanning *m/z* 50–800 at 1 scan s^{-1}. The compound assignment was performed by examination and comparison with literature mass spectra and NIST (National Institute of Standards and Technology) library.

RESULTS AND DISCUSSION

Yields of liquid pyrolysis products and solid residue characterization

Yields of liquid pyrolysates and sample characterization before and after hydrous pyrolysis experiments are shown in Table 1. Maximal yields of bitumen and expelled oils are obtained at 280ºC and 330ºC, respectively (Table 1). These trends are in agreement with previous observations reported for source rocks in natural (Tissot and Welte, 1984; Hunt, 1995; Farrimond *et al.*, 1998) and artificial systems (Lewan, 1993; Behar *et al.*, 2008, 2010). The obtained results reflect well three fundamental stages of the generation process: i) depolymerisation of kerogen to form bitumen plus gas, ii) the cracking of the bitumen to yield oil and gas and iii) the cracking of oil to yield gas (Braun and Rothman, 1975; Ungerer, 1990). Consequently, the highest yield of the bitumen is recorded at 280ºC, whereas the maximal yield of the expelled oil is observed at higher temperature of 330ºC (Table 1). Decrease of bitumen and expelled oil yields at 350ºC (Table 1) in part can be attributed to the cracking of kerogen, bitumen and expelled

TABLE 1. The yields of liquid pyrolysis products, total organic carbon content and Rock-Eval parameters of unheated sample and solid residues obtained after hydrous pyrolysis experiments

Experimental conditions (ºC/h)	Unheated	280ºC/20h	330ºC/20h	350ºC/20h
Yield of Expelled Oil[a] (mg HC/g original TOC)	-	193.6	446.2	362.9
Yield of Bitumen (mg HC/g original TOC)	548.5	1884.1	636.1	448.3
Solid sample				
TOC: total organic carbon (%wt.)	5.1	3.2	2.1	ND[g]
S1: free hydrocarbons (mg HC/g rock)	4.9	2.4	2.0	3.0
S2: pyrolysate hydrocarbons (mg HC/g rock)	24.9	20.2	10.0	7.4
S3: amount of CO_2 generated from oxygenated functional groups (mg CO_2/g rock)	18.2	1.9	1.3	0.8
T_{max}: temperature corresponding to S2 peak maximum (ºC)	413	429	442	441
HI[b] = S2 x 100/TOC (mg HC/g TOC)	485	642	470	212
OI[c] = S3 x 100/TOC (mg CO_2/g TOC)	354	59	60	23
PI[d] = S1/(S1+S2)	0.17	0.11	0.17	0.29
TR[e] = $[(S2_o\text{-}S2_r)/S2_o]$[f] x 100 (%)	NA[h]	19	60	70
Biopolymer composition				
Carbohydrates (mg/g)	16.11	10.73	5.98	7.18
Proteins (mg/g)	9.39	10.48	7.53	9.92
Lipids (mg/g)	7.58	5.99	7.56	22.03

[a]Expelled oil is a sum of free oil and rinse oil; [b]HI: Hydrogen Index; [c]OI: Oxygen Index; [d]PI: Production Index; [e]TR: Transformation Ratio; [f]$S2_o$: S2 peak in the unheated sample, $S2_r$: S2 peak in the solid residue obtained after hydrous pyrolysis; [g]ND: Not Determined; [h]NA: Not Applicable

oil into the gaseous products (not analyzed in this study). The formation of gases is particularly favored in the close system pyrolysis (Erdmann and Horsfield, 2006). On the other hand, it was also shown that thermal degradation of kerogen to volatile constituents is partly compensated by synthetic reactions, *e.g*. recombination processes (also facilitate in the close system pyrolysis; Erdmann and Horsfield, 2006), which generate a secondary kerogen from soluble organic matter (Oehler *et al*., 1974; Huc and Durand, 1977; Peters *et al*., 1981). These reactions, taking place at relatively low levels of maturation, resulted in the formation of a thermally stable secondary kerogen, which is degraded yet at higher maturities (Peters *et al*., 1981; Erdmann and Horsfield, 2006).

Generation of liquid hydrocarbons during hydrous pyrolysis experiments was followed by the decrease of TOC content and Rock-Eval parameters (S1, S2 and S3 peaks), comparing the solid residues with the unheated sample (Table 1). Marked decrease of S1 and S3 peaks and slight decrease of S2 peak in the solid residue obtained at 280°C (also expressed through the low Transformation Ratio, TR= 19%) indicate a release of free, adsorbed and occluded compounds and a degradation of labile heteroatomic structures, without significant cracking of the OM (Table 1). Releasing of high proportion of biogenic and diagenetic compounds, which have still not incorporated into macromolecular matrix, resulted in high bitumen yield at this temperature (1884.1mg HC/g original TOC; Table 1). The obtained result is consistent with high and bimodal S2 peak of the unheated sample. The first S2 peak maximum of the unheated sample with low T_{max} of 350ºC resulted from the presence of free and weakly adsorbed polar compounds which were not distilled within the S1 temperature interval. However these compounds were released during hydrous pyrolysis at 280ºC, resulting in very high bitumen yield (Table 1). Several other studies have also reported high bitumen yields for immature OM (Bechtel *et al*., 2005; Avramidis and Zelilidis, 2007; Vu *et al*., 2009).

An increase of HI from unheated sample to the solid residue at 280ºC (Table 1) could be attributed to the loss of carbon associated with non-lipid type material, rich in heteroatoms, particularly oxygen. This is documented by sharp decrease of Oxygen Index (OI; Table 1) from 354mg CO_2/g TOC (unheated sample) to 59mg CO_2/g TOC (pyrolysis product at 280ºC).

A notable decrease of S2 peak in the solid residue at 330ºC and a sharp increase of TR indicate an intense cracking of the OM in the temperature range of 280-330ºC (Table 1). The obtained result is in agreement with earlier studies which showed that even cracking of reactive OM rich in aliphatic structures requires a temperature above 300°C (Jaeschke *et al*., 2008). Moreover, a decrease of S2 peak and Hydrogen Index (HI), followed by an increase of TR and S1 peak confirm further OM cracking from 330ºC to 350ºC.

