

PIGMENT SUITES AND TAXONOMIC GROUPS IN PRASINOPHYCEAE¹

Mikel Latasa,² Renate Scharek³

Institut de Ciències del Mar, CSIC, Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

Florence Le Gall and Laure Guillou

Station Biologique, UMR 7127 CNRS et Université Pierre et Marie Curie, BP 74 29682 Roscoff, France

Pigment analysis performed on 30 Prasinophyceae strains revealed two main groups: the prasinoxanthin-containing and prasinoxanthin-less Prasinophyceae. Prasinoxanthin-containing Prasinophyceae comprised the orders Mamiellales, Pseudoscourfieldiales (*Pycnococcaceae*), and Prasinococcales. For this group, classification with pigment composition showed a good agreement with molecular phylogeny. Mamiellales, except *Crustomastix stigmatica*, accumulated uriolide, micromonal, dihydrolutein, and the pigment Unidentified M1 as characteristic pigments. Prasinococcales and Pseudoscourfieldiales (*Pycnococcaceae*) lacked micromonal and Unidentified M1. In addition, Pseudoscourfieldiales (*Pycnococcaceae*) lacked uriolide. A chl *c*₃-like pigment was present in prasinoxanthin-containing strains isolated from the deep sea. Common green algae pigments, a loroxanthin derivative, and siphonaxanthin plus derivatives were found in the prasinoxanthin-less Prasinophyceae, which included strains from Pyramimonadales, Pseudoscourfieldiales (*Nephroselmidiaceae*), Chlorodendrales, and a new order. Although some associations could be observed, the correspondence between pigments and molecular taxonomy was less clear for this group.

Key index words: chl *c*; HPLC; micromonal; prasinoxanthin; siphonaxanthin; Unidentified M1; uriolide; prasinophyte

The Prasinophyceae is an especially heterogeneous algal class in terms of pigments, probably because of its paraphyletic origin (Daugbjerg et al. 1995, Nakayama et al. 1998, Fawley et al. 2000). Pigment studies on Prasinophyceae started in earnest in the late 1960s with the pioneer work by Ricketts, who distinguished four major groups according to their pigment composition (Ricketts 1970). Many of those pigments were chromatographically and spectrophotometrically characterized and arbitrarily named. Fourteen years later,

Ricketts' xanthophyll K was structurally characterized, named as prasinoxanthin, and proposed as a pigment marker for Prasinophyceae (Foss et al. 1984). Foss et al. (1986) also described and named the pigment uriolide. Unknown 1 and Unknown A (Fawley 1992) were described as dihydrolutein and micromonal by Egeland and Liaaen-Jensen (1995). Jeffrey (1989) and Brown and Jeffrey (1992) showed the presence of a chl *c*₃-like pigment (chl *c*_{CS-170}) in a tropical strain of *Micromonas pusilla*. Unidentified M1 (Ricketts 1966, Fawley 1992) is a conspicuous pigment in Prasinophyceae that has not yet been characterized.

Parallel to the description of these pigments, an effort was made to use the information contained in these phenotypic characters to actualize the classification of Prasinophyceae. Thus, Hooks et al. (1988) subdivided the four groups proposed by Ricketts based on the prasinoxanthin-to-chl *a* ratio. However, Fawley (1992) dismissed this distinction. Mackey et al. (1996) also dismissed it but proposed another differentiation based on the chl *b*/chl *a* ratio. On the other hand, Guillard et al. (1991) proposed the presence of prasinoxanthin plus MgDVP as characteristic of the order Mamiellales in the Prasinophyceae. Fawley (1992), however, revealed the presence of this combination in *Pseudoscourfieldia marina*, a species belonging to Pseudoscourfieldiales, not Mamiellales. In short, thus far there have always been exceptions to proposed taxonomic classification of the Prasinophyceae based on their pigment composition.

Here we analyzed the pigment composition of 30 strains of Prasinophyceae, 12 named to species level, 17 known only to genus, and 1 unknown, grown in different culture media under the conditions indicated in Table 1. These strains were previously classified using 18S rRNA and represented seven different clades as defined by Guillou et al. (2004). The cultures were harvested in late exponential or early stationary phase, filtered onto glass fiber GF/F filters (Whatman, Maidstone, UK) with low vacuum (<0.3 atm) and immediately frozen in liquid nitrogen or at –80° C until analysis (less than 4 months). Pigments were extracted by placing the blotted dry filters in 90% acetone at –20° C overnight, followed by filter destruction with an ice-cooled cell mill (Vibrogen IV, Edmund Bühler, Tübingen, Germany) for 5 min. Samples were centrifuged for 10 min at 4° C. A total of 0.5 mL of the

¹Received 25 July 2003. Accepted 19 August 2004.

²Author for correspondence: e-mail latasa@icm.csic.es.

³Present address: Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Gijón, Avda. Príncipe de Asturias, 70 bis, 33212 Gijón, Spain.

TABLE 1. Strains, culture conditions, and depths of isolation.

