

Temporal variability of *Chlorobium phaeobacteroides* antenna pigments in a meromictic karstic lake

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ABSTRACT: The changes in the antenna pigment composition of *Chlorobium phaeobacteroides* growing in 2 meromictic basins of Lake Banyoles have been studied during a seasonal growth period. Changes involved both the increase in carotenoid content and the enrichment of the antenna in highly alkylated bacteriochlorophyll *e* (BChl *e*) homologs. Interestingly, the homolog composition at the end of the growth period was similar to those measured in laboratory cultures incubated at low light intensity, which are characterised by high amounts of BChl *e* homologs possessing larger alkyl substituents at the C-8 position of the macrocycle (i.e. isobutyl and neopentyl). Furthermore, differences in the vertical distribution of both pigments were also observed. Seasonal variation in the antenna pigment composition could be interpreted as a response to gradual changes in light regime during growth, mainly caused by self-shading and by the development of an overlying population of the purple sulphur bacterium *Chromatium minus*, which acts as a biological filter for light. The increase in both antenna pigments is discussed on the basis of recent findings on the spectral properties of BChl *e* and carotenoids and the role in the light adaptation processes is accordingly interpreted.

KEY WORDS: Bacteriochlorophyll *e* · *Chlorobium phaeobacteroides* · *Chromatium minus* · Isorenieratene · Light adaptation · Photosynthetic sulphur bacteria

INTRODUCTION

Occurrence of photosynthetic sulphur bacteria in nature is coupled to the simultaneous presence of light and sulfide (Pfennig 1978). In aquatic environments, opposite gradients of oxygen and sulfide generate oxic-anoxic boundaries where photosynthetic bacteria grow, forming coloured blooms (Pfennig 1989). These communities have been studied extensively in stratified lakes (van Gemerden 1967, Takahashi & Ichimura 1968, 1970, Trüper & Genovese 1968, Cohen et al. 1977, Parkin & Brock 1980a,b), including the Banyoles lacustrine system (Guerrero et al. 1980, 1985, 1987, Pedrós-Alió et al. 1983, 1984, Montesinos & Esteve 1984, van Gemerden et al. 1985, Garcia-Gil & Abella 1992, Garcia-Gil et al. 1993). Most of these studies have stressed the capacity of green photosynthetic sulphur bacteria, especially the brown species, to thrive

under low light intensities. However, there is little information concerning the response of these bacteria to low light intensities in natural conditions (Abella et al. 1980, Montesinos et al. 1983).

Sulphur bacteria usually develop under dim light of limited wavelengths (550 to 650 nm) (Parkin & Brock 1980a,b, Vila & Abella 1993). In these conditions, brown species of green sulphur bacteria become dominant in the photosynthetic community (Vila & Abella 1994, Vila et al. 1996, Borrego et al. 1997). This dominance has been attributed to the capacity of these species to adapt their pigment content to prevalent light conditions (Repeta et al. 1989, Overmann et al. 1992, Ormerod et al. 1993). Furthermore, it has been suggested that the enrichment of the antenna in highly alkylated homologs might be useful during episodes of light limitation (Smith & Bobe 1987, Borrego & Garcia-Gil 1995, Borrego et al. 1997).

We investigated the time-depth distribution of the antenna pigment composition in 2 populations of the

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brown photosynthetic sulphur bacterium *Chlorobium phaeobacteroides*. The main objective was to study the natural adaptation of the photosynthetic apparatus to prevalent light conditions. Our results show that natural populations of brown photosynthetic sulphur bacteria undergo substantial modifications in their antenna in response to light limitation. This adaptation involves both an increase in carotenoid content and an enrichment of the antenna in highly alkylated bacteriochlorophyll *e* (BChl *e*) homologs.

MATERIALS AND METHODS

Study area. Lake Banyoles is a karstic lake located in the NE of Spain (42° 07' N, 2° 45' E) and is composed of 6 main basins (Moreno-Amich & Garcia-Berthou, 1989). The study was carried out in 2 crenogenic, meromictic basins, basin C-III and basin C-IV, located at the northern area of the lake during the summers of 1994 and 1995, respectively. C-III is a circular, regular-shaped basin of 25 m in depth, whereas C-IV is an irregular-shaped basin, containing 3 sub-basins of 16, 18, and 19 m in depth. Meromixis is sustained in both the C-III and C-IV basins by a continuous influx of sulphate-rich water through 1 or several bottom springs (Garcia-Gil et al. 1996).

