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Variations in Pigment Contents of the Diatom *Phaeodactylum tricornutum* during Growth

J. I. Carreto[1]* and J. A. Catoggio[2]

[1] Instituto de Biología Marina; Mar del Plata, Argentina, and
[2] Departamento de Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata; La Plata, Argentina

Abstract

Changes in the amounts of chlorophyll *a*, chlorophyll *c*, fucoxanthin, diadinoxanthin and β-carotene were determined during *Phaeodactylum tricornutum* growth. The transition of the culture from the logarithmic to the stationary phase is accompanied by an increase in the carotenoids:chlorophyll *a* ratio, associated with variations in the percentage of individual carotenoids. While fucoxanthin content decreases with the age of the culture, diadinoxanthin content increases and β-carotene remains almost constant. The furanoid isomer of diadinoxanthin, absent during logarithmic growth, appears increasingly during the nutrient-deficient period. Changes in the amounts of chlorophyll *a*, chlorophyll *c* and fucoxanthin are quite similar.

Introduction

The pigment composition of marine algae is related to the taxonomic affinities of the species and their physiological state; therefore, for a given species, both the absolute and relative concentrations of its photosynthetic pigments depend on the conditions under which it develops (temperature, nutrient concentrations, light intensity, wavelength range, etc.).

From this point of view, many data in the literature on pigment composition of several species within the marine phytoplankton are meaningless, since growth conditions during collection were not precisely defined.

This dependence on environmental factors is of great importance, since some ratios among pigment concentrations may be used for indirect estimation of the main characteristics which define a phytoplanktonic population (Ketchum *et al.*, 1958; Yentsch and Vaccaro, 1958; Margalef, 1960, 1963, 1965).

Yentsch and Vaccaro (1958) have pointed to the quotient between chlorophyll concentrations and total carotenoids as a good physiological index, and Margalef (1960, 1963, 1965) has made use of the quotient between optical densities of the acetone extract at 430 and 665 nm, because of empirical relations he found between that quotient, inorganic carbon uptake per unit biomass, and specific diversity of phytoplanktonic populations. The biochemical significance of such an index is obscure, for the effect of environmental factors on the pigment composition of a species in culture is scarcely known, and furthermore, reported data are controversial (Mil'ko, 1963; Berland, 1966; Madgwick, 1966; Maestrini, 1966; Bunt, 1968; Bonin, 1969; Wallen and Geen, 1971).

In this paper, variations in the amounts and proportions of *Phaeodactylum tricornutum* pigments during its development in culture are presented; the data constitute part of a broader study undertaken to evaluate the effect of several environmental factors on the pigment composition of this alga.

Materials and Methods

Culture Conditions

The diatom selected was *Nitzschia closterium forma minutissima* Droop (No. 646 from the Indiana Culture Collection). This organism was reclassified by Lewin (1958) as *Phaeodactylum tricornutum*.

*Please address all correspondence to first author.

The culture medium employed is basically the ASP_2 of Provasoli *et al.* (1957), to which 2 mM of $NaHCO_3$ were added per liter; no vitamin enrichment was made, since this has been proved non-essential for this species (Droop, 1958; Hayward, 1968).

Unialgal (not bacteria-free) cultures were grown with continuous stirring (80 strokes/min) at 20°C ± 1.0 C° under constant light from 40 W "Sylvania White" fluorescent tubes (1700 lux) in cotton-stoppered Erlenmeyer flasks containing 150 ml medium. Light intensity was measured by means of a Norma (Austria) photometer (Series No. 847850) fitted with a barrier-layer-type photocell. The inoculum was obtained from stock cultures in the exponential phase of growth (initial concentration, 4 to 5 x 10^3 cells/ml of culture medium). Growth was determined by measuring the increase in cell number and measuring changes of optical density at 680 nm. The relative growth constant (k) and mean generation time (T) were calculated from the following formulae:

$$k = \frac{\log_e N - \log_e N_o}{t} = \frac{(\log_{10} N - \log_{10} N)\, 2.3}{t},$$

and $$T = 0.693/k,$$

where N = cell concentration at time t = 0; and t = time (h).

