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Variations in Chlorophyll and Carotenoid Pigmentation Among *Prochloron* (Prochlorophyta) Symbionts in Diverse Marine Ascidians

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Abstract

High-performance liquid chromatography (HPLC) was used to separate and quantify chlorophylls *a* and *b* as well as major carotenoid pigments present in freeze-dried preparations of diverse *Prochloron*- didemnid associations and in *Prochloron* cells separated from host colonies. Both chlorophyll *a* and *b* were consistently observed in each association. Chlorophyll *a* : *b* ratios ranged from 4.14 to 19.71. Ratio differences reflected species differences among didemnid hosts, which proved consistent over time and space. Generally good agreement was found between ratios determined in isolated cell preparations and in symbiotic colonies (*in hospite*). These values are 1.5 to 5-fold higher than ratios determined in a variety of eukaryotic green plants. The carotenoids in *Prochloron* are quantitatively and qualitatively similar to those found in various freshwater and marine cyanophytes from high-light environments. However, *Prochloron* differs from most cyanophytes in the absence of myxoxanthophyll and related glycosidic carotenoids. The consistent presence of chlorophyll *b* and individuality in carotenoid pigmentation render *Prochloron* biochemically distinct from cyanophytes, despite the fact that cells of both prokaryotic groups are often found in light-saturated environments.

Introduction

Among photosynthetic marine microorganisms, *Prochloron*, a genus of coccoid, non-motile, unicellular algae living as symbionts in or on tropical didemnid ascidians, is morphologically and biochemically unique (Lewin 1976). In cell structure it resembles cyanophyte algae, since no membrane-bound organelles or nuclei can be seen under the microscope, and its layered photosynthetic lamellae are not associated in chloroplasts. Nevertheless, *Prochloron* is distinguishable from cyanophytes by the absence of phycobilisomes and the presence of chlorophyll *b* (in addition to chlorophyll *a*) (Lewin and Withers 1975). In an earlier study based on spectrophotometric absorption scans and thin-layer chromatography (TLC), Withers *et al.* (1978) established the presence of chlorophyll *b* in *Prochloron* samples derived from didemnid hosts. They observed a similarity in the complement of carotenoid pigments between *Prochloron* and cyanobacteria, and confirmed the absence of phycobilisomes. In this study, we examined a broad range of *Prochloron* strains obtained from different hosts and habitats. Samples were collected in Palau, W. Micronesia, during 1979–83 and examined by high-performance liquid chromatography (HPLC). This study had two objectives: 1) to establish a set of quantitative data from which direct comparisons between eukaryotic plants, other prokaryotic algae and *Prochloron* could be made for ecological and taxonomic purposes, and 2) since *Prochloron* is almost always found in hosts which inhabit environments with saturating photosynthetically active radiation (PAR) levels, photosynthetic pigment characteristics in these high-light microorganisms were investigated and compared to other surface-dwelling microalgae, including cyanophyte bloom species.

Materials and Methods

Pigments of *Prochloron* cells, both associated in symbiosis and separated from their didemnid hosts, were extracted from a variety of samples; see Table I. For comparative purposes we included samples of freeze-dried and fresh cyanophytes and chlorophytes. Axenic clones of *Nostoc entophytum* and *Anacystis montana* were grown in the laboratories of the Culture Collection of Algae and Protozoa, Cambridge, U.K. The following algae were cultured at the laboratory in North Carolina under 400 μE m^{-2} s^{-1} PAR* illumination (cool white plus Gro-lux fluorescents). *Anabaena oscillarioides* (Cyanophyta) was grown in Chu-10 medium (minus combined nitrogen) (Chu 1942). *Chlorella vulgaris* and *Scenedesmus quadricauda* (Chlorophyta) were axenically grown in ASM-1 medium (Parker 1982). *Spirogyra* spp., and *Chlorococcum humicola* (Chlorophyta), obtained from Chowan River, N.C., were grown non-axenically in ASM-1 medium diluted 1:3. *Microcystis aeruginosa* (Cyanophyta) was collected from a surface bloom in the Neuse River, N.C., where it constituted over 95% of the phytoplankton biomass. *Codium decorticatum* and *Ulva lactuca* (Chlorophyta) were freshly sampled from Bogue Sound, N.C. Terrestrial spermatophytes were obtained from H.W.Paerl's garden. [Attempts were made to culture *Prochloron* sp. isolated from one of its hosts (*Lissoclinum patella*), following methods recently described by Patterson and Withers (1982) without success.]

