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A high resolution study of the chlorophyll and bacteriochlorophyll pigment distributions in a calcite/gypsum microbial mat

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Abstract

Application of a high resolution high performance liquid chromatography-mass spectrometry method to the study of a microbial mat system has permitted the identification of a greater number of pigments derived from green bacteria than reported in a previous study. Although the green bacteria found in the mat were identified as *Chloroflexus*-like, bacteriochlorophylls and bacteriophaeophytins *c* that can be attributed to Chloroflexaceae on the basis of literature reports account for less than 10% of the pigments derived from green bacteria in the mat. Analysis of the bacteriochlorophylls and bacteriophaeophytins *c* and *d* using atmospheric pressure chemical ionisation-liquid chromatography-tandem mass spectrometry reveals complex depth profiles, signalling inputs from a number of organisms. The pigment compositions provide evidence for green bacteria living in close proximity to the living cyanobacterial mat. Depth profiles of pigments derived from green, purple and cyanobacteria indicate that the remnants of mats present in the deeper part of the section contain a record dominated by signatures from anoxygenic photoautotrophs.

1. Introduction

Sedimentary tetrapyrroles provide a means to evaluate the composition of, and variations in, the primary producer community at the time a sediment sequence was deposited. Thus, variations in pigment abundance have been used to estimate changes in palaeoproductivity (Harris et al., 1996) and some distributions have provided clear evidence of contributions from specific groups of photoautotrophs (Ocampo et al., 1985; Verne-Mismer et al., 1990). A number of structures can reveal features of the palaeo-environmental conditions, for example photic zone anoxia from identification of pigments derived from anoxygenic photoautotrophs (Keely and Maxwell, 1993; Gibbison et al., 1995), and zooplankton grazing from recognition of phaeophorbides and steryl chlorin esters (Harradine et al., 1996).

Microbial mats are unique environments in which microbial growth on sediment surfaces allows the establishment of a community of organisms which essentially represents a discreet ecosystem inhabiting a narrow zone extending into the sediment on a scale of millimetres. Typically, the mat-building organisms are highly stratified and are dominated and controlled by the activities of phototrophic organisms. The positions and photosynthetic responses of the various organisms are closely controlled by steep and fluctuating gradients in physical and chemical variables, of which light, temperature, oxygen and sulfide concentration exert strong influences (see for example, Revsbech et al., 1983; Kühl et al., 1997; Wieland and Kühl, 2000; Epping and Kühl, 2000). Within the photoautotrophic members of the ecosystem it is usual for the living mat to be dominated by cyanobacteria at the surface with purple sulfur bacteria, if present, occupying a lower level at the interface between the oxygen and sulfide gradients. The deepest layers of the living mat may be host to obligate anaerobes, the green phototrophic bacteria, which utilise

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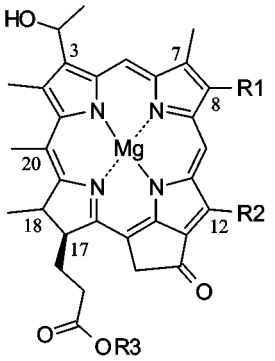
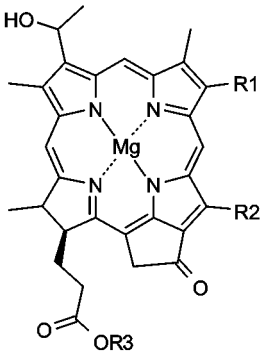


Bacteriochlorophyll *c*

R1: Et R2: Et
n-Pr Me
i-Bu

R3: Esterifying alcohol, see text

1



Bacteriochlorophyll *d*

R1: Et R2: Et
n-Pr Me
i-Bu
neo-Pent

R3: Esterifying alcohol, see text

2

their unique photosynthetic apparatus to harvest the highly attenuated and filtered light within this region (see Stal, 2001, for a more detailed review). Heterotrophic bacteria, particularly sulfate-reducing bacteria, within the mat are ultimately responsible for remineralisation of much of the organic carbon and for limiting the growth of the mat. Vertical growth may be stimulated by the constant trapping of small amounts of mineral grains at the surface of the mat or by more extensive coverage, for example by wind transport of sediments (Guerrero et al., 1993). Growth of the mat leads to expulsion of water from the lower layers and may be associated with growth of authigenic minerals. Consequently, photosynthetic activity in lower layers ceases to be viable and the organic content is subjected to the usual diagenetic processes through which recognisable signatures of the past community are preserved (see, for example, Keely et al., 1990; Keely and Maxwell, 1993).

Microbial mat systems exhibit very complex pigment distributions as a result of the wide diversity of pigments and the large number of possible degradation and transformation products (Villanueva et al., 1994). Although these environments may represent contemporary analogues of the ancient environments from which signatures of anoxygenic photoautotrophy have been detected, their pigment profiles are poorly characterised due mainly to their highly complex nature.

A calcite/gypsum microbial mat located in the Ebro Delta (South Catalonia, Spain) has been the subject of a previous study (Villanueva et al., 1994) where pigment distributions monitored by high performance liquid chromatography (HPLC) analysis were related to the organisms identified in the mat from microscopy. Although green non-sulfur bacteria, identified as *Chloroflexus*-like, were present and bacteriochlorophylls *c* were identified, detailed structural analysis of the pigments was not possible. Recent developments in the analytical techniques used to study pigment distributions (Airs and Keely, 2000; Airs et al., 2001a) permit more detailed analyses of such environments. Here, we report detailed HPLC and atmospheric pressure chemical ionisation-liquid chromatography tandem mass spectrometry (APCI-LC-MS/MS) analysis of the chlorophylls and bacteriochlorophylls from a second sample from the same calcite/gypsum microbial mat sampled at higher stratigraphic resolution.

2. Experimental

The section of calcite/gypsum microbial mat from les Salines de la Trinital, South Catalonia, Spain used in this study was a duplicate of that described previously (Villanueva et al., 1994) which had been stored at −20 °C since collection. The lithology was indistinguishable from the section analysed previously. The core was cut into 2 mm horizontal sections to a depth of 20 mm and two additional samples were taken at 24–26 and 28–30 mm depth Samples were extracted by sonication in acetone