Strain	Species	Order	Clade ^a	Temperature (°C)	Irradiance (μmol photons · m ⁻² · s ⁻¹)	Medium ^b	Depth (m)
CCMP 724	<i>Pyramimonas parkeae</i> Norris et Pearson	Pyramimonadales	I	15	100	f/2	?
CCMP 717	<i>Nephroselmis pyriformis</i> (Carter) Ettl 1982	Nephroselmidaceae	III	15	100	f/2	?
RCC 499 ^c	<i>Nephroselmis</i> sp.	Nephroselmidaceae	III	20	100	f/2	0
RCC 234	<i>Tetraselmis</i> sp.	Chlorodendratales	IV	20	100	k	15
RCC 500	<i>Tetraselmis</i> sp.	Chlorodendratales	IV	20	100	f/2	0
RCC 287	New species	New order	VII	20	100	k	120
	<i>Crustomastix stigmatica</i> Zingone	Mamiellales	II	19	30	f/2	?
RCC 116/OTTH 0595	<i>Ostreococcus tauri</i> Courties et Chrétiennot-Dinet	Mamiellales	II	20	100	k	Surface
RCC 501	<i>Ostreococcus</i> sp.	Mamiellales	II	20	100	f/2	0
RCC 344	<i>Ostreococcus</i> sp.	Mamiellales	II	20	100	k	5
RCC 371	<i>Ostreococcus</i> sp.	Mamiellales	II	20	100	k	65
RCC 343	<i>Ostreococcus</i> sp.	Mamiellales	II	20	100	k	40
RCC 356	<i>Ostreococcus</i> sp.	Mamiellales	II	15	100	k	0
RCC 393	<i>Ostreococcus</i> sp.	Mamiellales	II	20	25	k	90
RCC 141	<i>Ostreococcus</i> sp.	Mamiellales	II	20	25	k	105
RCC 143	<i>Ostreococcus</i> sp.	Mamiellales	II	20	25	k	120
CCMP 490	<i>Micromonas pusilla</i> (Butcher) Manton et Parke 1960	Mamiellales	II	20	100	k	Seawater tank
RCC 498	<i>Micromonas</i> (clade CCMP 490)	Mamiellales	II	20	100	f/2	0
RCC 372	<i>Micromonas pusilla</i>	Mamiellales	II	20	100	k	120 (for CCMP 489) ^d
RCC 418	<i>Micromonas</i> sp.	Mamiellales	II	20	100	k	0
RCC 391	<i>Mamiella</i> sp.	Mamiellales	II	20	100	k	5
CCMP 480	<i>Mantoniella squamata</i> (Manton et Parke) Desikachary 1972	Mamiellales	II	20	100	k	Salt marsh
RCC 395	<i>Mantoniella</i> sp.	Mamiellales	II	20	150	k	0
RCC 113	<i>Bathycoccus prasinos</i> Eikrem et Thronsdén	Mamiellales	II	20	100	k	?
RCC 244	<i>Pycnococcus provasolii</i> Guillard	Pseudoscourfieldiales	V	20	25	f/2	75
RCC 261	<i>Pseudoscourfieldia marina</i> (Thronsdén) Manton 1975	Pseudoscourfieldiales	V	25	40	k	20
CCMP 1192	<i>Prasinococcus capsulatus</i> Miyashita et Chihara	Prasinococcales	VI	20	100	k	25
CCMP 1407	<i>Prasinococcus cf. capsulatus</i>	Prasinococcales	VI	20	100	f/2	84
RCC 342	<i>Prasinococcus</i> sp.	Prasinococcales	VI	20	25	k	70
CCMP 1220	<i>Prasinoderma</i> sp.	Prasinococcales	VI	20	100	k	Net tow

Irradiance was provided in 12:12-h light:dark cycles.

^aClades were defined by Guillard et al. (2004).

^bk medium described by Keller et al. (1987) and f/2 medium by Guillard (1975).

^cStrain was lost.

^dThe depth of isolation of RCC 372 is unknown; the 120-m depth presented corresponds to CCMP 489, a phylogenetically related strain (Guillard et al. 2004).

TABLE 2. Chromatographic retention times and spectral characteristics of the main pigments obtained by on-line fast-scanning absorption spectra of the HPLC eluent.