The effect of experimental conditions on maturity parameters may be monitored through the Rock-Eval parameters, T_{max} and Production Index (PI) (Table 1). T_{max} increases from 413ºC for the unheated sample to 441ºC for the residual sample at 350ºC, while PI rises from 0.11 at 280ºC to 0.29 at 350ºC. The high value of S1 peak in the unheated sample (Table 1) implies a high proportion of biogenic and diagenetic compounds, and consequently the value of PI for the unheated sample is unrealistic and it was not considered. The values of T_{max} and PI strongly suggest that the OM at 280ºC is still immature. The temperature increase to 330ºC and 350ºC resulted in marked increase of the OM thermal maturity (Table 1).

Analyses of biopolymers in the unheated and residual samples after hydrous pyrolysis showed the effect of artificial maturation (thermal stress) on the concentrations of carbohydrates, proteins and lipids. The biopolymer proportions in the unheated microbial mat sample are carbohydrates > proteins > lipids, accounting for 49%, 28% and 23%, respectively (Fig. 3A). These biopolymer proportions are similar to those observed for OM in recent sediments (Dell'Anno *et al*., 2002; Pusceddu *et al*., 2003). High concentrations of biopolymers, especially carbohydrates, are directly related to the primary productivity of the autotrophic organisms present in microbial mats, since biochemical composition of OM in sediments depends on the origin of contributors (Danovaro *et al*., 2001; Pusceddu *et al*., 2003).

Changes in the relative abundance of biopolymers may be observed throughout the hydrous pyrolysis experiments (Fig. 3A). At 280ºC the carbohydrate concentration decreased with a relative increase of proteins (Table 1); however, the biopolymer groups kept the same trend as in the unheated sample, carbohydrates (39.5%) > proteins (38.5%) > lipids (22.0%) (Fig. 3A), which is consistent with very low OM cracking. At 330ºC an overall decrease of biopolymers concentrations was observed, especially of carbohydrates (Table 1). At this temperature the proportions are changed, showing the following trend: lipids > proteins > carbohydrates, whereas a significant increase in concentration of lipids over other biopolymers is observed at 350ºC (Fig. 3A; Table 1). This result is in accordance with higher thermal stability of lipids, in comparison with carbohydrates and proteins (Hunt, 1995).

The lipid composition (neutral-, phospho- and glycolipids) of the extracts of unheated and pyrolysed samples is shown in Figure 3B. Lipid composition of

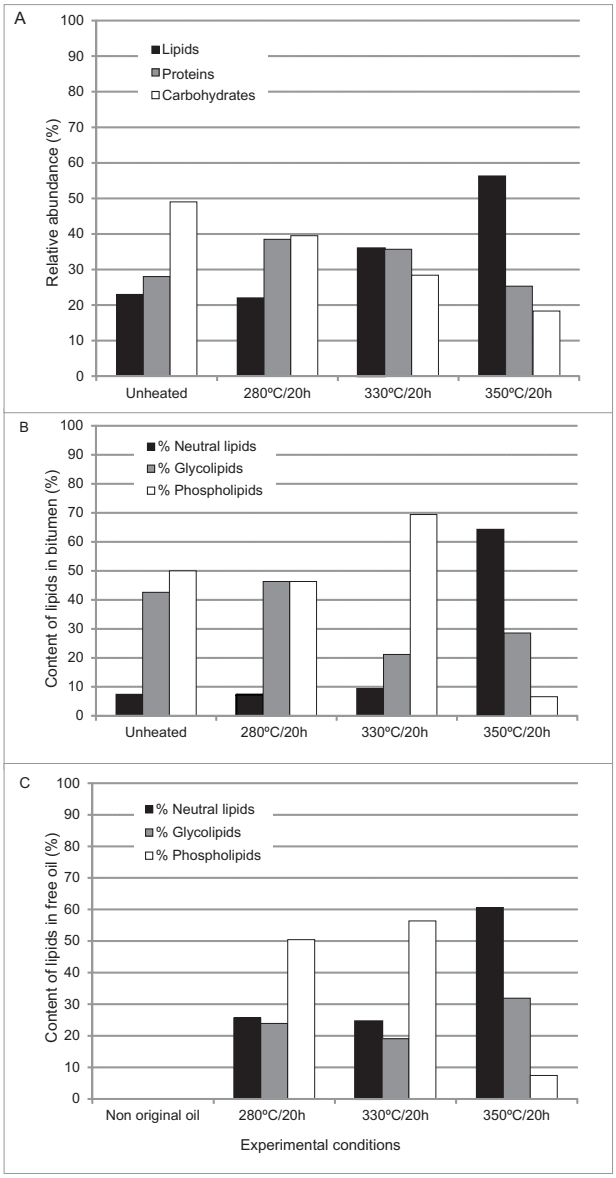

FIGURE 3. The relative abundance of A) biopolymers in unheated and residual samples, B) lipid composition of bitumen and C) free oil obtained by hydrous pyrolysis experiments.

bitumen generated at 280ºC is similar to composition of the extract of unheated sample, which suggests that at this temperature releasing of free and occluded compounds and degradation of labile fragments rich in heteroatoms are more significant than OM cracking. This observation is consistent with the results of Rock-Eval pyrolysis discussed above. In general, lipid composition of bitumen shows that phospholipids are the most abundant fraction at 280ºC and 330ºC, followed by glycolipids and neutral lipids (Fig. 3B). At the same temperatures in free oils, the most abundant phospholipids are followed by neutral lipids and glycolipids (Fig. 3C). Comparing the relative abundance of neutral lipids in free oils and corresponding bitumens obtained at 280ºC and 330ºC, free oils are enriched in this lipid fraction, which is consistent with the easier release of lower molecular weight compounds (Tissot and Welte, 1984; Hunt, 1995). With the temperature increase to 350°C, the composition of lipids is changed and shows marked predominance of neutral lipids over glyco- and phospholipids, both in bitumen and free oil (Fig. 3B, C). This result is in accordance with the higher thermal stability of neutral- over complex glyco- and phospholipids, reach in reactive functional groups.