Field measurements. Samples were taken fortnightly at around noon from the deepest point of each basin. Physical and chemical parameters relevant for the characterisation of the water column (temperature, conductivity, dissolved oxygen concentration, pH, and redox potential, E_H) were measured *in situ* using a Hydrolab DS3 multiparametric probe. Downwelling light intensity was measured using a Biospherical Instruments QSP-170 underwater quantum meter. For sulfide analysis, 10 ml water samples were fixed *in situ* with NaOH and zinc acetate (final conc. of 10 mmol l⁻¹) in screw-capped glass tubes. Sulfide concentration was then determined in the laboratory following the leucomethylene-blue method (Brock et al. 1971). Water samples were pumped through a plastic hose that was connected to a weighted double cone at one end, which was located at the sampling depth (Jørgensen et al. 1979). This system was designed to cause minimal mixing of microstratification. The water samples were stored in 1 l plastic bottles preserved from direct sunlight. Samples were processed in the laboratory within 6 h of sampling.

Pigment analyses. For pigment analyses, 500 ml of field samples were passed through a 47 mm, 0.45 µm pore diameter membrane filters (Gelman Sciences) previously covered with a thin layer of 2.5% Mg CO₃ (Guerrero et al. 1985). Bacterial cells retained on the filter were then recovered and placed in a light-pre-

Table 1. Physical and chemical characteristics of the basins studied

	Basin C-III	Basin C-IV
Maximal depth (m)	25	19
Bottom springs (number)	1	3
Meromixis	+	+
Anoxic monimolimnion	+	+
Depth of chemocline (m)	18	13.75
Depth of O ₂ /H ₂ S (m)		
Spring	18	18
Summer	15.25	11.75
Temperature (°C)		
Max. mixolimnion	27	26
Min. monimolimnion	12	16
Conductivity (µS cm ⁻¹)		
Min. mixolimnion	1200	1200
Max. monimolimnion	2300	2300
pH		
Highest (mixolimnion)	7.5	8.2
Lowest (monimolimnion)	6.8	6.7
E_H (mV)		
Highest (mixolimnion)	+300	+300
Lowest (monimolimnion)	-350	-250
Max. [O ₂] mixo. (% saturation)	161	137
Max. [H ₂ S] monimo. (µmol l ⁻¹)	1000	300

served, glass tube containing an appropriate volume of acetone (Scharlau, HPLC grade), which was used as the extraction solvent. Photosynthetic pigments were extracted by a 2 min sonication at 4°C using a B-Braun Labsonic 2000 sonic disrupter. To avoid pigment photo-oxidation, all manipulations were performed under dim light at room temperature. Samples were stored at -30°C for 24 h to ensure complete extraction of pigments. Pigment extracts were then centrifuged at 2500 × *g* for 15 min and passed through a 0.2 µm pore-size Dynagard syringe filters. Chromatographic analyses were performed as described by Borrego & Garcia-Gil (1994). Peaks were identified from retention times and absorption spectra, which were recorded between 350 and 800 nm at 4 s intervals with a Waters 996 photodiode array detector coupled to an HPLC system. Chromatograms were monitored at selected wavelengths where the absorption by each pigment was predominant, i.e. 453 nm for Isorenieratene (Isr), 473 nm for BChl *e*, and 770 nm for BChl *a*. Pigment concentrations were calculated from the total area of the corresponding peaks in the HPLC traces after injection of appropriate standards obtained from pure cultures of *Chlorobium phaeobacteroides* (BChl *e* and Isr) and *Chromatium minus* (BChl *a*). The extinction coefficient of BChl *a* was taken from Takahashi & Ichimura (1968). An average extinction coefficient $E^{1\%}_{1\text{cm}}$ of 3000 was used for Isr (Schmidt 1980). Since

