

**TEMPORAL VARIATION IN THE PIGMENT COMPOSITION OF
CAULERPA PROLIFERA (FORSSKÅL) LAMOUROUX MEADOWS IN
THE MAR MENOR LAGOON (SE SPAIN)**

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Abstract

Eighteen photosynthetic pigments (chlorophylls, carotenoids and degradation products) were separated and quantified from *Caulerpa prolifera* in a single-step procedure by reversed-phase high-performance liquid chromatography over the period from November 1995 to October 1996.

The chlorophyll *a* and chlorophyll *b*, are typical and characteristic pigments of the *Caulerpa prolifera*, appeared in a quantity very similar. In several months of the year the chlorophyll *b* appears in greater quantity than the chlorophyll *a*, especially during the months of June and July, coinciding with the greater biomass of the alga, and also with a great concentration of the carotenoid siphonein.

As regards the carotenoids, it was found that violaxanthin, siphonoxanthin and zeaxanthin-like were the most common carotenoids.

Key words : *Caulerpa prolifera*, seaweed, chlorophylls, carotenoids.

Introduction

Caulerpa prolifera (Forsskål) Lamouroux is widely distributed coenocytic species in the Western Mediterranean Sea. It is an invasive seaweed that has successfully colonized a great part of the Mar Menor lagoon (SE Spain) which cover more than 85 - 90 % of the bottom (Terrados, 1991 and Hegazi, 1999). The spreading of *Caulerpa* is still in progress and it is colonizing also rocky substrates. *Caulerpa* meadows and other marine plants are the main primary producers in the lagoon. They form the standing crop and determine the productivity of all communities in the lagoon. *Caulerpa* like all other species of algae are characterized by specific sets of pigment, chlorophyll *a* being the most abundant, while the other photosynthetic pigments are considered as accessories (Dawes, 1981 and Hegazi, *et al.* 1998). Variations in *Caulerpa* colour are related to varying amounts of pigment (chlorophylls, carotenoids and their breakdown products), with changes in colour during the growth and reproduction being caused by the accumulation of secondary carotenoids (Burczyk, 1987). Although many methods involving column and thin-layer chromatography have been described for the separation and identification of seaweed pigments (Jeffrey, 1969, Kleining, 1969, Czezug, 1975, 1976, 1986, Burczyk, 1987, Palermo *et al.* 1991). It is difficult to separate all the

photosynthetic pigments in a single-step procedure. However, while great efforts have been made to separate the photosynthetic pigments of phytoplankton (Mantoura and Llewellyn, 1983, Wright and Shearer, 1984, Kleppel, *et al.* 1988, Kohata, *et al.* 1991, Wright, *et al.* 1991, Kraay, *et al.* 1992, Van Heukelem, *et al.* 1992, 1994, Jeffrey *et al.* 1997). Little time has been dedicated to those of seaweeds until the work done by Hegazi *et al.* (1998) has led to the wide spread use of reverse phase high performance liquid chromatography (HPLC) techniques for the determination of seaweed pigments.

The aim of this work is to determine the concentrations of individual photosynthetic pigments in the *Caulerpa prolifera* meadows using a sensitive method of HPLC analysis in one year, for acquisition of data on the primary productivity of the lagoon and find a relationship between colour change and the concentration of photosynthetic pigments.

Material and Methods

Caulerpa prolifera was collected from the hyper saline coastal lagoon (Mar Menor) SE Spain over a period of one year from November 1995 to October 1996. The samples were done by skin and SCUBA diving. The algal thallus was gently washed by sea water and immediately transferred carefully to the laboratory in an ice box and stored at - 80 °C until the moment of chromatographic analysis. The algal thallus was dried gently with absorbent Whatman filter papers for a few seconds and weighed. The thallus was ground manually in a porcelain mortar in a cold and darkened fume cupboard to prevent photo-oxidative breakdown of the labile pigments (Jeffrey, 1961). In the present work 100 % acetone and small amount of magnesium carbonate were added in order to prevent the accidental formation of chlorophyll metabolites. This procedure was repeated until the algal thallus became colorless, at which point 5 ml of ethyl ether were added and the extract was filtered. The extract was concentrated by low pressure rotary evaporator at 25 °C. The dry extract was dissolved in 1 ml of acetone, microfiltered at 0.45 µm and 20 µl were injected into the chromatograph.

