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Studies of Phytoplankton Species and Photosynthetic Pigments in a Warm Core Eddy of the East Australian Current. II. A Note on Pigment Methodology

S. W. Jeffrey and G. M. Hallegraeff

CSIRO Division of Fisheries and Oceanography, P.O. Box 21, Cronulla, NSW 2230, Australia

ABSTRACT: Photosynthetic pigments in a warm core eddy of the East Australian Current were studied by *in vivo* fluorometry, spectrophotometry and thin-layer chromatography. The Variosens *in situ* fluorometer gave only an approximate guide to the location and quantity of phytoplankton in the water column; spectrophotometric curve analysis revealed the presence of intact or detrital chlorophylls, and quantitative thin-layer chromatography showed that in some samples intact chlorophyll *a* accounted for only 50–70 % of the total spectrophotometrically measured 'chlorophyll' In addition the full range of chlorophylls, carotenoids and chlorophyll degradation products was recorded from the chromatograms.

INTRODUCTION

In the previous paper (Jeffrey and Hallegraeff, 1980) the distribution of summer populations of phytoplankton at the centre and edges of a warm core eddy in the East Australian Current were summarized. In addition to species identifications, photosynthetic pigments were analysed by three methods – *in situ* fluorometry, spectrophotometry and thin-layer chromatography. The usefulness and limitations of these various techniques in studying field phytoplankton populations are briefly discussed below.

MATERIALS AND METHODS

The cruise track, sampling programme, preparation of water samples for pigment analysis and species identification, the use of the *in situ* Variosens fluorometer (Frünge1 and Koch, 1976), the spectrophotometric chlorophyll *a* analysis, and the thin-layer chromatographic procedures are as previously described (Jeffrey and Humphrey, 1975; Jeffrey and Hallegraeff, 1980, Jeffrey, in press).

For quantitative chromatographic analysis, the pigment spots were carefully scraped off the chromatograms, placed in centrifuge tubes or special elution tubes (Jeffrey, 1968) and eluted with about 0.5 ml 100 % acetone (chlorophylls) or ethanol (carotenoids). After 5 min, the cellulose powder was removed by centrifugation, the pigment solution transferred to a calibrated 0.7 ml capacity microcell, the volume accurately adjusted to a known volume, and the extinction measured using the 0.1 absorbance scale of the Cary 17 spectrophotometer. Pigment solutions containing only 0.2–0.7 μg per 0.5 ml solvent could thus be accurately measured. Extinction coefficients of pure pigments used for obtaining pigment concentrations are given in Jeffrey (1968, 1972), Jeffrey and Humphrey (1975) and Jeffrey et al. (1975).

RESULTS AND DISCUSSION

Comparison of *in Situ* Fluorescence Profiles with Extracted Chlorophyll *a*

The water column was always first profiled with the Variosens fluorometer to determine appropriate depths for discrete water sampling. Figure 1 shows the chlorophyll depth profiles by spectrophotometry of extracted chlorophyll *a*, compared with *in vivo* chlorophyll *a* fluorescence measured with the Variosens fluorometer. The best correspondence occurred

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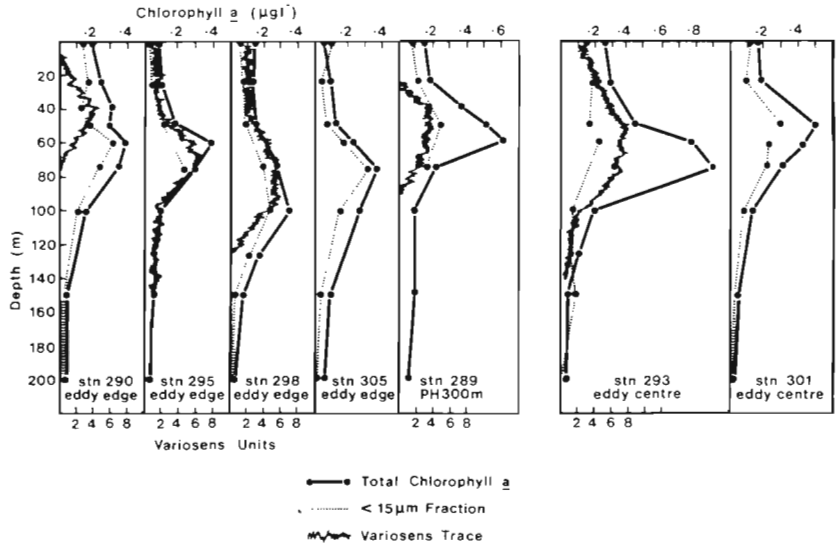