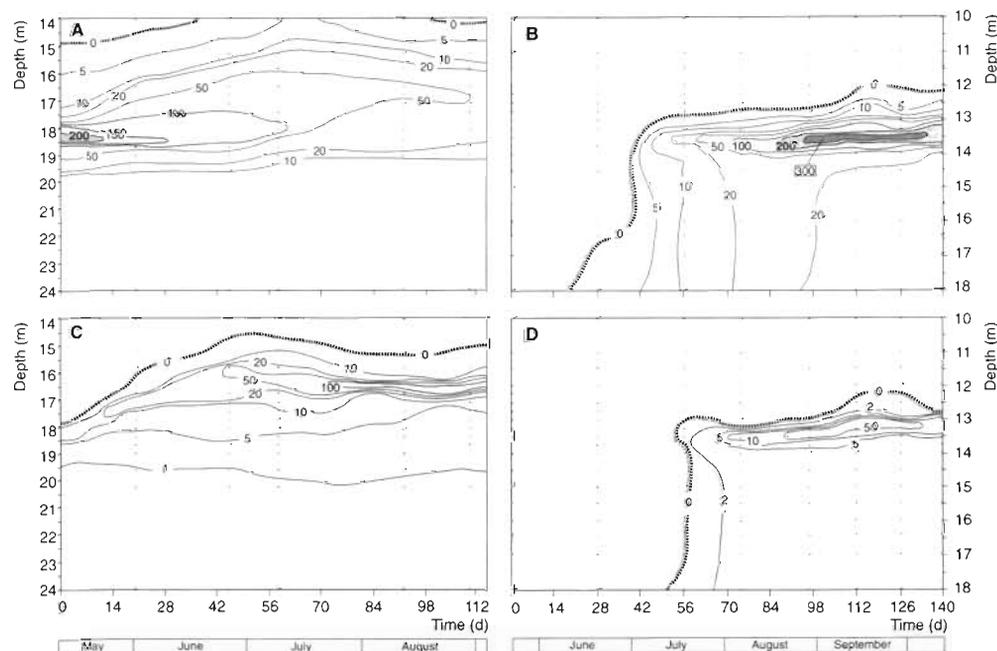


Fig. 1. Time-depth distribution of BChl *e* in (A) basin C-III and (B) basin C-IV during the study period. (C,D) The same for BChl *a*. Vertical dots represent sampling depths

there is no reliable extinction coefficient for BChl *e*, it was assumed to be equal to that of the related pigment chl *b* (Vernon 1960, Otte et al. 1993). Bacterial biomass (in mg fresh weight) was estimated from integrated pigment concentrations according to Montesinos (1982).

RESULTS

Physical and chemical properties of the basins

In both basins, thermal stratification began in early April with thermoclines spanning from 6 to 15 m in C-III and from 8 to 13 m in C-IV. Conductivity values were around 1200 and 2400 $\mu\text{S cm}^{-1}$ in the mixo- and the monimolimnion, respectively. Oxygen concentrations dropped drastically below the thermocline. The monimolimnion of C-III remained permanently anoxic, containing sulfide levels of up to 1 mmol l^{-1} . In basin C-IV, however, the monimolimnion became completely anoxic from July, with sulfide concentrations up to 300 $\mu\text{mol l}^{-1}$. The main physical and chemical features of the basins studied are compiled in Table 1.

An interesting feature observed in both basins was the upward displacement of the $\text{O}_2/\text{H}_2\text{S}$ boundary as stratification proceeded. It moved from 18 to 15.25 m and from 18 to 11.75 m in C-III and C-IV, respectively. This dynamic stratification behaviour strongly influences the development of photosynthetic sulphur bacteria mainly because of the progressive increase in light availability.

Population dynamics

In both basins, the photosynthetic bacterial assemblage was composed of *Chlorobium phaeobacteroides*, a brown-coloured species of the green sulphur bacteria group, and *Chromatium minus*, a purple sulphur bacterium member of the Chromatiaceae. In basin C-III, *C. phaeobacteroides* dominated the assemblage during the whole study period (Fig. 1A). Maximal BChl *e* concentrations of up to 200 $\mu\text{g l}^{-1}$ were measured at depths of 18 m during the initial phases of the study. As summer stratification proceeded, the *C. phaeobacteroides* population moved to a shallower position (17 m). This upward migration increased the intensity of the light at the bacterial plate from 0.02 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 18 m to 1.11 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 16.5 m, which corresponds to a change from 0.004 to 0.05% of the surface incident light. The integrated biomass of the *C. phaeobacteroides* population remained fairly constant until August, when it decreased considerably (Table 2). In basin C-IV, the distribution of *C. phaeobacteroides* was restricted to the period of complete anoxia, which lasted from July to September (Fig. 1B). In this same basin, the bacterial population was not well established until August, reaching a maximum of 300 $\mu\text{g l}^{-1}$ of BChl *e*. Furthermore, the bacterial plate did not change its position (13.5 m).