The analytical HPLC separation of seaweed pigments was carried out on a Hewlett Packard Series 1100 chromatograph and G1315 diode-array detector. Absorbance was registered at 430 nm (carotene detection) and 660 nm (chlorophyll detection). The complete spectrum of the photosynthetic pigments in the 400 to 700 nm range was saved in the computer memory for later interpretation.

The injection loop size was 20 µl. The method used consists of an elution gradient of methanol, acetone and ammonium acetate solution (1 M) Hegazi *et al.* (1998). Semi-preparative HPLC separation of the authentic standard pigments was carried out using a Shimadzu chromatographic system (LC-6A series) equipped with SPD-M6A photodiode array detector. The column was a spherisorb ODS-2, using 5 µm spherical particles (250 mm x 10 mm I.D.). The gradient program was similar to that used in the analytical column with a flow of 4 ml/min. The pigments were collected at the outlet of the detector, and the solvent was evaporated immediately under N₂ flow. The dry pigment was re-dissolved in acetone, benzene, diethyl ether, ethanol, or hexane.

All the separated photosynthetic pigments were identified according to their spectral characteristics and compared with the published data in different types of solvent (Dawson, *et al.* 1969, Foppen, 1971, Mantoura and Llewellyn, 1983, Wright and Shearer, 1984, Kleppel, *et al.* 1988, Kohata, *et al.* 1991, Wright, *et al.* 1991, Kraay, *et al.* 1992,

Van Heukelem, *et al.* 1992,1994, Jeffrey *et al.* 1997). The HPLC peaks were identified by comparing the retention times and spectral data with those of the authentic standards. The quantifications were done according to the methods used by Hegazi (1999).

Results and Discussion

In *Caulerpa prolifera* (chlorophyta), eighteen separate photosynthetic pigments were detected: chlorophyllide *a*, siphonein, neoxanthin, neoxanthin-like, violaxanthin, microxanthin, micronone, micronone-like, lutein-5,6-epoxide, siphonoxanthin, lutein, chlorophyll *b*, chlorophyll 'b', chlorophyll *a*, chlorophyll *a*, α -carotene, β -carotene, and phaeophytin *a*. Siphonoxanthin was seen to be as the most typical and distinct carotene in caulerpales. Chlorophylls *a* and *b* were the most common pigments in the green alga studied and in fact responsible for the green colour of this group of algae. The individual photosynthetic pigments of the *Caulerpa prolifera* studied had a broad spectrum polarity, ranging from the low polarity of carotenes to the very high polarity of chlorophyllides, which are dissociated at neutral pH according to the method of Hegazi *et al.*, 1998.

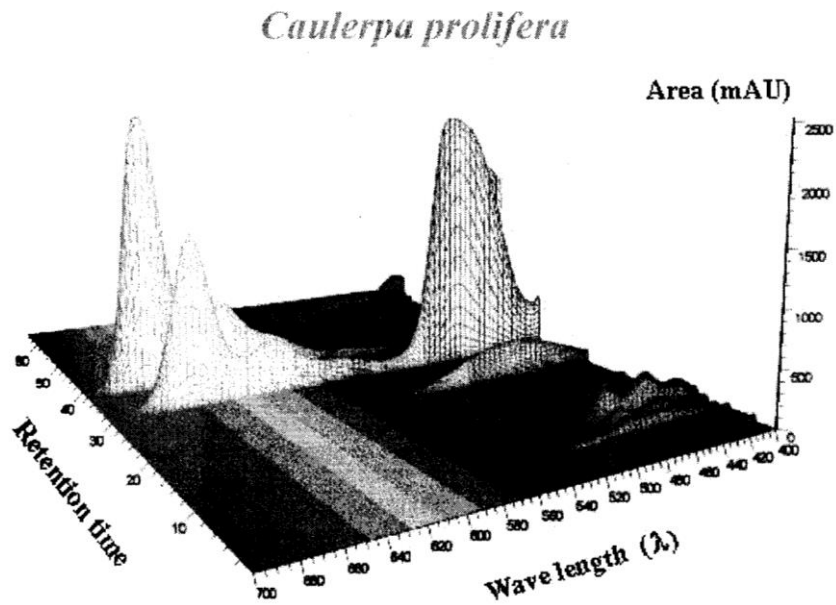


Fig. 1: Three dimensions chromatogram of the individual photosynthetic pigments of *Caulerpa prolifera*.