Fig. 1. Chlorophyll *a* profiles (total chlorophyll *a* and chlorophyll *a* from < 15 µm fraction) compared with *in vivo* chlorophyll fluorescence (Variosens) at all phytoplankton sampling stations. Variosens Units as defined in Tranter et al. (1979)

at Stations 295 and 298 (eddy edge); less close correspondence was found at Stations 290 (eddy edge) and 293 (eddy centre) respectively

Figure 2 shows a comparison between Variosens profiles, chlorophyll *a* and the depth distribution of the five dominant phytoplankton species categories, at one centre station (293) and one edge station (290). Both *Nitzschia* and *Rhizosolenia* showed a broad maximum from the surface down to about 75 m depth at the eddy centre (293), whereas the same species at the edge station (290) showed sharper subsurface maxima. The small flagellates (coccolithophorids, non-thecate dinoflagellates and green flagellates) were more uniformly distributed through the water column at the edges of the eddy, but at the centre stations the coccolithophorids and green flagellates showed sharper maxima with depth. At both stations the Variosens fluorescence profile corresponded more closely to the vertical distribution of *Nitzschia* than to any other species.

The Variosens *in situ* fluorometer gave fluorescence profiles which were a valuable but only approximate guide to the location and quantity of chlorophyll (i.e. phytoplankton) in the water column. The depths of the maxima were sometimes out of phase by up to 25 m (Fig. 1). Wire angle differences could account only for small discrepancies (not more than about 10 m). The fluorescence profiles correlated best with profiles of the larger diatoms, and less well with the flagellates (Fig. 2). It is possible that the instrument detects only a certain size range of alga with accuracy, and this range requires further definition. The Variosens fluorometer has also been evaluated against a Turner fluorometer measuring *in vivo* fluorescence (Herman and Denman, 1977) and against extracted chlorophyll *a* (Herman and Denman, 1977; Derenbach et al., 1979). Both authors recorded the necessity for frequent calibration.

