Vol. 35: 293–304, 1987 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published February 5

Chlorophyllase distribution in ten classes of phytoplankton: a problem for chlorophyll analysis

S. W. Jeffrey & G. M. Hallegraeff

CSIRO Marine Laboratories, Division of Fisheries Research, GPO Box 1538, Hobart, Tasmania 7001, Australia

ABSTRACT: Chlorophyllase activity, which degrades chlorophyll *a* to chlorophyllide *a*, was investigated in 93 species (113 isolates) of unicellular algae from 10 classes. Chlorophyllide *a* formation was measured by a simple incubation test in 50 % acetone, followed by chromatography and phase separation techniques. High activity of the acetone-activated chlorophyllase was found in one third of the 68 diatom isolates tested. These included the pennate diatoms *Asterionella glacialis, Cylindrotheca fusiformis, Delphineis* sp., *Grammatophora oceanica, Navicula* sp., *Nitzschia* (2 spp.), *Phaeodactylum tricornutum* and *Thalassionema nitzschioides*, and the important bloom-forming centric diatoms *Chaetoceros* (5 spp.), *Detonula pumila, Ditylum brightwellii, Eucampia zodiacus, Lithodesmium undulatum, Skeletonema costatum, Stephanopyxis turris* and *Streptotheca tamesis*. High activity was also found in 2 out of 3 chrysophyte isolates and 2 out of 7 chlorophyte isolates. In contrast, representatives of 7 other classes showed zero or low activity: dinoflagellates (11 isolates), most prymnesiophytes (9 isolates), cryptophytes (5 isolates), prasinophytes (4 isolates), chloromonads (2 isolates), 1 rhodophyte and 2 cyanophytes. Significant strain-specific differences in chlorophyllase activity in 6 out of 13 diatom species, 1 chrysophyte, 1 prymnesiophyte and 1 chlorophyte were also observed. A serious problem for chlorophyll analysis is that chlorophyllase can be activated by harvesting techniques *before* extraction takes place. Filtration causes the most extensive conversion of chlorophyll *a* to chlorophyllide *a*, particularly in old cultures, but harvesting by centrifugation also causes chlorophyllide formation in some species. The effects are more pronounced in diatoms than other algal groups. The further degradation of chlorophyllide *a* to pheophorbide *a* was seldom encountered. While spectrophotometric and fluorimetric methods would not be seriously affected by chlorophyllase activity (provided pheophorbide *a* is a minor end product), TLC and HPLC techniques for chlorophylls may be rendered invalid due to artefact formation.

INTRODUCTION

An active chlorophyllase (chlorophyll-chlorophyllide-hydrolase, EC 3.1.1.14) occurs in some species of unicellular marine algae (Barrett & Jeffrey 1964, 1971, Suzuki & Fujita 1986). Chlorophyllide *a*, the hydrolysis product of chlorophyll *a*, has also been reported from phytoplankton field samples (Patterson & Parsons 1963, Jeffrey 1965, 1974, Saijo & Kamiya 1973, Gieskes & Kraay 1980, Hallegraeff 1981, Gowen et al. 1983). Some of these studies claimed that as much as 50 % of the total chlorophyll *a* could be in this altered form. Chlorophyllase *in vivo* catalyses 3 types of reactions: hydrolysis of chlorophyll *a* to give phytol and chlorophyllide; transesterification; and under certain conditions, synthesis of chlorophyll *a* from chlorophyllide *a* and phytol (Beale 1984). Chlorophylls *a* and *b* and pheophytins *a* and *b* are all substrates for chlorophyllase (Holden 1963) forming the corresponding chlorophyllides and pheophorbides respectively. Chlorophyllase is a thylakoid-bound enzyme and in higher plants is thought to be primarily involved in chlorophyll catabolism (Beale 1984). Apparently the enzyme only catalyses chlorophyll breakdown when the disintegration of cell and chloroplast structures makes chlorophyll accessible to the thylakoid-bound enzyme (Holden 1961, Terpstra 1976).

Chlorophyllase is unusually active in aqueous organic solvent mixtures, and simple assays are usually carried out by incubation in 40 to 50 % aqueous solvents, which give maximum activity (Barrett & Jeffrey 1964, 1971). The chlorophyllide *a* formed from chlorophyll *a* can readily be separated from the parent compound by thin-layer chromatography (TLC) (Barrett & Jeffrey 1971), high-performance liquid chromatography (HPLC) (Mantoura & Llewellyn 1983)

0171-8630/87/0035/0293/$ 05.00

or solvent partition techniques (McFeeters et al. 1971, Terpstra & Goedheer 1975). Recently Suzuki & Fujita (1986) found pheophorbide *a* was a major end product of chlorophyllase activity in *Skeletonema costatum* cultures.

Chlorophyllase is active to a small extent even in 90 % acetone (Barrett & Jeffrey 1964), the recommended solvent for phytoplankton pigment analysis (SCOR-UNESCO 1966, Strickland & Parsons 1972, Parsons et al. 1984). To avoid extraction artefacts by inhibiting chlorophyllase activity, Jeffrey (1974) recommended that phytoplankton pigments be extracted in 100 % acetone. This is especially important when pigments are subsequently analysed by TLC or HPLC techniques, since the ratio of accurately determined chlorophylls and degradation products can be used as important physiological and ecological markers (e.g. Jeffrey 1974, Shuman & Lorenzen 1975, Hallegraeff 1981). The presence of chlorophyllide *a* in phytoplankton field samples as evidenced by chromatography may be an indication of senescent diatoms (Jeffrey 1974), an idea that is supported by the finding of significant concentrations of chlorophyllide *a* on chromatograms of phytoplankton taken during spring diatom blooms in Australian waters (Jeffrey 1974, Hallegraeff 1981).

These problems associated with chlorophyllase prompted further investigations of the extent of chlorophyllase distribution in phytoplankton, and of artefact formation during sample preparation. Ninety-three species (113 isolates) of cultured microalgae from 10 classes were surveyed. The activity of the enzyme was monitored in all species as the hydrolysis product of chlorophyll *a* (although in green algae chlorophyllide *b* would also have been formed). The active enzyme was present mainly in diatoms, but was randomly distributed among representatives of other algal classes. Tests of harvesting procedures (e.g. centrifugation and gentle filtration) have also shown that chlorophyllase in certain species can be activated by the harvesting procedures even *before* solvent extraction takes place. Both young and old cultures are affected by filtration and centrifugation, although old cultures are more susceptible.