The procedure used permitted the analysis of chlorophylls, carotenes and xanthophylls in a single-step and at a fixed temperature. Fig. 1 illustrates a well-resolved

three dimensions chromatogram corresponding to the separation of photosynthetic pigments from the green alga *Caulerpa prolifera*. In this chromatogram the separated factor (α) and peak resolution (R_s) were higher than 1. This indicates the absence of overlapping between peaks, while the R_s values demonstrate that resolution between adjacent bands was greater than 98 %.

Low values of the chlorophyll *a* in June and July, which gradually increased until October with 2.32 mg/g fresh weight, where the chlorophyll *b* oscillated between 1.40 mg/g in August and 2.33 mg/g in October. Low amounts of degradation product (chlorophyllide *a* and phaeophytin *a*) were obtained (Fig. 2.1). The carotenoids appeared with high value of siphonein in June and September with 0.27 and 0.29 mg/g respectively. Violaxanthin showed a greater quantity in the months of winter, oscillating between 0.1 and 0.21 mg/g during the study period, followed by siphonoxanthin ranged from 0.08 – 0.17 mg/g, and the β -carotene from 0.06 – 0.12 mg/g (Fig. 2.2).

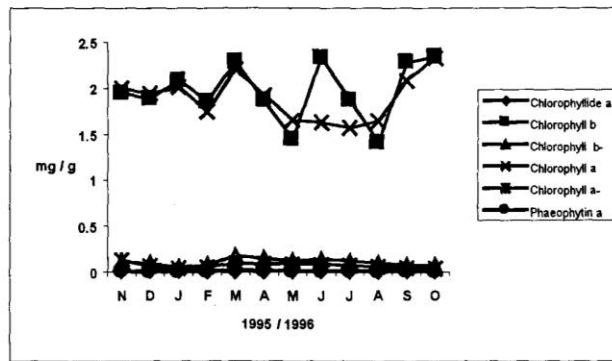


Fig. 2.1. Temporal variation of chlorophylls and degradation products values in mg/g fresh weight of *Caulerpa prolifera*

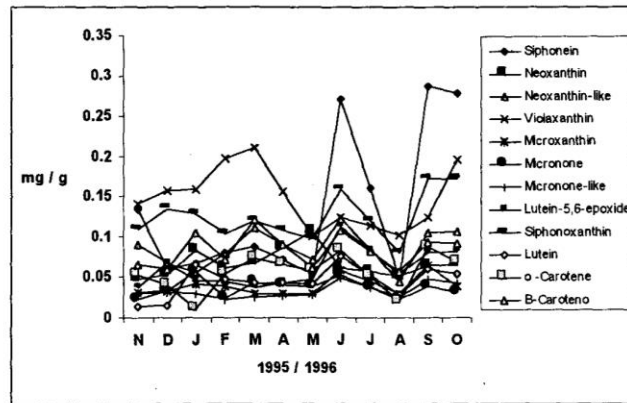


Fig. 2.2. Temporal variation of carotenoids values in mg/g fresh weight values of *Caulerpa prolifera*.

The breakdown of pigments and variations in algal colour were also observed in our study during the reproduction period and under severe environmental conditions in the intertidal zone of the lagoon.

We are agree that all taxonomic groups of algae have a specific sets of pigments which have a different concentration from one species to another. The balance between pigments values were occurred within a few margins in each group and the variability of the concentration must be related to the quantity and quality of the light in the marine environment (Dring, 1982). Nevertheless, Nakayama *et al.* (1983) reported that there is a fixed relationship in brown algae between fucoxanthin and total carotenoids (between 70-90%). Our results confirmed that *Caulerpa prolifera* have their own photosynthetic pigments, and the variation of their concentration values depend on a multiple factors such as depth, temperature, seasonality, habitat, availability of light, etc.,. With all these factors we can calculate the efficiency of photosynthetic process and no fixed proportion between all pigments during the work period.

Table 1 identifies the photosynthetic pigments according to the absorption maxima (nm) of each peak in the mobile phase. These are compared with spectral data in different types of solvents. All the absorption maxima of the spectral data coincide with the previous published data (Foppen, 1971, Burczyk, 1987, Shahidi, *et al.*, 1998 and Hegazi *et al.*, 1998).

Table 1. List of photosynthetic pigments of *Caulerpa prolifera* and spectral data in the mobile phase and in different solvents.