The carotenoid (mainly isorenieratene and β -isorenieratene) to bacteriochlorophyll ratio of *Chlorobium phaeobacteroides* populations also showed spatial and temporal variations (Fig. 2). As a general rule, higher amounts of Isr per unit BChl *e* were usually found on

Table 2. Location of the bacterial plate, light intensity reaching it, and main biological parameters of *Chromatium minus* and *Chlorobium phaeobacteroides* populations at the beginning and the end of the growing season. fw: fresh weight

	Basin C-III		Basin C-IV	
	11 May 1994	30 Aug 1994	20 Jul 1995	18 Oct 1995
<i>Chromatium</i> plate depth (m)	18.0	16.6	13.5	13.1
Incident light intensity at plate (% of transmission)	1.2	0.4	3.5	0.3
[BChl a] ($\mu\text{g l}^{-1}$)	12.7	125.5	2.28	4.8
[BChl a] (mg m^{-2}) ^a	9.9	51.4	1.0	25.6
[Biomass] (mg fw m^{-2}) ^b	1.5	5.2	0.2	3.0
<i>Chlorobium</i> plate depth (m)	18.3	16.8	13.5	13.4
Incident light intensity at plate (% of transmission)	0.04	0.05	3.5	0.07
[BChl e] ($\mu\text{g l}^{-1}$)	266.1	49.6	31.5	294.1
[BChl e] (mg m^{-2}) ^a	215.9	77.5	45.1	226.3
[Biomass] (mg fw m^{-2}) ^b	14.1	9.7	7.9	14.4

^aValues integrated within the bacterial plate
^bEstimated from integrated pigment values according to Montesinos (1982)

the bacterial plate. In basin C-III, however, the high Isr/BChl e ratios were found below the bacterial maximum.

The density of purple bacterial populations in lake Banyoles is often limited by the position of the O₂/H₂S boundary layer since it determines the light intensity that reaches sulfide-containing waters (Borrego et al. 1993). In basin C-III, *Chromatium minus* was only noticeable when the location of the oxic-anoxic interface reached its shallowest position (15 m), favouring an increase in the incident light from 0.2 to 3.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (from 0.16 to 0.40% of the surface incident light). Accordingly, the population maximum was observed at the end of the study period (Fig. 1C). The distribution of purple bacteria in basin C-IV was

Table 3. Light extinction coefficients (in m^{-1}) calculated from the PAR values (400 to 700 nm) in the mixolimnion and monimolimnion of basin C-III and C-IV at the beginning and the end of the growing season

Basin	Date	η_{mixo}	η_{mon}
C-III	11 May 1994	0.67	4.00
	30 Aug 1994	0.81	4.20
C-IV	25 May 1995	0.89	0.28
	18 Oct 1995	0.99	4.67

similar to that observed in C-III, with maxima located just below the oxic-anoxic boundary. The population maximum was measured during September, reaching a BChl a concentration of 50 $\mu\text{g l}^{-1}$ (Fig. 1D). In basin C-IV, growth of purple bacteria had severe effects on light extinction due to their effect as a biological filter for light (Table 3).