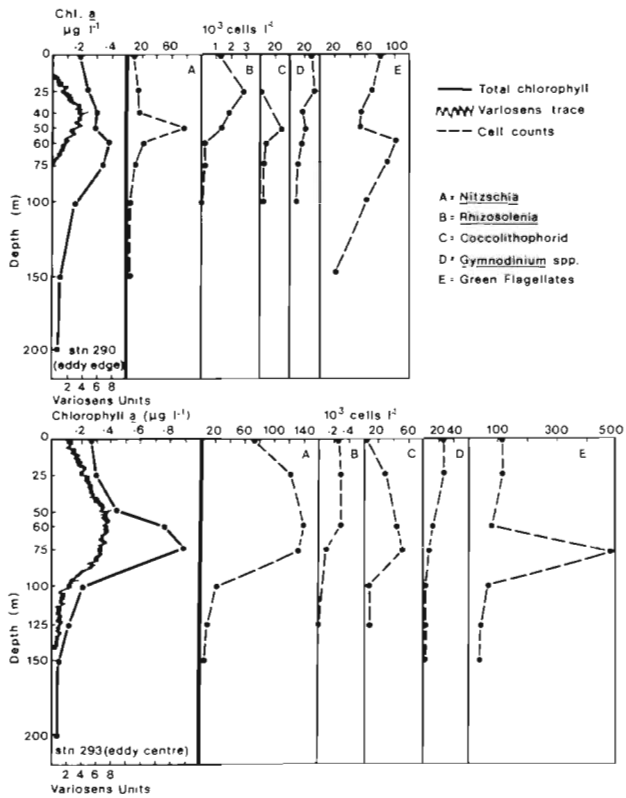


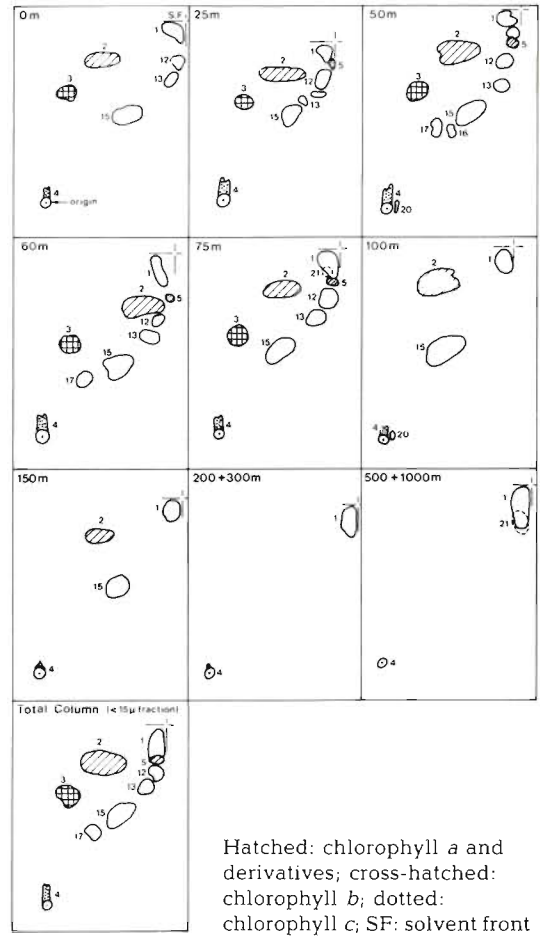
Fig. 2. Counts of dominant phytoplankton species compared with profiles of chlorophyll *a* and *in vivo* fluorescence at an eddy edge station (290 above) and an eddy centre station (293 below)

Comparison of Spectrophotometric Pigment Analyses with Thin-Layer Chromatography

Qualitative Aspects

The photosynthetic pigment profiles at the eddy centre Station (301) were evaluated both by thin-layer chromatography and by spectrophotometric curve analysis. Pigment chromatograms of discrete samples from surface waters to 1,000 m depth (Fig. 3) revealed chlorophylls *a* and *c*, carotene and fucoxanthin (diatoms and coccolithophorids) at all depths down to 150 m, chlorophyll *b* and lutein (green algae) down to 75 m, peridinin (dinoflagellates) at 50 m, and astaxanthin (copepods) at 75 m. An unidentified blue pigment, chromatographically identical to the acid rearrangement product of fucoxanthin, was found at both 50 and 100 m. Its presence may indicate an acidification history for fucoxanthin after passage through the gut of planktonic animals. The only chlorophyll *a* degradation products at this station were pheophytin *a* at 25 to 75 m and chlorophyllide *a* at 100 m. Deep samples (below 150 m) contained only chlorophyll *c*, carotene and detrital material at the origin of the chromatograms.

Absorption spectra of the phytoplankton pigment extracts taken from the same samples shown in Figure 3 revealed some interesting features. Figure 4 shows the non-quantitative family of curves from 0–1000 m using diethyl ether as solvent to enhance the spectral characteristics. Exact locations of peaks, inflections and minima of these curves are given in Table 1. The most obvious characteristic is the presence of a red maximum at all depths, even in the deepest samples which contained no chlorophyll *a* at all (Fig. 3). In these deep samples (200–1000 m) the red maxima were at 663–664 nm, indicating the presence of chlorophyll *a* degradation products. (Chlorophyll *a* in diethyl ether has its red maximum at 660 nm, Table 1). These degradation products were probably located in the detrital 'origin' spot on the chromatograms (Fig. 3). The positions of the blue maxima of the pigment extracts (Fig. 4, Table 1) show these trends even more clearly. Samples from the upper 150 m, which contained intact chlorophyll *a* (Fig. 3), and therefore came from living phytoplankton, had blue Soret maxima ranging from 427–430 nm (normal for the chlorophyll *a* Soret band, Table 1). However, in deep samples containing only detrital material the 430 nm (chlorophyll *a*) band had disappeared, and was replaced by one at 410–412 nm (similar to spectra of pheophytin *a* and pheophorbide *a*). At 150 nm, where living cells and detrital material occurred, both peaks were apparent (427 and 412 nm). A further distinction between living phytoplankton and detrital samples is the appearance of a well-marked blue minimum at 360–273 nm in the deep (150–1000 m) samples. The absorption beyond this minimum rose steeply into the U. V. region. This feature was also rather surprisingly found in the surface (0 m) sample, which not only showed a rather prominent 'origin spot' on the chromatogram (Fig. 3), but contained many disintegrating and senescent *Nitzschia* cells. These steeply rising detrital curves in the U. V. region are reminiscent of curves of pigment extracts of Antarctic sediments, some of which contain