Among the plant materials discussed, carotenoid and chlorophyll pigments were all examined by HPLC, and, in specified cases, also by TLC and scanning spectrophotometry. The pigments were extracted from centrifuged algal cells or freshly ground higher plants by sonication for 4 min in 90% v/v HPLC-grade acetone (Fisher) buffered with $MgCO_3$ to pH 8.0, and then allowed to stand at 4 °C in darkness for 30 min. For *Scenedesmus* and *Ulva* a second sonication was necessary in order to quantitatively remove the pigments, but for all freeze-dried samples only a single extraction was needed. Acetone volumes used for extraction were varied according to the quantities of plant material being analyzed. Dry weights of all plant materials were also determined. Extracts were centrifuged twice at 2600 RPM, and the supernatants were filtered through 25-mm diameter 0.2-μm porosity Nuclepore filters in preparation for both HPLC and TLC.

* PAR = photosynthetically active radiation (400–700 nm).

HPLC Methodology

For each HPLC analysis 20 μl of filtrate was injected into an Altex model 110-A liquid chromatograph equipped with dual pumps, a 25-cm long Ultrasphere ODS-18 reverse-phase column and a Hitachi 100–10 spectrophotometer (detector) having an 8-μl micro-flow cell. A linear solvent gradient program was used. The flow rate was 1.5 ml min^{-1}. The following mobile-phase gradient steps were employed: 0-7 min = 100% of a 90:10 methanol: acetonitrile mixture (solvent A), 7–11 min = a linear increase to 60% acetone and 40% of solvent A, 11–20 min = 60% acetone and 40% solvent A, 20–28 min = a linear decrease in 60% acetone until solvent A was again the sole solvent. Absorption peaks were graphically recorded and integrated, and total peak areas were determined with a Hewlett-Packard model 3390 A recording integrator-plotter. Integration analyses allowed for quantification both of specific pigments and of relative contributions of the individual pigments to total pigments detected.

Carotenoids and chlorophylls were identified and quantified by comparing their retention times and absorbance characteristics to known reference standards, which included: 1) purified chlorophyll *a* (from *Anacystis nidulans*) from Sigma Chemical Company, ii) purified β carotene, also from Sigma, iii) zeaxanthin, one sample from *Phormidium persicinum* (donated by Dr. D. Chapman, U.C.L.A.), a second from freeze-dried corn (*Zea mais*), and a third synthesized by Hoffman La Roche, Switzerland; iv) myxoxanthophyll, two samples respectively from *Anabaena oscillarioides* and *Microcystis aeruginosa* (from the Institute of Marine Sciences, U.N.C.), v) purified echinenone from Hoffman La Roche. Respective R_f values were also obtained by TLC separations (Paerl *et al.* 1983) and compared to corresponding values determined by other workers (Stransky and Hager 1970; Jeffrey 1975, 1981).

Chlorophylls *a* and *b* had absorption maxima at 663 nm and 642 nm respectively. Maximum absorption peaks for carotenoids were close to 475 nm; accordingly this wavelength was chosen for specifically detecting carotenoids. Together, chlorophyll *a* and carotenoids were effectively detected at 440 nm. We used molar extinction coefficients for chlorophylls *a* and *b* as published by Jeffrey and Humphrey (1975) and for pertinent carotenoid values as published by Davies (1976).

TLC Methodology

Two-dimensional TLC was used both for pigment purification and for identification of selected sam-

ples on Avicel microcrystalline cellulose-coated glass plates 25 × 20 × 20 cm (Analtech, Inc.). All TLC and HPLC reagents were Fisher HPLC or A. R. grade. The first-dimension separation was in n-propanol : ligroine (2.5 : 97.5 v/v), the second in ligroine : chloroform : acetone (70:30:0.5 v/v/v). The only deviation from Jeffrey's technique (1981) was in the use of ligroine instead of light petroleum, which was unavailable. Pigments from filtrates were initially concentrated by extraction and partitioning between equal volumes of petroleum ether and 10% aqueous NaCl at −20 °C for 20 min. The ether phase containing the pigments was collected and concentrated to 30 µl by volatilizing the ether under a stream of helium or argon. Any water remaining after this concentration step was further separated by centrifugation and discarded.

Concentrated pigment solutions were applied to plates. Clearly separated spots were localized and removed by carefully scraping them off the plates and preserving them in 90% acetone at −20 °C. (Reference R_f values were as follows: chlorophyll *a* = 0.843/0.239, chlorophyll *b*= 0.512/0.101). Pigments purified in this manner were processed further by HPLC for identification or purification.

Absorption characteristics of filtered extracts were determined with a Bausch and Lomb model 2000 U.V.-Vis. double-beam scanning spectrophotometer. We used quartz microcuvettes 1 cm in width.