Pigment	Abbreviation	Retention time (min)	Absorption maxima
Unknown chl c_3 -like	C_3 -like	7.1	<i>453, 583, (623)</i>
Mg-24-divinyl pheoporphyrin <i>a5</i> monomethyl ester	MgDVP	9.3	<i>440, 627</i>
Uriolide	Uri	14.1	<i>451, 477</i>
Violaxanthin-derivative	Vio-der	15	<i>423, 444, 473</i>
Siphonaxanthin	Sip	16.1	<i>467, (448)</i>
Neoxanthin	Neo	17.5	<i>412, 439, 467</i>
Prasinoxanthin	Pra	18.7	<i>468, (448)</i>
Violaxanthin	Vio	20.1	<i>442, 472, (416)</i>
Micromonal	Mmnal	22.3	<i>471, (448)</i>
Unidentified Z1	Uni Z1	23.7	<i>444, 466</i>
Unidentified Z2	Uni Z2	24.2	<i>444, 466</i>
Antheraxanthin	Ant	25.2	<i>446, 476, 424</i>
Zeaxanthin	Zea	30.3	<i>452, 479</i>
Lutein	Lut	30.6	<i>447, 476</i>
Dihydrolutein	Dihy	31.2	<i>431, 457</i>
Siphonaxanthin-derivative A1	Sip A1	31.6	<i>469, (448)</i>
Siphonaxanthin-derivative B1	Sip B1	32.1	<i>468, (448)</i>
Siphonaxanthin-derivative A2	Sip A2	32.7	<i>467, (448)</i>
Siphonaxanthin-derivative B2	Sip B2	33.7	<i>467, (448)</i>
Loroxanthin-derivative	Lor-der	34.3	<i>448, 477</i>
Chl <i>b</i>	Chl <i>b</i>	36.6	<i>464, 648</i>
Chl <i>a</i>	Chl <i>a</i>	39.5	<i>432, 664</i>
γ -Carotene-like	γ -car-like	41.3	<i>465, 493, (442)</i>
Unidentified M1	Uni M1	41.5	<i>440, 466, (412)</i>
α -Carotene	α -car	42.7	<i>446, 476</i>
β -carotene	β -car	42.9	<i>454, 480</i>

Absorption maximum are in italics, and shoulder are enclosed in parentheses.

supernatant was mixed with 0.1 mL of MilliQ water (Millepore, Billerica, MA, USA) and 100–150 μ L injected onto the HPLC chromatographic system using the method of Zapata et al. (2000) optimized for our system with minor modifications (Latasa et al. 2001).

Many pigments found in Prasinophyceae are not commercially available. Therefore, those pigments were identified by their chromatographic and spectral characteristics (Table 2) and by their presence in well-known strains analyzed by us. We found several peaks without a clear correspondence in the literature; these pigments were named according to their absorption characteristics and chromatographic behavior. Thus, those unknown pigments with absorption and chromatographic properties similar to known ones were named by adding the suffix “-like.” For instance, a pigment eluting before Unidentified M1 (Ricketts 1966, Fawley 1992) had very similar spectral and chromatographic characteristics to γ -carotene, and we named it γ -carotene-like. A pigment with chromatographic and spectrophotometric characteristics of a chl c_3 was named as unknown chl c_3 -like. On the other hand, there were some pigments with different chromatographic but similar spectral properties. We named these pigments as derivatives. For instance, the spectra of siphonaxanthin-derivative A1, A2, B1, and B2 were identical to that of siphonaxanthin. However, their retention time was clearly different, probably reflecting a different composition of their fatty acid esters (Ricketts 1971). The same reasoning was applied

to loroxanthin and violaxanthin derivatives. Other peaks we named were Unidentified Z1 and Z2, with spectral characteristics resembling, but not identical, to those of uriolide.

For pigment quantification, the system was calibrated with standards of chl c_3 , violaxanthin, prasinoxanthin, zeaxanthin, lutein, chl *a* and *b*, and α - and β -carotene from 14 Carbon Agency (Hørsholm, Denmark). We used extinction coefficients from Jeffrey et al. (1997), except for prasinoxanthin for which we applied the extinction coefficient of fucoxanthin (166 $L \cdot g^{-1} \cdot cm^{-1}$). These two pigments possess chromophores with carbonyl functions as terminal aliphatic ketones, a characteristic that normally causes a reduction of the extinction coefficient (Bjørnland 1997). Concentrations of uriolide, siphonaxanthin, micromonal, and related (-like) pigments were estimated based on the response factor of prasinoxanthin; the same procedure was applied to estimate Unidentified Z1 and Z2. Concentrations of antheraxanthin, dihydrolutein, and loroxanthin-derivative were estimated based on the response factor of lutein. Concentrations of neoxanthin were estimated based on the response factor of violaxanthin. Concentrations of Unidentified M1 and γ -carotene-like pigments were estimated based on the response factor of β -carotene. Concentrations of unknown chl c_3 -like and MgDVP were estimated based on the response factor of chl c_3 .

Special pigment features. Twenty-five pigments normalized to chl *a* concentration were considered in this

TABLE 3. Pigment-to-chl *a* ratios (w/w) of 30 strains of Prasinophyceae.

