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OCCURRENCE OF LOROXANTHIN, LOROXANTHIN DECENOATE, AND LOROXANTHIN DODECENOATE IN *TETRASELMIS* SPECIES (PRASINOPHYCEAE, CHLOROPHYTA)[1]

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The pigment composition of six species of *Tetraselmis* (Prasinophyceae) was analyzed using improved HPLC methods. All pigment extracts showed three peaks corresponding to unknown carotenoids. The isolated pigments were analyzed using UV–Vis spectroscopy, electrospray ionization–mass spectrometry (ESI–MS), and when carotenoid esters were suspected, gas chromatography–mass spectrometry (GC–MS) of the methyl ester and dimethyloxazoline derivative of the corresponding fatty acid. The new pigments were determined to be loroxanthin, loroxanthin 19-(2-decenoate), and loroxanthin 19-(2-dodecenoate); this is the first time these pigments have been described in the genus *Tetraselmis*. Moreover, this is the first report of esterification of 2-decenoic acid to loroxanthin. The relative contents of these pigments depended on the light regime, with the lowest proportions measured at the highest photon flux density assayed. The implications of the identification of these pigments in the genus *Tetraselmis* for the pigment types previously described in the class Prasinophyceae are discussed.

Key index words: **2-decenoic acid; carotenoids; loroxanthin; loroxanthin esters; mass spectrometry; Prasinophyceae; *Tetraselmis***

Abbreviations: **DMOX, 2-alkenyl-4,4-dimethyloxazoline; ESI–MS, electrospray ionization–mass spectrometry; FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry**

Prasinophyceans aggregate as a paraphyletic group with heterogeneous morphological and biochemical features and are considered representatives of the earliest green algae (Melkonian 1990, Sym and Pienaar 1993, Bhattacharya and Medlin 1998). Initially, the group was established to separate primitive scaly flagellates from the Chlorophyceae (Christensen 1962). However, the class Prasinophyceae now includes more than 20 genera and seven phylogenetic clades (Zingone et al. 2002, Guillou et al. 2004). Given their phylogenetic position and retention of ancestral characters, prasinophyceans have attracted much attention with respect to ultrastructural, phylogenetic, and biochemical studies that are helping to elucidate the evolutionary history of the green lineage (Sym and Pienaar 1993, Nakayama et al. 1998, Fawley et al. 2000, Yoshii et al. 2003, Kelly 2007).

Prasinophyceans have been grouped into three pigment types according to their different carotenoid composition (Egeland et al. 1997). Type 1 possesses the basic set of carotenoids present in chlorophyceans: neoxanthin, violaxanthin, lutein, zeaxanthin, and β,β-carotene; type 2 includes the basic set of carotenoids plus siphonaxanthin or loroxanthin and their esters; type 3 includes the most diverse group of xanthophylls with prasinoxanthin, uriolide, micromonol, micromonal, and dihydrolutein (Egeland et al. 1997). Recent studies employing improved HPLC methods have analyzed the carotenoid composition of prasinophyceans (Latasa et al. 2004) confirming previous results of Egeland et al. (1997). Different prasinophycean pigment types usually match phylogenetic clades (Guillou et al. 2004, Latasa et al. 2004).

Within the prasinophyceans, the order Chlorodendrales (genus *Tetraselmis*) was presented as a typical example of pigment type 1, whereas pigment type 2 comprises several genera from the orders Pyramimonadales (*Pterosperma*, *Pyramimonas*; Kohata and Watanabe 1989, Yoshii et al. 2002) and Pseudoscourfieldiales (*Nephroselmis*; Latasa et al. 2004).

The genus *Tetraselmis* comprises more than 26 marine and freshwater species. This genus has been exhaustively studied for pigment analysis using different chromatographic procedures, and most of

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the species tested have been reported to possess common green algae pigments (type 1) typical of the Chlorophyceae (Egeland et al. 1997). However, Egeland et al. (1995) had reported the occurrence of minor carotenoids that are less polar than zeaxanthin and with lutein-type absorption spectra. The occurrence of two peaks corresponding to unknown carotenoids in HPLC chromatograms of *Tetraselmis* species was also found by Zapata et al. (2000), Bustillos-Guzmán et al. (2002), and Yoshii et al. (2002).

In this study, we report for the first time the occurrence of loroxanthin and loroxanthin-ester carotenoids in six *Tetraselmis* species, including the description of a new carotenoid, loroxanthin 19-(2-decenoate). We also describe how light intensity can modulate the relative proportion of these pigments. The methodological and systematical implications associated with the identification of such compounds in the genus *Tetraselmis* are discussed.

MATERIALS AND METHODS

Algal cultures. *Tetraselmis suecica* (Kylin) Butcher (CCMP 904), *Tetraselmis marina* (Cienk.) R. E. Norris, T. Hori et Chihara (CCMP 898), *Tetraselmis rubens* Butcher (CCMP 899), and *Pyramimonas parkeae* R. E. Norris et B. R. Pearson (CCMP 724) strains were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). *Tetraselmis chui* Butcher (CCAP 8/6), *Tetraselmis subcordiformis* (Wille) Butcher (CCAP 161/1A), and *Tetraselmis tetrathele* Butcher (CCAP 66/1A) were obtained from the Culture Collection of Algae and Protozoa (Oban, Scotland, UK). Cultures were maintained at 15 ± 1°C in L1 enriched seawater medium (Guillard and Hargraves 1993) under 100 μmol photons · m^{-2} · s^{-1} of cool-white fluorescent light (Osram, Augsburg, Germany) on a 12:12 light:dark (L:D) cycle. Additional cultures of *T. suecica* were grown under 20 and 500 μmol photons · m^{-2} · s^{-1}.