Results

Evaluations of Analytical Procedures

Chlorophyll and carotenoid pigments remained well preserved in recently freeze-dried didemnid colonies and in isolated *Prochloron* samples. No degradation product of chlorophyll *a* or *b*, respectively pheophytin *a* (abs max = 667 nm) or pheophytin *b* (abs max = 655 nm), was detected by scanning spectrophotometry (400−700 nm) of extracts from HPLC separations. Quantitative values for chlorophylls *a* and *b* and for major carotenoids decreased less than 8% during 6 months of storage in the dark at −20 °C. From all samples except *Scenedesmus* and *Ulva*, complete pigment extraction was achieved by a single sonication. In these two algae, included for comparative purposes, approximately 70% of the pigment content was extractable after the first sonication and a further 20% was extracted after the second. Extraction efficiencies for chlorophylls and carotenoids were similar, ± 4.5%. The total elution time for all chlorophylls and carotenoids in the gradient described above was 19 min (Fig. 1). Baseline drift during a single run proved to be less than 3%. Replicate samples analyzed by HPLC during a 2-h period after pigment extraction and centrifugation had a standard error of ± 4.9%, indicating that extraction and storage procedures yielded pigment extracts stable for at least 2 h.

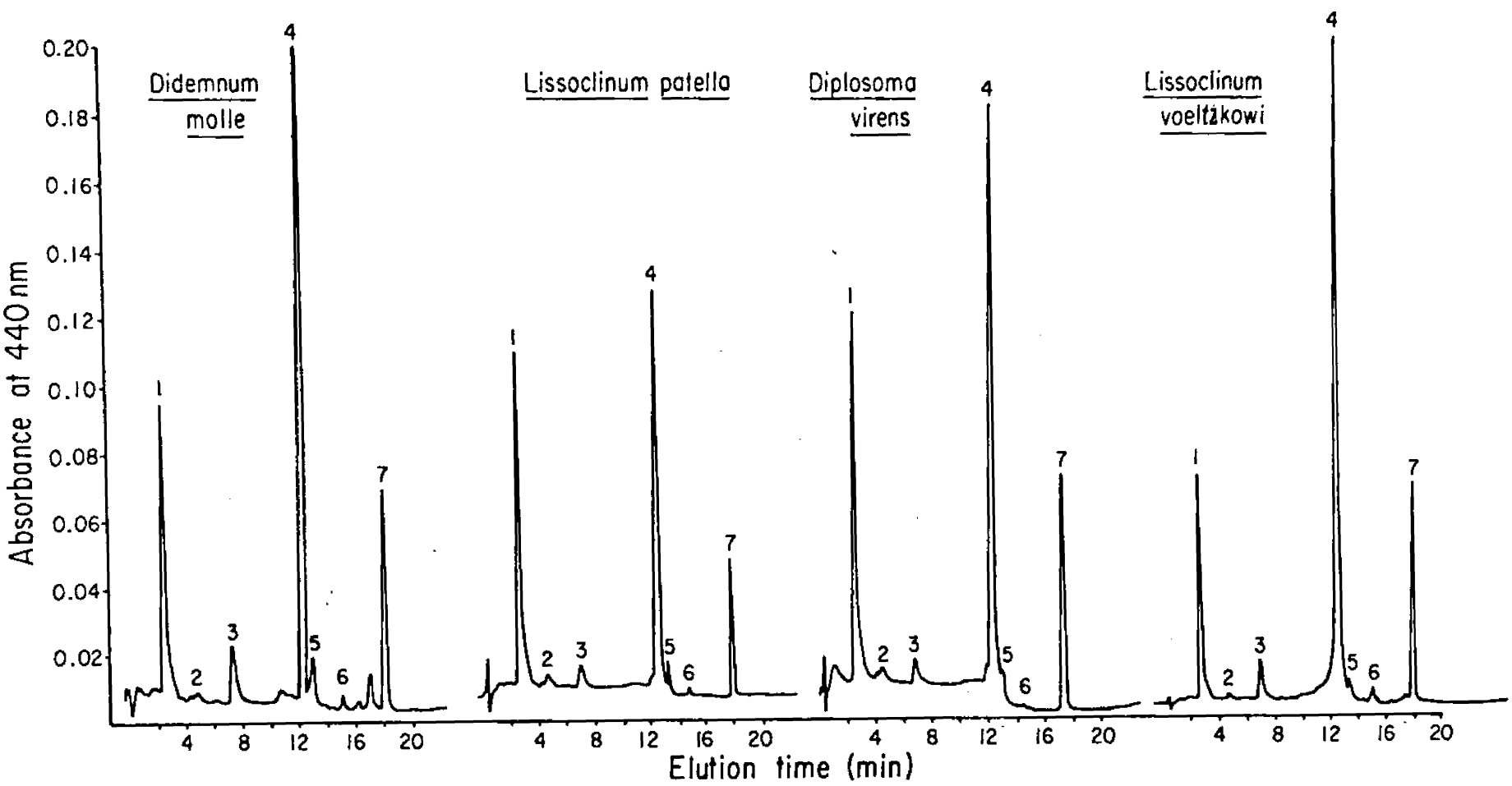

Fig. 1. HPLC elutions of *Prochloron* acetone extracts obtained from 4 separate didemnid hosts. This and subsequent (Fig. 2, 3) chromatographs were monitored at 440 nm in order to detect both chlorophylls *a* and *b* as well as major carotenoid pigments. The following absorption peaks were identified: 1 = zeaxanthin, 2 = cryptoxanthin, 3 = chlorophyll *b*, 4 = chlorophyll *a*, 5 = echinenone, 6 = unidentified carotenoid pigment, 7 = β carotene.