MATERIALS AND METHODS

Algal cultures. Algae were grown in stationary 250 ml Erlenmeyer flasks at 18 °C under 40 to 60 µE m^{-2} s^{-1} white fluorescent light and 12 · 12 h light : dark cycles. Media were as designated in Jeffrey (1980) which included various dilutions of medium f (Guillard & Ryther 1962), medium G (Loeblich & Smith 1968) and for freshwater algae, medium MBL/MB2 (Guillard's medium in Nichols 1973). Algae were harvested during log phase growth (5 to 10 d). 'Old' cultures were harvested in stationary phase after 5 wk. Except for 15 species studied at the Scripps Institution of Oceanography, California, USA, all other species came from the CSIRO Algal Culture Collection (Jeffrey 1980). A total of 88 marine and 5 freshwater species were examined, which included more than one strain of 16 species (a total of 113 isolates; Table 1). Previous analyses of chlorophyllase activity in a few marine species (Barrett & Jeffrey 1964, 1971) are included for completeness (see Table 2).

Table 1. Number of species and total isolates (by class) examined. (Includes for completeness species previously examined by Barrett & Jeffrey 1964, 1971)

Algal class	Species	Total isolates	Species with 2 or more strains
Chloromonads (raphidophytes)	2	2	0
Chrysophytes	2	3	1
Cryptophytes	5	5	0
Cyanophytes	2	2	0
Diatoms: centric	29	43	11
Diatoms: pennate	22	25	2
Dinoflagellates	11	11	0
Euglenophytes	1	1	0
Green algae:			
Chlorophytes	6	7	1
Prasinophytes	4	4	0
Prymnesiophytes	8	9	1
Rhodophytes	1	1	0
Total	93	113	16

Chlorophyllase enzyme assays. For routine tests of chlorophyllase activity (Table 2) approximately 100 ml cultures were harvested by centrifugation at 2000 g for 2 to 5 min, the supernatant was discarded, and the algal pellet containing the enzyme resuspended in 2 ml 50 % aqueous acetone (Barrett & Jeffrey 1971). After 15 min incubation in the dark at 20 °C the reaction was stopped by adding 9 ml 100 % acetone (Barrett & Jeffrey 1964) to give a final concentration >90 %. The extract was cleared by centrifugation, and the pellet re-extracted with 2 ml portions of 90 % acetone until colourless (1 or 2 times). The combined supernatants were used for chlorophyllide *a* analysis (see below). The accuracy of this enzyme assay (expressed as % chlorophyll conversion) was ± 5 %.

Effect of harvesting procedures (filtration and centrifugation) on chlorophyllase activity. About 75 ml culture was diluted with 500 ml filtered seawater. One 250 ml portion was filtered under slight negative pressure (250 to 380 mm Hg) onto Whatman GF/C filters. This step took 1 to 2 min, and the filters contain-

Table 2. Chlorophyllase activity in unicellular marine algae from 10 classes. Cells were incubated in 50 % acetone for 15 min at 20 °C and % conversion determined by chromatography and spectrophotometry

Species	Isolator, source or deposition*	Chlorophyllide *a* formed as % of total chlorophyll *a*
Bacillariophyceae		
Pennales		
Amphiprora hyalina Eul. ex Van Heurck	J. B. Jordan, FCRG 70, CS-28	5
Amphiprora sp.	J. C. Eyles (PH), CS-11	0
Amphora sp.	J. C. Eyles (PH), CS-10c	5
Asterionella glacialis Castr.	BR. Grant (PH), CS-15	95
	J. L. Stauber (PH), CS-90c	36
	J. L. Stauber (PH), CS-135	73
Cylindrotheca closterium var. *californica* (Meres.) Reim. & Lewin	G. F. Humphrey (PH), CS-1c	5••
Cylindrotheca closterium Reim. & Lewin	T. J. Smayda, FCRG	0
	J. L. Stauber, Coral Sea, CS-107	0
Cylindrotheca fusiformis Reim. & Lewin	Halifax, CS-13c	20; 50••
Delphineis sp.	J. C. Eyles (PH), CS-12	96
Grammatophora oceanica Ehrenb.	D. Frood (PP), CS-84	100
Navicula sp.	J. L. Stauber, Coral Sea, CS-112	1
Navicula sp.	J. C. Eyles (PH), CS-46c	95••
Nitzschia frustulum (Kütz.) Grun.	J. L. Stauber, Coral Sea, CS-115	30
Nitzschia frustulum var. *perminuta* Grun.	L. de Phillipa, Georges R. (NSW) CS-60c	5
Nitzschia fraudulenta Cleve	J. L. Stauber, Port Hacking, CS-101	65
Nitzschia seriata Cleve	J. B. Jordan, FCRG 52	5
Nitzschia cf. *bilobata* W. Smith	J. C. Eyles (PH), CS-47	0
Nitzschia sp.	J. L. Stauber, Coral Sea, CS-105	95
Nitzschia sp.	M. Wootton (PH), CS-5c	0
Nitzschia sp.	J. L. Stauber, Coral Sea, CS-106	30
Phaeodactylum tricornutum Bohl.	Plymouth, 1052/1, CS-29c	100••
Pleurosigma delicatulum W. Smith	D. Frood (PP), CS-83	9
Thalassionema nitzschioides Grunow	J. C. Eyles (PH), CS-61	75
Centrales		
Chaetoceros affinis Lauder	J. C. Eyles (PH), CS-78	100
Chaetoceros calcitrans (Paulsen) Takano	Cambridge LB 1001/1, CS-55	1
Chaetoceros didymus Ehr.	B. R. Grant (PH), CS-2	0••; 12
	T J. Smayda, FCRG	50
Chaetoceros difficile Cleve	J. C. Eyles (PH), CS-8	100
Chaetoceros cf. *mitra* (Bail.) Cleve	J. C. Eyles ((PH), CS-70	0
Chaetoceros cf. *radians* Shütt	J. C. Eyles (PH), CS-68	5
Chaetoceros sp.	J. L. Stauber, Coral Sea, CS-119	100
Chaetoceros sp.	N. Central Pacific Gyre, FCRG-56	90
Coscinodiscus sp.	J. L. Stauber (NW Shelf), CS-150	0
Detonula pumila Schütt	J. C. Eyles (PH), CS-72	57
Ditylum brightwellii (T. West) Grun. ex van Heurck	J. C. Eyles (PH), CS-74	75
	J. L. Stauber (PH), CS-131	48
	J. B. Jordan, FCRG-64	0
Minutocellus polymorphus (Hargr. & Guillard) Hasle, Von Stosch & Syvertsen	J. C. Eyles (PH), CS-3c	10
Eucampia zodiacus Ehr.	J. C. Eyles (PH), CS-71	45
Lauderia annulata Cleve	J. B. Jordan, FCRG-14, CS-30	50
	J. B. Jordan, FCRG-14	10
Lithodesmium undulatum Ehr.	J. B. Jordan, FCRG-25	100
Odontella aurita (Lyngb.) de Breb.	S. McGill (PP), CS-19	9
Odontella mobiliensis (Bail.) Grun. ex Van Heurck	D. Frood, Great Barrier Reef, CS-82	0
	J. L. Stauber (PH), CS-133	22
Odontella regia (Schulze) Simonsen	J. C. Eyles (PH), CS-65	0
Rhizosolenia fragilissima Berg.	FCRG	0
Rhizosolenia setigera Brightw.	D. Frood (PP), CS-62	20
	J. L. Stauber (NW Shelf), CS-147	20