Mass cultures of *T. suecica* and *P. parkeae* were grown in 25 L polycarbonate flasks, each containing 2 L of inoculum obtained from an exponentially growing culture maintained in the same conditions and diluted to 20 L with L1 medium. These cultures were grown at 15 ± 1°C under 150 μmol photons · m^{-2} · s^{-1} of cool-white fluorescent light on a 12:12 L:D cycle, with aeration during the light period.

Sample preparation. Cells were harvested at 4–6 h into the light cycle from exponentially growing cultures. Ten milliliters of each culture was filtered onto 25 mm Whatman GF/F (Whatman International, Maidstone, UK) filters using less than 20 kPa vacuum. Filters were frozen immediately at −25°C and analyzed within 12 h.

Pigment extraction. Frozen filters were extracted under low light in Teflon-lined screw-capped tubes with 5 mL 90% acetone using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 min in an ultrasonic bath. Extracts were then filtered through 25 mm diameter hydrophilic Teflon (PTFE) syringe filters (MFS HP020, 0.2 μm pore size, Advantec MFS Inc., Dublin, CA, USA) to remove cell and filter debris. An aliquot (0.5 mL) of the acetone extract was mixed with 0.2 mL of water, and 200 μL was injected immediately into the HPLC. This procedure avoids peak distortion of early eluting peaks (Zapata and Garrido 1991) and prevents the loss of nonpolar pigments before injection (Latasa et al. 2001).

HPLC pigment analysis. Two HPLC methods were used: the C_8 method of Zapata et al. (2000) and a modified version of this method, optimized for the analysis of the pigment suite of *Tetraselmis* species. The chromatographic equipment was a Waters (Milford, MA, USA) Alliance HPLC System consisting of a 2695 separation module, a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector using a Sat/In analog interface. The chromatographic system was controlled using Millenium[32] software (Waters). The stationary phase was a C_8 column (Waters Symmetry, 150 × 4.6 mm, 3.5 μm particle size, 100 Å pore size, Waters) thermostated at 25°C with a circulating water bath (Neslab RTE 200, Thermo Electron Corporation, Newington, NH, USA). Mobile phases were: A = methanol:acetonitrile:aqueous pyridine solution (0.025 M pyridine, pH adjusted to 5.0 with acetic acid) in the proportions 50:25:25 (v/v/v), and B = acetonitrile:acetone (80:20 v/v). A segmented linear gradient was (min, %B): 0 min, 0%; 22 min, 40%; 28 min, 95%; 38 min, 95%. Initial conditions were reestablished by reversing the linear gradient (4 min), and the flow rate was 1 mL · min^{-1}. A modified protocol optimizing the separation of loroxanthin employed the same column, temperature, and gradient, but mobile phases were A = methanol:acetonitrile:aqueous pyridine solution (0.025 M pyridine, pH adjusted to 5.0 with acetic acid) in the proportions 50:40:10 (v/v/v), and B = acetonitrile:acetone (80:20 v/v). Solvents were HPLC grade (Romil-*SpS*™ Ltd., The Source Convent Waterbeach, Cambridge, UK). Pyridine was reagent grade (Merck KGaA, Darmstadt, Germany).

Pigment identification. Pigments were identified either by cochromatography with authentic standards obtained from SCOR reference cultures and diode-array spectroscopy (see Zapata et al. 2000) or by mass spectrometry (HPLC–MS). After checking for peak purity, spectral information was compared with a library of chl and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see Jeffrey and Wright 1997).

Isolation and purification of unknown pigments. Mass cultures were harvested by continuous centrifugation at 4°C using a Beckman Avanti J-25 centrifuge with a JCF-Z rotor (Coulter-Beckman, Fullerton, CA, USA). The freshly packed biomasses were extracted with nine times their volume of cold acetone and centrifuged, and the cell pellets were reextracted several times with acetone:methanol (7:3 v/v) until colorless. The successive extracts were mixed, and each sample was injected into the optimized C_8 HPLC system under semipreparative conditions. Loroxanthin and loroxanthin-related pigments were isolated by collecting the corresponding chromatographic fractions from several runs at the detector outlet.

Visible spectroscopy. Aliquots of purified pigments were dried under a gentle stream of nitrogen at low temperature. Dried residues were redissolved in adequate volumes of analytical grade acetone, methanol, or hexane, and the corresponding spectra (350–600 nm) measured at room temperature with a Perkin-Elmer Lambda 35 UV/Vis spectrophotometer (Perkin-Elmer, Waltham, MA, USA). The coefficient percentage III/II, describing spectral fine structure, was calculated in the three solvents as described by Ke et al. (1970). All these operations were performed under subdued light.

Mass spectrometry. Electrospray mass spectra (ES–MS) were obtained with an LCQ Deca XP mass spectrometer coupled to a Thermo Finnigan liquid chromatograph (Thermo Electron Corporation, San Jose, CA, USA). The purified pigments in acetone were infused at 5 to 15 μL · min^{-1} into a mobile phase of 95% aqueous methanol. Mass spectra of the unknown carotenoids were acquired in positive ion mode. The compounds were analyzed using full MS scans and tandem mass spectrometry (MS/MS) experiments. Significant ions of the

direct mass spectrum that were abundant enough, were isolated in the ion trap and subjected to MS/MS fragmentation.