Effect of temperature on the generation of liquid hydrocarbons n-Alkanes and isoprenoids

n-Alkanes range from *n*-C_{17} to *n*-C_{33} in the extract from the unheated sample (Fig. 4A), with an odd-carbon number predominance (Carbon Preference Index (CPI) for full range of *n*-alkanes = 2.74; Table 2). This distribution is sharply dominated by *n*-C_{17}, typical of lipids from cyanobacteria (Shiea *et al.*, 1990; Kenig *et al.*, 1995; Thiel *et al.*, 1997; Summons, 2008), which resulted in notably high CPI value for short chain *n*-alkanes (C_{14}-C_{20}), reaching the value of 12.55 (Table 2). CPI value for mid- and long chain n-alkanes (C_{22}-C_{34}) in the extract of immature unheated sample and all pyrolysates is close to 1 (Table 2), indicating no contribution of terrestrial OM.

n-Alkane distributions in liquid pyrolysates differ from that of the unheated sample; however, in almost all the pyrolysates *n*-C_{17} is a dominant *n*-alkane homologue (Fig. 4C-G), emphasizing the influence of parent OM on the biomarker distributions. Figure 4B shows that bitumen obtained at 280ºC is dominated by C_{26} *n*-alkane, whereas low molecular weight *n*-alkanes (*n*-C_{15} to *n*-C_{17}) are absent. On the other hand, bitumens obtained at 330ºC and 350ºC show comparable abundances of *n*-alkane homologues in *n*-C_{17} to *n*-C_{35} range, with a bimodal distribution (Fig. 4D, F). Free oils obtained at 280ºC and 330ºC (Fig. 4C, E) have similar *n*-alkane distributions, which are dominated by low molecular weight *n*-alkanes (*n*-C_{15} to *n*-C_{19}) and with a maximum at *n*-C_{17}. Distribution of *n*-alkanes in free oil obtained at 350ºC differs from those observed in free oils at lower temperatures, and clearly is bimodal with maxima at *n*-C_{17} and *n*-C_{34} (Fig. 4G). Predominance of *n*-C_{17} in the extract of unheated sample and the liquid pyrolysis products, followed by bimodal distributions of *n*-alkanes in bitumens obtained at 330ºC and 350ºC and free oil obtained at 350ºC (which showed elevated contents of *n*-C_{26} and *n*-C_{34}), confirmed a domination of *Microcoleus* taxon in the sample investigated in this study (Paoletti *et al.*, 1976; Grimalt *et al.*, 1992; Rontani and Volkman, 2005; Allen *et al.*, 2010). However, the contribution of other cyanobacterial taxa could not been ruled out, since it was shown that some cyanobacteria (*e.g. Nostoc* and *Spirulina* sp.) contain a second series

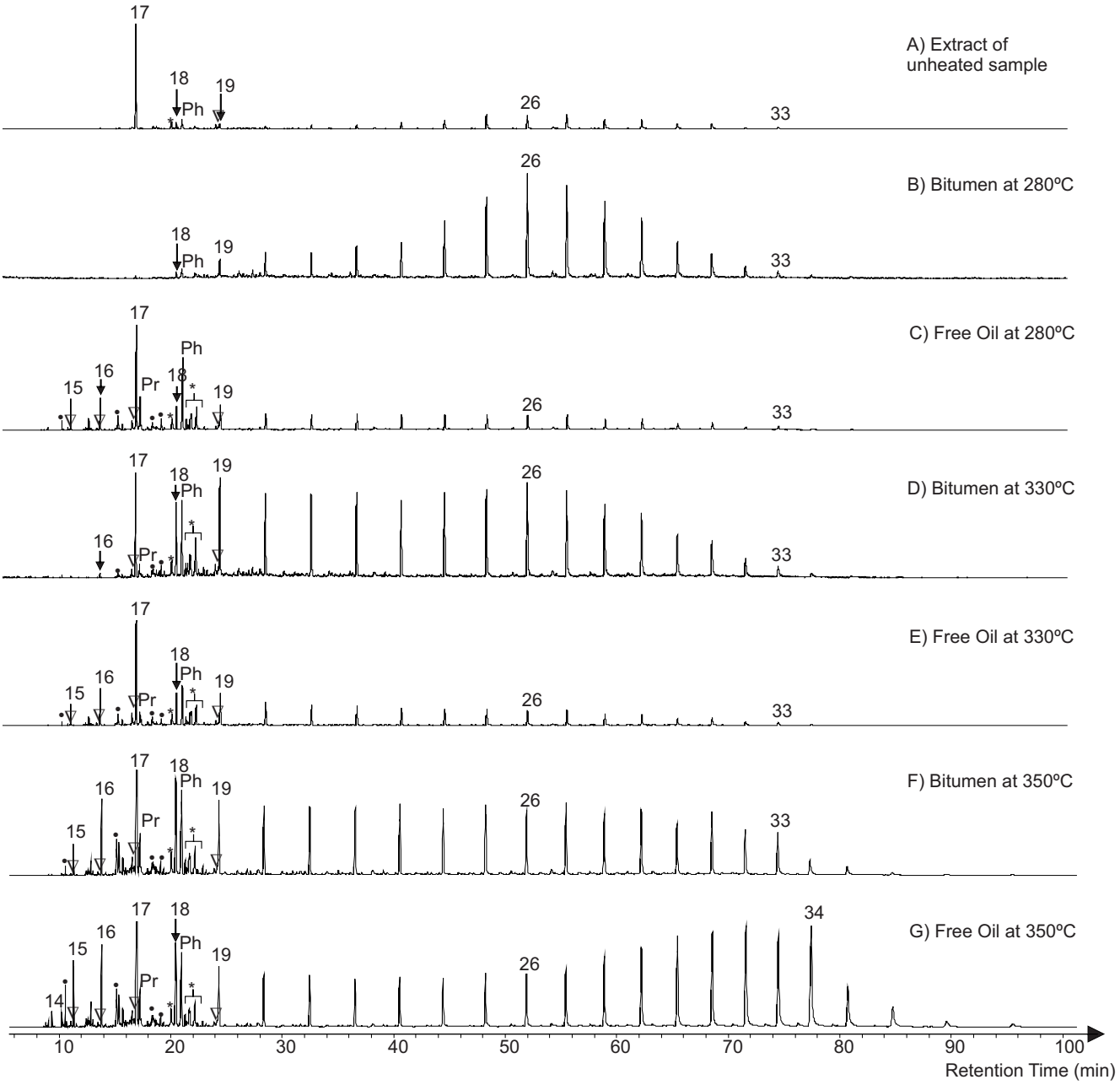

FIGURE 4. Mass chromatogram of *n*-alkanes (*m/z* 85) in A) the extractable organic matter of unheated sample, B, C) bitumens and free oils obtained at 280ºC, D, E) 330ºC and G, F) 350ºC, respectively. *n*-Alkanes are labeled according to their carbon number; Pr: Pristane; Ph: Phytane; ▽: *n*-alkenes, with the same number of carbon atoms as the adjacent *n*-alkanes; •: isoprenoids; *: isomeric phytenes.