Hatched: chlorophyll *a* and derivatives; cross-hatched: chlorophyll *b*; dotted: chlorophyll *c*; SF: solvent front

Fig. 3. Cellulose thin-layer chromatograms of photosynthetic pigments throughout water column (0–1000 m) at Station 301 (eddy centre). Solvent systems, 1st Dimension: *n*-propanol in light petroleum (60–80 °), 2.5:97.5 (v/v); 2nd Dimension: chloroform, light petroleum (60–80 °), acetone, 25:75:0.5 (v/v/v). 1: carotenes (yellow); 2: chlorophyll *a* (blue-green); 3: chlorophyll *b* (olive green); 4: chlorophyll *c* (light green); 5: pheophytin *a* (grey); 6, 7, 8: unknown chlorophyll *a* derivatives (blue-green); 9, 10: chlorophyllide derivatives (blue-green); 11, 12, 13, 14: yellow xanthophylls; 15: fucoxanthin (orange); 16: peridinin (brick-red); 17: neofucoxanthin (orange); 18: unknown xanthophyll (orange); 19: pheophorbide *a* (grey); 20: unknown pigment (blue); 21: unknown carotenoid (orange); 22, 23: chlorophyll derivatives (blue-green)

Table 1. Spectral characteristics of pigment extracts of phytoplankton from 0–1000 m depth (Station 301) compared to pigment extracts of a diatom *(Stephanopyxis turris)*, a green flagellate *(Tetraselmis suecica)*, a dinoflagellate *(Amphidinium carterae)*, and pure chlorophylls *a*, *b*, c_1 and c_2. Solvent diethyl ether ~ indicates an inflection

Depth (m)	Maxima (nm)										Blue minimum	Blue:red ratio
0	660		611	573			~445	428	~410		387	3.5
60	661		612	577	531	~470	~450	428	~410	~387		2.76
0–100 (< 15 µm fraction)	661		616	580	534		~450	430	~410	~390		2.31
125	662		616	580	534		~450	430	~410			3.62
150	662.5		608		530	~500 ~470	~450	427	412		360	4.0
200 + 300	664		605			~502 ~470			410		373	5.95
500 + 1000	663		606			~470			412		372	6.15
Diatom *Stephanopyxis turris*	662		615	578		465–473	~447	430	~415			2.73
Green flagellate *Tetraselmis suecica*	662	~646	~615			~468	452	432	~413			2.84
Dinoflagellate *Amphidinium carterae*	662	628		578		~475	447	430	~415			2.96
Pure pigments												
Chlorophyll *a*•	660							428				1.30
Chlorophyll *b*•		643					453					2.82
Chlorophyll c_1••		628		580			445					10.10
Chlorophyll c_2••		629		580			448					14.50
Pheophytin *a*•••	665								410			2.26

• Jeffrey (unpublished); •• Jeffrey (1969); ••• Jeffrey and Lorenzen (unpublished)

no red band at all (Jeffrey and Holm-Hansen, unpublished).

This U. V. absorbance has been recorded previously for acetone extracts of particulate material from 300–1000 m depths in the Sargasso Sea (Yentsch and Ryther, 1959), and extracts of lake sediments (Gorham, 1960).