Table 2 (continued)

Species	Isolator, source or deposition*	Chlorophyllide *a* formed as % of total chlorophyll *a*
Bacillariophyceae (continued)		
Skeletonema costatum (Grev.) Cleve	M. Wootten (PH), CS-6	80**; 100**
	J. C. Eyles (PH), CS-76	100
	CCMP, Boothbay Harbour, USA, CS-181	100
Stephanopyxis turris (Grev.) Ralfs in Pritch	L. Provasoli, FCRG, CS-31	50
	J. L. Stauber (PH), CS-100	44
Streptotheca tamesis Shrubs	J. C. Eyles (PH), CS-81	100
	J. L. Stauber (PH), CS-129	46
Thalassiosira eccentrica (Ehr.) Cleve	B. R. Grant (PP), CS-17	0
	J. L. Stauber (NW Shelf), CS-148	0
Thalassiosira stellaris Hasle & Guillard	S. McGill (PP), CS-16	5
Thalassiosira pseudonana Hasle & Heimd.	R. Swan, Perth, CS-20c	3
	R. Guillard (Long Island) CS-173	0
Thalassiosira oceanica Hasle	J. C. Eyles (PH), CS-67	3
Thalassiosira rotula Meun.	J. Jordan, FCRG-10, CS-32	10
	J. C. Eyles (PH), CS-77	6
	FCRG	5
Thalassiosira sp.	Origin unknown	31**
Dinophyceae		
Amphidinium carterae Hulb.	Halifax, CS-21	0**
Amphidinium klebsii Kof. & Swez.	Cambridge 1102/3, CS-33	0
Amphidinium hoefleri Schiller & Diskus	FCRG	0
Gymnodinium simplex (Lohm.) Kof. & Swez.	Origin unknown	0**
Gymnodinium sanguineum Hirasaka	F. T. Haxo, PY-14, CS-35	0
Gymnodinium sp.	Plymouth (1117/2), CS-36	2
Gyrodinium resplendens Hulb.	FCRG	0
Peridinium balticum (Levan.) Lemm.	F. T. Haxo, La Jolla, PY-14, CS-38	1
Kryptoperidinium foliaceum (Stein) Lindeman	UTEX 1688, CS-37	1
Prorocentrum gracile Schütt	D. Frood, CS-80	0
Prorocentrum micans Ehr.	W. H. Thomas, La Jolla, FCRG, CS-27	0
Raphidophyceae		
Fibrocapsa japonica Toriumi & Takano	J. B. Jordan, Pt. Loma, FCRG-51, CS-34	7
Heterosigma akashiwo (Hada) Hada	M. Parke, CS-39	0**
Chrysophyceae		
Pelagococcus subviridis Norris	B. C. Booth, North Atlantic, CS-58	100***
	J. L. Stauber (PH), CS-99	0
Sphaleromantis sp.	Origin unknown	100**
Prymnesiophyceae		
Cricosphaera carterae (Braar. & Fag.) Braarud	L. Provasoli, CS-40	0
Emiliania huxleyi (Lohm.) May & Mohl.	R. Guillard, Sargasso Sea, W. H. Thomas BT6, CS-57	0
Isochrysis galbana Parke	Halifax, Canada, CS-22	0**
Pavlova lutheri (Droop) Green	Halifax, Canada, CS-23	5**
Pavlova sp.	D. Frood (PP), CS-63	15
Pavlova sp.	R. Guillard, Sargasso Sea, Woods Hole, CS-50	20
Pavlova sp.	R. Guillard, Sargasso Sea, CS-49	18
Phaeocystis pouchetii (Hariot) Lagerheim	J. L. Stauber (PH), CS-165	8
	S. I. Blackburn (Southern Ocean), CS-188	40
Cryptophyceae		
Chroomonas sp.	Port Hacking, CS-24	5**
Cryptomonas maculata Butcher	L. Borowitzka, Dee Why, Roche, CS-85	0
Unidentified crypto F 5a	Woods Hole	0**
Unidentified crypto 3c	Woods Hole	0**
STX-157 (*Chroomonas* sp.?)	K. C. Haines, St Croix, CS-48	0
Rhodophyceae		
Porphyridium purpureum (Bor.) Drev & Ross	Halifax, Canada, CS-25c	0**

Table 2 (continued)

Species	Isolator, source or deposition*	Chlorophyllide *a* formed as % of total chlorophyll *a*
Chlorophyceae		
Chlorella stigmatophora Butch.	Origin unknown	0**
Dunaliella tertiolecta Butch.	Halifax, Canada, CS-14c	79**
	CCMP, CS-175	12
Nannochloris atomus Butch.	Origin unknown	0**
Prasinophyceae		
Micromonas pusilla (Butcher) Manton and Parke	UTEX LB 991, CS-86	0
Tetraselmis chui Butch.	G. W. Griffith, Galverston, CS-26	1
Tetraselmis suecica (Kyl). (Butch)	Cambrigde 66/4, CS-56	0**
Unidentified prasinophyte, omega 48	CCMP, omega 48-23AX	1
Cynanophyceae		
Oscillatoria cf. *woronichinii*	T. J. Smayda, Mass., FCRG SM-24, CS-52	20