Chlorophyll Analyses

Table I summarizes the chlorophyll *a* : *b* ratios and specific chlorophyll *a* contents of the materials examined. The chl *a* : *b* ratios in whole colonies were very close to those in separated cells of *Prochloron*, indicating that no significant amounts of these pigments were contributed by other epiphytic or endozoic algae associated with the didemnids, and that, for chlorophyll determinations by HPLC, prior isolation of *Prochloron* is necessary only for determining pigments per unit of algal biomass.

The specific chlorophyll *a* content of *Prochloron* appears close to the mean value for most of the green plant materials examined, but the chl *a* : *b* ratios are generally higher (Table I). While in eukaryotic plants these ratios ranged from 1.76 to 4.45, those of *Prochloron* ranged from 4.14 to 19.71. *Prochloron* derived from diverse didemnid hosts revealed dramatic, but consistent, differences in chl *a* : *b* ratios (Table I). For example, samples from *L. voeltzkowi* and *D. molle* obtained during different years show consistent differences in chl *a* : *b* ratios. *L. voeltzkowi* sampled in 1982 yielded ratios of 8.89 to 10.41, compared with 9.10 to 10.23 from 1983 samples, while *D. molle* ratios ranged from 4.90 to 5.95 in 1982 and 4.08 to 5.30 in 1983. *D. virens* consistently yielded higher chl *a* : *b* ratios (14.05 to 15.23 in 1982 and 12.80 to 14.95 in 1983).

Carotenoid Analyses

The types of major carotenoid pigments found in *Prochloron* were in good agreement with previous data of Withers *et al.* (1978). The following major carotenoids were identified: zeaxanthin ($R_f = 0.380/0.340$), echinenone ($R_f = 0.860/0.415$), and β carotene ($R_f = 0.960/0.890$). A minor carotenoid, possibly cryptoxanthin ($R_f = 0.72/0.41$), was also consistently detected by both TLC and HPLC, but we had no reference standards for its confirmation (cf. Hertzberg and Liaaen Jensen 1966; Stransky and Hager 1970). Myxoxanthophyll, which is found in diverse cyanophytes (Goodwin 1980), was conspicuously absent from *Prochloron* (Figs 1, 2), a finding previously documented by Withers *et al.* (1978). In other respects the carotenoid pigment types in *Prochloron* (Fig. 1) proved more similar to those of cyanophytes (Fig. 2) than to those of eukaryotic green plants (Fig. 3). Carotenoids typically found in eukaryotes, including lutein, neoxanthin, violaxanthin and antheraxanthin (Hager and Stransky 1970, Jeffrey 1981), have not been detected in *Prochloron*. Likewise, the relative proportions of carotenoids, estimated from integrated peak data (Table II), indicate that in its carotenoid composition *Prochloron* more closely resembles cyanophytes than chlorophytes (Figs 1, 2, 3), and like cyanophytes, has a ra-

Table I. Average chlorophyll *a* : *b* ratios and chlorophyll *a* content of *Prochloron* (determined *in hospite* in all but 3 samples) and various eukaryotic plants.

Class and species	Source	Chl *a* : *b*	Chl *a* (mg g^{-1} dry wt)
Prochlorophyta			
Prochloron	in *Didemnum molle* (1982)	5.23	4.94
	in *Didemnum molle* (1983)	4.98	5.01
	from *Diplosoma similis* (1982)	4.14	6.32
	in *Diplosoma virens* (1982)	14.05	5.41
	in *Diplosoma virens* (1979)	13.20	5.03
	in *Diplosoma virens* (1983)	13.65	4.78
	in *Lissoclinum bistratum*	7.2	3.28
	in *Lissoclinum patella* (1983)	10.41	4.21
	from *Lissoclinum patella* (1982)	10.62	6.15
	in *Lissoclinum voeltzkowi* (1982)	9.29	5.05
	in *Lissoclinum voeltzkowi* (1983)	9.36	5.12
	from *Lissoclinum voeltzkowi* (1982)	10.41	7.05
	in *Tridemnum clinides* (1982)	19.71	4.77
	in *Tridemnum cyclops* (1982)	5.83	3.49
Chlorophyta	*Chlorococcum humicola*	2.25	7.41
	Codium decorticatum	2.09	7.52
	Scenedesmus quadricauda	1.96	6.05
	Spirogyra sp.	1.76	5.90
	Ulva lactuca	2.61	4.48
Spermatophyta	*Brassica oleracea*	3.22	4.25
	Spinacia oleracea	2.29	8.26
	Cichorium endivia	2.41	8.19
	Impatiens sp.	3.85	5.23