	ϵ_3 -like	MgDVP	Uri	Vio-der	Sip	Neo	Pra	Vio	Mmmal	Uni Z1	Uni Z2	Ant	Zea	Lut	Dihy	Sip Al	Sip B1	Sip A2	Sip B2	Lor-der	Chl b	γ -like	Uni M1	α -car	β -car	
<i>Pyramimonas parkeae</i>	—	0.001	—	0.021	—	—	—	0.077	—	—	—	0.002	0.002	0.070	—	—	—	—	—	0.027	0.677	0.011	—	—	0.088	
CCMP 724	—	0.015	—	0.044	—	0.021	—	0.077	—	—	—	0.016	0.019	0.022	—	—	0.059	—	0.125	—	0.698	0.005	—	—	0.040	0.025
<i>Nephroselmis pyriformis</i>	—	0.007	—	0.023	—	0.030	—	0.053	—	—	—	0.036	0.033	0.018	—	—	0.071	—	0.083	—	0.723	0.020	—	—	0.041	0.075
CCMP 717	—	0.022	—	0.039	—	0.081	—	0.057	—	—	—	0.006	0.005	0.096	—	0.043	—	0.048	—	—	0.812	0.009	—	—	0.020	0.062
<i>Nephroselmis</i> sp. RCC 499	—	—	—	0.018	—	0.031	—	0.024	—	—	—	0.008	0.014	0.138	—	—	—	—	—	—	0.655	0.062	—	—	0.000	0.191
<i>Tetraselmis</i> sp. RCC 234	—	—	—	0.018	—	0.074	—	0.131	—	—	—	0.051	0.042	0.382	—	—	—	—	—	—	1.313	—	—	—	0.026	0.053
<i>Tetraselmis</i> sp. RCC 500	—	—	—	0.018	—	0.074	—	0.131	—	—	—	0.017	0.065	0.038	—	—	—	—	—	—	0.786	—	—	—	0.034	0.271
RCC 287	—	0.023	—	0.008	—	0.170	—	0.055	—	—	—	—	—	—	—	—	—	—	—	—	0.633	—	—	—	0.000	0.098
<i>Crustonastix stigmatica</i>	—	0.061	0.079	0.016	—	0.045	0.191	0.044	0.039	0.014	0.025	0.051	0.050	0.003	0.032	—	—	—	—	—	—	—	—	0.028	0.000	0.098
<i>Ostreococcus tauri</i> RCC	116	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Ostreococcus</i> sp. RCC 501	—	0.062	0.073	0.022	—	0.039	0.136	0.047	0.038	0.010	0.023	0.019	0.020	0.005	0.032	—	—	—	—	—	0.627	—	—	0.027	0.000	0.044
<i>Ostreococcus</i> sp. RCC 344	—	0.057	0.068	0.015	—	0.041	0.154	0.101	0.040	0.015	0.022	0.031	0.025	0.002	0.027	—	—	—	—	—	0.618	—	—	0.033	—	0.039
<i>Ostreococcus</i> sp. RCC 371	—	0.060	0.074	0.015	—	0.044	0.156	0.105	0.043	0.018	0.021	0.016	0.028	0.001	0.031	—	—	—	—	—	0.625	—	—	0.037	—	0.039
<i>Ostreococcus</i> sp. RCC 343	—	0.063	0.077	0.016	—	0.043	0.186	0.055	0.043	0.023	0.027	0.047	0.033	0.001	0.028	—	—	—	—	—	0.648	—	—	0.030	—	0.036
<i>Ostreococcus</i> sp. RCC 356	—	0.067	0.086	0.019	—	0.045	0.177	0.102	0.042	0.004	0.010	0.035	0.075	0.007	0.039	—	—	—	—	—	0.637	—	—	0.031	0.003	0.042
<i>Ostreococcus</i> sp. RCC 393	0.020	0.024	0.080	0.010	—	0.060	0.212	0.123	0.033	0.020	0.022	0.055	0.038	0.006	0.045	—	—	—	—	—	0.672	—	—	0.035	—	0.046
<i>Ostreococcus</i> sp. RCC 141	0.020	0.056	0.076	0.043	—	0.037	0.144	0.019	0.059	0.009	0.033	—	—	0.004	0.025	—	—	—	—	—	1.032	—	—	0.027	0.014	0.016
<i>Ostreococcus</i> sp. RCC 143	0.029	0.036	0.072	0.036	—	0.038	0.146	0.028	0.059	0.009	0.030	—	—	0.004	0.030	—	—	—	—	—	1.022	—	—	0.032	0.012	0.018
<i>Micromonas pusilla</i> CCMP	490	—	0.072	0.096	0.026	—	0.045	0.191	0.103 ^a	0.053	0.012	0.032	0.019	0.013	0.006	0.010	—	—	—	—	0.768	—	—	0.031	0.002	0.056
<i>Micromonas</i> sp. RCC 498	—	0.067	0.086	0.033	—	0.037	0.160	0.149	0.047	0.015	0.036	0.014	0.012	0.003	0.005	—	—	—	—	—	0.797	—	—	0.022	0.003	0.035
<i>Micromonas pusilla</i> RCC	372	0.015	0.045	0.084	0.025	—	0.044	0.168	0.139 ^a	0.057	0.011	0.029	0.013	0.016	0.006	0.018	—	—	—	—	0.810	—	—	0.026	—	0.031
<i>Micromonas</i> sp. RCC 418	—	0.080	0.085	0.016	—	0.036	0.130	0.089 ^a	0.038	0.011	0.020	0.020	0.060	0.010	0.007	—	—	—	—	—	0.812	—	—	0.028	—	0.032
<i>Mamiella</i> sp. RCC 391	—	0.060	0.072	0.021	—	0.043	0.189	0.074	0.010	0.015	0.024	0.011	0.019	0.008	0.052	—	—	—	—	—	0.896	—	—	0.034	0.027	0.055
<i>Mantoniella squamata</i>	—	0.068	0.075	0.029	—	0.037	0.150	0.068 ^a	0.045	0.013	0.033	0.006	0.010	0.003	0.021	—	—	—	—	—	0.644	—	—	0.026	0.020	0.101
CCMP 480	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Mantoniella</i> sp. RCC 395	—	0.065	0.073	0.028	—	0.031	0.123	0.056	0.043	0.008	0.028	0.003	0.006	0.004	0.009	—	—	—	—	—	0.622	—	—	0.021	0.013	0.049
<i>Bathycoccus prasinos</i> RCC	113	0.041	0.047	0.018	—	0.018	0.097	0.078	0.030	0.007	0.023	0.002	0.017	0.003	0.018	—	—	—	—	—	0.462	—	—	0.013	—	0.029
<i>Pycnococcus provasolii</i>	—	0.080	—	0.017	—	0.061	0.305	0.024	—	0.019	0.031	—	0.009	0.002	0.021	—	—	—	—	—	0.971	—	—	—	0.013	0.019
RCC 244	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Pseudocosticoidia marina</i>	—	0.071	—	0.006	—	0.057	0.287	0.036	—	0.018	0.025	—	0.078	0.003	0.023	—	—	—	—	—	0.871	—	—	—	0.006	0.027
RCC 261	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Prasinococcus capsulatus</i>	—	0.098	0.097	0.017	—	0.076	0.183	0.035	—	0.020	0.023	0.001	0.008	0.015	0.005	—	—	—	—	—	0.620	—	—	—	0.006	0.019
CCMP 1192	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Prasinococcus cf. capsulatus</i>	0.020	0.025	0.081	0.015	—	0.060	0.192	0.047	—	0.008	0.016	—	0.016	0.180	0.053	—	—	—	—	—	0.713	—	—	—	0.024	0.030
CCMP 1407	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Prasinococcus</i> sp. RCC 342	—	0.098	0.102	0.021	—	0.068	0.209	0.035	—	0.008	0.019	—	0.008	0.014	0.111	—	—	—	—	—	0.953	—	—	—	0.032	0.011
<i>Prasinoderma</i> CCMP 1220	—	0.109	0.064	0.015	—	0.069	0.195	—	—	0.019	0.026	0.011	0.045	0.025	0.005	—	—	—	—	—	0.882	—	—	—	0.009	0.027