of *n*-alkanes, additional to the common predominance of *n*-C_{17} (Paoletti *et al.*, 1976). This is particularly related to *Spirulina* because it was observed in studied mats (see section Sample description). In addition, unimodal long-chain *n*-alkane distributions have also been observed in a broad variety of other anoxygenic phototrophic and heterotrophic bacteria (with a maximum at *n*-C_{26} or *n*-C_{27}; Han and Calvin, 1969; Shiea *et al.*, 1990) and in the common sulfate-reducer *Desulfovibrio desulfuricans* (Davis, 1968). Considering that cyanobacteria play an important role in microbial mats by establishing oxygen gradients and/or supplying nutrients for heterotrophic bacteria (Al-Hasan *et al.*, 1998; Cohen, 2002), we could suppose that long chain *n*-alkanes present in all samples in some extent can also be attributed to heterotrophic and/or sulfate-reducing bacteria. The possible impact of the latter is also assumed based on prominent C_{30} hop-17(21)-ene in the unheated sample (see further below section Hopanoid biomarkers).

Differences in distribution of *n*-alkanes between free oil and bitumen obtained at 280ºC (Fig. 4B, C) are consistent

TABLE 2. Values of parameters calculated from distributions and abundances of *n*-alkanes and isoprenoids

Experimental conditions (ºC/h)	Unheated	280ºC/20h		330ºC/20h		350ºC/20h	
	Extract	Bit[a]	Oil[b]	Bit	Oil	Bit	Oil
Pr[c]/Ph[d]	-	-	0.37	0.13	0.26	0.29	0.32
Pr/*n*-C_{17}	-	-	0.24	0.12	0.09	0.18	0.17
Ph/*n*-C_{18}	1.70	2.04	3.98	1.23	1.36	0.94	0.95
n-C_{17}/*n*-C_{31}	10.50	-	13.91	1.59	12.17	1.73	0.89
CPI (*n*-C_{16}-*n*-C_{34})[e]	2.74	0.98	1.97	1.16	1.62	1.21	1.03
CPI (*n*-C_{14}-*n*-C_{20})[f]	12.55	1.98	3.30	1.97	2.35	1.29	1.47
CPI (*n*-C_{22}-*n*-C_{34})[g]	1.34	1.00	1.17	1.09	1.14	1.19	0.94

[a]Bit: Bitumen; [b]Oil: Free oil; [c]Pr: Pristane; [d]Ph: Phytane

[e]CPI (C_{16}-C_{34}) = 1/2 x [Σodd(*n*-C_{17} - *n*-C_{33})/Σeven(*n*-C_{16} - *n*-C_{32}) + Σodd(*n*-C_{17} - *n*-C_{33})/Σeven(*n*-C_{18} - *n*-C_{34})]

[f]CPI (C_{14}-C_{20}) = 1/2 x [Σodd(*n*-C_{15} - *n*-C_{19})/Σeven(*n*-C_{14} - *n*-C_{18}) + Σodd(*n*-C_{15} - *n*-C_{19})/Σeven(*n*-C_{16} - *n*-C_{20})]

[g]CPI (C_{22}-C_{34}) = 1/2 x [Σodd(*n*-C_{23} - *n*-C_{33})/Σeven(*n*-C_{22} - *n*-C_{32}) + Σodd(*n*-C_{23} - *n*-C_{33})/Σeven(*n*-C_{24} - *n*-C_{34})]

n-C_x: designates *n*-alkane homologue, where x represents the number of carbon atoms

with previous discussion that under such conditions, free, weakly adsorbed and occluded compounds are released, without significant OM cracking. Throughout this process, low molecular weight *n*-alkanes are more easily released and concentrated in free oil, whereas long chain homologues remain adsorbed and are released into bitumen during the extraction. Distribution of *n*-alkanes in bitumen obtained at 330ºC suggests cracking of OM. However, differences in distributions of *n*-alkanes between free oil and bitumen obtained at 330ºC clearly indicate the enrichment of low molecular weight compounds within free oil. This is also observed at 280ºC. At 350ºC distributions of *n*-alkanes in free oil and bitumen are similar, indicating not only the cracking of the OM, but also sufficient thermal conditions for the uniform release of low- and high molecular weight *n*-alkanes into free oil.

The effect of temperature was also noticed in the appearance of *n*-alkenes, ranging from *n*-C_{15} to *n*-C_{19}, methylalkanes and isoprenoids in liquid pyrolysates (Figs. 4B-G; 5). High amounts of C_{17}-C_{19} *n*-alkanes, the presence of *n*-alkenes in the same range and some methylalkanes, especially 8-methylhexadecane (which has been reported in other hypersaline *Microcoleus* mats; Grimalt *et al.*, 1992; Rontani and Volkman, 2005; Allen *et al.*, 2010) represent additional confirmation of cyanobacterial OM (Shiea *et al.*, 1990; Kenig *et al.*, 1995; Thiel *et al.*, 1997). The presence of phytane and isomeric phytenes could be related to a direct contribution and/or thermal maturation of methanogenic bacteria (Risatti *et al.*, 1984; Rowland, 1990) or to phytol degradation (Grossi *et al.*, 1998).

The low values of pristane (Pr) to phytane (Ph) ratio in all liquid pyrolysates (Table 2), followed by the presence of isomeric phytenes (Figs. 4B-G; 5), are consistent with the bacterial origin of OM, deriving from hypersaline environments (ten Haven *et al.*, 1987; Killops and Killops, 2005). Namely, bacteria from such environments contain complex lipids with a high amount of phytanyl moieties, resulting in low Pr/Ph ratio.

The Pr/*n*-C_{17} and Ph/*n*-C_{18} ratios generally showed a decreasing trend with the increase of temperature, thereby confirming an increase of thermal maturity. A slight increase of Pr/*n*-C_{17} and Pr/Ph ratios from 330ºC to 350ºC is consistent with the observation that the early formation rates of pristane and phytane during pyrolysis are somewhat the same, but the decomposition of phytane may be slightly faster than pristane, resulting in an increase of the pristane over phytane ratio with increasing maturity (Tang and Stauffer, 1995).