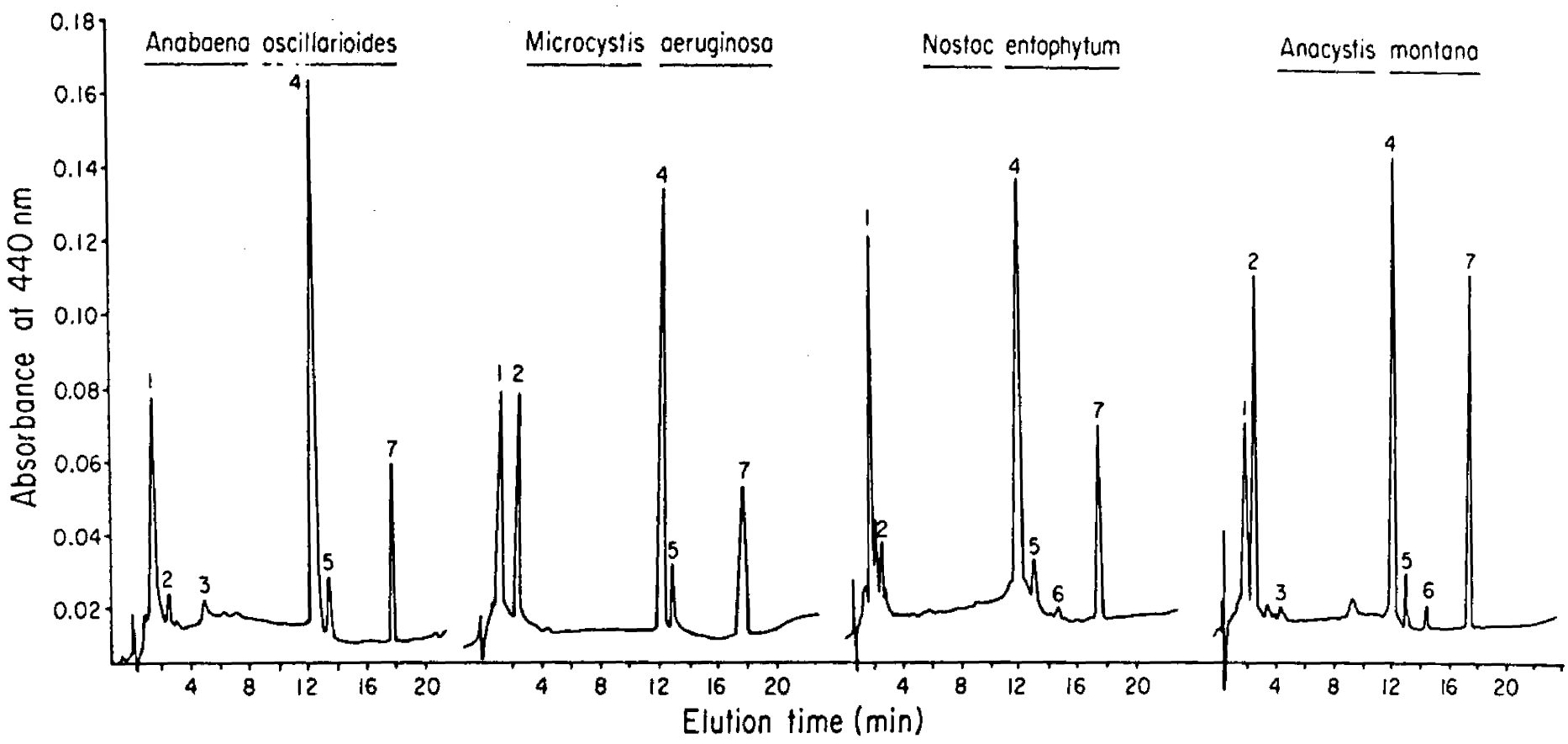

Fig. 2. HPLC elutions of surface-dwelling cyanophyte genera. The following absorption (at 440 nm) peaks were identified: 1 = myxoxanthophyll, 2 = zeaxanthin, 3 = cryptoxanthin, 4 = chlorophyll *a*, 5 = echinenone, 6 = unidentified carotenoid pigment, 7 = β carotene.