Pigment abbreviations are provided in Table 2.

^aExact quantification is difficult because of contamination with micromonol.

work to compare 30 strains of Prasinophyceae (Table 3). In phytoplankton ecology, the pigment-to-chl *a* ratio is a required parameter to estimate the relative contribution of the different phytoplankton groups (Mackey et al. 1996). The ratios presented here correspond to a single growth condition for each strain. Although these ratios can be affected by the physiological status, we did not observe strong divergences among strains despite the important differences in irradiance (25 to 150 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) experienced by the cultures.

The carotenoid composition of *Bathycoccus prasinos*, *Mantoniella squamata*, *Micromonas pusilla*, *Prasinococcus capsulatus*, *Pycnococcus provasolii*, *Nephroselmis pyriformis*, *Pseudoscourfieldia marina*, *Tetraselmis* sp., *Pyramimonas parkeae*, and *Ostreococcus tauri*, summarized by Egeland et al. (1997), and that of *Crustomastix stigmatica*, described by Zingone et al. (2002), are almost identical to those found in our study for those species.

The main discrepancy between our results and published work was the presence of prasinoxanthin instead of siphonaxanthin (Chrétiennot-Dinet et al. 1995) in all our *Ostreococcus* strains. In our system, siphonaxanthin and prasinoxanthin eluted with more than a 1-min interval (Table 2). This and the injection of a prasinoxanthin standard confirmed the identification of prasinoxanthin in *Ostreococcus*.

An unknown chl *c*₃-like pigment similar to chl *c*_{CS-170} of *Micromonas pusilla* CS-170 (Jeffrey 1989) was found in *Prasinococcus* cf. *capsulatus* CCMP 1407, in strains RCC 393, RCC 141, and RCC 143 of *Ostreococcus* sp., all four isolated from deep waters, and in *Micromonas pusilla* RCC 372 (Table 1). Whereas four of the five strains had been grown at low light levels (25 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, blue light), one of them was grown at 100 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, which suggests this pigment does not accumulate solely because of very low growth irradiances. In this sense, the most striking result was the presence of unknown chl *c*₃-like in *Micromonas pusilla* RCC 372. This strain, isolated from an unknown, but probably shallow, depth in the Gulf of Naples was linked by molecular methods to CCMP 489 (Guillou et al. 2004), a strain isolated from the Sargasso Sea at 120 m depth. Therefore, the presence of this pigment might not be only a plastic and temporary adaptation to low irradiance levels, but it may reflect some phylogenetic traits related to deep ocean habitats.