Fatty acid analysis. The fatty acid moieties of esterified carotenoids were converted into fatty acid methyl ester (FAME) by transesterification using methanolic H_2SO_4 as described by Christie (1989) or converted into 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives by direct reaction of the isolated pigment with 2-amino-2-methylpropanol as described by Garrido and Medina (1994). Both FAME and DMOX derivatives were analyzed by GC–MS using a Hewlett-Packard Model 5890 gas chromatograph equipped with a split/splitless injection system in combination with a Hewlett-Packard 5971 mass-selective detector (Agilent Technologies Inc., Santa Clara, CA, USA). A fused silica capillary column (0.25 mm i. d. × 30 m) coated with 68% cyanopropyl silicone (SP-2330, Supelco Inc., Bellefonte, PA, USA) was employed. Helium was used as carrier gas at a column head pressure of 5 psi. The oven temperature was maintained at 80°C for 2 min, raised at 40°C · min^{-1} to 150°C and then to a final temperature of 210°C (at a rate of 1°C · min^{-1}). The mass spectrometer was operated in the electron impact ionization mode (70 eV). The transfer line and ion source temperature were both 280°C.

RESULTS

HPLC analysis. When a pigment extract of *T. suecica* was analyzed using the HPLC method of Zapata et al. (2000), two carotenoid peaks (Fig. 1C, peaks g and h) eluting between lutein (peak f) and chl *b* (peak i) were observed. These peaks, which had already been observed during the development of the HPLC method (Zapata et al. 2000), showed online spectra, identical to that of lutein (Table 1). This spectral similarity should correlate with the same conjugated double bond system, and suggested the possible occurrence of loroxanthin related carotenoids that share the chromophore with β,ε-carotene and lutein. As the occurrence of loroxanthin dodecenoate had been previously described in *P. parkeae* (Kohata and Watanabe 1989), we analyzed this species with the same HPLC method. The result (Fig. 1A) shows the same retention time and online spectral features for peak h in both species. Free loroxanthin, previously described in *P. parkeae* (Kohata and Watanabe 1989), was not detected by this method in *T. suecica* or *P. parkeae* extracts. However, its presence cannot be completely discounted since the online spectrum corresponding to 9′-*cis*-neoxanthin (peak b) is altered in its chromatographic retention time and has a slightly higher wavelength maxima (Table 1) and

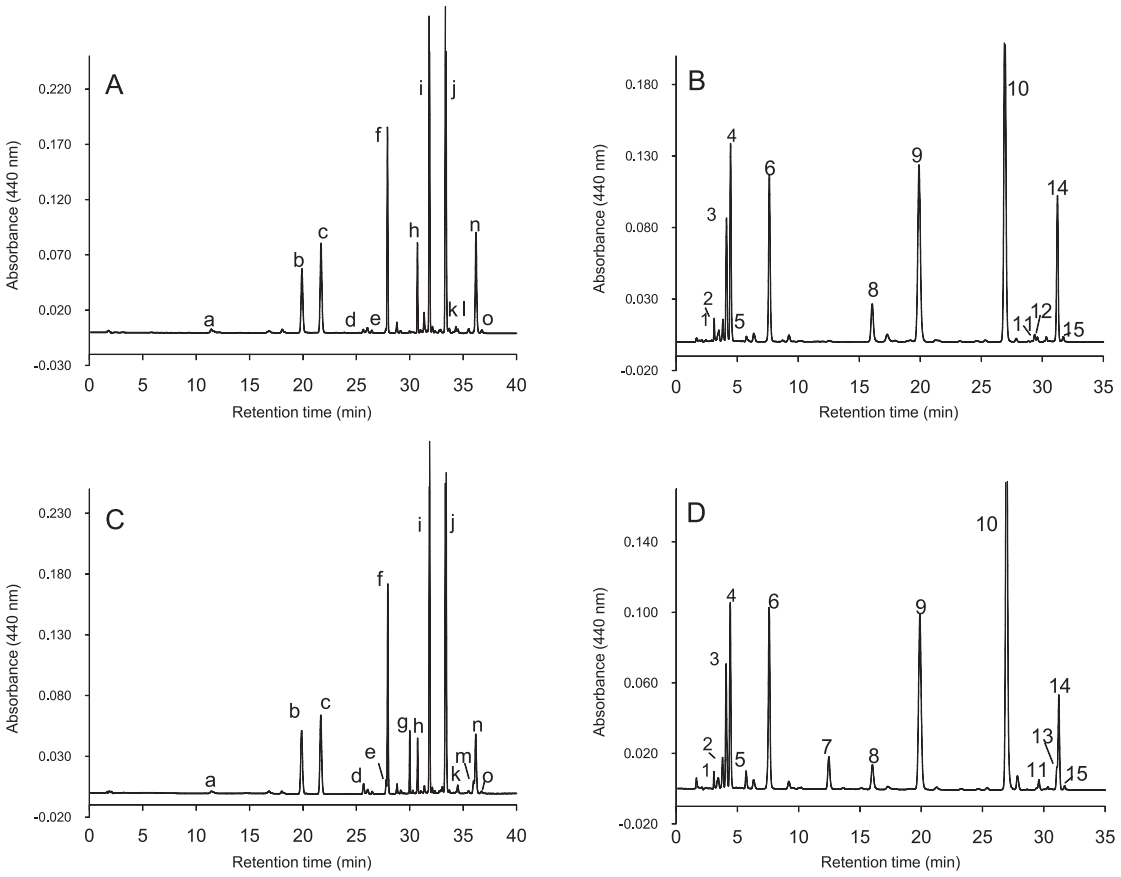

FIG. 1. HPLC chromatograms of pigment extracts from (A, B) *Pyramimonas parkeae* (CCMP 724) and (C, D) *Tetraselmis suecica* (CCMP 904), obtained either with the method of Zapata et al. (2000) (A and C) or the modified method described in this work (B and D). Peak identification as in Table 1.