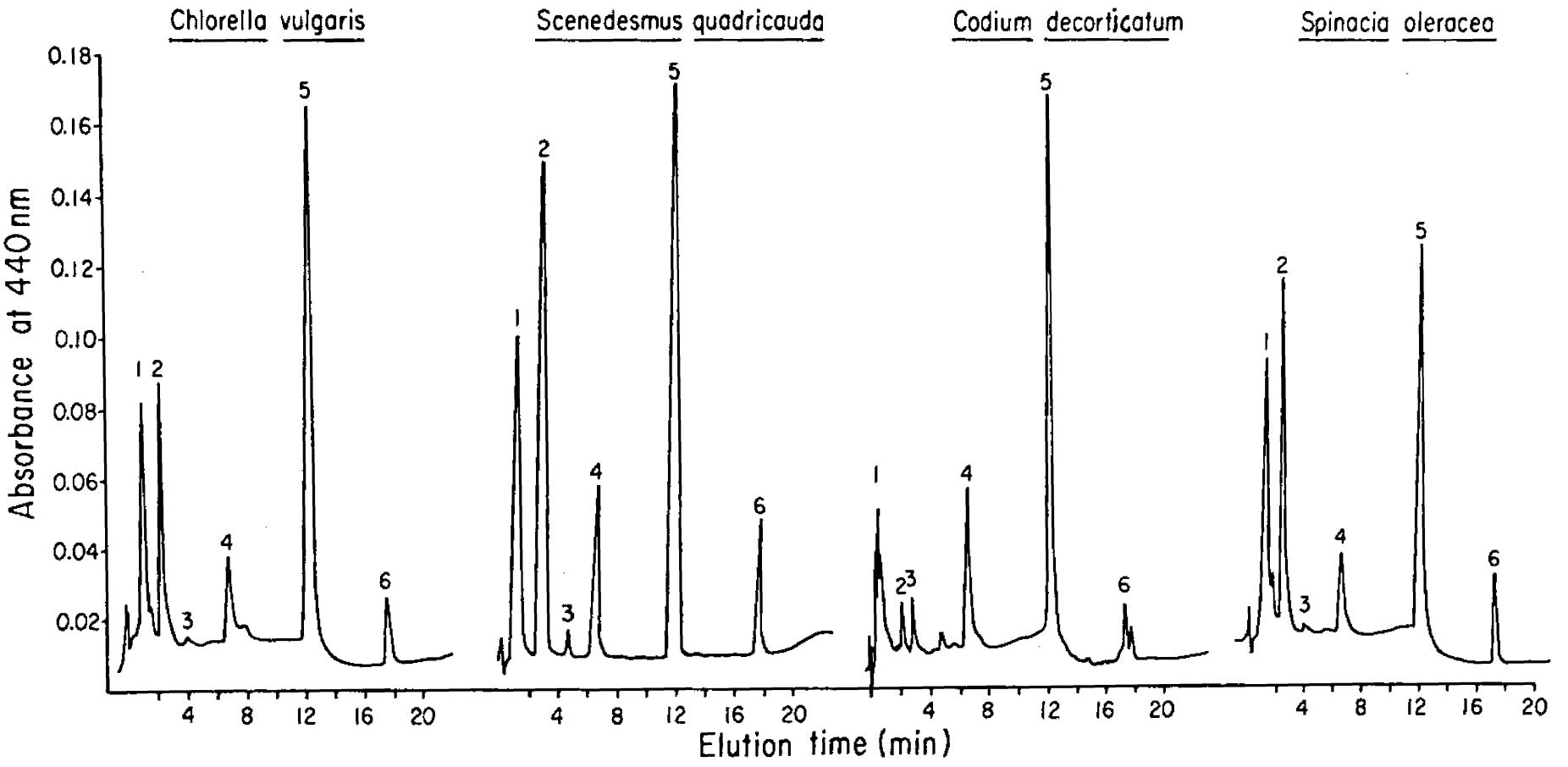

Fig. 3. Representative HPLC elutions of eukaryotic plants monitored at 440 nm. The following peaks were identified: 1 = lutein, 2 = zeaxanthin, 3 = unidentified carotenoid pigment, 4 = chlorophyll *b*, 5 = chlorophyll *a*, 6 = β carotene.

Table II. Relative proportions of major carotenoids in various *Prochloron* strains, as determined by HPLC.

Prochloron from:	Carotenoids (% of total) Zeaxanthin	Echinenone	Cryptoxanthin	β-carotene
Didemnum molle	26	12	6	52
Diplosoma similis	29	9	3	50
Diplosoma virens	20	10	4	58
Lissoclinum patella	24	9	2	63
Lissoclinum voeltzkowi	31	6	3	59

tio of β carotene to chl *a* higher than that of chlorophytes (Table III, Figs 1, 2) (cf. Stransky and Hager 1970, Hager and Stransky 1970 and Goodwin 1980). Echinenone, reported as an extra-plastidic carotenoid produced under unfavorable environmental conditions in certain chlorophytes (Goodwin 1980), occurs in *Prochloron* and cyanophytes (Figs 1, 2) but not in any of the samples of eukaryotes examined here. Lutein, commonly present in eukaryotic chlorophytes (Fig. 3), was not found in *Prochloron* or in any of the cyanophytes examined (Figs 1, 2). *Prochloron* contains rather large quantities of zeaxanthin, as do most prokaryotic and eukaryotic plants (Figs 2, 3).