* Code: CS = CSIRO Culture Collection number; Cambridge = The Culture Collection of Algae and Protozoa, Cambridge; FCRG = Food Chain Research Group, Scripps Oceanography, USA; Halifax = Halifax Regional Laboratory, Nova Scotia, Canada; Mass. = Massachusetts, USA; PH = Port Hacking isolate, Australia; PP = Port Phillip Bay isolate, Australia; Plymouth = The Marine Laboratory, Plymouth, UK; Syd. Uni. = Sydney University, Australia; UNSW = University of New South Wales, Australia; UTEX = Culture Collection of Algae at the University of Texas, USA; Woods Hole = Woods Hole Oceanographic Institution, USA (now CCMP, Bigelow Oceanographic Laboratory)
** Barrett & Jeffrey 1964, 1971
*** Lewin et al. 1977

ing the algae were then placed immediately in centrifuge tubes containing 100 % acetone to inactivate the chlorophyllase. The other 250 ml portion was harvested by gentle centrifugation (2000 g for 5 min), and the cell pellet immediately extracted with 100 % acetone. The extracts were then assayed for chlorophyll *a* and chlorophyllide *a* by chromatography and spectrophotometry.

Assay of chlorophyllide *a* by thin-layer chromatography. The pigments were transferred from acetone to diethyl ether (peroxide-free) for chromatographic analysis, by adding an equal amount of diethyl ether to the acetone extract and gently mixing with 10 volumes 5 % NaCl solution. The ether hyperphase was collected, concentrated under a stream of nitrogen, and chromatographed on thin layers of specially purified cellulose (Jeffrey 1981), using a solvent system of 2.5 % *n*-propanol in petroleum ether (ligroine [USA]; 60 to 80 °C fraction). After development in one dimension (approximately 5 min; Fig. 1) the chlorophyll *a* (R_f = 0.75) and chlorophyllide *a* (R_f = 0.28) zones were collected and eluted quantitatively with 100 % acetone. Extinctions were read at 664 nm in a Unicam SP500 or Cary Model 17 spectrophotometer. Chromatography plus elution gave recoveries of 90 to 92 % (Jeffrey 1981).

Assay of chlorophyllide *a* formation by solvent partition. A rapid method for the assay of chlorophyllide *a* was frequently used, after the formation of chlorophyllide *a* had been confirmed by chromatography. Four ml hexane (68 to 70 °C) and 1 ml 0.05 % NaCl were added to 3 ml of the original 90 % acetone extract in a glass-stoppered 10 ml graduated centrifuge tube, which was gently mixed by inverting 20 times (cf. Parsons 1963). Chlorophyll *a* migrated to the hexane hyperphase, and chlorophyllide *a* was retained in the aqueous hypophase. The relative amount of chlorophyllide *a* in the pigment mixture was then estimated from the difference in extinction at 664 nm of the original acetone extract and the hexane fraction. A correction was made for the larger volume of the hexane layer (4 ml, compared to 3 ml acetone) and for the slightly higher extinction coefficient of chlorophyll *a* in the hexane/acetone mixture compared to 90 % aqueous acetone as solvent (Whitney & Darley 1979). Provided the chlorophyll content of the original acetone extract was not too low (>0.2 μg ml^{-1}) the method agreed with the results obtained by chromatography to within ± 5 %.

Zero time controls. Controls with species containing highly active chlorophyllase were difficult to perform in the absence of a good enzyme inhibitor which could be added to the culture before sample preparation commenced. However, in certain cultures of pennate diatoms which adhered to the culture walls, the culture medium was decanted, and the cells extracted either in 100 % acetone (without filtration or centrifugation), or frozen in ether-dry ice mixtures at −70 °C, and then

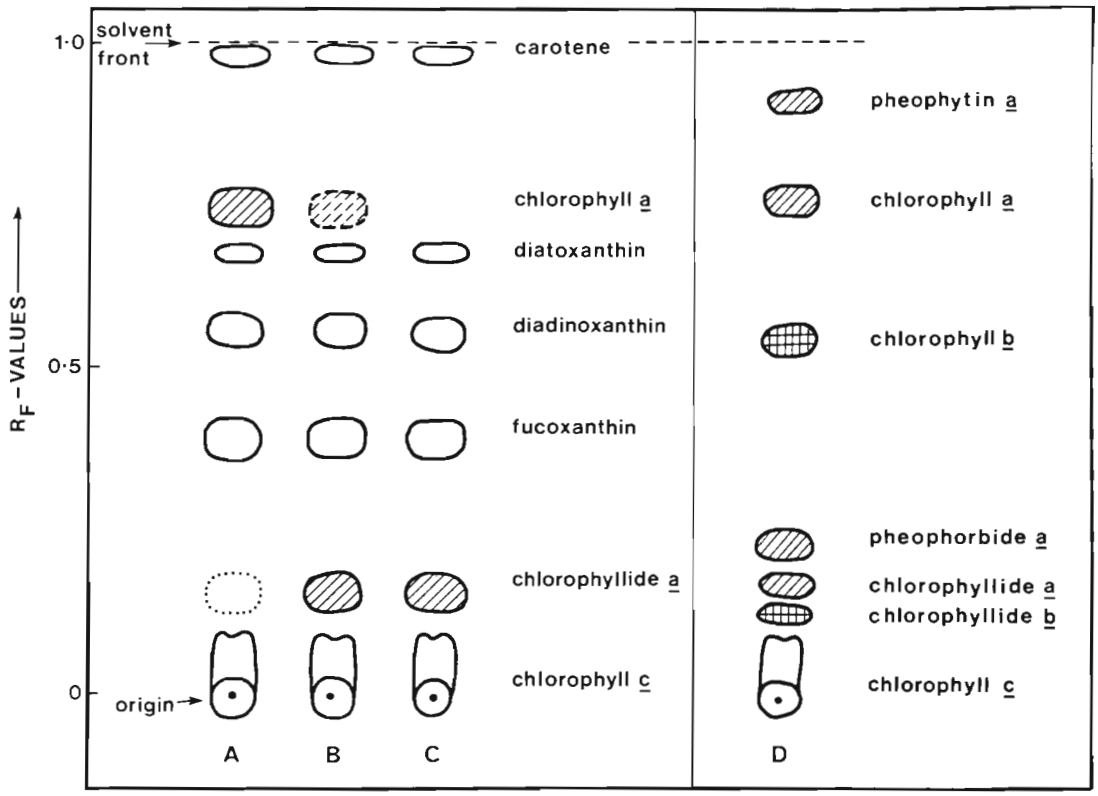

Fig. 1. *Skeletonema costatum*. Cellulose thin-layer chromatogram of pigments from a 9 d old culture. Incubation conditions: (A) no incubation, cells harvested by low-speed centrifugation (2000 rpm) and extracted with 100 % acetone; (B) no incubation, cells harvested by filtration (250 mm Hg) and extracted with 100 % acetone; (C) Cells incubated in 50 % acetone for 15 min, reaction stopped with 90 % acetone. Chlorophyllide *a* accounted for 3, 74 and 100 % of the total chlorophyll *a*, in Treatments 1, 2 and 3 respectively. (D) Standard chromatogram showing position of chlorophylls and derivatives. Solvent system 2.5 % *n*-propanol in petroleum ether (60 to 80 °C fraction)