Spatial and temporal variations in the distribution of BChl e homologs

The HPLC method used provides high resolution in the separation of different photosynthetic pigments, in particular BChl homologs from green sulphur bacteria (Borrego & Garcia-Gil 1994). BChl e eluted as several peaks grouped into 2 subsets (Fig. 3). The main group (from 22 to 30 min) consisted of a 4-peak cluster assigned to the farnesyl-esterified BChl e homologs (BChl e_F). Different molecules eluted

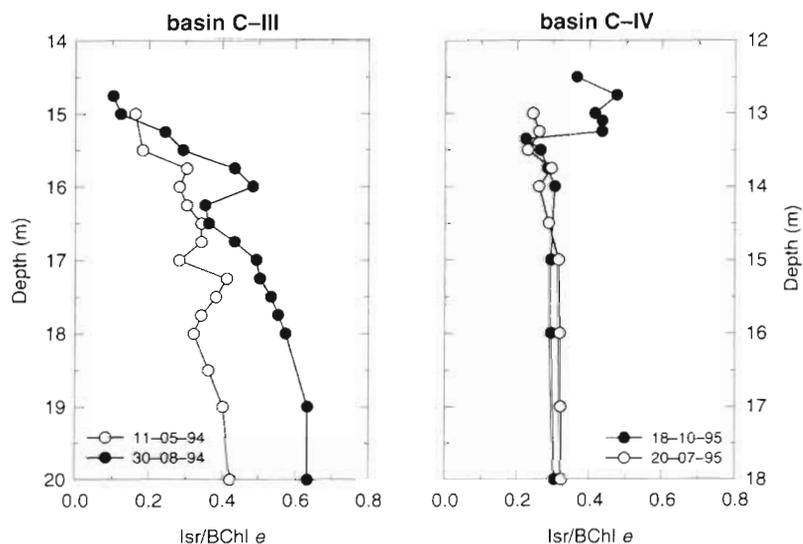


Fig. 2. Vertical profiles of Isr/BChl e ratios in the basins studied at the initial and final stages of the growing season. Dates given as d/mo/yr

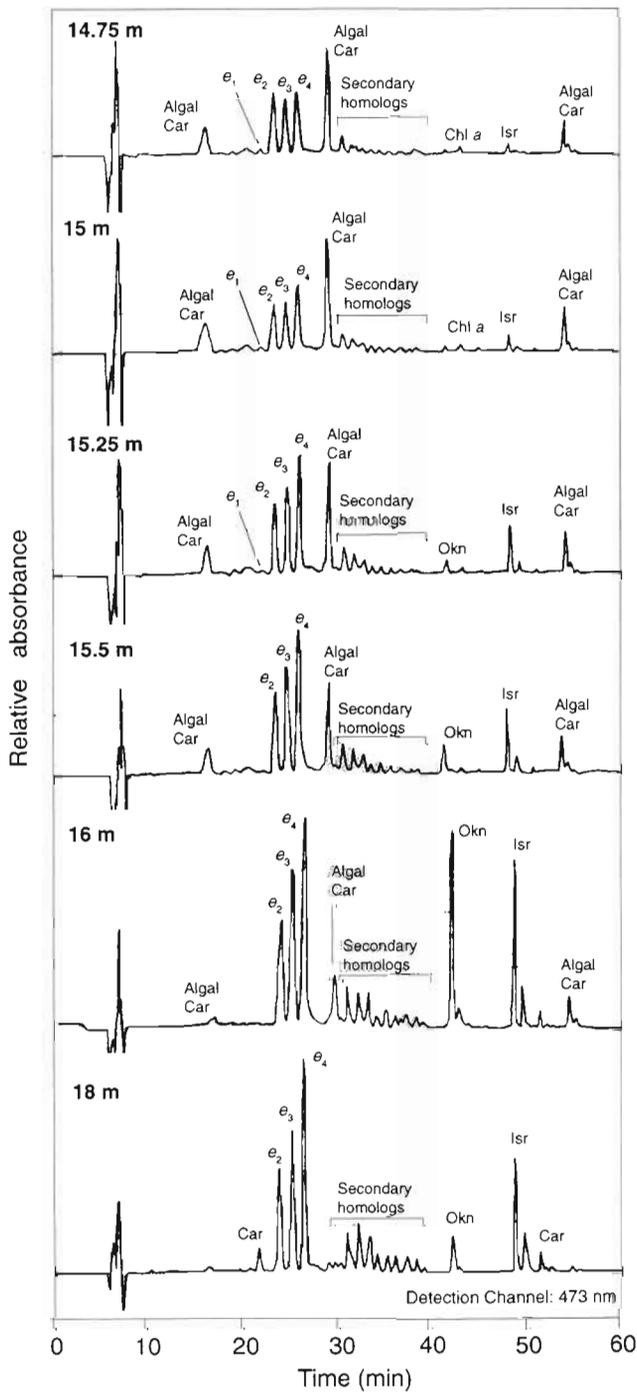


Fig. 3. Vertical profile of HPLC traces corresponding to the photosynthetic assemblage of basin C-III showing the progressive loss of BChl e_1 and the increase of BChl e_3 and BChl e_4 homolog content. Okn: okenone, main carotenoid (Car) from *Chromatium minus*