The presence of both chlorophylls *a* and *b* as well as carotenoid pigments is reflected in spectrophotometric absorption scans of 90%-acetone extracts. Characteristic features in the spectra appear in the 350–475 nm region, where *Prochloron* exhibits a relatively wide absorbance band (Fig. 4). Per unit of chlorophyll, the absorption of near-UV light was higher in acetone extracts of whole didemnid colonies than in extract of the algae alone, indicating the presence of some UV-absorbing material originating from the animal (host) cells.

Discussion

Comparative HPLC analyses of *Prochloron*, cyanophytes, chlorophytes and higher-plant materials revealed both unique and common pigment characteristics of *Prochloron*. Although its specific chlorophyll *a* content is not strikingly different from that of other prokaryotic or eukaryotic algae, the relative content of chlorophyll *b* is generally lower by a factor of 2 to 5. We cannot yet say whether the observed variations in chl *a*/chl *b* ratios reflect genetic differences or contrasting environmental conditions in the different didemnid hosts.

Table III. Chlorophyll *a* and carotenoid contents of various algae. (Published data were recomputed for this comparison).

Organisms (by genus)	Chl *a* (mg g^{-1})	Total carotenoids (mg g^{-1})	Carotenoids: chl *a*	β-carotene: total carotenoids	Reference
Cyanophyta					
Anacystis	9.46	7.67	0.81	0.24	1
Synechoccocus	3.96	3.59	0.91	0.22	1
Microcystis	3.91	3.14	0.80	0.42	2
Merismopedia	6.11	3.64	0.60	0.40	1
Phormidium	2.99	2.95	0.99	0.28	1
Anabaena	3.95	2.82	0.71	0.40	
Aphanizomenon	3.25	2.19	0.67	0.45	
Oscillatoria	6.88	2.32	0.34	0.59	1
Calothrix	3.06	2.63	0.86	0.19	1
Chlorophyta					
Dunaliella	8.60	3.39	0.40	0.26	3
Eudorina	4.55	2.85	0.63	0.20	3
Ankistrodesmus	22.02	4.10	0.19	0.02	4
Scenedesmus	12.16	5.12	0.42	0.13	
Chlorella	6.95	2.96	0.43	0.09	
Zygnema	6.33	3.56	0.56	0.18	3
Chara	2.05	0.84	0.41	0.31	3
Prochlorophyta					
Prochloron					
in *Did. molle*	5.01	2.91	0.58	0.51	
in *Dip. similis*	4.14	2.43	0.59	0.50	
in *Dip. virens*	4.78	2.86	0.60	0.58	
in *L. patella*	4.21	2.77	0.66	0.63	
in *L. voeltzkowi*	5.12	2.30	0.45	0.59	

[1] Stransky and Hager 1970
[2] Paerl *et al.* 1983
[3] Hager and Stransky 1970
[4] Goodwin 1980

Prochloron contains a range of carotenoid pigments; with the exception of the absence of myxoxanthophyll, the spectrum is like that of most cyanophytes thus far examined (Stransky and Hager 1970, Goodwin 1980). Carotenoids may serve for photosynthetic energy transfer (as an accessory pigment: Warburg and Negelein 1922, Emerson and Lewis 1943, Haxo and Blinks 1950, Goedheer 1959, Cho and Govindjee 1970, Cox and Bendall 1974) and/or for photoprotection. In *Prochloron*, which may inhabit didemnids in shallow oligotrophic tropical waters exposed to high levels of irradiation, accessory carotenoids might serve in either role.