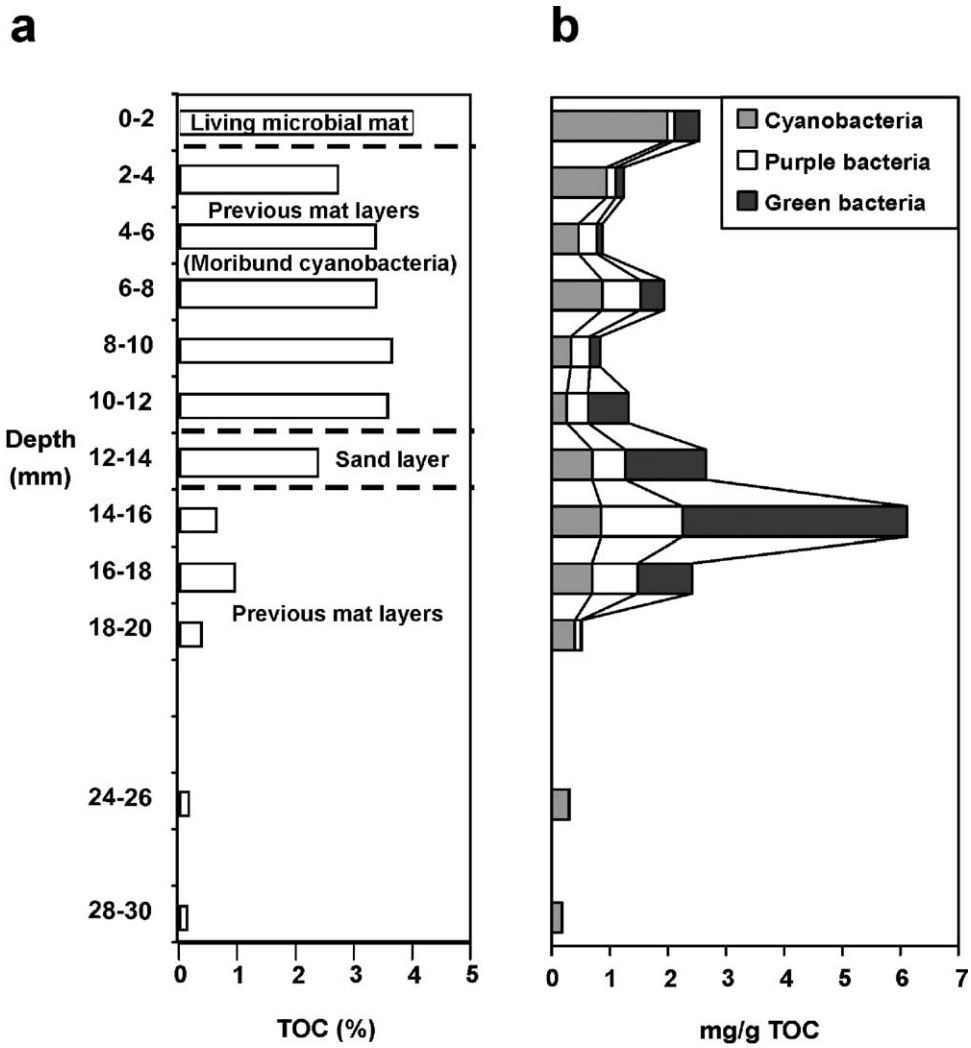


Fig. 1. Depth profiles showing (a) lithology with bars representing total organic carbon, (b) abundances of pigments derived from green bacteria (sum of all Bchl *c* and *d* derivatives), purple bacteria (sum of all Bchl *a* derivatives) and cyanobacteria (sum of all chlorophyll *a* derivatives).

(see Naylor and Keely, 1998) and dry extracts were methylated using diazomethane before analysis by reversed-phase HPLC as described by Airs et al. (2001a). Pigment concentrations were estimated by comparison of HPLC peak areas with those of standards. Standard concentrations were determined using the following extinction coefficients: chlorophyll *a* 88.15 l g^{-1} cm^{-1}, phaeophytin *a* 51.2 l g^{-1} cm^{-1}, phaeophytin *b* 31.8 l g^{-1} cm^{-1} (Jeffrey et al., 1997) bacteriochlorophyll *a* 59.4 l g^{-1} cm^{-1}, bacteriophaeophytin *a* 40.0 l g^{-1} cm^{-1}, bacteriochlorphyll *d* 98.0 l g^{-1} cm^{-1} and bacteriochlorophyll *c* 92.6 l g^{-1} cm^{-1} (Oleze, 1985). Bacteriophaeophytins *c* and *d* were quantified by reference to a standard of bacteriophaeophytin *c* prepared by demetallation (HCl, 2M) of bacteriochlorophyll *c*. Selected horizons were analysed using APCI-LC-MS/MS (for conditions see Airs et al., 2001a) with post-column addition of acid to improve the limit of detection for chlorophylls (Airs and Keely, 2000). Total organic carbon contents were measured after acid dissolution of carbonates. The total organic carbon contents of the sediments indicate the sandy layer (2.4%) marks a transition between high values above and low values below, with the two deepest samples having very low values (Fig. 1a).

3. Results and discussion

3.1. Identifications of pigments

The HPLC-photodiode array (PDA) chromatograms of the microbial mat extracts (plotted at the λ_{max}