extracted in cold 100 % acetone. In the case of *Cylindrotheca fusiformis* (CS-13c), *Navicula* sp. (CS-46c) and *Dunaliella tertiolecta* (CS-14c) no chlorophyllide *a* was present; traces of chlorophyllide *a* were present in *Phaeodactylum tricornutum* (CS-29c) and *Skeletonema costatum* (CS-6), but in *Pelagococcus subviridis* (CS-58) 60 % of the chlorophyll *a* was in the form of chlorophyllide *a* (Lewin et al. 1977) following the above procedures.

RESULTS

Chlorophyllase activity in algal species

Chlorophyllase activity in the 88 marine species tested are listed in Table 2, which includes for completeness results of 21 species previously examined (Barrett & Jeffrey 1964, 1971). Results of the 5 freshwater species (3 chlorophytes, 1 euglenoid and 1 cyanophyte) are given in Table 3.

The pennate and centric diatoms tested showed the highest chlorophyllase activity (Table 4). Thirteen out of 43 centric diatom isolates and 10 out of 25 pennate diatom isolates showed greater than 50 % conversion of chlorophyll *a* to chlorophyllide *a* under the test conditions, while a number of other diatoms showed less but significant conversion (11 to 50 %). In contrast, only 3 out of 11 dinoflagellates tested showed any chlorophyllase activity, and this was always less than 10 % conversion.

Low or negligible activity occurred in most of the prymnesiophytes, cryptophytes, prasinophytes, chloromonads, cyanophytes, the euglenoid and the rhodophyte. However, 2 out of 3 chrysophyte isolates showed high activity (>50 % conversion) and 5 out of 7 chlorophyte isolates (marine and freshwater) showed from 10 to >50 % conversion.

Species with high chlorophyllase activity (>50 % conversion under test conditions) are listed in Table 5. The greatest activity occurred in diatoms commonly found in bloom situations: the pennate diatoms *Asterionella glacialis, Cylindrotheca fusiformis, Delphineis* sp., *Grammatophora oceanica, Navicula* sp., *Nitzschia* (2 spp.), *Phaeodactylum tricornutum* and

Table 3. Chlorophyllase activity in unicellular freshwater algae. Analyses as for Table 2

Species	Isolator, source or deposition*	Chlorophyllide *a* formed as % of total chlorophyll *a*
Chlorophyceae		
Chlamydomonas reinhardii Dang.	Syd. Uni. 137 C+, CS-51	47
Chlorella pyrenoidosa Chick	Cambridge (211/8?), CS-41	15
Chlorella vulgaris Beijer.	Cambridge (211/11?), CS-42	15
Euglenophyceae		
Euglena gracilis Klebs	UNSW Strain 'Z', CS-66	10
Cyanophyceae		
Anabaena cylindrica Lemm.	N. Weare, FCRG B-1, CS-53	7

* Code as in Table 1

Table 4. Summary of chlorophyllase activity in classes of unicellular algae (marine and freshwater)

Algal class	No. of isolates tested	No. of isolates showing the listed % conversion chlorophyll *a* to chlorophyllide *a* under test conditions				
		0 %	1–10 %	11–20 %	21–50 %	> 50 %
Chloromonads (raphidophytes)	2	1	1			
Chrysophytes	3	1				2
Cryptophytes	5	4	1			
Cyanophyte (freshwater)	1		1			
Cyanophyte (marine)	1		1			
Diatoms: centric	43	8	10	3	9	13
Diatoms: pennate	25	5	7	0	3	10
Dinoflagellates	11	8	3			
Euglenophyte (freshwater)	1		1			
Green algae:						
Chlorophytes (freshwater)	3			2	1	
Chlorophytes (marine)	4	2		1		1
Prasinophytes	4	3	1			
Prymnesiophytes	9	3	2	3	1	
Rhodophyte	1	1				
Total	113	36	28	9	14	26

Thalassionema nitzschioides, and the centric diatoms *Chaetoceros* (5 spp.), *Detonula pumila, Ditylum brightwellii, Eucampia zodiacus, Lithodesmium undulatum, Skeletonema costatum, Stephanopyxis turris* and *Streptotheca tamesis*.

Strain-specific differences in chlorophyllase activity were found in 6 out of 13 diatoms, 1 chrysophyte, 1 prymnesiophyte and 1 chlorophyte (Table 6). Accepting a maximum analytical error of ± 10 %, significant strain-specific differences were observed in *Asterionella glacialis, Chaetoceros didymus, Ditylum brightwellii, Lauderia annulata, Odontella mobiliensis, Streptotheca tamesis, Pelagococcus subviridis, Phaeocystis pouchetii* and *Dunaliella tertiolecta*. Strain differences were negligible in *Cylindrotheca closterium, Rhizosolenia setigera, Skeletonema costatum* (all strains highly active), *Stephanopyxis turris, Thalassiosira eccentrica, T. pseudonana* and *T. rotula*.

Effect of harvesting procedures on chlorophyllase activity

The effects of gentle centrifugation and filtration on the stability of chlorophyll *a* are compared in Table 7. Both young (10 d) and old (5 wk) cultures of 10 species known to contain the enzyme were studied.

Centrifugation caused little or no formation of chlorophyllide *a* in 8 out of 10 young cultures, but in 2 species (*Ditylum brightwellii* and *Chaetoceros difficile*), centrifugation caused 18 and 26 % conversion of chlorophyll *a* to chlorophyllide *a* respectively. Old cultures were more susceptible to centrifugal damage than young cultures, 8 out of 10 showing between 13 and 60 % conversion of chlorophyll *a* to chlorophyllide *a*. The 2 exceptions were *Navicula* sp. and *Cylindrotheca fusiformis*.