The identitiy of minor peaks and inflections in field samples due to accessory chlorophylls and carotenoids may be compared with absorption spectra of ether extracts of pigments from a cultured diatom *Stephanopyxis turris*, a green flagellate *Tetraselmis suecica* and a dinoflagellate *Amphidinium carterae* (Fig. 5), and with absorption maxima of ether solutions of pure chlorophylls *a*, *b*, c_1 and c_2 (Table 1). For example, in the *Tetraselmis* curve the 646 nm inflection is due to chlorophyll *b*, and the 452 and the 430 nm peaks, the blue (Soret) absorption bands of chlorophylls *b* and *a*, respectively. In the *Stephanopyxis* curve the inflection at 465–473 nm is mainly fucoxanthin, and the inflection at 447 nm and the peak at 430 nm are the blue maxima of chlorophyll *c* and *a*, respectively. In the *Amphidinium* curve, the small red maximum of chlorophyll *c* is revealed at 628 nm, the presence of peridinin by the inflection at 475 nm, and the blue maxima of chlorophylls *c* and *a* at 447 and 430 nm, respectively. Peaks and inflections in these areas in the phytoplankton curves (Fig. 4) may be interpreted similarly, and the identity of the pigments present confirmed from the chromatograms.

The ratio of blue to red bands taken at the maxima (Table 1) show further characteristics of phytoplankton pigments. Pure chlorophyll *a* in diethyl ether has a blue:red ratio of 1·30. The presence of carotenoids absorbing in the blue-green region in extracts of diatoms, green flagellates and dinoflagellates, in addition to chlorophylls *a* and *c*, raises this ratio to 2.73–2.96. Natural phytoplankton samples containing mostly living cells (0–125 m) showed blue:red ratios of 2.31–3.62. With more detritus and less living cells in the water column these ratios rose to 4.0 (150 m), 5.95 (200 + 300 m) and 6.15 (500 + 1000 m). Thus simple inspection of pigment curves in diethyl ether can give useful information concerning their phytoplankton or detrital origin.

Quantitative Aspects

Five samples, collected at the Variosens fluorescence maxima were pooled and used for quantitative pigment analyses. Chromatograms of these pooled samples are shown in Figure 6A (Stations 300 + 302, eddy centre) and Figure 6B (Stations 291 + 294 + 299, midway between eddy centre and eddy edge). Quan-

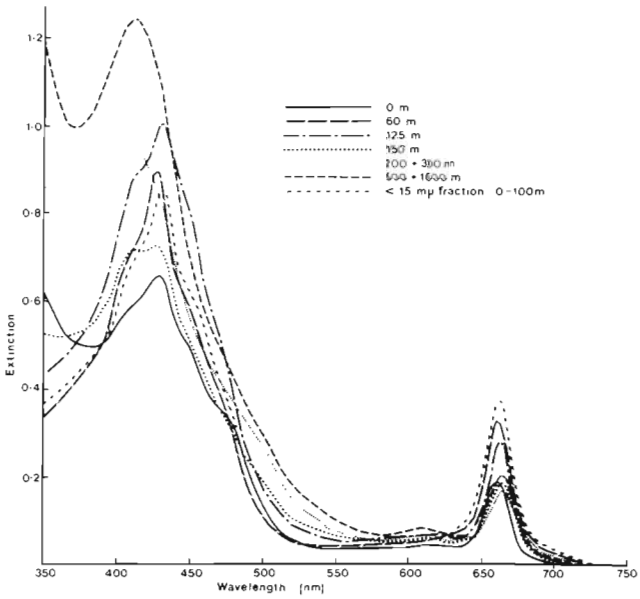


Fig. 4. Absorption spectra of phytoplankton pigments from 0–1000 m at Station 301. These spectra display characteristics associated with the presence of living phytoplankton or detrital material; no quantitative significance is intended. Solvent: diethyl ether

titative analyses of the eluted pigments are given in Table 2. Because much more pigment was available for analysis in these pooled samples, all chlorophyll breakdown products were more clearly identified. These were pheophytin *a* (Fraction 5), chlorophyllide *a* (9), pheophorbide *a* (19), and the unidentified derivatives (6, 7, 8, 10, 22 and 23). The quantitative analyses in Table 2 show that intact chlorophyll *a* in these pooled samples amounted to only 50–70 % of the total material absorbing at 664 nm. The unidentified blue-green chlorophyll *a* derivatives amounted to 20–24 % of the total 'chlorophyll *a*', the 'origin' material accounted for 8 %, and pheophytin *a*, pheophorbide *a*, and chlorophyllide *a* together reached 19 % of the total in these pooled samples. In simple spectrophotometric and fluorescence methods all these derivatives would be measured as chlorophyll *a*.