CPI values for the full range of *n*-alkanes (C_{16}-C_{34}) and CPI values for short chain n-alkanes (C_{14}-C_{20}) showed a decreasing trend with temperature increase, thus also indicating an increase of OM maturity (Table 2).

Hopanoid biomarkers

Hopane distribution in the extract of unheated sample is dominated by C_{27} hop-17(21)-ene and C_{30} hop-17(21)-ene, whereas C_{27} 17β(H)-hopane, C_{30} 17β(H)21β(H)-hop-22(29)-ene (diploptene), C_{30} 17β(H)21α(H)-hop-22(29)-ene and C_{30} neohop-13(18)-ene are found in lower amounts

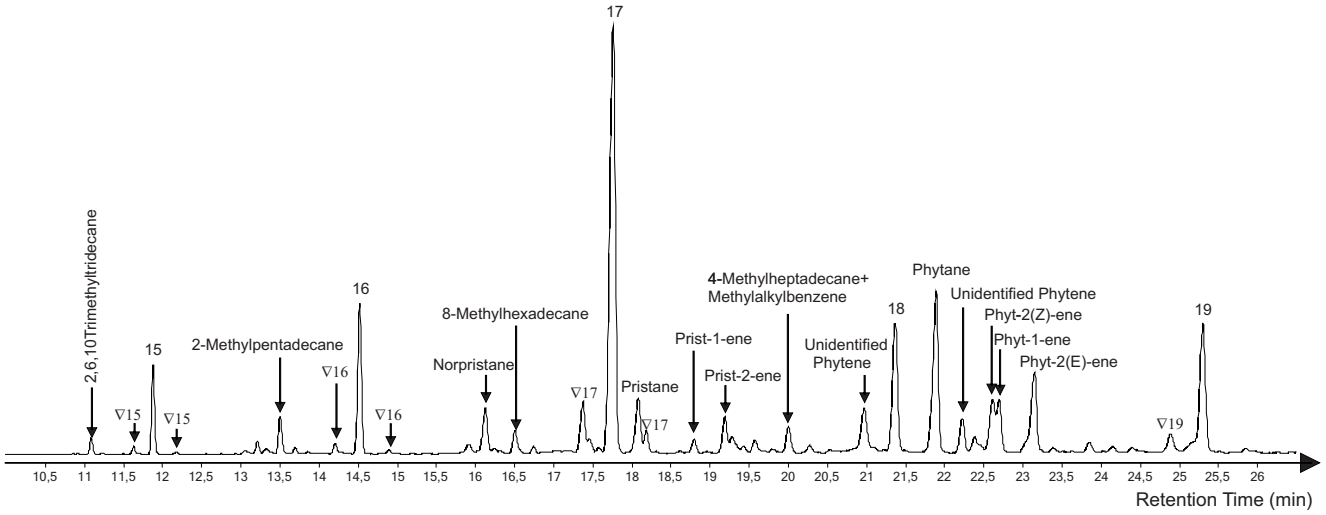

FIGURE 5. Partial *m/z* 85 mass chromatogram of the free oil expelled during hydrous pyrolysis at 330ºC, showing the distribution of low molecular weight alkanes. For peak assignments, see legend of Figure 4.

(Fig. 6A). C_{30} Hop-17(21)-ene, diploptene and C_{30} neohop-13(18)-ene are present in almost all hopanoid containing prokaryotes. The biological source of C_{30} hop-17(21)-ene has not yet been clarified, although the compound is commonly present in immature sediments. A direct input to the sediment by bacteria or in some cases by ferns and moss (Bottari *et al.*, 1972; Volkman *et al.*, 1986; Wakeham, 1990), as well as a diagenetic origin from hop-22(29)-ene, has been proposed (Brassell *et al.*, 1980). Wolff *et al.* (1992) established high concentrations of C_{30} hop-17(21)-ene in early Jurassic diagenetic concretions, containing a high amount of reduced iron, and hence the authors suggested that the most probable source of this hopanoid biomarker were sulfate reducing bacteria. The presence of diploptene in the extract of unheated sample (Fig. 6A) is consistent with cyanobacterial origin of the OM, since it was shown that concentration of diploptene is especially high in cyanobacteria (Rohmer *et al.*, 1984; Yamada *et al.* 1997), where this compound may reach up to 86% of the total lipid fraction (De Rosa *et al.*, 1971). C_{30} 17β(H)21α(H)-hop-22(29)-ene was found in lake sediments (Uemura and Ishiwatari, 1995), although the origin of this hopene remains unclear. However, isomerisation of pure diploptene under acidic conditions did not result in the formation of C_{30} 17β(H)21α(H)-hop-22(29)-ene and therefore the origin of C_{30} 17β(H)21α(H)-hop-22(29)-ene was related to direct biogenic bacterial input (Uemura and Ishiwatari, 1995). C_{27} Hop-17(21)-ene and C_{27} 17β(H)-hopane are widespread in immature OM. Also, these hopanoids were obtained in high amounts by flash pyrolysis of several families of Alphaproteobacteria, Betaproteobacteria and Cyanobacteria (Sugden *et al.*, 2005).

Hopane distributions in the liquid pyrolysates differ from that observed in the extract of unheated sample (Fig. 6). Bitumen and free oil obtained at the same temperature showed almost identical distribution of hopanoids; therefore, only mass chromatograms of free oils are shown in Figure 6, as representatives. In general, the hopanoid patterns in liquid pyrolysates are characterized by the occurrence of 17α(H)21β(H)-, 17β(H)21α(H)- and 17β(H)21β(H)-hopanes from C_{27} to C_{33}, with the C_{28} hopanes being absent. Hopenes were also found in liquid pyrolysates, however in considerably lower amounts than hopanes. In addition to C_{27} hop-17(21)-ene and C_{30} hop-17(21)-ene (which are also present in the extract of unheated sample), C_{29} hop-17(21)-ene was identified in all liquid pyrolysates (Fig. 6B-D).