Filtration was even more damaging than centrifuga-

Table 5. List of species with high chlorophyllase activity (> 50 % conversion of chlorophyll *a* to chlorophyllide *a* under test conditions)

Species	CSIRO or other Code No.
Bacillariophyceae	
Pennales	
Asterionella glacialis	CS-15; CS-135
Cylindrotheca fusiformis	CS-13c
Delphineis sp.	CS-12
Grammatophora oceanica	CS-84
Navicula sp.	CS-46c
Nitzschia fraudulenta	CS-101
Nitzschia sp.	CS-105
Phaeodactylum tricornutum	CS-29c
Thalassionema nitzschioides	CS-61
Centrales	
Chaetoceros affinis	CS-78
Chaetoceros didymum	FCRG-strain
Chaetoceros difficile	CS-8
Chaetoceros sp.	FCRG-56
Chaetoceros sp.	CS-119
Detonula pumila	CS-72
Ditylum brightwellii	CS-74
Eucampia zodiacus	CS-71
Lithodesmium undulatum	FCRG-25
Skeletonema costatum	CS-6; CS-181; CS-76
Stephanopyxis turris	CS-31
Streptotheca tamesis	CS-81
Chrysophyceae	
Pelagococcus subviridis	CS-58
Sphaleromantis sp.	Origin unknown
Chlorophyceae	
Chlamydomonas reinhardii	CS-51
Dunaliella tertiolecta	CS-14c

Table 6. Strain-specific differences in chlorophyllase activity

Species	Code No.	% conversion chlorophyll *a* to chlorophyllide *a* under test conditions
Bacillariophyceae		
Asterionella glacialis	CS-90c	36
	CS-135	73
	CS-15	95
Chaetoceros didymus	CS-2	0; 12
	FCRG	50
Cylindrotheca closterium	FCRG	0
	CS-107	0
Ditylum brightwellii	FCRG	0
	CS-131	48
	CS-74	75
Lauderia annulata	FCRG-14	10
	CS-30	50
Odontella mobiliensis	CS-82	0
	CS-133	22
Rhizosolenia setigera	CS-62	20
	CS-147	20
Skeletonema costatum	CS-6	80; 100
	CS-181	100
	CS-76	100
Stephanopyxis turris	CS-100	44
	CS-31	50
Streptotheca tamesis	CS-129	46
	CS-81	100
Thalassiosira eccentrica	CS-17	0
	CS-148	0
Thalassiosira pseudonana	CS-173	0
	CS-20c	3
Thalassiosira rotula	FCRG	5
	CS-77	6
	CS-32	10
Chrysophyceae		
Pelagococcus subviridis	CS-99	0
	CS-58	100
Prymnesiophyceae		
Phaeocystis pouchetii	CS-165	8
	CS-188	40
Chlorophyceae		
Dunaliella tertiolecta	CS-175	12
	CS-14c	79

tion to both young and old cultures. Four out of 10 young cultures showed substantial conversion of chlorophyll *a* to chlorophyllide *a* (40 to 74 %) after harvesting by filtration (see Fig. 1 for details of *Skeletonema costatum*). In old cultures, the chloroplast membranes of all species were damaged by filtration, the formation of chlorophyllide *a* ranging from 13 to 81 %.

DISCUSSION

Distribution of chlorophyllase in unicellular algae

The present survey indicates that diatoms are the major carriers of the acetone-activated chlorophyllase. In 23 of the 68 diatom isolates tested, over 50 % of the chlorophyll *a* was converted to chlorophyllide *a* under the test conditions. The most active diatom species are all common bloom-forming organisms: *Asterionella glacialis, Chaetoceros didymus, Detonula pumila, Ditylum brightwellii, Eucampia zodiacus, Lithodesmium undulatum, Navicula* sp., *Nitzschia* spp., *Skeletonema costatum, Stephanophyxis turris, Streptotheca tamesis* and *Thalassionema nitzschioides*. Within the other classes, only 2 green flagellates (*Dunaliella tertiolecta* and the freshwater *Chlamydomonas reinhardii*) and 2 chrysophytes (*Pelagococcus subviridis* [1 strain] and *Sphaleromantis* sp.) showed high activity. Representatives of 7 other algal classes showed zero or low activity (0 to 10 % conversion of chlorophyll *a* to chlorophyllide *a*). These included dinoflagellates, most prymnesiophytes, cryptophytes, prasinophytes, chloromonads, cyanophytes, a euglenophyte and a rhodophyte.

Table 7. Effect of cell harvesting techniques (centrifugation and filtration) and culture age on chlorophyllase activity in some unicellular marine algae

Algae	CSIRO Culture No.	Young culture (1 wk)		Old culture (5 wk)	
		Centrifugation	Filtration	Centrifugation	Filtration
Dunaliella tertiolecta	CS-14c	0	6	13	15
Navicula sp.	CS-46c	0	0	3	13
Cylindrotheca fusiformis	CS-13c	0	0	0	20
Detonula pumila	CS-72	0	3	60	50
Stephanopyxis turris	CS-31	5	0	37	32
Phaeodactylum tricornutum	CS-29c	0	7	13	44
Streptotheca tamesis	CS-81	8	40	22	81
Skeletonema costatum	CS-6	3	74	40	75
Ditylum brightwellii	CS-74	18	55	21	49
Chaetoceros difficile	CS-8	26	70	24	60

Strain-specific differences in chlorophyllase activity were seen in 6 out of 13 diatom cultures, as well as different isolates of *Pelagococcus subviridis* (chrysophyte), *Phaeocystis pouchetii* (prymnesiophyte) and *Dunaliella tertiolecta* (chlorophyte). The differences seen may reflect inherent strain (i.e. genetic) differences, or physiological changes in the cells imposed by the isolation and culture conditions. These observations caution against generalizing from the present work to phytoplankton species in the world ocean, even though the chlorophyllase activity in isolates of other species was similar (Table 6).

Activation of chlorophyllase during harvesting procedures

The finding in the present work that membrane-bound chlorophyllase can be activated by cell-harvesting techniques poses a major problem for chlorophyll analysis. In intact cells, chlorophyllase is inactive as a hydrolytic enzyme, since it is bound at sites inaccessible to its chlorophyll substrate (Terpstra 1976). Therefore, gentle centrifugation of young cultures would be expected to cause little damage, and except for 2 very fragile diatoms (Table 7) this was the case. However, in older cultures, which contained a higher proportion of senescent cells, centrifugation caused greater cell damage, and therefore greater conversion of chlorophyll *a* to chlorophyllide *a*.