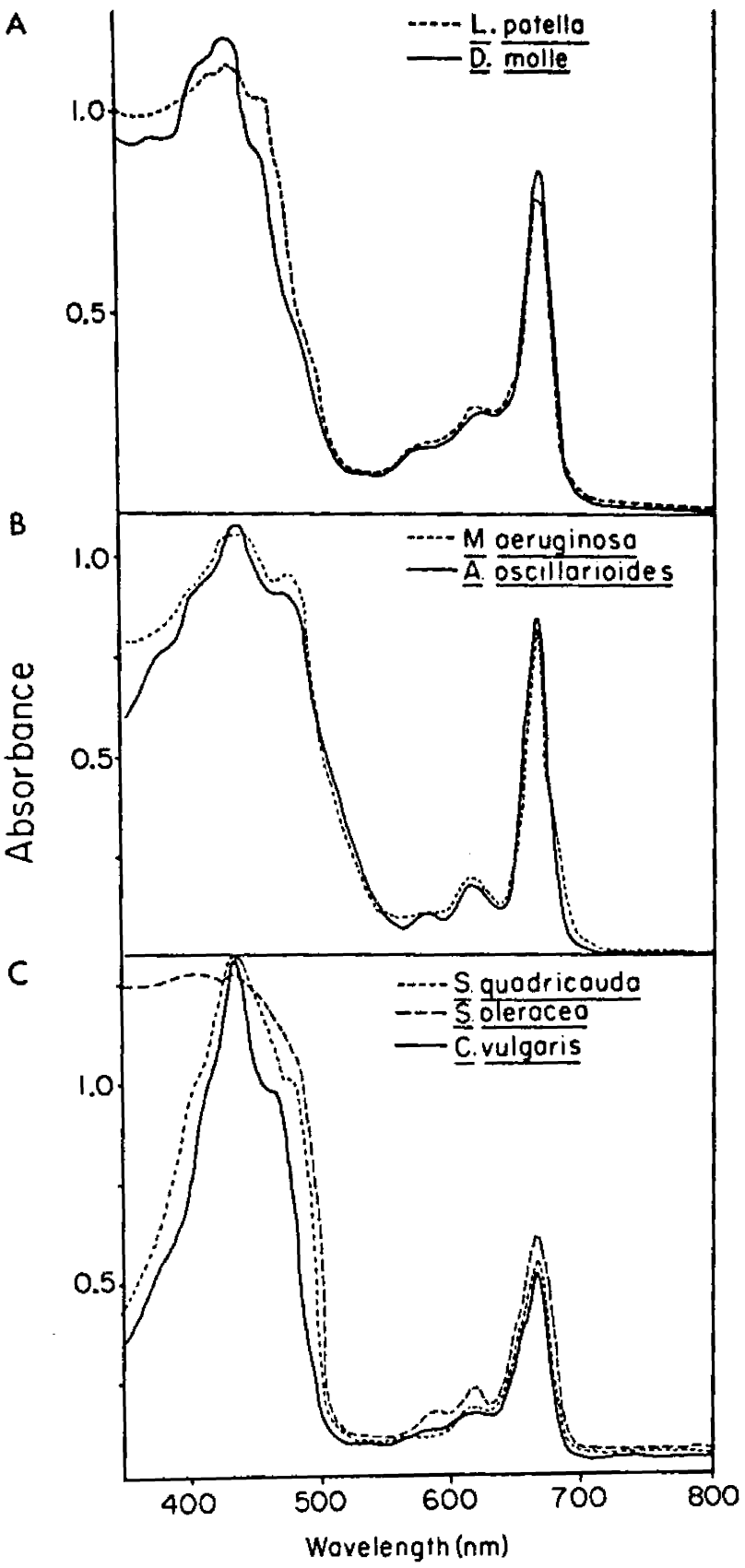

Fig. 4. Absorbance scans of representative prochlorophytes (A), cyanophytes (B), a terrestrial green plant (*S. oleracea*) and 2 chlorophytes (*S. quadricauda* and *C. vulgaris*) (C). There were large differences in near-U.V. (350–450 nm) absorbance characteristics among these plants. Surface-dwelling cyanophytes, terrestrial eukaryotes as well as prochlorophytes revealed strong near-U.V. absorption while subsurface- dwelling chlorophytes revealed a noticeable decline in near-U.V. absorbance.

Spectral absorbance curves of acetone extracts of *Prochloron* resemble those of surface-dwelling cyanophytes (*Microcystis aeruginosa*, *Anabaena oscillarioides*) and terrestrial plants (*Spinacia oleracea*) (Fig. 4), with strong absorption maxima in the 350–450 nm region, as reported earlier by Withers *et al.* (1978). In contrast, absorption scans of chlorophytes present in subsurface waters with low PAR regimes reveal a distinctly reduced absorption in the 350–450 nm region (Fig. 4). The presence of abundant carotenoids and the strong low-wavelength absorption may reflect *Prochloron*'s well-illuminated habitats, where it might be able to exploit the light at relatively high PAR saturation levels ($P_{opt.}$ = 700 μE m^{-2} s^{-1} PAR; R. Alberte, personal communication). It has been shown that energy from low-wavelength radiation captured by β-carotene may be efficiently transferred to chlorophyll in the photosystem I of cyanophytes (Goedheer 1964), whereas xanthophylls may serve more as photoprotective pigments (Goodwin 1980). The relatively high proportion of β-carotene in this alga may therefore be of ecological relevance. *Prochloron* thus shows several well-defined biochemical and ecological similarities to cyanophytes, complementing the more obvious similarities in their structural features.

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