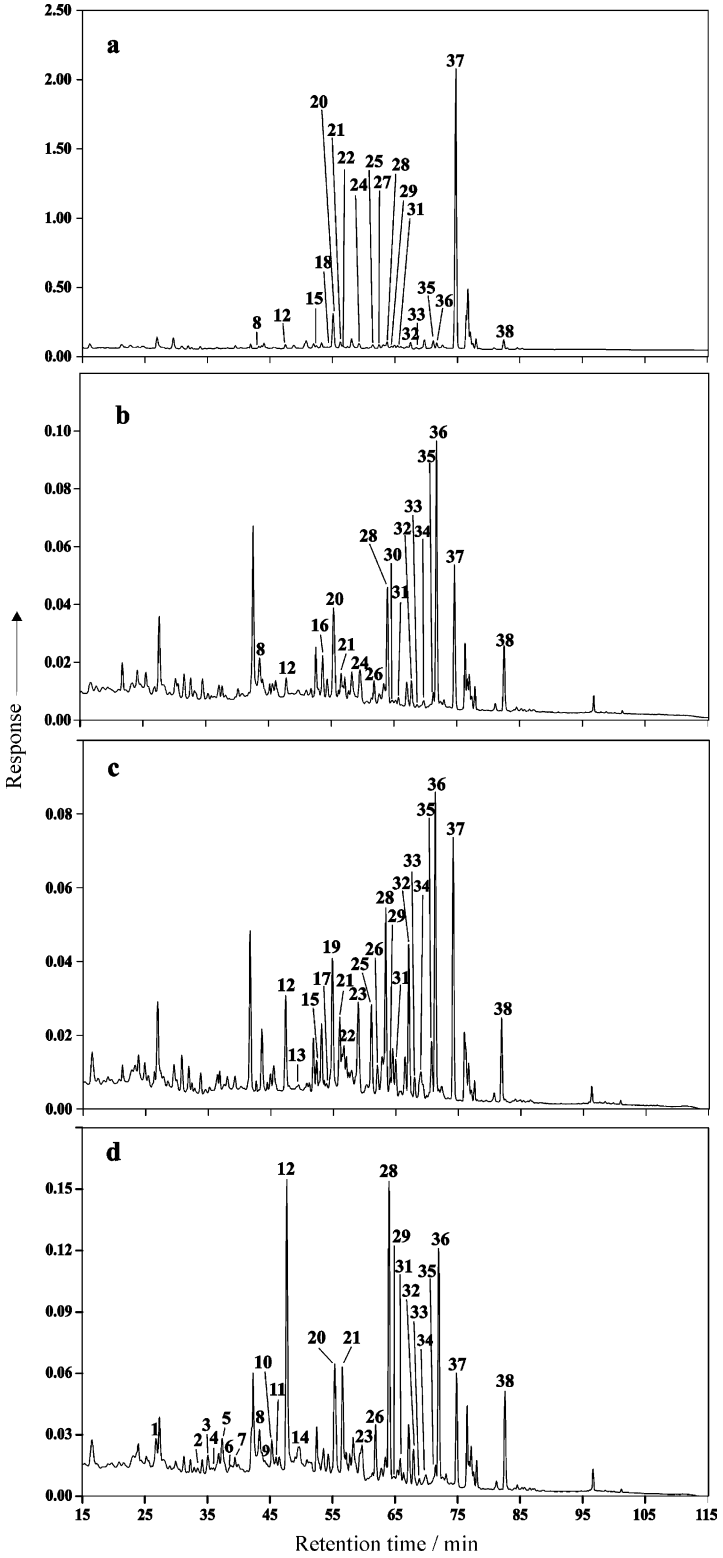


Fig. 2. HPLC-PDA chromatograms (300–800 nm plotted at the λ_{max}) of microbial mat extracts from (a) the living cyanobacterial mat, (b) 8–10 mm depth, (c) 10–12 mm depth and (d) 12–14 mm depth. Numbers refer to the peak identities detailed in Table 1.

between 300 and 800 nm) reveal complex distributions (Fig. 2a–d). A number of pigments commonly reported in sediments, and observed in the previous study (Villanueva et al., 1994), were identified on the basis of UV/vis spectra obtained on-line using photodiode array detection, HPLC retention time and LC-MS/MS analysis. These include chlorophyll *a*, phaeophytin *a*, phaeophorbide *a* and their pyro-derivatives and β-carotene, zeaxthanthin and myxoxanthophyll. The previous analysis of this microbial mat indicated that the cyanobacterium *Microcoleus chthonoplastes* was the main mat building organism and significant contributions from algae were not apparent. It appears, therefore, that the contributions from chlorophyll *a* and its transformation products are derived mainly from cyanobacteria. Pigments derived from purple sulfur bacteria were also identified (bacteriochlorophyll *a*, bacteriophaeophytin *a* and their pyro-derivatives, together with the carotenoids spirilloxanthin and rhodopin). The purple sulfur bacteria that inhabit microbial mats are essentially anaerobic photoautotrophs whose bacteriochlorophylls (mostly bacteriochlorophyll *a*) enable them to harvest long wavelengths of light that penetrate the cyanobacterial mat (Van Gemerden, 1983; Stal, 2001). Although bacteriochlorophyll *a* also occurs as an accessory pigment in green bacteria, it is only ever present in minor amounts. Clearly, therefore, bacteriochlorophyll *a* and its derivatives in the mat reflect a signature of the purple sulfur bacteria.

A number of components gave on-line UV/vis spectra characteristic of bacteriopigments from green bacteria. Bacteriochlorophylls *c* (structure **1**; 435, 665 nm) and *d* (structure **2**; 429, 660 nm) eluted between t_R 26 and 51 min and bacteriophaeophytins *c* (412, 667 nm) and *d* (407, 660 nm) eluted between t_R 44 and 71 min. The original investigation identified the green bacteria in the mat as *Chloroflexus*-like (Villanueva et al., 1994). Chloroflexaceae are green filamentous bacteria that are capable of anoxygenic photosynthesis (Pfennig, 1989) using bacteriochlorophyll *c* (*Chloroflexus* and *Oscillochloris*), or bacteriochlorophyll *d* (*Chloronema*—planktonic species only reported to occur in freshwater; Borrego et al., 1998) as their main light harvesting pigments. *Chloroflexus aurantiacus*, which inhabits thermal springs, is the most comprehensively studied species of the genus *Chloroflexus* having been successfully maintained in culture (Pierson and Castenholz, 1974a,b). Although bacteriochlorophyll *c* is the main chlorophyll in *C. aurantiacus*, traces of bacteriochlorophyll *d* have been reported to occur (Brune et al., 1987; Larsen et al., 1994, 1995), and are attributed to this being a biosynthetic precursor of bacteriochlorophyll *c* (Brune et al., 1987). A recent analysis of amplified 16S rRNA genes, using primers specific to the green non-sulfur bacteria, revealed high diversity among *Chloroflexus*-related organisms from microbial mats in Guerrero Negro, Mexico (Nübel et al., 2001). Notably, the only species enriched from the mat were reported to contain bacteriochlorophyll *c*. In the previous study of the Ebro delta microbial mat only three bacteriochlorophylls *c* were recognised and bacteriochlorophyll *d* was not detected (Villanueva et al., 1994). Thus, the presence of bacteriochlorophyll *d* as a major component of the pigments from green bacteria is unprecedented in a microbial mat environment. Although it was established that the bacteriochlorophylls *c* occurred as mixtures of homologues, structural assignment was limited to molecular mass determination (Villanueva et al., 1994). Given the substantial number of components resolved by HPLC in the present study, and the recognition of bacteriochlorophyll *d* and derivatives, a detailed examination of the pigments specific to the green bacteria was warranted.

3.2. Detailed analysis of bacteriopigments from green bacteria

The bacteriochlorophyll *c* and *d* derived pigments were examined using APCI-LC-MS/MS. Bacteriochlorophylls were detected as the bacteriophaeophytins in MS as a consequence of the application of post-column addition of acid to enhance response for the chlorophylls (Airs and Keely, 2000). Peak 1 ($t_R = 26.7$ min; Fig. 2), assigned as bacteriochlorophyll *c* (435, 666 nm), gave an ion at *m*/*z* 799 corresponding to MH^+. On MS/MS fragmentation, the protonated molecule gave rise to an abundant ion at *m*/*z* 595, resulting from the loss of 204 daltons (Da) and corresponding to the loss of farnesyl (Airs et al., 2001b; Airs and Keely, 2002). A bacteriophaeophytin *c* macrocycle at *m*/*z* 595 corresponds to a 36 carbon skeleton with two additional carbons extending the alkyl chains at C-8 and/or C-12 (see structure **1**) from the [Et,Me] configuration inherited by all tetrapyrroles from biosynthesis. In studies of the MS/MS behaviour of bacteriochlorophylls under APCI conditions, the alkyl side chains have been shown to fragment mainly by β-cleavage (Airs and Keely, 2002). The prominence of ions arising from the loss of 29 and 15 Da in MS^4 and MS^5, and absence of ions due to the loss of 43 Da, identifies the parent bacteriochlorophyll as farnesol esterified bacteriochlorophyll *c* [*n*-Pr,Et]. Peak 5 also gave UV/vis absorption maxima at 435 and 665 nm, in agreement with bacteriochlorophyll *c*, and gave an ion at *m*/*z* 867 in full MS. Fragmentation of this ion gave a prominent MS^2 product ion at *m*/*z* 595. The difference of 272 Da corresponds to loss of geranyl geranyl with proton transfer to the macrocycle (Airs et al., 2001b), identifying the esterifying alcohol as geranyl geraniol. Peak 14 gave a UV/vis spectrum consistent with bacteriochlorophyll *d* (429, 660 nm) and a protonated molecule at *m*/*z* 819. Fragmentation of *m*/*z* 819 in the ion trap induced formation of a product ion at *m*/*z* 567. The relative abundance of this