The presence of these products on chromatograms provides clues to the physiological state of phytoplankton (Jeffrey, 1974; Lorenzen and Jeffrey, in press). Pheophorbide *a*, a marker of zooplankton grazing (Jeffrey, 1974; Shuman and Lorenzen, 1975), and chlorophyllide *a*, a marker of chlorophyllase-containing diatoms (Jeffrey, 1974), were previously found in Australian coastal waters. However, these compounds were only detected occasionally in the present work. Instead, pheophytin *a* was the most common degradation product encountered. Its presence was not entirely due to storage artefacts (unpublished experiments), and it may have originated from faecal material from salps and small copepods (Hallegraeff, in press) which were abundant at some eddy stations (Heron, pers. comm.). Neveux (1975) has also recognised pheophytin *a* (from chromatograms) as a major chlorophyll degradation product in Mediterranean and coastal Atlantic waters.

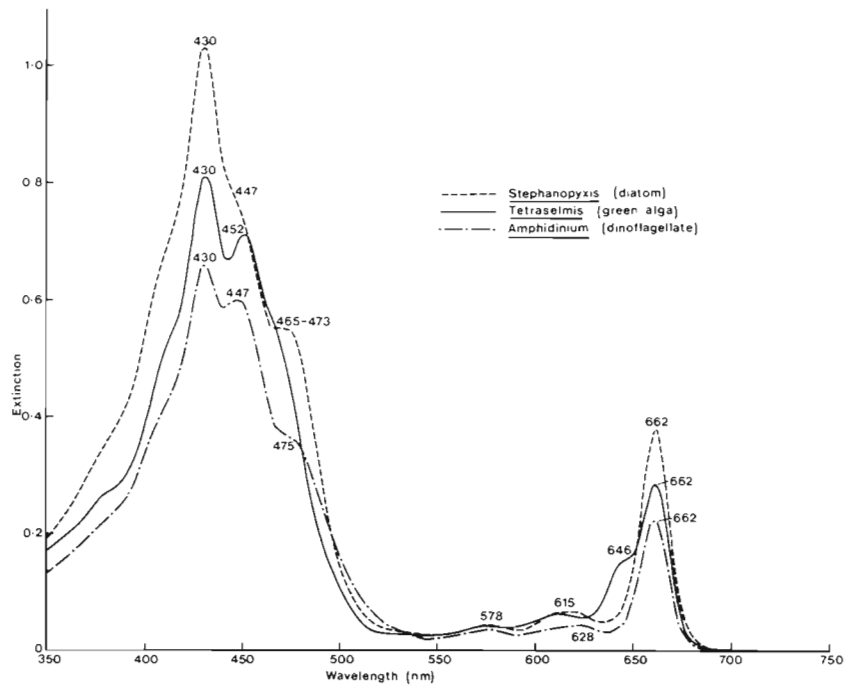


Fig. 5. Pigment absorption spectra of diatom *Stephanopyxis turris*, green flagellate *Tetraselmis suecica* and dinoflagellate *Amphidinium carterae*. Solvent: diethyl ether

Table 2. Relative proportions of pigment fractions eluted quantitatively from cellulose thin-layer chromatograms for two grouped sets of 10 l samples collected at Variosens maxima. A: Stations 300 + 302 (eddy centre), 20 l sample; B: Stations 291 + 294 + 299 (midway stations), 30 l sample

Pigments	Total pigment fraction per chromatogram (μg)		Chlorophyll *a* derivatives as % total 'chlorophyll *a*'		Pigment ratios	
	A	B	A	B	A	B
Chlorophyll *a*	1.50	2.55	50.2	71.0		
Chlorophyll *b*	0.26	0.30				
Chlorophyll *c*	0.47	0.63				
Chlorophyll *a* derivatives*	0.72	0.73	24.1	20.3		
Chlorophyllide *a* derivatives**	0.19	0.18	6.3	5.1		
Pheophytin *a*	0.32	–	10.7	–		
Fucoxanthin + neofucoxanthin	1.48	1.30				
Peridinin	0.10	0.13				
'Origin' material	0.26	–	8.7	–		
Pheophorbide *a*	–	0.13	–	3.6		
Total chlorophyll *a* + derivatives	2.99	3.59				
Ratio chl. *a*:*b*					5.77	8.5
chl. *a*:*c*					3.19	4.1
chl. *a*:fucoxanthin					1.02	1.96
chl. *a*:peridinin					15.15	19.6