TABLE 1. Peak identification, retention, and online spectral characteristics of the major pigments from *Tetraselmis* and *Pyramimonas* extracts.

Peak	Pigment	Ret. time	λ max in eluent		
HPLC method of Zapata et al. (2000)					
a	Chlorophyllide *a*	11.44	430	618	663
b	Loroxanthin +9′-*cis*-neoxanthin	19.88	413	439	466
c	Violaxanthin	21.68	415	441	470
d	Antheraxanthin	25.68	(422)	447	475
e	Zeaxanthin	27.78	(428)	453	480
f	Lutein	27.95	(423)	447	475
g	Loroxanthin decenoate	30.00	(425)	448	476
h	Loroxanthin dodecenoate	30.74	(422)	448	476
i	Chl *b*	31.85	462	599	649
j	Chl *a*	33.38	431	617	663
k	β,ψ-carotene	34.33	436	463	493
l	β,ψ-carotene-like	34.49	436	463	493
m	β,ε-carotene	35.98	(423)	447	475
n	ββ-carotene	36.18	(426)	453	478
o	*cis*-ββ-carotene	36.76	(418)	446	469
Modified HPLC method					
1	Chlorophyllide *a*	3.12	434	623	665
2	Loroxanthin	3.84	(423)	447	475
3	9′-*cis*-neoxanthin	4.13	413	437	465
4	Violaxanthin	4.46	415	441	470
5	Antheraxanthin	5.76	(422)	447	475
6	Zeaxanthin + lutein	7.64	(423)	447	475
7	Loroxanthin decenoate	12.46	(423)	448	475
8	Loroxanthin dodecenoate	16.02	(422)	448	476
9	Chl *b*	19.90	463	599	649
10	Chl *a*	26.93	431	617	663
11	β,ψ-carotene	29.37	436	463	493
12	β,ψ-carotene-like	29.66	436	463	493
13	β,ε-carotene	31.07	(423)	448	475
14	ββ-carotene	31.23	(426)	453	478
15	*cis*-ββ-carotene	31.72	(418)	446	469

less-pronounced fine structure (% III/II = 84) relative to the 9′-*cis*-neoxanthin of our pigment data base (% III/II = 88).

Pigment extracts of *P. parkeae* and *T. suecica* were analyzed using a modified method to optimize separation of loroxanthin (see Materials and Methods section). In this procedure, the mobile A phase contained more acetonitrile relative to water content. A new peak with a lutein-type chromophore appeared in both chromatograms (peak 2, Fig. 1, B and D). The peak corresponding to 9′-*cis*-neoxanthin (peak 3) exhibited expected spectral features. Again, in the retention period between lutein and chl *b*, the peak attributed to loroxanthin dodecenoate of *P. parkeae* (peak 8) eluted at the same retention time in the chromatograms of both species. The slightly more polar compound with an identical online spectrum (peak 7) was detected only in the chromatogram of *T. suecica* (Fig. 1D). This modified HPLC method was also employed for the semi-preparative separation and purification of compounds in peaks 2, 7, and 8 (Fig. 1, B and D).

Mass spectrometry. When submitted to ESI–MS analysis, the carotenoid isolated in peak 2 from *T. suecica* afforded (Table 2) a clear ion at m/z = 584 attributed to loroxanthin molecular ion ($[M]^+$), which was further isolated and fragmented in the ion trap (MS/MS experiment). The fragment ions are coherent with the loroxanthin structure and can be explained as originating from the elimination of water from hydroxyl groups ($[M\text{-}18]^+$) or the elimination of an in-chain fragment with a formal loss of toluene ($[M\text{-}92]^+$), or resulting from an elimination reaction at the ε terminal group ($[M\text{-}138]^+$) (Enzell and Back 1995), and their combinations. Most of these fragment ions coincide with those obtained for loroxanthin by Kohata and Watanabe (1989) in an electron impact MS experiment.

The mass spectra obtained for the carotenoids in peak 8 isolated either from *T. suecica* or from *P. parkeae* were identical, confirming that the same compound occurs in both species. The direct mass spectrum shows peaks corresponding to the molecular ion (m/z = 764, $[M]^+$) and two fragment ions (Table 2). The protonated adduct could not be detected in the spectra, so it is supposed to readily undergo cleavage to produce the fragment ions that are explained by formal losses of water (m/z = 747, $[M+H\text{-}18]^+$) and of dodecenoic acid (m/z = 567, $[M+H\text{-}198]^+$). The MS/MS spectra (parent ion m/z = 719, $[M+H\text{-}18]^+$) showed ions resulting from

TABLE 2. Major fragments in the mass spectra of selected pigments isolated from *Tetraselmis suecica* (peaks 2, 7, and 8) and *Pyramimonas parkeae* (peaks 2 and 8). Figures in bold indicate the base peak in the direct spectrum. Arrows indicate the fragments that were isolated in the ion trap and submitted to MS/MS fragmentation.