Pigment Analysis

Direct Spectrophotometry

Homogenized cultures were filtered through Whatman GF/C glass-fiber filters covered by a layer of $MgCO_3$. Cells thus collected were disrupted in a Potter homogenizer with 4 to 5 ml cold 90% acetone, protected from light. The suspension was transferred to a centrifuge tube, kept 30 min in the dark, and centrifuged for 10 min at 3,000 revs/min. Supernatant liquid together with two washings of the residue were gathered in a centrifuge 0.1 ml-graduated tube and diluted to volume (15 ml) with 90% acetone. Absorbances were immediately read at 750, 663, 645, 630, 480, 430 and 410 nm in a Uvispek (Hilger-Watts) spectrophotometer. Chlorophyll concentrations were calculated using the equations recommended by the SCOR-UNESCO Working Group 17 (1966). Total carotenoids were calculated using the equation proposed by Parsons and Strickland (1963). To avoid or reduce errors in the estimation of chlorophyll *c*, concentrations were handled so as to keep absorbances at 630 nm within 0.1 to 0.2 units. Standard deviation was 0.09 mg/l for chlorophyll *a* at the 2 mg/l level, and 0.09 mg/l for chlorophyll *c* at the 0.8 mg/l level.

Spectrophotometry After Separation by Thin-Layer Chromatography

The acetone extract, obtained as described for the direct determination, was shaken in a separatory funnel with an equal volume of peroxide-free ethyl ether and 25 ml of 10% NaCl aqueous solution saturated with $MgCO_3$. Once a neat separation was obtained, the clear aqueous phase was discarded and the ethereal extract was shaken twice with salt solution. The extract was evaporated to dryness by bubbling nitrogen, and the final residue was dissolved in 100 µl acetone. Fifty microliters were seeded as a 4 cm-length, 3 mm-wide stroke on a 250 µ-thick Merck 2% $MgCO_3$ silica gel plate using a 70:30 (v/v) hexane-acetone mixture as a solvent for 30 min. The coloured bands were removed from the plate and the pigments contained therein eluted with 90% acetone for further examination. The absorbances of the clear extracts resulting from centrifugation were measured spectrophotometrically. The following specific extinction coefficients were used for calculation: chlorophyll *a* at 663 nm, 89.3 l/g cm (SCOR-UNESCO Working Group 17, 1966), and fucoxanthin at 450 nm, 88.0 l/g cm (Rabinowitch, 1951; Strickland, 1965). The extinction coefficient of β-carotene was used for the minor xanthophylls, as their coefficients are not known. Chlorophyll *a* concentration was found by resorting to a two-component equation (Humphrey and Jeffrey, unpublished) to avoid the effect of chlorophyllide *a* traces.

Pigments were basically identified by comparing their spectra and absorption maxima in the visible range with those mentioned in the literature. Another criterium applied was the relative chromatographic sequence of isolated pigments, coincident with that described by Healey *et al.* (1967).

Results

Pigment Composition

It was possible to identify the following pigments in *Phaeodactylum tricornutum*: chlorophyll *a*, chlorophyll *c*, fucoxanthin, diadinoxanthin and β-carotene, in