according to their increasing alkylation order at position C-8 of the macrocycle, that is: BChl e_1 , e_2 , e_3 , and e_4 correspond to [ethyl, ethyl], [propyl, ethyl], [isobutyl, ethyl], and [neopentyl, ethyl] BChl e_F , respectively

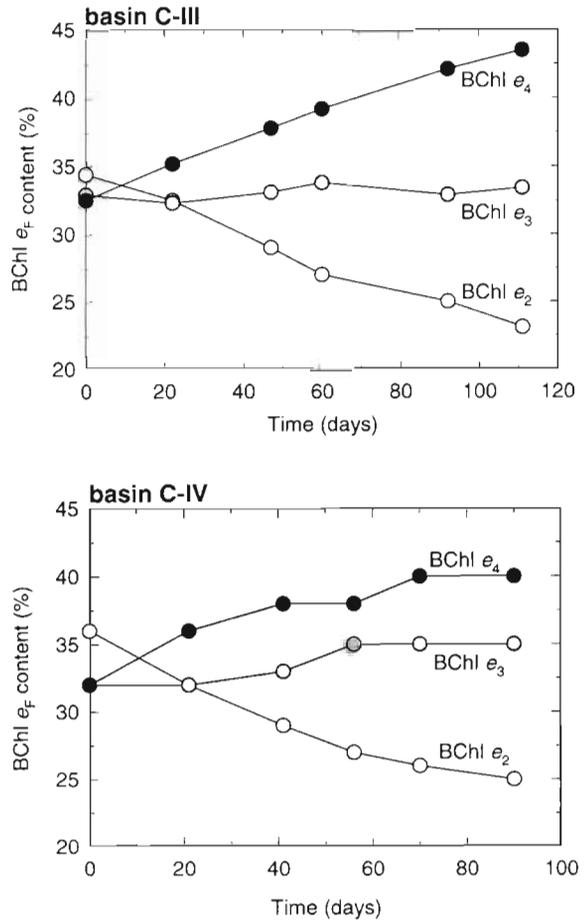


Fig. 4. Evolution of the relative content of the different BChl e_F homologs in the *Chlorobium phaeobacteroides* populations from the basins studied

(IUPAC numbering system, Smith 1994). The secondary set (from 32 to 40 min) was composed of several minor BChl e homologs, tentatively identified as end products of BChl biosynthesis with esterifying alcohols other than farnesyl (Caple et al. 1978). Carotenoids (mainly isorenieratene and β -isorenieratene) eluted as a 2-peak cluster at the end of the run (48 to 50 min).

HPLC pigment traces from natural samples were virtually devoid of BChl e_1 , whereas the amounts of the BChl e_2 , e_3 , and e_4 homologs exhibited both spatial and temporal changes. Vertical variations were observed in both basins. Concentrations of BChl e_3 and e_4 were higher at deeper positions, whereas small amounts of BChl e_1 in the uppermost layers were detectable (Fig. 3). Temporal variations in BChl e homolog distribution mainly affected BChl e_2 content, which decreased, and BChl e_4 , which progressively increased during seasonal growth from 30 to 44% and from 32 to 41% in C-III and C-IV, respectively (Fig. 4). In addition, the enrichment in BChl e_4 content was found to be related to the growth of the *Chromatium minus* popu-