Table 1
Assignment of chlorin components in microbial mat

Peak number[a]	t_R (min)[b]	Main UV/vis absorption bands (nm)	Assignment[c]	$[MH]^+$[d]	Diagnostic fragment ions[e]	Esterifying alcohol[f]	[C-8,C-12] substituents[g]
1	26.2	435, 665	Bchl *c*	799	595	Farnesol	[*n*-Pr,Et]
2	32.9	427, 660	Bchl *d*	839	567	Geranyl geraniol	[*n*-Pr,Me]
3	34.5	429, 660	Bchl *d*	763	567	Tetradecanol	[Et,Et]/[*n*-Pr,Me]
4	35.6	428, 660	Bchl *d*	853	581	Geranyl geraniol	[*n*-Pr,Et]/[*i*-Bu,Me]
5	37.3	435, 663	Bchl *c*	867	595	Geranyl geraniol	[*n*-Pr,Et]/[*i*-Bu,Me]
6	38.6	427, 660	Bchl *d*	777	581	Tetradecanol	[*n*-Pr,Et]/[*i*-Bu,Me]
7	39.4	430, 660	Bchl *d*	791	595	Tetradecanol	[*i*-Bu,Et]/[*neo*-Pent,Me]
8	43.2	363, 606, 767	Bchl *a*	889	611	Phytol	[Et,Me]
9	44.1	430, 660	Bchl *d*	805	581	Hexadecanol	[*n*-Pr,Et] /[*i*-Bu,Me]
10	45.2	412, 667	Bph *c*	785	581	Farnesol	[Et,Et]/[*n*-Pr,Me]
11	45.9	430, 660	Bchl *d*	819	595	Hexadecanol	[*i*-Bu,Et] /[*neo*-Pent,Me]
12	47.4	412, 668	Bph *c*	799	595	Farnesol	[*n*-Pr,Et]/[*i*-Bu,Me]
13	49.4	413, 667	Bph *c*	813	609	Farnesol	[*i*-Bu,Et]
14	49.4	427, 660	Bchl *d*	819	567	Octadecanol	[Et,Et]/[*n*-Pr,Me]
15	52.4	409, 660	Bph *d*	839	567	Geranyl geraniol	[*n*-Pr,Me]
16	53.6	413, 668	Bph *c*	839	567	Geranyl geraniol	[Et,Me]
17	53.8	413, 664	Bph *c*	853	581	Geranyl geraniol	[*n*-Pr,Me]
18	54.1	417, 660	Bph *d*	853	581	Geranyl geraniol	[*n*-Pr,Et]/[*i*-Bu,Me]
19	54.8	407, 660	Bph *d*	763	567	Tetradecanol	[Et,Et]/[*n*-Pr,Me]
20	55.0	433, 664	Chl *a*	871	593	Phytol	[Et,Me]
21	56.2	412, 668	Bph *c*	867	595	Geranyl geraniol	[*n*-Pr,Et]/[*i*-Bu,Me]
22	56.6	409, 660	Bph *d*	777	581	Tetradecanol	[*n*-Pr,Et]/[*i*-Bu,Me]
23	58.9	409, 660	Bph *d*	791	595	Tetradecanol	[*i*-Bu,Et]/[*neo*-Pent,Me]
24	59.2	411, 665	Bph *c*	791	595	Tetradecanol	[*n*-Pr,Et]/[*i*-Bu,Me]
25	61.4	406, 660	Bph *d*	791	567	Hexadecanol	[Et,Et]/[*n*-Pr,Me]
26	62.0	405, 660	Bph *d*	791	567	Hexadecanol	[Et,Et]/[*n*-Pr,Me]
27	62.4	400, 665	Bph *c*	791	567	Hexadecanol	[Et,Me]
28	63.7	358, 527, 747	Bph *a*	889	611	Phytol	[Et,Me]
29	64.4	410, 660	Bph *d*	805	581	Hexadecanol	[*n*-Pr,Et]/[*i*-Bu,Me]
30	64.6	410, 664	Bph *c*	805	581	Hexadecanol	[Et,Et]/[*n*-Pr,Me]
31	65.4	406, 660	Bph *d*	819	595	Hexadecanol	[*i*Bu,Et]/[*neo*-Pent,Me]
32	67.4	406, 660	Bph *d*	819	567	Octadecanol	[Et,Et]/[*n*-Pr,Me]
33	68.4	409, 664	Bph *c*	819	567	Octadecanol	[Et,Me]
34	68.9	407, 660	Bph *d*	833	581	Octadecanol	[*n*-Pr,Et]/[*i*-Bu,Me]
35	71.0	409, 660	Bph *d*	847	595	Octadecanol	[*i*-Bu,Et]/[*neo*-Pent,Me]
36	71.6	358, 531, 749	Pbph *a*	831	553	Phytol	[Et,Me]
37	74.7	409, 665	Ph *a*	871	593	Phytol	[Et,Me]
38	82.4	411, 665	Pph *a*	813	535	Phytol	[Et,Me]

[a] Peak numbers refer to peaks in Fig. 1.
[b] Retention times not all obtained from a single sample.
[c] Bchl = bacteriochlorophyll; Bph = bacteriophaeophytin; Chl = chlorophyll; Ph = phaeophytin; Pbph = pyrobacteriophaeophytin; Pph = pyrophaeophytin.
[d] All chlorophyll derivatives appear as Ph MH^+ due to post column demetallation prior to sequential mass scanning (cf. Airs and Keely, 2000).
[e] Obtained by resonance excitation during LC-MS analysis.
[f] Assigned according to mass loss from MH^+ (cf. Airs et al., 2001a,b).
[g] Assigned according to fragment ions in MS^4 and MS^5 (Airs and Keely, 2002).

ion was much lower than those formed during MS/MS of peaks 1 and 5, but was similar to that seen for bacteriochlorophylls esterified by saturated straight chain alcohols (Airs and Keely, 2002). The mass of the alcohol fragment (252 Da) corresponds to an 18 carbon alkyl chain, presumed to be octadecanol from its known occurrence in phototrophic bacteria (Airs et al., 2001b, and references therein). The macrocycle at m/z 567 corresponds to an alkylation pattern of [Et,Et] or [*n*-Pr,Me] for bacteriochlorophyll *d* (35 carbon macrocycle). The

esterifying alcohols of the remaining bacteriochlorophylls and bacteriophaeophytins were assigned using the same approach (Table 1). The ions in MS^2 arising from loss of the esterifying alcohol from the bacteriochlorophylls and bacteriophaeophytins *c* and *d* form a homologous series, differing by 14 Da, consistent with additional CH_2 groups in the macrocycle. Where diagnostic ions were seen in MS^4 and MS^5 (cf. Airs and Keely, 2002), the alkyl side chains at positions C-8 and C-12 have been assigned (see Table 1).