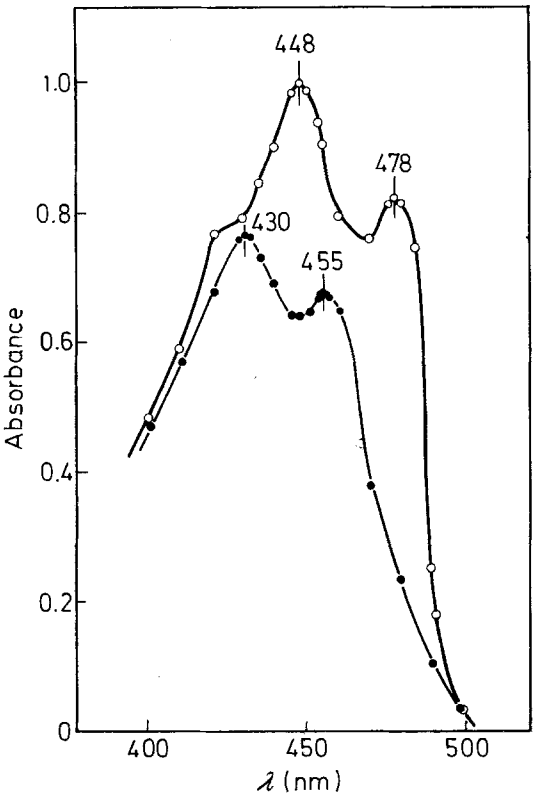

Fig. 1. *Phaeodactylum tricornutum.* Absorption spectra for isolated pigments in ehtanol. Open circles: diadinoxanthin; filled circles: neodiadinoxanthin

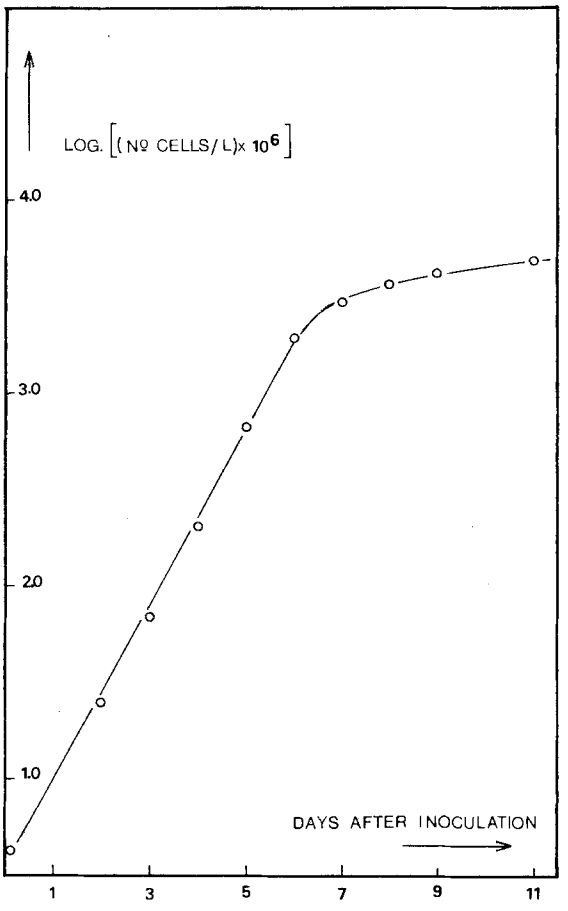

Fig. 2. *Phaeodactylum tricornutum.* Growth curve over 11 days at 1700 lux and 20°C

agreement with most literature records (Jeffrey, 1961; Parsons, 1961; Strain, 1966; Riley and Wilson, 1967; Hayward, 1968, Mann and Myers, 1968); experimental results nevertheless show diatoxanthin to be absent, at least within the detection limits of the applied technique which is not as sensitive as those used by authors who claim the presence of traces of this pigment (Parsons, 1961; Strain, 1966; Mann and Myers, 1968).

It should be pointed out here that, when handling extracts from rather old cultures, small amounts of a sixth component were detected; this has been identified as neodiadinoxanthin (the furanoid isomer of diadinoxanthin) because of the coincidence of its absorption maxima with those quoted by Aitzetmüller *et al.* (1968), and the similarity of its spectrum and Rf value with those of the product obtained by acetic acid treatment of an ethanolic extract of diadinoxanthin (Fig. 1). In connection with the absence of diatoxanthin reported in the preceding paragraph, it should be pointed out that in the separation system applied, neodiadinoxanthin (not registered in the literature as a normal component for this species) exhibits the same Rf as diatoxanthin from *Navicula pelliculosa*.

Growth

Fig. 2 shows a typical growth curve of *Phaeodactylum tricornutum* under our experimental conditions; note the absence of a lag phase and the exponential growth during the first 6 days, after which the stationary phase begins. The specific growth rate is $k = 0.042\ h^{-1}$, corresponding to a doubling time of $T = 16.4$ h.

Variations in Pigment Contents During Growth

Variations in the amount per liter of chlorophyll *a*, chlorophyll *c*, fucoxanthin, diadinoxanthin, β-carotene and neodiadinoxanthin during growth, are shown in Fig. 3.