Siphonaxanthin was a major pigment in *Crustomastix stigmatica* and *Tetraselmis* sp. RCC 234. In addition, siphonaxanthin derivatives were found in *Tetraselmis* sp. RCC 234 (A1 and A2) and *Nephroselmis pyriformis* and *Nephroselmis* sp. RCC 499 (B1 and B2). Although *Tetraselmis* possessing siphonaxanthin had not been reported before, *Tetraselmis* is included in the order Chlorodendrales with species that do have siphonaxanthin (Egeland et al. 1997). Besides the pigments normally associated with Chlorophyceae, only lutein-like (Egeland et al. 1995) and loroxanthin-like pigments (Rodríguez 2001) were found in this genus. The pigment composition of *Tetraselmis* RCC 234 was

clearly different from other species of this genus described before, and its closest pigment suite was that of *Pyramimonas propulsa*, formerly *Asteromonas propulsa* (Ricketts 1970). Siphonaxanthin derivatives named as xanthophylls K1S and K2S had been reported by Ricketts (1970) in *Heteromastix longifilis* and *Heteromastix* sp. P198. This genus seems to be quite close to *Nephroselmis* because *Heteromastix minuta* was changed to *Nephroselmis minuta* by Butcher 1959 (Thronsdren 1997).

All the prasinoxanthin-containing strains, except *Ostreococcus* sp. RCC 356, exhibited two chromatographic peaks that eluted between micromonal and antheraxanthin in our system. Their spectral characteristics were very similar and resembled those of uriolide. Remarkably, these peaks were also present in *Pycnococcus provasolii* and *Pseudoscourfieldia marina*, two strains where we could not find uriolide, and consequently we named them as Unidentified Z1 and Z2.

Guillou et al. (2004) demonstrated that RCC 287 was phylogenetically related to *Picocystis salinarium* (Levin et al. 2000). However, the pigment composition of RCC 287 was typical of Chlorophyceae (Table 3), and it did not possess alloxanthin or monadoxanthin (typical of cryptophytes) or diatoxanthin (found in chromophytes and euglenophytes), which are the main xanthophylls of *P. salinarium*. Other differences between our results and those provided in the literature were as follows: 1) the presence of α -carotene in *Bathycoccus prasinos* reported by Egeland and Liaaen-Jensen (1995) but not found in our analysis, and 2) the presence of uriolide and micromonal in *Pseudoscourfieldia marina* reported by Egeland et al. (1995) but not found in our analysis. This last result agrees with the findings of Fawley (1992).

Prasinoderma sp. CCMP 1220 was the only strain without violaxanthin, whereas Hasegawa et al. (1996) reported violaxanthin in *Prasinoderma coloniale*. All the species without exception (even *Prasinoderma* sp. CCMP 1220, without violaxanthin) had a violaxanthin-derivative with a shorter retention time (Tables 2 and 3). We could not confirm the presence of neoxanthin in *Pyramimonas parkeae*. A pigment eluted with the same retention time as neoxanthin but with maxima at 440 and 468 nm, which was different from neoxanthin or loroxanthin (Table 2), pigments with similar retention times. The prasinoxanthin-less strains, except RCC 287 and *Crustomastix stigmatica*, contained the γ -carotene-like pigment. We could not confirm the presence of MgDVP in strain RCC 287 and *Tetraselmis* sp. RCC 500, indicating that if present, its concentration was below our detection limits.

Pigment suites and taxonomic affinities. The pigment composition of the 30 strains studied showed a very clear division in two main groups as proposed by Egeland et al. (1995): the prasinoxanthin-containing and prasinoxanthin-less Prasinophyceae (Table 3). The prasinoxanthin-containing Prasinophyceae were represented by the phylogenetic clades II, VI, and V (Mamiellales, Prasinococcales, and Pseudoscourfieldiales)

determined by small subunit rDNA phylogeny (Guillou et al. 2004). These phylogenetic groups can be classified based on the presence/absence of uriolide, micromonal, and Unidentified M1. Thus, Mamiellales could be clearly set apart because of the absence of micromonal and Unidentified M1 in the other two orders (Table 3). Prasinococcales and Pseudoscourfieldiales are again clearly discriminated from each other because of the absence of uriolide in the Pseudoscourfieldiales. Among the Mamiellales, some additional differentiation could be distinguished. Thus, *Ostreococcus* sp. RCC 141 and RCC 143, both isolated from the deep ocean, lacked zeaxanthin and antheraxanthin and possessed an unknown chl c_3 -like pigment.

We can extend and validate this classification with results from the literature. Thus, Fawley (1992) found uriolide but neither Unidentified M1 nor micromonal (Unknown A in Fawley's work) for strain IV E5G, which is now *Prasinococcus* CCMP 1194 (in clade VI). On the other hand, clones Ω 48-23 (CCMP 1203), BT-5 (CCMP 2194), and 1326.1 (CCMP 1199), described as, or very similar to, *Pycnococcus provasolii* (Guillard et al. 1991), did contain prasinoxanthin but not uriolide (Foss et al. 1986). These results confirm the correspondence between the proposed pigment key and taxonomical clades.