The bacteriochlorophylls and bacteriophaeophytins *c* and *d* in the mat exhibit variation both in the degree of alkylation at C-8 and C-12, and in the esterifying alcohol, with farnesol, geranyl geraniol, tetradecanol, hexadecanol and octadecanol being present (Table 1). In the limited studies available, the bacteriochlorophylls of benthic green non-sulfur bacteria have been reported to occur with an alkylation pattern of [Et,Me] at C-8 and C-12 respectively. Octadecanol is the main esterifying alcohol of the C-17 propionic acid substituent, although phytol, geranyl geraniol, hexadecanol or octadec-9-en-1-ol also occur (Risch et al., 1979; Fages et al., 1990; Blankenship et al., 1995). Recent studies of natural populations of planktonic green non-sulfur bacteria have revealed a range of esterifying alcohols and C-8/C-12 alkylation patterns and the presence of both bacteriochlorophylls *c* and *d* (Borrego et al., 1998; Gich et al., 2001). The occurrence of bacteriochlorophylls *c* and *d* with extended alkylation at C-8 and C-12 in the Ebro delta mat suggests either that the pigments of the green non-sulfur bacteria present in this environment can, like those of the green sulfur bacteria and planktonic green non-sulfur bacteria, be modified in response to light limitation or that a highly diverse range of green bacteria are present in this mat environment (cf. Nübel et al., 2001).

3.3. Pigment profiles

As discussed earlier, the majority of chlorophyll *a*-derived pigments in this mat originate from cyanobacteria. The depth profile of the summed abundances of chlorophyll *a* and its transformation products reveal contributions from cyanobacteria in all of the sections of the mat examined (Fig. 1b). The contribution is greatest in the top 2 mm, coinciding with the position of the living cyanobacterial mat. Whereas the TOC values show relatively little variation within the upper 12–14 mm, the chlorophyll *a*-derived pigments show significant variations indicative of changes in the signature from cyanobacteria. This could have resulted from changes either in cyanobacterial productivity or in pigment preservation. In particular, laboratory studies of the short-term influences of environmental factors such as temperature (Wieland and Kühl, 2000) and irradiance levels (Epping and Kühl, 2000) have demonstrated the close coupling

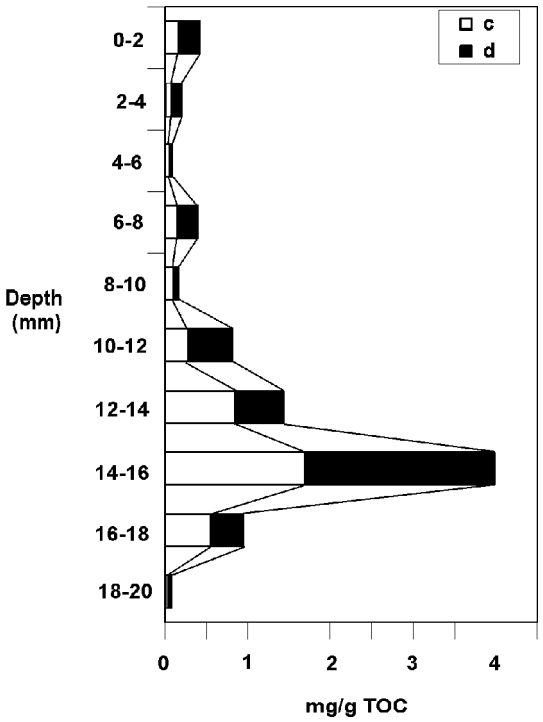


Fig. 3. Depth profiles of the sum of all bacteriochlorophyll *c* and *d* derived pigments.

of oxygen and sulfur cycling in microbal mats and the increased photosynthetic activity associated with elevated temperature and irradiance levels.

Pigments derived from purple bacteria are present in low abundance near the surface of the mat and show greater relative abundance deeper in the section, particularly at 6–8 and between 12 and 18 mm depth (Fig. 1b). The contribution to the pigment profile at 24–26 mm was very small and no contribution was detected at 28–30 mm depth. Thus, the maxima for the pigments derived from purple bacteria coincide with the regions of the sediment containing moribund cyanobacterial mats and the relative contribution from the purple bacteria is low in, and just below, the living cyanobacterial mat. It is likely, given the negligible influence of grazers in the mat environment (Villanueva et al., 1994), that the profiles reflect changes in the photosynthetic activity among the purple bacteria. Pigments derived from green bacteria are present in low abundance in close proximity to the cyanobacterial mat. The higher relative abundance in mat layers between 12 and 18 mm depth (Fig. 1b), indicates a greater relative contribution from anoxygenic photoautotrophs in past mat communities and the near or total absence of bacterial pigments in the deepest sections indicates that the environment was dominated by oxygenic photosynthetic organisms at the time the sediments were deposited. Clearly, therefore, changes in the extent of photic zone

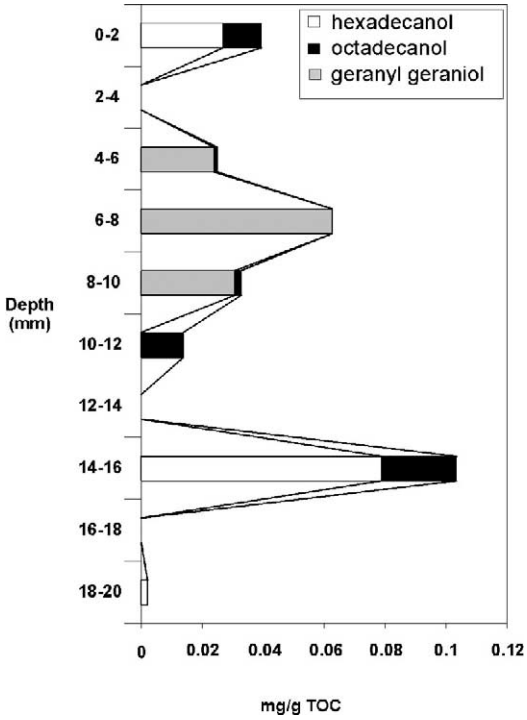


Fig. 4. Depth profiles of the sum of all bacteriochlorophyll *c*-derived [Et,Me] components having octadecanol, hexadecanol and geranyl geraniol esterifying alcohols.

anoxia are evident, most likely as a consequence of a changing mat environment. For example, if the mat experienced shading or submersion, changes in the intensity and quality of available light could influence the populations of all of the phototrophic prokaryotes in the mat. Also, if a high sulfide concentration influenced early mat communities, such an environment would be more tolerable in general to the obligate anaerobic green bacteria than to purple bacteria (Guerrero et al., 1987).