Neohopene series, comprising C_{27} neohop-13(18)-ene, C_{29} neohop-13(18)-ene and C_{30} neohop-13(18)-ene, was also detected in liquid pyrolysates in low amount (Fig. 6B-D). These rearranged hopanoids have been reported as trace constituents in some bacteria, together with C_{30} hop-17(21)-ene and diploptene (Howard *et al.*, 1984; Douka *et al.*, 2001). Possible formation of these neohopenes through an isomerisation reaction of hop-17(21)-enes was reported by Moldowan *et al.* (1991). However, based on isotopic $\delta^{13}C$ values of precursor hopanoids and neohopene, Sinninghe-Damsté *et al.* (2014) observed that the latter cannot be formed solely by an isomerisation reaction and that the other sources must be involved. The presence of C_{27} neohop-13(18)-ene and C_{30} neohop-13(18)-ene in liquid pyrolysates could be explained by the isomerisation of C_{27} hop-17(21)-ene and C_{30} hop-17(21)-ene, found in the extract of unheated sample. However, the presence of C_{29} neohop-13(18)-ene in pyrolysis products cannot be explained by this reaction and indicates a different source.

While the high relative abundances of C_{30} and C_{27} homologues in liquid pyrolysates are consistent with the

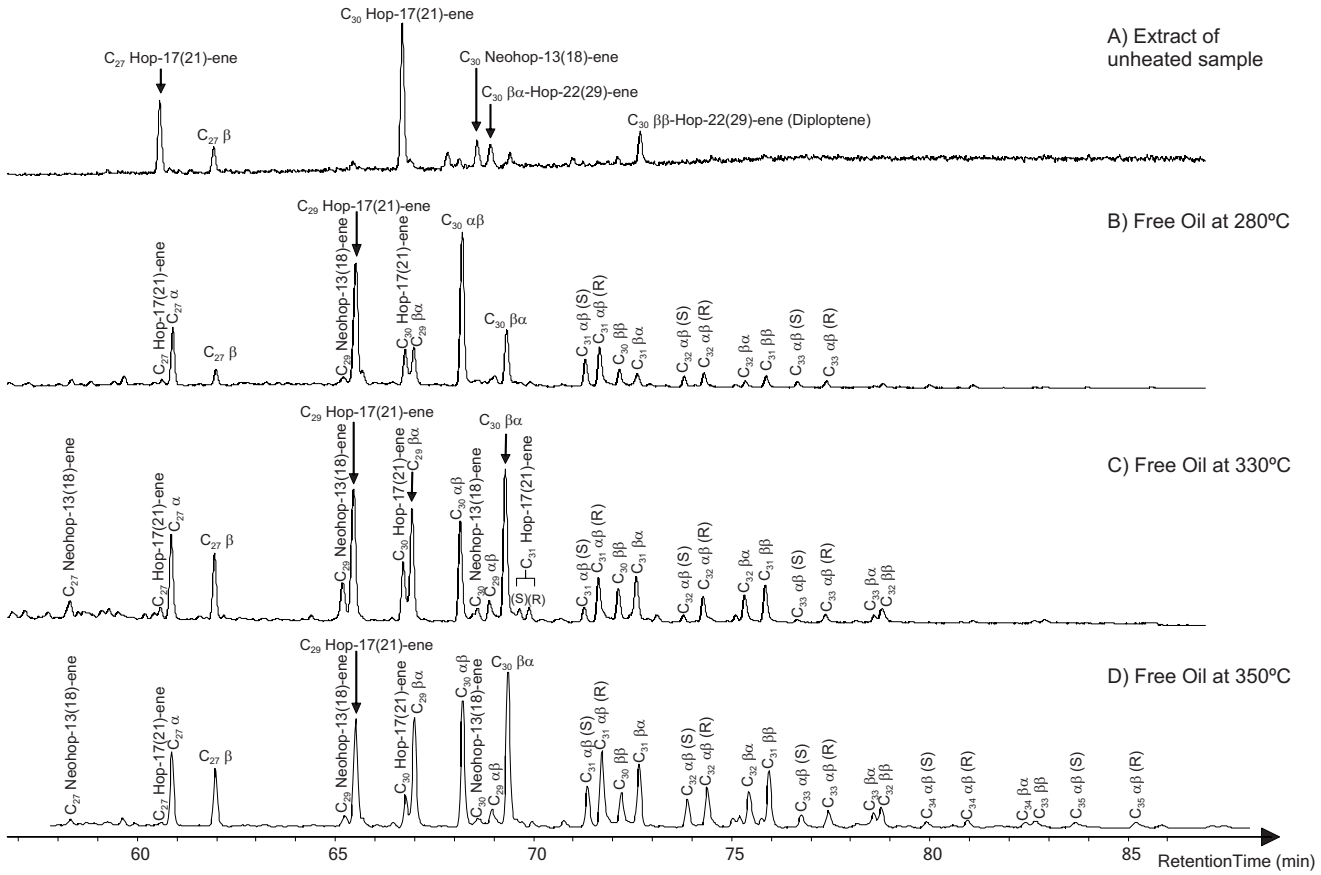

FIGURE 6. Mass chromatogram of hopanoids (m/z 191) in the A) extractable Organic Matter (OM) of unheated sample and B) free oils obtained at 280ºC, C) 330ºC and D) 350ºC, respectively. ββ, βα and αβ designate configurations at C-17 and C-21 in hopanes; (S) and (R) designate configuration at C-22 in hopanes.

presence of hopanoids with the same number of carbon atoms in the extract of unheated sample (Fig. 6), relatively high amounts of C_{29} hopenes and hopanes in liquid pyrolysates may indicate that the precursor hopanoid lipids were functionalized at position 29, as in the case of aminobacteriohopanepentol, which is abundant in methanotrophic bacteria (*e.g. Methylococcus capsulatus* or *Methylomonas methanica*) (Neunlist and Rohmer, 1985). However, this particular hopanoid cannot be taken as the exclusive precursor of C_{29} hopanes, considering the high sensitivity of side chains from biological hopanoids (Burhan *et al.*, 2002).