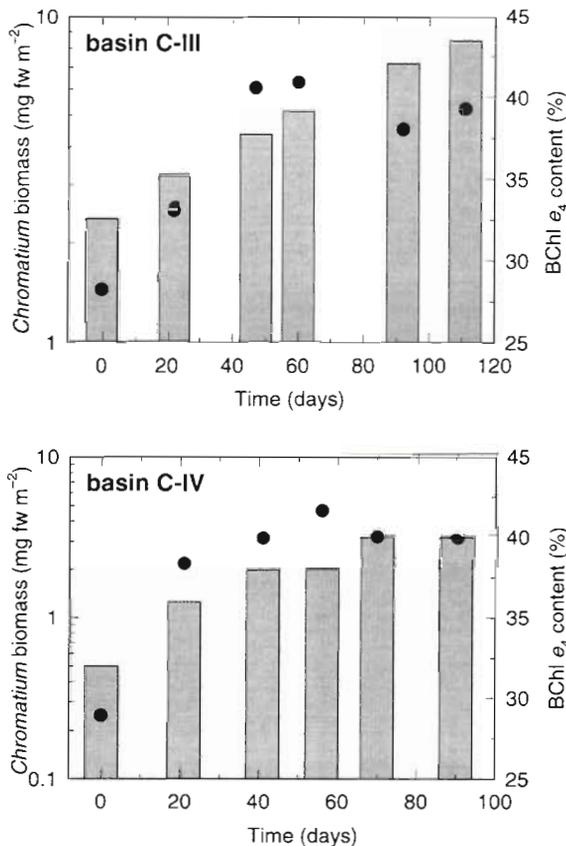


Fig. 5. Evolution of the *Chromatium* biomass (●) and the relative content of BChl e₄ (bars) during the study period

lation (Fig. 5). The maximum relative content of BChl e₄ was always found to be associated to the bulk of the bacterial plate, decreasing both above and below it (data not shown). Interestingly, elution patterns from natural *Chlorobium phaeobacteroides* populations

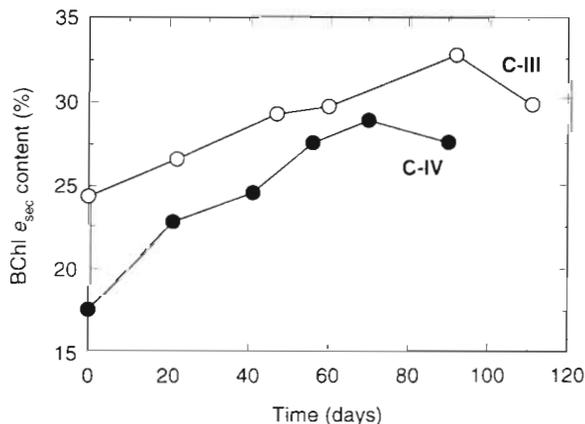


Fig. 6. Evolution of the relative content of BChl e secondary homologs (BChl e_{sec}) in the *Chlorobium phaeobacteroides* populations from the basins studied

with high amounts of BChl e₃ and BChl e₄ were identical to those obtained from pure cultures of the same species growing at low light intensity (0.5 μmol m⁻² s⁻¹) at the laboratory (Borrego & Garcia-Gil 1995). Elution patterns from secondary homologs did not show any qualitative variation although the BChl e_{sec}/BChl e_{total} ratio increased with depth and time (Fig. 3 and Fig. 6, respectively).

DISCUSSION

In stratified lakes, vertical distribution of photosynthetic bacterial cells is rather heterogeneous due to quantitative and qualitative modifications of light as a result of self-shading and selective light absorption (Guerrero et al. 1985, Vila et al. 1996). These alterations of light generate significant differences in the physiological status of cells, involving both photosynthetic activity and antenna pigment composition (Pedrós-Alió et al. 1983, Montesinos & Esteve 1984, Guerrero et al. 1985, van Gemerden et al. 1985).

The natural populations of *Chlorobium phaeobacteroides* reported here exhibited both quantitative and qualitative changes in their light-harvesting pigments throughout the water column as well as during seasonal growth. As stated above, vertical changes are mainly attributable to dramatic light extinction within the bacterial plate. Although some strains of *C. phaeobacteroides* are adapted to survive at extremely low light intensities (Overmann et al. 1992), the populations studied were strongly light-limited below the bacterial plate (<0.05% of the surface incident light). Under these ultra-dim light conditions, the antenna pigment composition remains unchanged since cell growth has been suggested as a key factor to trigger changes in BChl homolog distribution (Borrego & Garcia-Gil 1995). Accordingly, cells in these deeper, darker layers may constitute a residual, non-growing, depth-confined population in transit of sedimentation, and probably under different stages of degradation (Guerrero et al. 1985). This observation is supported by the increase of both the Isr/BChl e ratio and the content of BChl e secondary homologs (BChl e_{sec}) in the deeper layers of the basins studied.