Within the top 20 mm where bacterial pigments occur, the summed abundance profiles of bacteriochlorophylls and bacteriophaeophytins *c* are similar to those of bacteriochlorophylls and bacteriophaeophytins *d* (Fig. 3). Although *Chloroflexus*-like organisms were the only green bacteria identified in this mat, bacteriochlorophyll *c* is not the major bacteriochlorophyll in the top 4 mm which contains the living section. It is likely, therefore, that the green bacteria represented throughout the mat are more diverse than was recognised previously on the basis of microscopy. The differences in the relative proportions of bacteriochlorophylls *c* and *d* in the deeper section of the mat indicates either different populations of green bacteria, or changes in physiology among the population. The only alkylation at C-8 and C-12 reported for benthic *Chloroflexus* is [Et,Me]. A depth plot displaying abundances of [Et,Me] bacteriochlorophylls and bacteriophaeophytins *c* throughout the

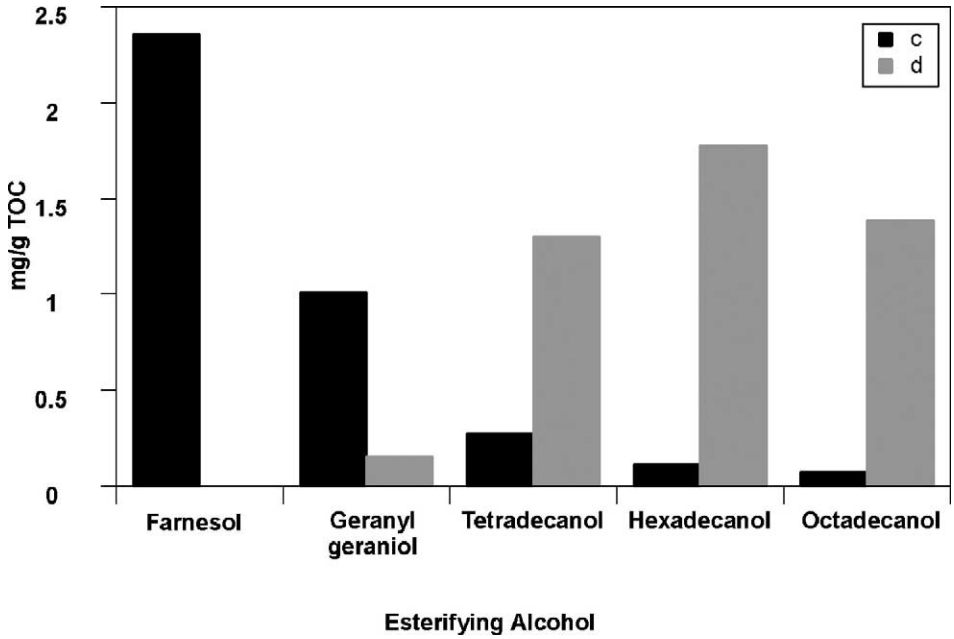


Fig. 5. Distribution of the esterifying alcohols among all bacteriochlorophyll *c*- and *d*-derived pigments.

mat (Fig. 4) reveals a maximum at 6–8 mm depth for geranyl geraniol esters, and at 14–16 mm depth for octadecanol and hexadecanol esters. It is interesting to note that while octadecanol and geranyl geraniol esters were both found in the presence of hexadecanol esters, they were never found to occur together. Furthermore, geranyl geraniol and octadecanol esters showed concentration maxima in sections where hexadecanol esters were not detectable. To date, cultures of green bacteria producing predominantly geranyl geraniol esterified bacteriochlorophylls *c* have not been reported. Experiments in which a culture of *C. aurantiacus* was grown anaerobically under different illumination intensities showed that the distribution of bacteriochlorophylls *c* can change with light intensity (Larsen et al., 1994). Specifically, the abundance of octadecanol esters was approximately constant at the light intensities used and the relative proportions of geranyl geraniol and hexadecanol esters varied but were detectable throughout the experiment. The distribution of bacteriochlorophylls *c* in the microbial mat differs markedly from those seen in cultures of *C. aurantiacus*. This difference between culture and the sedimentary profile could be due to variations caused by environmental factors other than light intensity, more extreme variation in light intensity than that used in laboratory experiments, changes in the wavelength range of penetrating light, differences in the pigment compositions of mat dwelling *Chloroflexus*-related species to those of *C. aurantiacus,* or contribution from other groups of phototrophic bacteria to the total bacteriochlorophyll *c* and *d* content in the mat. Based on literature reports of the bacteriochlorophylls of cultured strains of *C. aurantiacus*, the bacteriochlorophylls and bacteriophaeophytins *c* in the mat that are known to occur in Chloroflexaceae make up less than 10% of the total bacteriochlorophylls *c* and *d* derived pigment pool. It is probable, therefore, that other phototrophic prokaryotes are present, or that the filamentous bacteria found in the mat biosynthesise a wider range of bacteriochlorophylls than have been