The prasinoxanthin-less strains were included in phylogenetic clades I, III, IV, and VII corresponding to Pyramimonadales, Nephroselmidaceae, Chlorodendrales, and a new clade, respectively (Guillou et al. 2004). The only clear exception to this rule was *C. stigmatica*, a species of the Mamiellales without prasinoxanthin but with siphonaxanthin (Zingone et al. 2002). In this sense, *C. stigmatica* was more similar to *Tetraselmis* sp. RCC 234, a species of the Chlorodendrales with siphonaxanthin plus derivatives A1 and A2 (Tables 2 and 3). The two Nephroselmidaceae analyzed could be distinguished because they possessed siphonaxanthin-derivatives B1 and B2 (Table 3). Despite this last result, the presence of siphonaxanthin derivatives A1 and A2 in only one of the two strains of *Tetraselmis* (Table 3) makes it doubtful that siphonaxanthin and derivatives could have a determinant phylogenetic meaning. *Pyramimonas parkeae*, the only strain belonging to the order Pyramimonadales, was unique because it contained a loroxanthin-derivative pigment. However, Fawley (1991) reviewed the presence of loroxanthin in green algae and concluded that this pigment had no taxonomic value.

In this work we presented an analysis of the pigment suites of 30 strains that included seven phylogenetic clades of the Prasinophyceae class. The pigment composition for the prasinoxanthin-containing Prasinophyceae offered a perspective very much coherent with the molecular phylogenetic classification and, in some cases, with the ecological affinities of the strains. Such a clear correspondence between pigment and small subunit rRNA classification was not found for the prasinoxanthin-less Prasinophyceae analyzed. In fu-

ture studies, we suggest that stronger emphasis should be placed on the prasinoxanthin-less Prasinophyceae, a very diverse group in terms of pigments, a fact that probably reflects their taxonomic diversity.

We thank A. Zingone for graciously providing the culture of *Crustomastix stigmatica* and F. Rodríguez and J. L. Garrido for sharing information on the unknown chl c_3 -like pigment. Two anonymous reviewers provided extended comments that improved the original manuscript. This work has been carried out in the framework of the EU project PICODIV (EVK3-CT-1999-00021) with additional support from the Spanish Ministry of Science and Technology (REN2000-2913-E). L. G. was supported by the EU Marie Curie Grant (EVK3-CT-1999-50004).

- Bjørnland, T. 1997. UV-vis spectroscopy of carotenoids. In Jeffrey, S. W., Mantoura, R. F. C. & Wright, S. W. [Eds.] *Phytoplankton Pigments in Oceanography*. UNESCO Publishing, Paris, pp. 578–594.
- Brown, M. R. & Jeffrey, S. W. 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. *J. Exp. Mar. Biol. Ecol.* 161:91–113.
- Chrétiennot-Dinet, M. J., Courties, C., Vaquer, A., Neveux, J., Claustre, H., Lautier, J. & Machado, M. C. 1995. A new marine picoeukaryote: *Ostreococcus tauri* gen. et sp. nov. (Chlorophyta, Prasinophyceae). *Phycologia* 34:285–92.
- Daugbjerg, N., Moestrup, O. & Arctander, P. 1995. Phylogeny of genera of Prasinophyceae and Pedinophyceae (Chlorophyta) deduced from molecular analysis of the rbcL gene. *Phycol. Res.* 43:203–13.
- Egeland, E. S., Eikrem, W., Thronsdén, J., Wilhelm, C., Zapata, M. & Liaaen-Jensen, S. 1995. Carotenoids from further Prasinophytes. *Biochem. Syst. Ecol.* 23:747–55.
- Egeland, E. S., Guillard, R. R. L. & Liaaen-Jensen, S. 1997. Additional carotenoid prototype representatives and a general chemosystematic evaluation of carotenoids in prasinophyceae (Chlorophyta). *Phytochemistry* 44:1087–97.
- Egeland, E. S. & Liaaen-Jensen, S. 1995. Ten minor carotenoids from prasinophyceae (Chlorophyta). *Phytochemistry* 40:515–20.
- Fawley, M. W. 1991. Disjunction distribution of the xanthophyll loroxanthin in the green algae (Chlorophyta). *J. Phycol.* 27:544–8.
- Fawley, M. V. 1992. Photosynthetic pigments of *Pseudoscourfieldia marina* and select green flagellates and coccoid ultraphytoplankton: implications for the systematics of the Micromonadophyceae (Chlorophyta). *J. Phycol.* 28:26–31.
- Fawley, M. W., Yun, Y. & Qin, M. 2000. Phylogenetic analyses of 18S rDNA sequences reveal a new coccoid lineage of the Prasinophyceae (Chlorophyta). *J. Phycol.* 36:387–93.
- Foss, P., Guillard, R. R. L. & Liaaen-Jensen, S. 1984. Prasinoxanthin, a chemosystematic marker for algae. *Phytochemistry* 23:1629–33.
- Foss, P., Guillard, R. R. L. & Liaaen-Jensen, S. 1986. Carotenoids from eucaryotic ultraplankton clones (Prasinophyceae). *Phytochemistry* 25:119–24.
- Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. In Smith, W. L. & Chanley, M. H. [Eds.] *Culture of Marine Invertebrate Animals*. Plenum Press, New York, pp. 29–60.
- Guillard, R. R. L., Keller, M. D., O'Kelly, C. J. & Floyd, G. L. 1991. *Pycnococcus provasolii* gen. et sp. nov., a coccoid prasinoxanthin-containing phytoplankton from the western North Atlantic and Gulf of Mexico. *J. Phycol.* 27:39–47.
- Guillou, L., Eikrem, W., Chrétiennot-Dinet, M. J., Le Gall, F., Massana, R., Romari, K., Pedrós-Alió, C. & Vaulot, D. 2004. Diversity of picoplanktonic Prasinophyceae assessed by direct SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. *Protist* 155:193–214.