During annual growth, the increase in bacterial biomass results in a progressive reduction of light intensity mainly due to self-shading. This effect is even greater if other photosynthetic populations (phytoplankton or purple bacteria) thrive in upper zones where they selectively absorb light (Vila & Abella 1994, van Gemerden & Mas 1995). In C-III and C-IV, especially during late summer, growth of overlying *Chromatium minus* populations critically shaded the *Chlorobium phaeobacteroides* plate. The progressive

attenuation of light intensity was the main factor responsible for changes in the antenna pigment content of *C. phaeobacteroides* cells, which were mainly characterised by a selective enrichment of BChl e_4 . During the final stages of the growth cycle, pigment content should reach an optimum composition for the prevalent light conditions.

The severe light limitation at the end of the study period also affected the content of BChl e_{sec} homologs. These pigment molecules are assumed to be end products of anomalous biosynthetic pathways produced by senescent cells (Caple et al. 1978). An increase in the relative content of these pigment forms suggests, therefore, a precarious physiological status of cells during the final stages of the growth cycle. However, these secondary homologs are likely to maintain their light-harvesting properties because the different chemical substitutions do not affect their absorption properties (Caple et al. 1978, Borrego & Garcia-Gil 1994).

It is of interest to consider whether or not the enrichment of the antenna in carotenoids and in highly alkylated homologs confers some advantages for success in environments where light conditions are critical. It has been generally accepted that Isr is responsible for the shoulder in the 520 nm region in the *in vivo* absorption spectrum of brown Chlorobiaceae, which provides an active window from which light can be selectively absorbed (Abella et al. 1980, Gorlenko et al. 1983, Montesinos et al. 1983, Pfennig 1989, Vila & Abella 1994). According to recent findings on carotenoid-depleted chlorosomes from *Chloroflexus aurantiacus* (Foidl et al. 1997, Frese et al. 1997) and *Chlorobium phaeobacteroides* (J. Aschenbrücker pers. comm.), carotenoids are suspected to be unessential for photosynthetic growth. Furthermore, it has recently been demonstrated that BChl e , and not Isr, accounts for the shoulder at the 520 nm region (Cox et al. 1998). Therefore, the idea that brown Chlorobiaceae use a carotenoid-based strategy to thrive in deep layers of stratified lakes should be reconsidered. However, our results argue against the negligible role of Isr in both light absorption and energy transfer to BChls. Furthermore, carotenoids have also been implicated in photoprotection of chlorophylls and membrane enzymes against photodynamic damage, by efficiently quenching highly reactive oxygen radicals, in phototropism, and in phototaxis (Liaaen-Jensen & Andrewes 1972). The protective function of carotenoids against oxygen radicals is, however, unlikely in the natural habitats where green photosynthetic bacteria usually develop since molecular oxygen is almost permanently absent. Further efforts should attempt to elucidate the importance of these pigments in the development of natural populations of Chlorobiaceae.

The increase in the content of highly alkylated BChl e homologs (BChl e_4 , [N,E] BChl e_F) results in a displacement of the *in vivo* BChl e Q_y absorption maxima towards longer wavelengths (Borrego & Garcia-Gil 1994). Although a red-shift from 720 nm to 725 nm of the Q_y maximum was measured in the populations studied, it is unlikely to produce any benefit in the overall absorption range since light reaching the bacterial population comprises a narrow waveband between 520 and 600 nm (Vila & Abella 1994). However, an increase in the amount of homologs with larger alkyl substituents can contribute to the formation of different types of BChl aggregates (Huster & Smith 1990). This modification would permit either the packaging of higher amounts of light-harvesting molecules inside the chlorosomes or an increase in the efficiency of antenna complexes in terms of energy transfer. The study of both chlorosomes containing high amounts of carotenoids and also highly alkylated homologs may provide further information about the role of different pigments in the adaptation of the brown species of green sulphur bacteria to extremely low light intensities.

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