Pigment (peak no, retention time in Fig. 1)	MS signals	
	MS	MS/MS
Loroxanthin (peak 2, retention time = 3.84 min)	**584** $[M]^+$ →	566 $[M\text{-}18]^+$, 492 $[M\text{-}92]^+$, 474$[M\text{-}92\text{-}18]^+$, 446$[M\text{-}138]^+$, 428 $[M\text{-}138\text{-}18]^+$
Loroxanthin derivative (peak 7, retention time = 12.46 min)	736 $[M]^+$ **719** $[M+H\text{-}18]^+$ → 567 $[M+H\text{-}170]^+$	701 $[M+H\text{-}18\text{-}18]^+$, 549 $[M+H\text{-}18\text{-}170]^+$, 531 $[M+H\text{-}18\text{-}18\text{-}170]^+$
Loroxanthin derivative (peak 8, retention time = 16.02 min)	764 $[M]^+$ **747** $[M+H\text{-}18]^+$ → 567 $[M+H\text{-}198]^+$ 549 $[M+H\text{-}18\text{-}198]^+$	729 $[M+H\text{-}18\text{-}18]^+$, 549 $[M+H\text{-}18\text{-}198]^+$, 531 $[M+H\text{-}18\text{-}18\text{-}198]^+$

MS, mass spectrometry.

combinations of the mentioned losses (Table 2). This fragmentation pattern is in agreement with that shown by Kohata and Watanabe (1989) for loroxanthin dodecenoate employing both electron impact and field desorption MS.

The mass spectrum of the carotenoid corresponding to peak 7 in *T. suecica* (Table 2) can be explained in a similar way. Peaks in the direct MS spectrum can be attributed to the molecular ion ($m/z = 736$, $[M]^+$) and to fragment ions originated from a protonated adduct ($m/z = 719$, $[M+H-18]^+$) and $m/z = 567$, $[M+H-170]^+$). This latter ion could be explained by the loss of decenoic acid. The combination of these losses constituted the fragmentation pattern when the base peak ($m/z = 719$, $[M+H-18]^+$) was submitted to MS/MS.

Both pigments (peaks 7 and 8 in Fig. 1, B and D) showed common fragment ions once the lateral chain was lost (at $m/z = 567$, 549, and 531, corresponding to the successive losses of three hydroxyl groups), confirming that loroxanthin is the esterified carotenoid in both cases.

Fatty acid analysis. As expected, the FAMEs derived from peak 8 (Fig. 1, B and D) isolated either from *P. parkeae* or from *T. suecica* showed an identical mass spectrum (Fig. 2A), which is diagnostic for 2-dodecenoic acid methyl ester. The presence of an ion at $m/z = 87$ as the base peak together with a distinctive ion at $m/z = 113$ is characteristic of the occurrence of a double bond at position 2 (Christie 2008). Although the expected molecular ion ($m/z = 212$) was not detected, other diagnostic peaks confirm the molecular mass. Thus, ions representing the loss of methoxyl ($m/z = 181$, $[M-31]^+$), and the loss of the McLafferty ion ($m/z = 138$, $[M-74]^+$) are abundant, as is the McLafferty ion per se ($m/z = 74$). A characteristic ion (Christie 2008) at $[M-116]^+$ ($m/z = 96$) is also abundant.

The mass spectrum of the FAME derived from the additional carotenoid in *T. suecica* (peak 7), shown in Figure 2B, shows similar features and can be interpreted in the same way. Again, ions at $m/z = 87$ and $m/z = 113$ suggest a double bond at position 2. The expected molecular mass of this acid, deduced from the loss of 170 Da in the electrospray mass spectrum of the pigment, is compatible with a structure with 10 carbon atoms and one double bond. This finding implies a molecular ion for the methyl ester at $m/z = 184$, which cannot be observed in our spectrum but can be confirmed by ions at $m/z = 153$ ($[M-31]^+$), $m/z = 110$ ($[M-74]^+$), and $m/z = 68$ ($[M-116]^+$). This evidence strongly suggests that 2-decenoic acid is esterified to loroxanthin in this pigment.

The analysis by GC–MS of the DMOX derivatives served as an additional confirmation of the identity of these acids. The mass spectra of DMOX derivatives of 2-monoenoic acids show clear peaks for molecular ions, the $[M-15]^+$ ion as the base peak and a prominent peak at $m/z = 110$, while the ions at $m/z = 113$ and 126 (usually very abundant in the spectra of DMOX derivatives of other fatty acids)