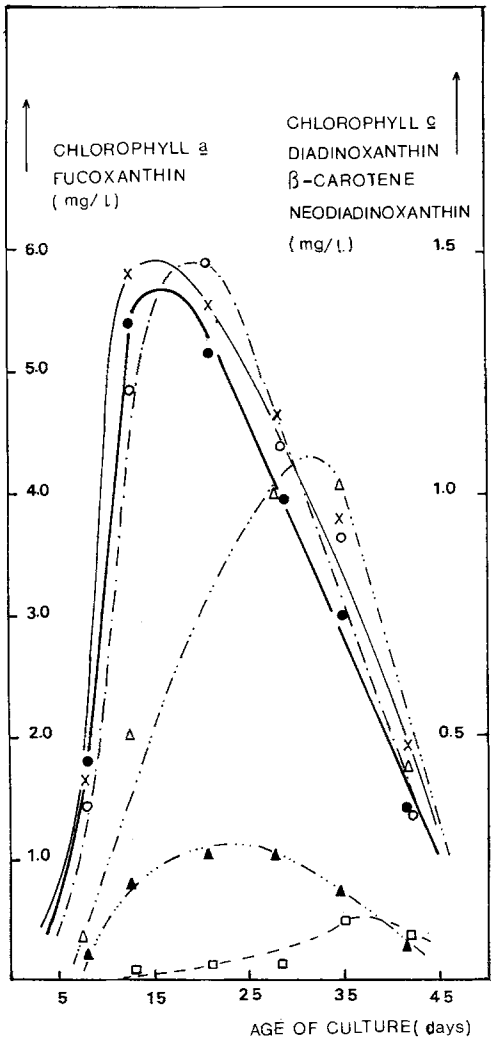

Fig. 3. *Phaeodactylum tricornutum*. Pigment concentrations at various times during a 42-day growth period. Filled circles: chlorophyll *a*; crosses: chlorophyll *c*; open circles: fucoxanthin; open triangles: diadinoxanthin; closed triangles: β-carotene; squares: neodiadinoxanthin

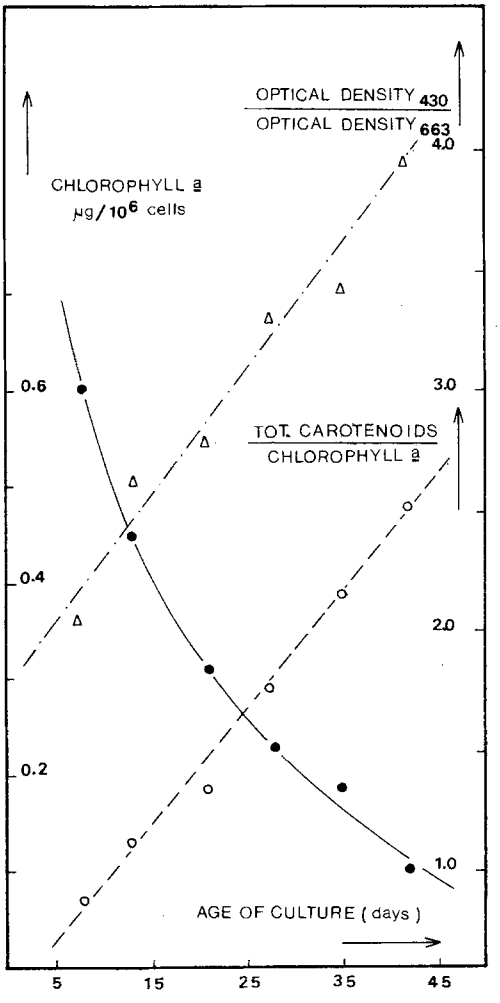

Fig. 4. *Phaeodactylum tricornutum*. Changes in chlorophyll *a* concentration per 10^6 cells (filled circles); total carotenoids:chlorophyll *a* ratio (open circles) and quotient of optical densities at 430 and 663 nm (triangles) during growth

During growth of cultures, the amount of chlorophyll *a* per cell continually decreases (Fig. 4) as a result of nutritional limitations of the medium. As these decreases in chlorophyll *a* content are accompanied by a similar decrease in chlorophyll *c* content, the ratio between the two pigments remains constant with time (0.31 ± 0.03 in 95% of the cases). Table 1 shows values obtained during a representative experiment.