All liquid pyrolysates contain homohopanes with more than 30 carbon atoms (Fig. 6B-C), which indicates that bacteriohopanepolyols were constituents of the precursor biomass. With an increase of the temperature and thermal maturity, content of homohopanes increases (Fig. 6B-D), which results in a continuous decrease of the C_{30} αβ-hopane to sum of C_{31}-C_{35} αβ-hopanes ratio (Table 3). This result is consistent with well known data that bacteriohopanepolyols (which are main sources of homohopanes) owing to the presence of functional groups in their molecules are bounded into macromolecular matrix during very early diagenesis by a weak (sulfur) and strong covalent (ether) bonds (Mycke *et al.*, 1987; Sinninghe-Damsté *et al.*, 1995; Köster *et al.*, 1997; Farrimond *et al.*, 2003). On the other hand, C_{30} homologues such as C_{30} hop-17(21)-ene, diploptene and C_{30} neohop-13(18)-ene do not possess hydroxyl groups and consequently mostly remained free in the soluble OM or have been weakly adsorbed on the macromolecular matrix. Therefore, with increasing of the thermal stress bounded homohopanes are released due to the cleavage of covalent (mostly ether) bonds which resulted in decrease of C_{30} αβ-hopane/ΣC_{31}-C_{35}(S+R) αβ-hopanes ratio (Table 3; Fig. 6).

In general, chromatograms shown in Figure 6B-G and biomarker ratios such as C_{30} βα-hopane/C_{30} αβ-hopane, C_{31} αβ(S)-hopane/C_{31} αβ(S+R)-hopanes and C_{27} β-hopane/C_{27} α-hopane (Table 3) indicate that hopane distributions obtained at 280ºC show higher thermally maturity than those obtained at 330ºC and 350ºC. This result can be explained by the fact that at 280ºC no or very little OM cracking was observed and that hopane maturity ratios mainly reflect the thermal effect on the free and weakly adsorbed hopanes,

TABLE 3. Values of parameters calculated from distributions and abundances of hopanoids and steroids

Experimental conditions (ºC/h)	Unheated	280ºC/20h		330ºC/20h		350ºC/20h	
	Extract	Bit[a]	Oil[b]	Bit	Oil	Bit	Oil
C_{30} αβ-Hopane/ΣC $_{31}$-C_{35}(S+R) αβ -Hopanes	-	4.25	1.34	1.29	1.19	0.74	0.48
C_{30} βα-Hopane/ C_{30} αβ-Hopane	-	0.59	0.37	1.49	1.50	1.69	1.16
C_{27} β-Hopane/ C_{27} α-Hopane	-	0.38	0.27	0.75	0.77	0.94	0.85
C_{35} αβ-Hopane/C_{31} (S+R) αβ -Hopanes		0.28	0.39	0.23	0.28	0.22	0.33
% C_{27} Ster[c]	82	39	45	46	47	57	56
% C_{28} Ster[d]	10	23	19	22	21	18	18
% C_{29} Ster[e]	8	38	36	32	32	25	26
C_{27} βαα(R) -Sterane/C_{27} ααα(R) -Sterane	-	0.22	0.24	0.14	0.13	0.15	0.15

[a]Bit: Bitumen; [b]Oil: Free oil

[c]% C_{27} Ster = 100 x C_{27}5α(H)14α(H)17α(H)20(R)-Sterane/[Σ(C_{27}-C_{29})5α(H)14α(H)17α(H)20(R)-Steranes] in bitumen and free oil % C_{27} Ster = 100 x C_{27}-Ster-2-ene/[Σ(C_{27}-C_{29})-Ster-2-enes] in the extract of unheated sample

[d]% C_{28} Ster = 100 x C_{28}5α(H)14α(H)17α(H)20(R)-Sterane/[Σ(C_{27}-C_{29})5α(H)14α(H)17α(H)20(R)-Steranes] in bitumen and free oil, % C_{28} Ster = 100 x C_{28}-Ster-2ene/[Σ(C_{27}-C_{29})-Ster-2-enes] in the extract of unheated sample

[e]% C_{29} Ster = 100 x C_{29}5α(H)14α(H)17α(H)20(R)-Sterane/[Σ(C_{27}-C_{29})5α(H)14α(H)17α(H)20(R)-Steranes] in bitumen and free oil, % C_{29} Ster = 100 x C_{29}-Ster-2-ene/[Σ(C_{27}-C_{29})-Ster-2enes] in the extract of unheated sample

For other peak assignments, see legends of Figures 6 and 7.

present in the original, unheated sample. On the other hand, at 330ºC and 350ºC distributions of hopanes reflect the thermal maturity of the OM at the moment when bitumen and free oil were generated by cracking processes. During this thermal cracking process, hopanes are released from the macromolecular matrix. Since isomerisation processes leading to the formation of thermodynamically more stable compounds are faster on free than on bounded biomarkers (Radke, 1987; Peters and Moldowan, 1991; Farrimond *et al.*, 2003), hopanes which are released by cracking (at 330ºC and 350ºC) are enriched in compounds with thermodynamically less stable configuration in comparison to free and weakly adsorbed hopanes present in pyrolysis products at 280ºC, which resulted in decrease of maturity ratios with temperature increase (Table 3).

In accordance to previous results, the slightly higher maturity of free oil than of bitumen is also apparent from hopane maturity ratios at 280ºC and 350ºC (at 330ºC the values of parameters are almost equal).

Values of hopane maturity parameters indicate higher maturity of pyrolysates obtained at 350ºC than at 330ºC, as expected. The relatively low values of hopane maturity ratios in liquid pyrolysates (Table 3), compared to crude oils, could be partly attributed to the source material. Namely, it was shown that isomerisation processes require the presence of an acidic clay catalyst and that at the same maturity level OM from clay rich sources generally show higher values of biomarker maturity parameters than OM from carbonate/evaporite sources (Rullkötter and Marzi, 1988; Peters *et al.*, 2005). Moreover, it is well known that isomerisation processes in biomarkers require a long time.

Steroid biomarkers

Steroid distribution in the extract of unheated sample is characterized by a prevalence of C_{27} ster-2-ene. Other steroid type constituents are C_{28} 4-methylsterene, C_{28} ster-2-ene and C_{29} ster-2-ene (Fig. 7A). The presence of sterenes is consistent with detection of numerous sterols in cyanobacteria-dominated microbial mats (Jungblut *et al.*, 2009). Both algae and bacteria are considered as precursors of 4-methylsteroids (Peters *et al.*, 2005), therefore an estimation of the exact origin of C_{28} 4-methylsterene in the extract of unheated sample requires further investigations.