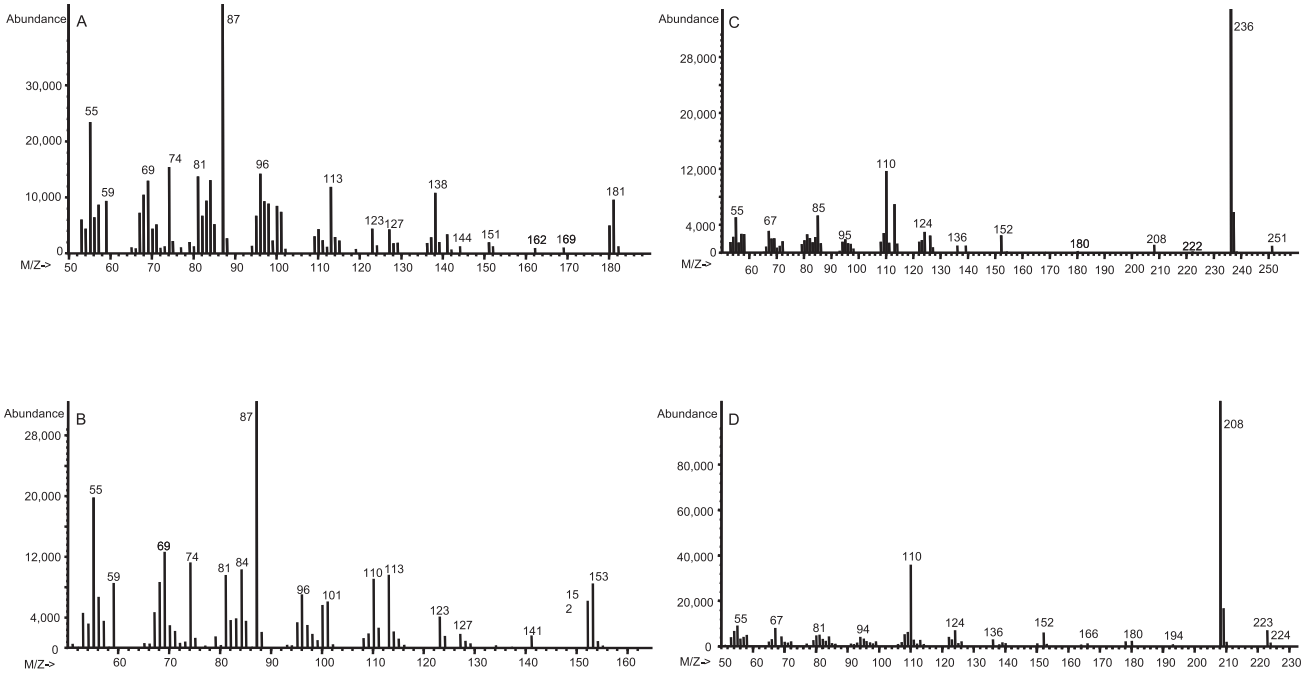

FIG. 2. Mass spectra of derivatives of 2-dodecenoic acid (A and B) and of 2-decenoic acid (C and D). The acids were analyzed by GC–MS of their methyl esters (A and C) or their corresponding DMOX derivatives (B and D). DMOX, 2-alkenyl-4,4-dimethyloxazoline; GC–MS, gas chromatography–mass spectrometry.

TABLE 3. Visible absorption maxima and spectral fine structure in standard solvents of loroxanthin and loroxanthin esters isolated from *Tetraselmis suecica*.

Pigment	Acetone				Diethyl ether				Hexane			
	λ max.			%III/II	λ max.			%III/II	λ max.			%III/II
Loroxanthin (peak 2)	(421.5)	447.0	474.4	42.7	(422.0)	444.4	472.2	48.4	422.5	444.6	473.0	57.5
Loroxanthin-decenoate (peaks g and 7)	(422.3)	449.9	477.1	41.2	(422.3)	446.5	474.4	49.6	422.2	445.4	473.9	62.7
Loroxanthin-dodecenoate (peaks h and 8)	(422.8)	449.2	476.7	42.3	(422.3)	446.2	474.3	48.9	422.4	445.5	474.1	64.2

Wavelengths given in parentheses denote shoulders in the absorption spectrum.

TABLE 4. Pigment to chl *a* molar ratios of *Tetraselmis* species and *Pyramimonas parkeae* (ND = not detected).

	Loro	Neo	Viola	Anth	Lut	Loro-Dec	Loro-Dodec	Chl *b*	βψ-car	βε-car	ββcar	Total Loro pigments	Total βε-pigments
Tetraselmis chui (CCAP 8/6)	0.096	0.195	0.222	0.007	0.360	0.069	0.051	0.517	0.010	0.083	0.121	0.216	0.576
T. marina (CCMP 898)	0.154	0.222	0.360	0.026	0.305	0.088	0.026	0.624	0.008	0.062	0.146	0.268	0.573
T. rubens (CCMP 899)	0.037	0.248	0.308	0.020	0.430	0.041	0.013	0.623	0.037	0.032	0.193	0.091	0.521
T. subcordiformis (CCAP 161/1A)	0.058	0.220	0.263	0.009	0.410	0.086	0.071	0.560	0.010	0.053	0.193	0.215	0.625
T. suecica (CCMP 904)	0.037	0.168	0.254	0.026	0.350	0.061	0.051	0.527	0.020	0.027	0.175	0.149	0.499
T. tetrathele (CCAP 66/1A)	0.038	0.207	0.291	0.014	0.518	0.062	0.050	0.543	0.012	0.018	0.090	0.150	0.668
Pyramimonas parkeae (CCMP 724)	0.034	0.196	0.334	0.011	0.375	ND	0.097	0.660	0.017	ND	0.334	0.131	0.506

TABLE 5. Pigment to chl *a* molar ratios of *Tetraselmis suecica* at different light intensities (ND = not detected).