The ratio total carotenoids:chlorophyll *a* (obtained from the spectrophotometric values), and the absorbance ratios of the acetone extract at 430 and 663 nm vary linearly with the age of the culture (Fig. 4). The increase in the percent of total carotenoids does not, however, indicate simultaneous increases in each individual carotenoid; the differences detected seem consistent with the role each carotenoid plays in photosynthesis. Thus, while fucoxanthin content, as percent of total carotenoids, decreases (on the basis of "chromatographic values") with the age of the culture, that of diadinoxanthin increases, and β-carotene remains almost constant (Table 2).

Variations in fucoxanthin and diadinoxanthin (as percentage of total carotenoids calculated by combining the individual species determined on fractions from chromatographic separation) are closely related to variations in the total carotenoids:chlorophyll *a* ratio and to the quotient between the absor-

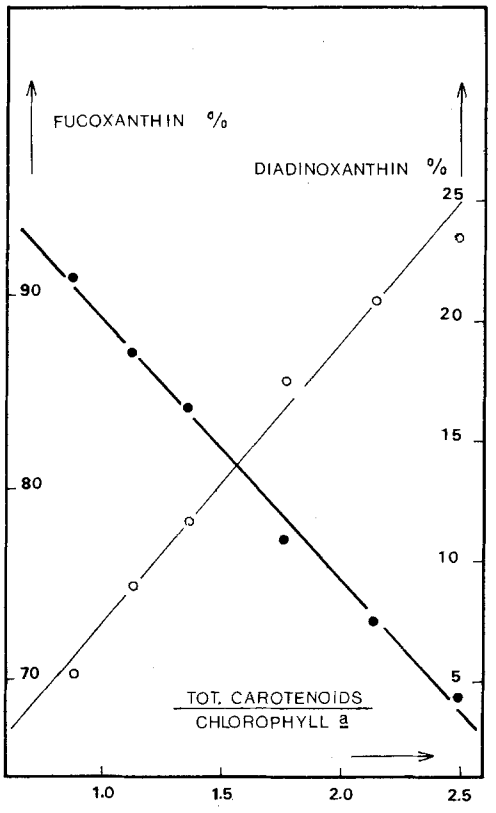

Fig. 5. *Phaeodactylum tricornutum.* Correlations between total carotenoids:chlorophyll *a* ratio and fucoxanthin (filled circles) and diadinoxanthin (open circles) as percentage of total carotenoids

Table 1. *Phaeodactylum tricornutum.* Chlorophyll *c*:chlorophyll *a* ratio at various times during a representative experiment

Time (days)					
8	14	22	29	36	43
0.31	0.28	0.26	0.26	0.29	0.29

Table 2. *Phaeodactylum tricornutum.* Changes in amounts of individual carotenoids (as percentage of total carotenoids) at various times during a 42-day growth period. nt: No trace

Carotenoid	Time (days)					
	8	13	21	28	35	42
Fucoxanthin	91.1	87.2	84.2	77.3	73.1	69.4
Diadinoxanthin	5.2	8.9	11.6	17.5	20.8	22.3
β-carotene	3.7	3.5	3.7	4.6	3.7	3.8
Neodiadinoxanthin	nt	0.4	0.5	0.6	2.4	4.5

bances of the acetone extracts at 430 and 663 nm (Fig. 5).

Finally, the furanoid isomer of diadinoxanthin, apparently absent (or present in amounts too low for detection) during the logarithmic growth, appears increasingly during the nutrient-deficient period, even though (perhaps because of its low concentration) the values obtained display a somewhat large dispersion. During the numerous experiments conducted, no chlorophyll decomposition products, i.e., phaeophytin and phaeophorbide, were found. The presence of chlorophyllide *a* traces at the origin of the chromatogram is attributed to enzymatic activity of chlorophyllase during extraction; it is absent from old cultures, when extraction was made at -77°C.