Bitumen and free oil obtained at the same temperature showed almost identical distribution of steroids; therefore, only mass chromatograms of free oils are shown in Figure 7, as representatives. Distributions of steroid biomarkers in liquid pyrolysates (Fig. 7B-D) are similar and characterized by the occurrence of C_{27}-C_{29} 5α(H)14α(H)17α(H)- and 5β(H)14α(H)17α(H)-steranes, whereas C_{27} ster-2-ene is identified as a minor component. A significant difference in steroid distribution between pyrolysates and the unheated sample extract suggests a maturity increase during the heating experiments. Although the liquid pyrolysates are dominated by C_{27} steranes among the steroid compounds, they contain a notably higher amount of C_{29} homologues (particularly those obtained at 280ºC) in comparison to the unheated sample (Fig. 7B-D; Table 3). Elevated contents of C_{29} steranes could be attributed to C_{29} sterols, *e.g.* β-sitosterol, detected in a relatively high amount in the alcohol fraction of unheated sample. This result is in agreement with the observation of Jungblut *et al.* (2009), who also reported C_{29} sterols as important constituents of cyanobacteria-dominated microbial mats.

The values of C_{27} βαα-sterane to thermodynamically more stable C_{27} ααα-sterane ratio decrease from 280ºC to 350ºC, indicating an increase of maturity. However, steranes with 14β(H)17β(H) configuration, as well as steranes having 20(S) configuration, both typical for crude oils, were not identified in any of the pyrolysates. This result is consistent with a well known fact that isomerisation processes in steranes, in addition to thermal stress, require a long (geological) time and the presence of an acidic clay catalyst (Peters *et al.*, 2005). On the other hand, despite the relatively low values of hopane maturity ratios (Table 3), hopanes with 17α(H)21β(H), as well as those with 22(S) configuration, both usually present in crude oils, were actually found in the liquid pyrolysates (Fig. 6; Table 3). This result is in agreement with the experimental data (Kvenvolden *et al.*, 1988; Rullkötter and Marzi, 1988) that isomerisations in hopanes require less energy than isomerisation processes in the ring and the side chain of steranes. Therefore, the artificial maturation performed on the microbial mat showed a good concordance with natural biomarker maturation processes.

CONCLUSIONS

The investigated immature microbial mat showed high liquid hydrocarbons generation potential.

Temperature of 280ºC is sufficient for the almost complete release of free, weakly adsorbed and occluded compounds, as well as for the degradation of labile fragments, rich in heteroatoms. No OM cracking was observed at this temperature.

At 330ºC the generation of free oil and bitumen is mostly related to OM cracking, with a transformation ratio of 60%. The highest yield of free oil was recorded at this temperature, although distributions of *n*-alkanes in bitumen and free oil obtained at 330ºC suggest an insufficient thermal stress for the uniform release of low and high molecular weight *n*-alkanes into free oil. However, at 350ºC the sufficient thermal conditions are achieved and the transformation ratio rises to 70%.

Rock-Eval maturity parameters, biopolymer and biomarker compositions indicate a significant increase of

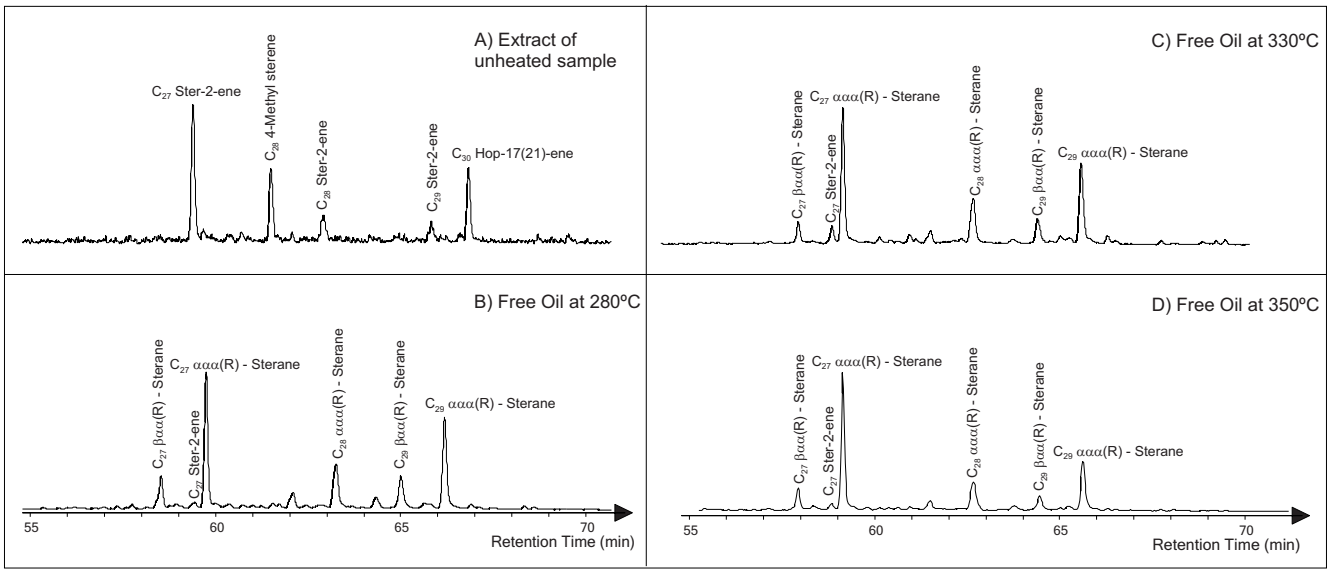

FIGURE 7. Mass chromatogram of steroids (*m/z* 217) in the A) extractable organic matter of unheated sample and B) free oils obtained at 280ºC, C) 330ºC and D) 350°C, respectively. βαα and ααα designate configurations at C-5, C-14 and C-17 in steranes; (R) designates configuration at C-20 in steranes.

the OM maturity during hydrous pyrolysis experiments. Artificial maturation of the microbial mat showed a good agreement with natural biomarker maturation processes.

Distributions of biomarkers in the extract of unheated sample and liquid pyrolysates confirm a domination of cyanobacteria in the microbial mat. The presence of long chain *n*-alkane homologues with a maximum at C_{26} and prominent C_{30} hop-17(21)-ene imply the contribution of sulfate reducing bacteria. Elevated contents of C_{29} hopanoids in liquid pyrolysis products could be attributed to the presence of methanotrophic bacteria.

Based on the obtained results, our future work will be focused on a detailed microbial study, usage of some other procedures (*e.g.* direct pyrolysis-GC-MS of original samples), as well as on performing the experiments using other microbial mat samples.

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