	Light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Loro	Neo	Viola	Anth	Zea	Lut	Loro-dec	Loro-dodec	Chl *b*	βψ-car	βε-car	ββ-car	Total βε-pigment
Tetraselmis suecica	20	0.113	0.195	0.241	0.006	0.003	0.240	0.052	0.055	0.506	0.035	0.097	0.121	0.557
(CCMP 904)	100	0.037	0.168	0.254	0.026	0.020	0.320	0.061	0.051	0.527	0.020	0.027	0.175	0.496
	500	0.011	0.256	0.220	0.165	0.185	0.801	0.034	0.033	0.568	0.175	ND	0.333	0.879

are inconspicuous (Christie 2008). All of these features are shown in the spectra of the acids under study (Fig. 2, C and D), with diagnostic peaks at m/z = 251 ($[M]^+$), 236 ($[M\text{-}15]^+$, base peak), and 110 for 2-dodecenoate (Fig. 2C) and at m/z = 223 ($[M]^+$), 208 ($[M\text{-}15]^+$, base peak), and 110 for 2-decenoate (Fig. 2D).

Visible spectroscopy. The spectral maxima and fine structure of the isolated pigments in three standard solvents are shown in Table 3.

Pigment composition of Tetraselmis *species.* Once the new pigments of *T. suecica* had been identified, other *Tetraselmis* species were analyzed (Table 4). Loroxanthin and both loroxanthin esters occurred in all the species analyzed. The proportion of loroxanthin decenoate was higher than that of loroxanthin dodecenoate. Except for *T. marina*, the sum of esterified forms exceeded the proportion of free loroxanthin. Other carotenoids are typical of green algae, with lutein, violaxanthin, and neoxanthin in the highest proportions. β,ψ-carotene also appeared in small proportions.

Influence of irradiance on T. suecica *pigment composition.* The molar ratios of β,ε-carotene, loroxanthin, and loroxanthin esters to chl *a* decreased with increasing light intensities (Table 5), with the nonesterified pigments suffering the highest variations. Other carotenoids (lutein, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, β,β-carotene, and β,ψ-carotene) showed the reversed trend, with highest pigment/chl *a* molar ratios at high-light (500 μmol photons $\cdot m^{-2} \cdot s^{-1}$).

DISCUSSION

Although *Tetraselmis* species have been widely analyzed (Brown and Jeffrey 1992, Egeland et al. 1995, Zapata et al. 2000, Bustillos-Guzmán et al. 2002, Latasa et al. 2004, Sigaud-Kutner et al. 2005), the presence of loroxanthin in this genus has never been reported. This is probably due to either the coelution of loroxanthin with neoxanthin in most of the chromatographic systems employed or to the low abundance of loroxanthin at high light (Table 5). For example, when analyzing *P. parkeae* (CCMP 724), the expected spectrum for neoxanthin was altered to the extent that the authors proposed the presence of an unknown carotenoid that ''eluted at the same retention time as neoxanthin but with maxima at 440 and 468 nm, which was different from neoxanthin or loroxanthin'' (Latasa et al. 2004). These maxima are intermediate between those of loroxanthin and neoxanthin and are produced when these pigments coelute in the same peak (Table 1).

However, loroxanthin was separated from neoxanthin in the pigment extracts of other species

when acetonitrile-enriched mobile phases were employed with a C_{18} stationary phase (Kohata and Watanabe 1989, Fawley 1991, Schagerl and Angeler 1998, Pineau et al. 2001). By increasing the acetonitrile content and reducing the water proportion of mobile phase A in our HPLC method, loroxanthin and neoxanthin were also successfully separated on a C_8 stationary phase (Fig. 1).

Mass spectrometry and GC–MS results confirm the presence of loroxanthin and loroxanthin 19-(2-dodecenoate) (Fig. 3) in *P. parkeae*, as described by Kohata and Watanabe (1989) and Sasa et al. (1992), and demonstrate the occurrence of these pigments in *T. suecica*.

The occurrence of 2-decenoic acid esterifying loroxanthin (Fig. 3) is reported here for the first time. The mass spectrum of the DMOX derivative of 2-decenoate here reported (Fig. 2D) shows a fragmentation pattern similar to that reported by Luthria and Sprecher (1993) for the DMOX derivative of 3-decenoate. However, our spectrum correlates with the fragmentation patterns of other 2-monounsaturated fatty acid derivatives (2-dodecenoate: this work; 2-octadecadecenoate: Christie et al. 2000), and the position of the double bond is also confirmed by the mass spectrum of the corresponding methyl ester (Fig. 2B). Our results confirm that the spectrum reported by Luthria and Sprecher (1993) does not correspond to 3-decenoate, but to 2-decenoate, probably having isomerized during derivatization, as suggested by Christie (2008).

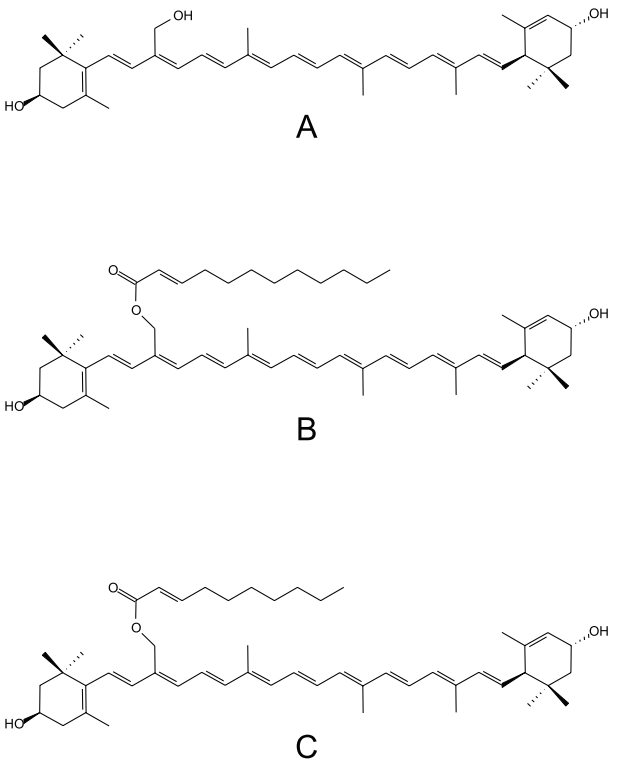

FIG. 3. Structures of the new carotenoids in *Tetraselmis* species tentatively identified in this work. (A) Loroxanthin, (B) loroxanthin 19-(2-dodecenoate), and (C) loroxanthin 19-(2-decenoate).