- Hasegawa, T., Miyashita, H., Kawachi, M., Ikemoto, H., Kurano, N., Miyachi, S. & Chihara, M. 1996. *Prasinoderma coloniale* gen. et sp. nov., a new pelagic coccooid prasinophyte from the western Pacific Ocean. *Phycologia* 35:170–6.
- Hooks, C. E., Bidigare, R. R., Keller, M. D. & Guillard, R. R. L. 1988. Coccooid eukaryotic marine ultraplankters with four different HPLC pigment signatures. *J. Phycol.* 24:571–80.
- Jeffrey, S. W. 1989. Chlorophyll c pigments and their distribution in the chromophyte algae. In Green, J. C., Leadbeater, B. S. C. & Diver, W. L. [Eds.] *The Chromophyte Algae: Problems and Perspectives*. Clarendon Press, Oxford, pp. 13–36.
- Jeffrey, S. W., Mantoura, R. F. C. & Bjørnland, T. 1997. Data for the identification of 47 key phytoplankton pigments. In Jeffrey, S. W., Mantoura, R. F. C. & Wright, S. W. [Eds.] *Phytoplankton Pigments in Oceanography*. UNESCO Publishing, Paris, pp. 449–559.
- Keller, M. D., Selvin, R. C., Claus, W. & Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23:633–8.
- Latasa, M., van Lenning, K., Garrido, J. L., Scharek, R., Estrada, M., Rodriguez, F. & Zapata, M. 2001. Losses of chlorophylls and carotenoids in aqueous acetone and methanol extracts prepared for RPHPLC analysis of pigments. *Chromatographia* 53:385–91.
- Lewin, R. A., Krienitz, L., Goericke, R., Takeda, H. & Hepperle, D. 2000. *Picocystis salinarum* gen. et sp. nov. (Chlorophyta)—a new picoplanktonic green alga. *Phycologia* 39:560–5.
- Mackey, M. D., Mackey, D. J., Higgins, H. W. & Wright, S. W. 1996. CHEMTAX—a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. *Mar. Ecol. Progr. Ser.* 144:265–83.
- Nakayama, T., Marin, B., Kranz, H. D., Surek, B., Huss, V. A. R., Inouye, I. & Melkonian, M. 1998. The basal position of scaly green flagellates among the green algae (Chlorophyta) is revealed by nuclear-encoded SSU rRNA sequences. *Protist* 149:367–80.
- Ricketts, T. R. 1966. The carotenoids of the phytoflagellate *Micromonas pusilla*. *Phytochemistry* 5:571–80.
- Ricketts, T. R. 1970. The pigment of Prasinophyceae and related organisms. *Phytochemistry* 9:1835–42.
- Ricketts, T. R. 1971. Identification of xanthophylls KI and KIS of the prasinophyceae as siphonein and siphonaxanthin. *Phytochemistry* 10:161–4.
- Rodríguez, F. J. 2001. Aplicación del análisis de pigmentos por cromatografía líquida de alta eficacia (HPLC) al estudio de la composición y distribución del fitoplancton marino. Ph.D. Thesis, University of Vigo, Spain, 264 pp.
- Thronsen, J. 1997. The planktonic marine flagellates. In Tomas, C. R. [Ed.] *Identifying Marine Phytoplankton*. Academic Press, San Diego, pp. 591–729.
- Zapata, M., Rodriguez, F. & Garrido, J. L. 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C-8 column and pyridine-containing mobile phases. *Mar. Ecol. Progr. Ser.* 195:29–45.
- Zingone, A., Borra, M., Brunet, C., Forlani, G., Kooistra, W. H. C. F. & Procaccini, G. 2002. Phylogenetic position of *Crustomastix stigmatica* sp. nov. and *Dolichomastix tenuilepis* in relation to the mammillales (Prasinophyceae, Chlorophyta). *J. Phycol.* 38:1024–39.