Filtration, even at low suction pressures, was more damaging than centrifugation to thylakoid membranes. In young cultures filtration severely damaged thylakoid membranes of some large centric diatoms and led to significant formation of chlorophyllide *a*, but caused negligible damage to other species. However, in old cultures of *all* species tested, filtration caused significant thylakoid disorientation, the percentage conversion of chlorophyll *a* to chlorophyllide *a* ranging from 13 to 81 %.

Implications for chlorophyll analysis

Many of the diatoms with high chlorophyllase activity examined in the present work are common bloom-forming organisms in coastal waters throughout the world. When harvesting these algae by usual filtration procedures, a considerable part of their chlorophyll *a* may be converted to chlorophyllide *a*.

If chlorophylls are measured by simple routine spectrophotometric (Jeffrey & Humphrey 1975) or fluorimetric (Holm-Hansen et al. 1965) methods, which do not distinguish between chlorophyll *a* and chlorophyllide *a*, artificially-produced chlorophyllide *a* would not affect the accuracy of the results, since the visible absorption and fluorescence spectra of chlorophyllide *a* are identical to those of chlorophyll *a* and would be measured as such. The presence or absence of the phytol chain does not influence molar absorption coefficients of the chlorophyll molecule (Holt & Jacobs 1954). However, if the final product of chlorophyllase activity is pheophorbide *a*, as found in tests with cultured *Skeletonema costatum* (Suzuki & Fujita 1986), then chlorophyll *a* could be underestimated by the above methods because of the differences in the pheophorbide *a* absorption and fluorescence spectra (Lorenzen & Jeffrey 1980).

Techniques such as phase separation (Whitney & Darley 1979) can result in serious underestimation of chlorophyll *a* because the artificially formed chlorophyllide *a* would be removed from the chlorophyll measurement. Further, TLC (Jeffrey 1981, Hallegraeff & Jeffrey 1985) and HPLC techniques (Mantoura & Llewellyn 1983, Wright & Shearer 1984, Bidigare et al. 1985, Murray et al. 1986), which sepa-

rate and quantitate chlorophylls and a wide spectrum of chlorophyll derivatives, may be rendered invalid if chlorophyll breakdown products seen on chromatograms are formed during the harvesting and extraction procedures. The present work and that of Suzuki & Fujita (1986) show that the possibility of artefacts occurring during harvesting procedures is particularly acute when diatoms are present in the samples.

Owens & Falkowski's (1982) work on chlorophyllase activity in isolated thylakoid preparations supports these conclusions. They found high chlorophyllase activity in thylakoids from several diatoms and minor activity in preparations from several flagellate groups. They also obtained evidence for a 'magnesium-removal' enzyme in thylakoids of some diatoms. This enzyme detaches magnesium from the chlorophyllide *a* formed from the chlorophyllase reaction resulting in the production of pheophorbide *a*.

In a study of chlorophyllase in *Skeletonema costatum* cultures, Suzuki & Fujita (1986) found, as we did, that both centrifugation and filtration activated the enzyme. They also found some enzyme activity in 100 % acetone, and recommended heat treatment (65 °C for 2 min in 100 % acetone) for complete enzyme inactivation. In our study, 100 % acetone, without heat treatment, caused complete enzyme inhibition. Suzuki & Fujita (1986) also described the formation of both chlorophyllide *a* and pheophorbide *a*, as end products of enzyme activity. In our study, pheophorbide *a* was seldom detected. In a related study in our laboratory, pigments in 51 diatom species (Stauber 1984) were analysed by 2-dimensional TLC after cell harvesting by centrifugation and pigment extraction in 100 % acetone, and traces of pheophorbide *a* were detected in only 2 species, *Nitzschia fraudulenta* and a tropical *Chaetoceros*. The discrepancies in the results of the 2 laboratories may be attributable to differences in the culture conditions used, to strain-specific enzyme differences, and possibly also to activation of the Owens & Falkowski 'Mg-removal' enzyme in Suzuki & Fujita's work. The *S. costatum* cultures used by Suzuki & Fujita were aerated with 0.5 % CO_2 in air, under continuous light. This may have caused sufficient acid to accumulate in the cells' vacuoles to form pheophorbide *a* from chlorophyllide. In our study, non-aerated stationary cultures were grown under 12 : 12 light : dark cycles and the pH of the growth medium was maintained between 7.9 and 8.5. Possibly our cultured algae resemble some field situations, for in field studies we identified chlorophyllide *a* but not pheophorbide *a* as a major chlorophyll degradation product during diatom blooms (Jeffrey 1974, Hallegraeff 1981).

In the 50 % acetone assay test, 36 out of 113 algal isolates tested in our experiments showed no chlorophyllase activity (Table 4). The enzyme may be absent, or it may be less accessible to solvent activation, and therefore does not pose a problem for chlorophyll analysis.

The present work, showing formation of chlorophyllide *a* during filtration of algal cells, particularly diatoms, cautions against the earlier suggestion (Jeffrey 1974) that chlorophyllide *a* seen on chromatograms is a marker for senescent diatoms. Until methods are found to inactivate the enzyme during harvesting procedures, chlorophyllide *a* presently found on chromatograms must be considered a possible artefact. Suzuki & Fujita (1986) suggested the use of a hypertonic medium in the final stages of filtration to prevent chloroplast damage, but neither they nor we have been able to achieve consistent success with this method. Perhaps the problem will be solved by adding a suitable chlorophyllase inhibitor prior to filtration or centrifugation. p-Cloro-mercuribenzoate, which inhibits chlorophyllase from rye seedlings (Klein & Vishniac 1961) and *Euglena gracilis* (Terpstra 1977), but not *Ailanthus altissima* (McFeeters et al. 1971) or *Phaeodactylum tricornutum* (Terpstra 1977), might deter artefact formation in some species. However, the general toxicity of this enzyme inhibitor would caution against its use in routine field studies.