Discussion

The pigments found in *Phaeodactylum tricornutum* are essentially the same as those quoted in previous papers for the same species (Jeffrey, 1961; Parsons, 1961; Strain, 1966; Riley and Wilson, 1967; Hayward, 1968; Mann and Myers, 1968), except for the diatoxanthin mentioned as occurring in very small amounts by some authors (Parsons, 1961; Strain, 1966; Mann and Myers, 1968) which has not been detected in our chromatograms.

The steady increase in the furanoid isomer of diadinoxanthin observed during

Table 3. *Phaeodactylum tricornutum*. Comparison of results obtained during logarithmic growth (a) and at end of stationary phase (b) with data published by other authors. Medium values of 35 experiments. nd: No data

Pigment	Source				
	Parsons (1961)	Jeffrey (1961)	Riley and Wilson (1967)	Present study (a)	(b)
Chlorophyll *c*: chlorophyll *a*	0.32	0.36	0.34	0.31 ± 0.03	
Fucoxanthin (%)	78	71	91	95	65
Neofucoxanthin *a* and *b* (%)	nd	20	nd	nd	nd
Diadinoxanthin (%)	16	4	6	3	25
β-carotene (%)	6	5	3	3	3
Neodiadinoxanthin (%)	nd	nd	nd	0	3

the stationary phase of growth suggests that its formation cannot be ascribed to faulty methodological techniques, but that it results from diadinoxanthin degradation with senescence.

The quantitative pigmentary composition of *Phaeodactylum tricornutum* given by different authors varies as to proportions of individual pigments (mainly fucoxanthin and diadinoxanthin percentages). The present results suggest that these differences do not arise from different analytical techniques, but are due to different physiological stages of the cultures analyzed (Table 3), since we have demonstrated than in *P. tricornutum* nutrient limitation – because of consumption during growth – results in increasing diadinoxanthin content with a corresponding decline in fucoxanthin content.

The chlorophyll *c*:chlorophyll *a* ratios given by most authors do not show important discrepancies once the chlorophyll *c* data have been corrected using the extinction coefficient of Jeffrey (1963). Some authors, nevertheless, have found for this (Berland, 1966; Maestrini, 1966) and other diatom species (Castelví, 1963; Bonin, 1969) varying proportions of chlorophyll *a* and *c*, with a definite trend for chlorophyll *c* to increase up to values high enough to attain a chlorophyll *c*:chlorophyll *a* ratio much greater than unity. Our results, instead, are in agreement with those of Madgwick (1966) for several unialgal cultures: the ratio of both chlorophylls remaining constant, and the contents of both varying to a similar degree in accordance with the age of the culture.

This study on *Phaeodactylum tricornutum* shows that while changes in chlorophyll *a*, chlorophyll *c* and fucoxanthin proportions are fairly parallel, changes in diadinoxanthin are not.

The differences observed between diadinoxanthin and fucoxanthin can be correlated with the functions ascribed to both pigments. While fucoxanthin is photosynthetically active, being the most efficient carotenoid in transferring energy to chlorophyll *a* (Dutton and Manning, 1941; Tanada, 1951; Teale, 1958) and apparently an important component of photosystem 2 (Emerson and Rabinowitch, 1960; Fork, 1963; Mann and Myers, 1968), diadinoxanthin seems to be related to photoprotection (Healey *et al.*, 1967).

Healey *et al.* (1967), working with *Navicula pelliculosa* in silicon-starvation synchrony, found during the silicon-deficient period that while the synthesis of chlorophylls *a* and *c* and fucoxanthin ceases, that of diadinoxanthin proceeds; these authors interpreted this as a mechanism induced by conditions favouring the photo-oxidation of chlorophylls.

On the other hand, Schwenker (1971) observed in *Euglena gracilis* a remarkable decrease in the concentration of diadinoxanthin together with an increase in the amount of zeaxanthin during the stationary growth phase.