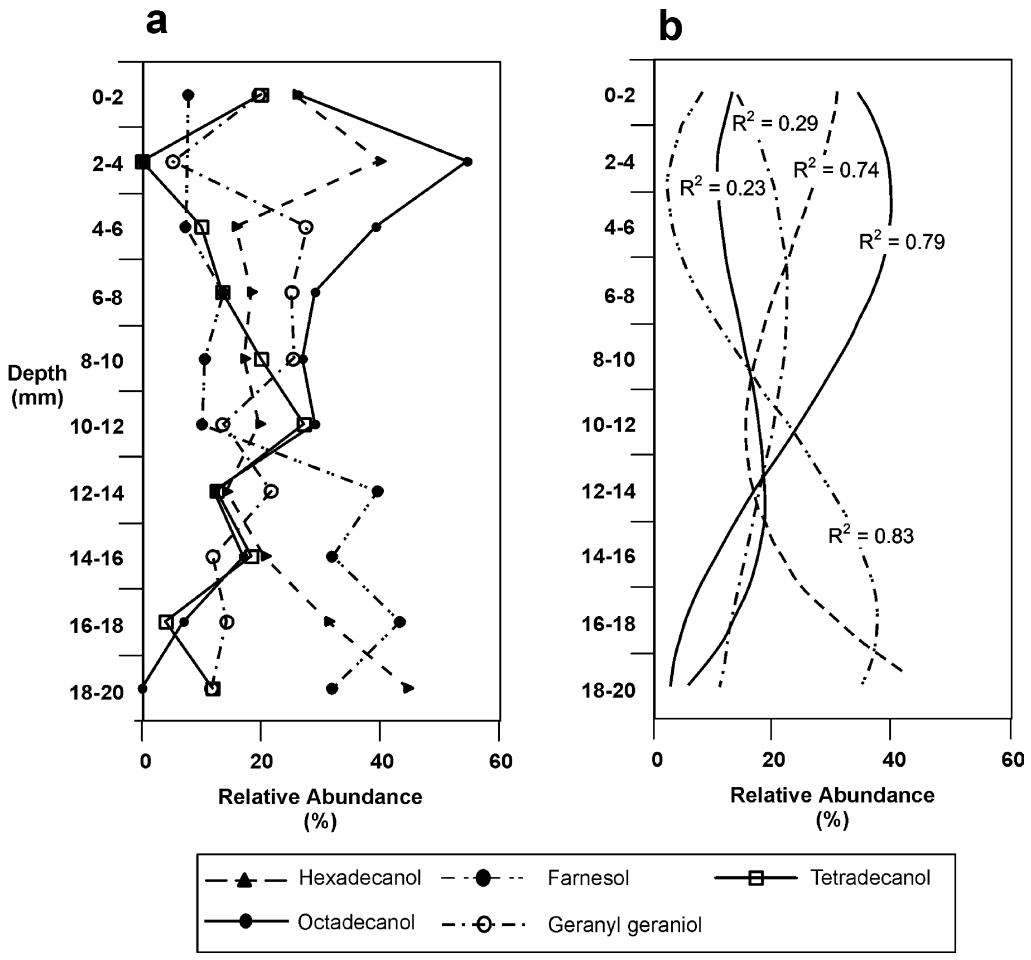


Fig. 6. Sum of all bacteriochlorophyll *c* and *d* derived pigments grouped according to the esterifying alcohol at position C-17 (a) Depth profiles (b) third order polynomial regression lines indicating coefficients of determination (R^2).

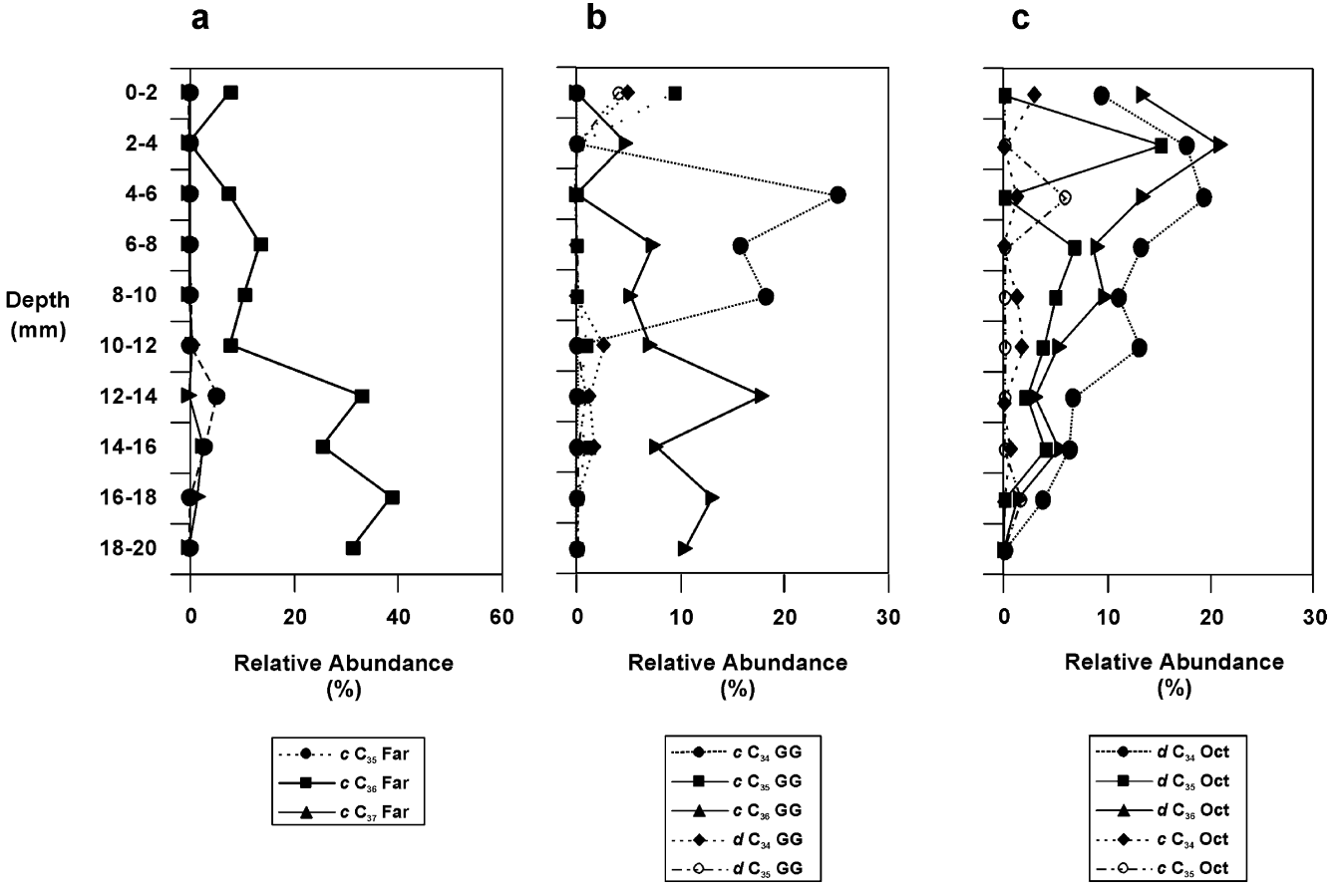


Fig. 7. Depth profiles for individual bacteriochlorophyll *c* and *d* components according to the number of carbon atoms in the macrocycle (differences resulting from the extent of alkylation at C-8, and C-12) and esterifying alcohol (Far = farnesol, GG = geranyl geraniol, Oct = octadecanol).

recognised previously in cultures of benthic Chloroflexaceae. Notably, farnesol has not been reported as an esterifying alcohol of the bacteriochlorophylls of Chloroflexaceae, but dominates the bacteriochlorophyll distributions of Chlorobiaceae (Caple et al., 1978; Nozawa et al., 1991; Otte et al., 1993), suggesting that these organisms may also be present. The previous study did not report the occurrence of Chlorobiaceae from microscopy, and limitation in analytical techniques prevented the assignment of any of the bacteriochlorophyll structures, preventing them being attributed to particular green bacteria (Villanueva et al., 1994). The chromatograms obtained in the present study contain late-eluting carotenoids (seen between peaks numbered 37 and 38 in Fig. 2) including a component exhibiting a retention time and on-line UV/Vis spectrum that are consistent with isorenieratene, an aromatic carotene common in Chlorobiaceae. This component was observed in the top nine samples, but not in the lowest three. The tentative identification could not be confirmed by LC-MS due to the low ionisation efficiency of carotenes under the APCI conditions used.