No	Pigment	Acetone	Benzene	Diethyl ether	Ethanol	Hexane	Eluent
1	Chlorophyllide a	428, 616,652		428, 662			408, 432, 508, 536, 580, 608, 664
2	Siphonoin				455		452
3	Neoxanthin		423, 448, 478		415, 438, 467	416, 437, 466	416, 440, 468
4	Neoxanthin-like		423, 448, 478		415, 438, 467	416, 437, 466	416, 436, 464
5	Violaxanthin		428, 454, 483		419, 441, 471	443, 472	416, 440, 472
6	Microxanthin				396, 420, 447	396, 418, 446	424, 448
7	Micronone				445	419, 440,467	440, 468
8	Micronone-like				445	419, 440,467	440, 464
9	Lutein-5,6-epoxide				424, 444,483		420, 440, 468
10	Siphonoxanthin				448		448, 464
11	Lutein				420, 445,475		424, 448, 472
12	Chlorophyll b	454, 596,644		453, 593,642			468, 548, 596, 652
13	Chlorophyll 'b	454, 596,644		453, 592,642			468, 548,596, 652
14	Chlorophyll a	428, 616,662		430, 615,661			412, 432, 532, 580, 616, 664
15	Chlorophyll á	428, 616,662		428, 614,661			412, 432, 532, 580, 616, 664
16	α-Carotene					420, 442,472	420, 448, 476
17	β-Carotene					425, 449,477	428, 452, 476
18	Phaeophytin a	410, 468,668		408, 503,667			412, 448, 472, 508, 536, 560, 608, 668

Our results are more efficient than that of Barlow *et al.* (1997), who used a C₈ column of greater polarity than C₁₈, which would explain the low resolution for polar pigments and reduced retention times. They are also more efficient than those of Van

Heukelem *et al.* (1994), who used more than one run with different temperatures to separate the photosynthetic pigments of phytoplankton.

Although all the steps of the procedure were carried out rapidly using fresh algae in cool, dark fume cupboards to prevent breakdown of the photosynthetic pigments, only small quantities of phaeophytin *a* were detected, while no phaeophytins were detected from other chloropigments or magnesium-free metabolites such as phaeophorbides. The results, therefore, do not agree with those of Zapata *et al.* (1987), who used dried algae in a dessicator at 4 °C for pigment extraction or Henley and Ramus (1989), who analysed pigments extracted over a period of 24 h from *Ulva rotundata* in N, N-dimethyl formamide (DMF) at room temperature. In our opinion, when dried algae are used or the extracts are kept at in room temperature for 24 h, the photosynthetic pigments are broken down and incorrect analytical results ensue.

The advantage of the chromatographic separation technique and quantification used by in this study compared with the other published works is that we used a sensitive method and worked over a period of one year.

Finally all the photosynthetic pigments of *Caulerpa prolifera* listed here have been reported by Hegazi *et al.* (1998) and the quantification is considered a preliminary data for further investigations for seaweed pigments.

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التغيرات الوقتية فى التركيب الصبغى لطحلب الكوليريا بروليفرا فى بحيرة البحر الصغير
(جنوب شرق اسبانيا)

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تم فصل ٨ نوع من الأصباغ المسئولة عن البناء الضوئى (الكلوروفيلات و الكاروتينينات ومشتقاتها) فى طحلب كوليريا بروليفرا فى خطوة واحدة وتقديرها كيميا باستخدام جهاز كروماتوجرافيا السوائل ذو الكفاءة العالية ذى الحالة العكسية فى الفترة من نوفمبر ١٩٩٥ الى أكتوبر ١٩٩٦ .
ظهر الكلوروفيل (أ) و الكلوروفيل (ب) وهما من أنواع الأصباغ المميزة للنوع كوليريا بروليفرا بكمية متقاربة . وقد ظهر الكلوروفيل (ب) بكمية كبيرة عن الكلوروفيل (أ) فى العديد من شهور السنة وخصوصا أثناء شهرى يونيو ويوليو حيث يتطابق مع أعلى معدل للكتلة الحيويه للطحلب خلال هذه الفترة ، وأيضاً مع أعلى تركيز للكاروتين السيفونين .
وبالنسبة للكاروتينات الأخرى فقد وجد أن الفيولاكسانثين و السيفونواكسانثين ونظير-
النيواكسانثين هم أكثر الكاروتينات كيميا خلال مدة الدراسة .