In nitrogen-deficient cultures, Yentsch and Vaccaro (1958) demonstrated a parallel decrease in chlorophyll *a* and cellular nitrogen content, ascribing this to the decomposition of the chlorophyll-protein complexes of the chloroplast. The fact that the maximum wave-

length of the fucoxanthin spectrum in organic solvents shows a shift of 40 nm toward lower wavelength values compared to its *in vivo* spectrum points to strong binding forces between the pigment and the proteic fraction of the lamellar structure of the chloroplast; both the synthesis and decomposition of chlorophylls and fucoxanthin would, thus, be linked to nitrogen metabolism through the synthesis and decomposition of chloroplast proteins. Results herein can be interpreted, by analogy, as nitrogen being the limiting factor in our experiments.

Another point deserving discussion is the correlation observed between the variation in pigment ratios and the quotient of optical densities of the total acetone extract at 430 and 663 nm. Margalef (1960, 1963, 1965) insisted on the value of this quotient, on the basis of empirical connections found between such an index and chlorophyll *a* content per cell, inorganic carbon uptake per unit biomass and specific diversity of phytoplanktonic communities, without attempting to interpret its biochemical significance.

Variations observed in our *Phaeodactylum tricornutum* cultures show that for a unispecific population, such index describes in a simple way the variations in pigment proportions, increasing in value as the proportion of photosynthetically active pigments (chlorophyll *a*, chlorophyll *c* and fucoxanthin) decreases and that of diadinoxanthin, a pigment apparently related to photoprotective functions, concomitantly increases; this index might consequently be considered as a quotient between photosynthetically active pigments and pigments with other functions.

Literature Cited

Aitzetmüller, K., W.A. Svec, J.J. Katz and H.H. Strain: Structure and chemical identity of diadinoxanthin and the principal xanthophyll of *Euglena*. Chem. Commun. *1017*, 32-33 (1968)

Berland, B.: Contribution à l'étude des cultures des diatomées marines. Recl Trav. Stn mar. Endoume *40*, 3-82 (1966)

Bonin, D.J.: Influence de differents facteurs écologiques sur la croissance de la diatomée marine *Chaetoceros affinis* Launder en culture. Téthys *1*, 173-238 (1969)

Castelví, J.: Pigmentos de la diatomea marina *Skeletonema costatum* (Grev.) en su dependencia de los factores ambientales y de la dinámica de las poblaciones. Investigación pesq. *24*, 129-137 (1963)

Bunt, J.S.: Some characteristics of microalgae isolated from Antarctic sea ice. Antarctic Res. Ser. *11*, 1-14 (1968)

Droop, M.R.: Optimum relative and actual ionic concentrations for growth of some euryhaline algae. Verh. int. Verein. theor. angew. Limnol. *13*, 722-730 (1958)

Dutton, H.J. and W.M. Manning: Evidence for carotenoid-sensitized photosynthesis in the diatom *Nitzschia closterium*. Am. J. Bot. *28*, 516-526 (1941)

Emerson, R. and E. Rabinowitch: Red drop and role of auxiliary pigments in photosynthesis. Pl. Physiol., Lancaster *33*, 477-485 (1960)

Fork, D.C.: Observations on the function of chlorophyll *a* and accessory pigments in photosynthesis. *In*: Photosynthetic mechanisms of green plants, pp 353-361. Ed. by B. Kok and A.T. Jagendorf. Washington: National Academy of Sciences 1963

Hayward, J.: Studies on the growth of *Phaeodactylum tricornutum*. IV. Comparison of different isolates. J. mar. biol. Ass. U.K. *48*, 657-666 (1968)

Healey, F.P., J. Coombs and B.E. Volcani: Changes in pigment content of the diatom *Navicula pelliculosa* (Bré.) Hilse in silicon-starvation synchrony. Arch. Mikrobiol. *59*, 131-142 (1967)

Jeffrey, S.W.: Paper chromatographic separation of chlorophylls and carotenoids from marine algae. Biochem. J. *80*, 336-342 (1961)

- Purification and properties of chlorophyll *c* from *Sargassum flavicans*. Biochem. J. *86*, 313-318 (1963)