Although the total abundances of the five esterifying alcohols found in the mat are very similar, they are not evenly distributed between the bacteriochlorophylls *c* and *d* through the mat (Fig. 5). Hexadecanol and octadecanol are the dominant esterifying alcohols of the bacteriochlorophylls *d* whereas farnesol and geranyl geraniol are dominant in the bacteriochlorophylls *c* (Fig. 5). By contrast, tetradecanol is prominent in both bacteriochlorophylls *c* and *d* (Fig. 5). Depth plots of the relative abundances of the structures containing the different esterifying alcohols show a major change from octadecanol-dominated in the top section to farnesol-dominated below 11 mm depth (Fig. 6a). Third order polynomial fits to the depth profiles gave good coefficients of determination for farnesol, octadecanol and hexadecanol esters and poor coefficients for tetradecanol and geranyl geraniol esters (Fig. 6b). Thus, the fitted lines represent a simplified model for the changes in the relative contributions of farnesol, octadecanol and hexadecanol esters. A strong inverse relationship is evident in the profiles of farnesol and octadecanol (Fig. 6b). Given that farnesol esters in this mat are associated exclusively with bacteriochorophyll *c* and octadecanol esters mainly with bacteriochlorophyll *d* (Fig. 5), it is most likely that the profiles reflect changes in the relative proportions of two different green bacteria and not an adaptative response involving modification of both the esterifying alcohol and methylation at C-20. The profile for hexadecanol esters suggests either a mixed contribution from bacteriochlorophyll *c* and *d* producing organisms, or contributions from a third type of green bacteria. Examination of the individual profiles for bacteriochlorophylls *c* and *d* esterified with hexadecanol esters (not shown) revealed that the overall profile is dominated by the contribution from the bacteriochlorophyll *d*. Thus, it appears that a contribution from a third type of green bacteria is evident. The profiles for tetradecanol esterified bacteriochlorophylls show similar maximum abundances for the bacteriochlorophyll *c*- and bacteriochlorophyll *d*-derived components but exhibit marked differences with depth. The most likely explanations are either adaptative response with conversion of bacteriochlorophyll *d* to *c* by methylation, or competition between different species.

As well as showing different distributions for the bacteriochlorophylls *c* and *d*, the esterifying alcohols also show differences for the extended alkylation at C-8/C-12 (C_{34-37} macrocycles). Thus, hexadecanol and octadecanol are predominant for the C_{34} macrocycles of both bacteriochlorophylls whereas farnesol and geranyl geraniol are predominant for the C_{35} through to C_{37} macrocycles of bacteriochlorophyll *c*. Since it is known that adaptative response to light limitation influences either the nature of the esterifying alcohol (Airs et al., 2001b) or the extent of alkylation at C-8 and C-12 (Smith and Bobe, 1987), it appears likely that several distinct biological sources account for the different bacteriochlorophylls present in the mat.

Farnesol has only been reported to esterify bacteriochlorophylls of Chlorobiaceae. Accordingly, it is tempting to suggest that these organisms were significant contributors to the sedimentary organic carbon pool throughout the period in which the top 20 mm of the section was deposited. Geranyl geraniol has been reported as an esterifying alcohol of the bacteriochlorophylls from members of Chlorobiaceae, but only in minor amounts (Caple et al., 1978; Airs et al., 2001b), suggesting its presence in the mat to be linked to *Chloroflexus*-related organisms. It is entirely possible, however, that the distributions of bacteriochlorophylls identified in the microbial mat originate from green bacteria that have not been isolated in pure culture, and hence the characteristics of their bacteriochlorophylls are unknown.

Plots of the relative abundances of each of the individual bacteriochlorophyll homologues show distinct differences in their depth profiles. These differences are apparent both among homologues possessing the same esterifying alcohol and among components possessing the same number of carbon atoms in the macrocycle (Fig. 7). In general, the profiles for the macrocycles with fewer carbon atoms at C-8 and C-12 (for example [Et,Me] and [Et,Et]/[*n*-Pr,Me]) appear to be inversely correlated with the structures having extended alkylation (for example [*n*-Pr,Et]/[*i*-Bu,Me] and [*i*-Bu,Et]/[*neo*-Pent,Me]). Knowing that members of the green sulfur bacteria adapt their pigments as a result of changes in irradiance (Smith and Bobe, 1987; Airs et al., 2001b and references therein), it is tempting to suggest

that the differences observed may reflect, in part, changes over time in the irradiance levels and spectral cross-section that reached the green bacteria. Such changes would most likely be associated with changes in the temperature/light intensity experienced by the overlying phototrophic populations.

For example, shading by a dense population of purple bacteria would attenuate the light available for photosynthetic organisms underneath them and would restrict the wavelengths available for photosynthesis in the deeper layers. Although the purple bacteria harvest light at longer wavelengths (800–1050 nm, Imhoff, 1995), than the green bacteria (700–800 nm, Imhoff, 1995), the intensity of light available for photosynthesis would be greatly reduced by an overlying population of purple sulfur bacteria (Montesinos et al., 1983), and could induce the green bacteria to biosynthesise bacteriochlorophyll pseudo-homologues with a higher degree of alkylation, as reported for laboratory cultures grown under low light intensity (Smith and Bobe, 1987; Borrego and Garcia-Gil, 1995).

4. Conclusions

Improvements in HPLC and APCI-LC-MS methodology have permitted the separation and identification of a greater number of pigment components in a complex mat system than identified in a previous study. MS/MS studies permitted the identification of structural variations between the large number of bacteriochlorophylls *c* and bacteriochlorophylls *d* found in the mat. Analysis of the bacteriochlorophyll derived pigments reveals that those commonly associated with green filamentous bacteria contributed less than 10% to the total pigments derived from green bacteria, while green filamentous bacteria were the only green bacteria identified by microscopy in the original study of the mat. Depth plots of the remaining bacteriochlorophyll derived pigments reveal complex distributions, and indicate changing populations of green bacteria. The summed contribution of pigments derived from green, purple and cyanobacteria reveal that anoxygenic phototrophs were the main primary producers in the mat in times past.

Because of the overlap between the pigments biosynthesised by species of cultured phototrophic prokaryotes (Borrego and Garcia-Gil, 1994), and the occurrence in nature of distributions more complex than those seen in laboratory cultures (Borrego et al., 1998), studying the pigment distributions alone can point to the complexity of a system but cannot provide conclusive species assignments. Future studies will focus on the identification of species present, as well as the identifications of the pigments.

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