Although further studies would be needed to assess the *cis-trans* isomerism of the Δ2 double bond in the esterifying acids, the *trans* configuration seems to be most probable, considering that all loroxanthin and siphonaxanthin fatty acid esters heretofore reported in prasinophyceans have been Δ2 monounsaturated esters with a *trans* form (Yoshii 2006). This has been the case in *P. parkeae* (loroxanthin-*trans* Δ2 dodecenoic acid ester, Sasa et al. 1992), in *Pyramimonas amilifera* (siphonaxanthin-*trans* Δ2 dodecenoic acid ester and siphonaxanthin-*trans* Δ2 decenoic acid ester, Egeland et al. 1997), and in *Pterosperma cristatum* (-*trans* Δ2 tetradecenoic and -*trans* Δ2 dodecenoic acid esters of both siphonaxanthin and 6-OH siphonaxanthin, Yoshii et al. 2002). Only *Mesostigma viride* contains siphonaxanthin esters with the saturated dodecenoic and tetradecanoic acids (Yoshii et al. 2003).

Several studies have found loroxanthin associated with light-harvesting antennas in chlorophyte algae (Plumley and Schmidt 1995, Pineau et al. 2001). Niyogi et al. (1997) suggested that α-carotene-derived xanthophylls like lutein and loroxanthin can contribute to the dissipation of excess absorbed light energy. However, our results point out that loroxanthin and loroxanthin esters could have a light-harvesting role in *Tetraselmis* species, as they accumulate in *T. suecica* at low light intensity (Table 5). The decrease of loroxanthin pigments and the increase of lutein as light intensity increases seem to restrict the photoprotective role to the last one. It has been suggested that pigments can bind proteins in pigment-protein complexes as specific pairs, both as end products and their biosynthetic precursors (Grossman et al. 2004). This possibility would be the case for the pair loroxanthin-lutein, in which loroxanthin is derived from lutein by hydroxylation of the methyl group at C19 (although the nature of the reaction and the putative loroxanthin synthase are not known, Grossman et al. 2004). Our results suggest that loroxanthin and lutein could interconvert as a response to changes in light intensity, but de novo synthesis of lutein seems to be necessary at high light to explain the dramatic increase (> 3-fold) in the lutein/chl *a* ratio (Table 5). The very low proportion of loroxanthin under high light could hamper its correct identification even when HPLC methods with enough resolution are employed.

It has to be noted that while ''free'' loroxanthin undergoes a dramatic decrease with light (the loroxanthin to chl *a* ratio at 500 μmol photons $\cdot m^{-2} \cdot s^{-1}$ is only 10% of the same ratio at 20 μmol photons $\cdot m^{-2} \cdot s^{-1}$), the variation of loroxanthin esters to chl *a* ratio is much smaller (Table 5). This fact could be explained if these pigments were also involved in a structural function

that would require specific stoichiometries in pigment-protein complexes. The uncommon structural features of the esterifying fatty acid seem to be related to a possible structural role. It has been suggested that, as the double bond at the Δ2 position of the fatty acid is rigid, the Δ2 unsaturated fatty acid esters of carotenoids are likely to play a role in the structural stability of the antenna system (Yoshii 2006). Moreover, Yoshii et al. (2002) showed that in *Pterosperma cristatum* Δ2 unsaturated fatty acids are synthesized only as components of pigments (siphonaxanthin esters).

In a recent review, Yoshii (2006, p. 224) examined *T. suecica* and determined that its carotenoid composition ''is very similar to *Nephroselmis olivacea* and typical green plants (Egeland et al. 1997, Yoshii et al. 2002).'' However, the new pigment composition here described for six *Tetraselmis* species would shift this genus from pigment type 1 (common green algal carotenoids) to pigment type 2 (loroxanthin-siphonaxanthin series, sensu Egeland et al. 1997). The results obtained in this work encourage a reexamination of the pigment composition of other non-prasinoxanthin-containing prasinophyceans (e.g., members of the Chlorodendraceae like other *Tetraselmis* species and representatives of the genus *Scherffelia*) using adequate HPLC methods.

On the other hand, loroxanthin has been detected not only in prasinophyceans but also in other classes of Chlorophyta: Charophyceae, Chlorophyceae, Pleurastrophyceae, and Ulvophyceae (Fawley 1991). The systematic significance of loroxanthin in the class Chlorophyceae has been discussed (Fawley 1991, Fawley and Buchheim 1995, Schagerl and Angeler 1998, Yoshii et al. 2004). No loroxanthin esters had been described in these algal classes until Laza-Martinez et al. (2007), applying modern HPLC methods, detected both loroxanthin and loroxanthin esters in the Ulvophyceae *Oltmannsiellopsis* sp. This result emphasizes the need for the reexamination of all these taxa to determine the presence of loroxanthin esters and their chemotaxonomical significance.

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