Ketchum, B.H., J.H. Ryther, C.S. Yentsch and N. Corwin: Environmental conditions for primary production. D. 1. Productivity in relation to nutrients. Rapp. P.-v. Réun. Cons. perm. int. Explor. Mer *144*, 132-140 (1958)

Lewin, J.C.: The taxonomic position of *Phaeodactylum tricornutum*. J. gen. Microbiol. *18*, 427-432 (1958)

Madgwick, J.C.: Chromatographic determination of chlorophylls in algal cultures and phytoplankton. Deep-Sea Res. *13*, 459-466 (1966)

Maestrini, S.: Etude de l'influence de quelques facteurs de milieu sur la productivité d'une algue planctonique en culture. Recl Trav. Stn mar. Endoume *41*, 33-108 (1966)

Mann, J.E. and J. Myers: On pigments, growth, and photosynthesis of *Phaeodactylum tricornutum*. J. Phycol. *4*, 349-355 (1968)

Margalef, R.: Valeur indicatrice de la composition des pigments du phytoplancton sur la productivité, composition taxonomique et propriétés dynamiques des populations. Rapp. P.-v. Réun, Commn int. Explor. scient. Mer Méditerr. *15*, 277-281 (1960)

- Modelos simplificados del ambiente marino para el estudio de la sucesión y distribución del fitoplancton y del valor indicador de sus pigmentos. Investigación pesq. *23*, 11-52 (1963)

- Ecological correlations and the relationship between primary productivity and community structure. Proc. I. B. P. Symp., Pallanza, Italy. Memorie Ist. Ital. Idrobiol. *18*, 355-364 (1965)

Mil'ko, E.S.: Effect of various environmental factors on pigment production in the alga *Dunaliella salina*. Microbiology *32*, 256-261 (1963)

Parsons, T.R.: On the pigment composition of eleven species of marine phytoplankton. J. Fish. Res. Bd Can. *18*, 1017-1025 (1961)

- and J.D.H. Strickland: Discussion of spectrophotometric determination of marine plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J. mar. Res. *21*, 155-163 (1963)

Provasoli, L., J.J.A. McLaughlin and M.R. Droop: The development of artificial media for marine algae. Arch. Mikrobiol. *25*, 392-428 (1957)

Rabinowitch, E.I.: Photosynthesis and related processes, Vol. II. 661 pp. New York: Interscience 1951

Riley, J.P. and T.R.S. Wilson: The pigments of some marine phytoplankton species. J. mar. biol. Ass. U.K. *47*, 351-362 (1967)

Schwenker, U.: Einfluß des Stoffwechsels auf die Pigmentzusammensetzung in alternden Kulturen von *Euglena gracilis*. Planta *101*, 101-116 (1971)

SCOR-UNESCO Working Group 17: Determination of photosynthetic pigments. 1. *In*: Determination of photosynthetic pigments in sea water, pp 9-18. Paris: UNESCO 1966

Strain, H.H.: Fat-soluble chloroplast pigments: their identification and distribution in various Australian plants. *In*: Biochemistry of chloroplasts, Vol. I. pp 387-406. Ed. by T.W. Goodwin. London: Academic Press 1966

Strickland, J.D.H.: Production of organic matter in the primary stages of the marine food chain. *In*: Chemical oceanography, Vol. I. pp 477-610. Ed. by J.P. Riley and B. Skirrow. London: Academic Press 1965

Tanada, T.: The photosynthetic efficiency of carotenoid pigments in *Navicula minima*. Am. J. Bot. *38*, 276-283 (1951)

Teale, F.W.J.: Carotenoid-sensitized fluorescence of chlorophyll *in vitro*. Nature, Lond. *181*, 415-416 (1958)

Wallen, D.G. and G.H. Geen: Light quality and concentration of proteins, RNA, DNA and photosynthetic pigments in two species of marine plankton algae. Mar. Biol. *10*, 44-51 (1971)

Yentsch, C.S. and R.F. Vaccaro: Phytoplankton nitrogen in the oceans. Limnol. Oceanogr. *3*, 443-448 (1958)

Dr. José Ignacio Carreto
Instituto de Biología Marina
Casilla de Correo 175
Mar del Plata
Argentina

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