* Fractions 6, 7, 8 (Fig. 6A); 22, 23 (Fig. 6 B)
** Fractions 9, 10 (Fig. 6A, B)

Table 3. Concentrations and ratios of chlorophylls *a*, *b* and *c* at selected stations, using trichromatic spectrophotometry

Station	Depth (m)	Chlorophylls ($\mu g\ l^{-1}$)				
		a	*b*	*c*	*a*:*b*	*a*:*c*
301	0	0.14	0.03	0.04	5.36	3.19
(Eddy centre)	25	0.17	0.02	0.04	7.00	4.45
	50	0.49	0.08	0.13	6.49	3.72
	60	0.43	0.08	0.12	5.49	3.52
	75	0.30	0.06	0.08	5.42	3.82
305	50	0.10	0.007	0.03	14.30	3.90
(Eddy edge)	60	0.21	0.046	0.054	4.56	3.89
	75	0.36	0.13	0.008	2.73	4.43
	100	0.25	0.11	0.07	2.26	3.49

Several unidentified blue-green chlorophyll *a* derivatives were abundant on chromatograms of eddy samples (Fig. 6). These have also been observed at inshore stations of the East Australian Current (Jeffrey, 1974; Hallegraeff, in press), and in senescent blue-green light diatom cultures (Jeffrey, unpublished). Their absorption spectra, chromatographic properties and possible identity will be discussed elsewhere.

Pigment Ratios

Ratios of chlorophylls *a*:*b* and *a*:*c* calculated from trichromatic equations (Jeffrey and Humphrey, 1975) for two stations are given in Table 3. These ranged from 2.26 to 7.00 (excluding one very low *b* sample) and 3.19 to 4.45, respectively. Pigment ratios for other stations calculated from quantitative thin-layer chromatography (Table 2) gave similar values: chlorophyll *a*:*b*, 5.77 and 8.5; chlorophyll *a*:*c*, 3.19 and 4.1; chlorophyll *a*:fucoxanthin, 1.02 and 1.96; and chlorophyll *a*:peridinin, 15.15 and 19.6. The low content of peridinin was surprising, considering the abundance of the small dinoflagellates, and suggests that many of the dinoflagellates observed microscopically must have been colourless, non-photosynthetic forms.

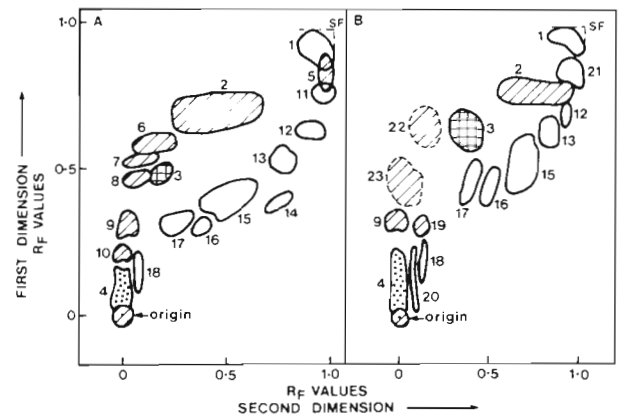


Fig. 6. Cellulose thin-layer chromatograms of samples. A: Variosens maximum at Stations 300 + 302 combined (eddy centre); B: Variosens maximum at Stations 291, 297 + 299 combined (midway between eddy centre and eddy edge). Solvent systems and identity of pigment fractions as for Figure 3. SF: solvent front

CONCLUSIONS

In situ fluorometry, spectrophotometry and thin-layer chromatography gave increasing detail of pigments in phytoplankton samples. *In situ* fluorescence located the phytoplankton populations in the water column, spectrophotometry gave accurate data on chlorophyll *a* concentrations providing there was no interference from chlorophyll degradation products, curve analysis detected the presence of detrital chlorophylls, and thin-layer chromatography revealed the full pigment complement and allowed accurate quantitative analysis of all pigment fractions.

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