HPLC and TLC are now the methods of choice for accurate analysis of all chlorophylls and derivatives in phytoplankton samples. These methods are being used increasingly in oceanography for water-column work (Gieskes & Kraay 1983a, b, Mantoura & Llewellyn 1983, Hallegraeff & Jeffrey 1985, Murray et al. 1986, Jeffrey & Hallegraeff in press), sediment-trap experiments (Repeta & Gagosian 1982) and grazing experiments (Kleppel & Pieper 1984). The value of increased sensitivity and refinement of separation procedures may be rendered useless if artefact formation during sample preparation is not prevented. Use of 100 % instead of 90 % acetone as extraction solvent should prevent chlorophyllase activity during extraction, although Suzuki & Fujita (1986) found that 100 % acetone was not sufficient and recommended heat treatment (65 °C for 2 min) for complete inactivation of their *Skeletonema costatum* chlorophyllase. Clearly, now that the problem of chlorophyllase activity has been identified as seriously invalidating results of pigment analysis using separation techniques, appropriate procedures must be found to inactivate it during sample preparation and analysis.

Acknowledgements. We thank Mrs J. C. Eyles and Mrs J. L. Stauber (1978–1980) and Dr. S. Blackburn (1986) for expert maintenance of the CSIRO Algal Culture Collection while these studies were carried out, and Mr J. Jordan for provision of cultures from the FCRG Culture Collection at the Scripps Institution of Oceanography in California in 1973. SWJ also wishes to thank Dr. Paul Nival, Deputy Director, Station

Zoologique, Villefranche-sur-Mer, France, for providing peaceful visitor facilities (April 1986), that made completion of this manuscript possible. We are also indebted to Dr. Colin Gibbs for valuable comments on the paper.

LITERATURE CITED

Barrett, J., Jeffrey, S. W. (1964). Chlorophyllase and formation of an atypical chlorophyllide in marine algae. Pl. Physiol. Lancaster 39: 44–47

Barrett, J., Jeffrey, S. W. (1971). A note on the occurrence of chlorophyllase in marine algae. J. exp. mar. Biol. Ecol. 7: 252–262

Beale, S. I. (1984). Biosynthesis of photosynthetic pigments in chloroplast biogenesis. In: Baker, N. R., Barber, J. (ed.) Elsevier Science Publishers, Amsterdam, p. 135–205

Bidigare, R. R., Kennicutt II, M. C., Brooks, J. M. (1985). Rapid determination of chlorophylls and their degradation products by high-performance liquid chromatography. Limnol. Oceanogr. 30: 432–435

Gieskes, W. W. C., Kraay, G. W. (1980). Primary production and phytoplankton pigment measurements in the northern North Sea during FLEX-76. Meteor Forsch.-Ergebnisse A 22: 105–112

Gieskes, W. W. C., Kraay, G. W. (1983a). Unknown chlorophyll *a* derivatives in the North Sea and the tropical Atlantic Ocean revealed by HPLC analyses. Limnol. Oceanogr. 28: 757–766

Gieskes, W. W. C., Kraay, G. W. (1983b). Dominance of Cryptophyceae during the phytoplankton spring bloom in the central North Sea detected by HPLC analysis of pigments. Mar. Biol. 75: 179–185

Gowen, R. J., Tett, P., Wood, B. J. B. (1983). Changes in the major dihydroporphyrin plankton pigments during the spring bloom of phytoplankton in two Scottish sea lochs. J. mar. biol. Ass. U.K. 63: 27–36

Guillard, R. R. L., Ryther, J. H. (1962). Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8: 229–239

Hallegraeff, G. M. (1981). Seasonal study of phytoplankton pigments and species at a coastal station off Sydney: importance of diatoms and the nanoplankton. Mar. Biol. 61: 107–118

Hallegraeff, G. M., Jeffrey, S. W. (1985). Description of new chlorophyll *a* alteration products in marine phytoplankton. Deep Sea Res. 32: 697–705

Holden, M. (1961). The breakdown of chlorophyll by chlorophyllase. Biochem. J. 78: 350–364

Holden, M. (1963). The purification and properties of chlorophyllase. Photochem. Photobiol. 2: 175–180

Holm-Hansen, O., Lorenzen, C. J., Holmes, R. W., Strickland, J. D. H. (1965). Fluorometric determination of chlorophyll. J. Cons. perm. int. Explor. Mer 30: 3–15

Holt, A. S., Jacobs, E. E. (1954). Spectroscopy of plant pigments 1. Ethyl chlorophyllides *a* and *b* and their pheophorbides. Am. J. Bot. 41: 710–722

Jeffrey, S. W. (1965). Paper chromatographic separation of pigments in marine phytoplankton. Aust. J. mar. Freshwat. Res. 16: 307–313

Jeffrey, S. W. (1974). Profiles of photosynthetic pigments in the ocean using thin-layer chromatography. Mar. Biol. 26: 101–110

Jeffrey, S. W. (1979). Cultivating unicellular marine plants. In: CSIRO Fish and Oceanogr., Hobart, A. Rep. 1977–1979, p. 22–43

Jeffrey, S. W. (1981). An improved thin-layer chromatography technique for marine phytoplankton pigments. Limnol. Oceanogr. 26: 191–197

Jeffrey, S. W., Hallegraeff, G. M. (in press). Phytoplankton pigments, species and light climate in a complex warm-core eddy of the East Australian Current. Deep Sea Res.

Jeffrey, S. W., Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz. 167: 191–194

Klein, A. O., Vishniac, W. (1961). Activity and partial purification of chlorophyllase in aqueous systems. J. biol. Chem. 236: 2544–2547

Kleppel, G. S., Pieper, R. E. (1984). Phytoplankton pigments in the gut contents of planktonic copepods from coastal waters off southern California. Mar. Biol. 78: 193–198

Lewin, J. C., Norris, R., Jeffrey, S. W., Pearson, B. (1977). An abberrant chrysophycean alga *Pelagococcus subviridis* gen. nov. et sp. nov. from the north Pacific Ocean. J. Phycol. 13: 259–266

Loeblich, A. R. III, Smith, V. E. (1968). Chloroplast pigments of the marine dinoflagellate *Gyrodinium resplendens*. Lipids 3: 5–13

Lorenzen, C. J., Jeffrey, S. W. (1980). Determination of chlorophyll in seawater. UNESCO Tech. Pap. Mar. Sci. 35: 1–20

Mantoura, R. F. C., Llewellyn, C. A. (1983). The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. Analytica chim. Acta 151: 297–314

McFeeters, R. F., Chichester, C. O., Whitaker, J. R. (1971). Purification and properties of chlorophyllase from *Ailanthus altissima* (Tree-of-Heaven). Pl. Physiol. 47: 609–618

Murray, A. P., Gibbs, C. F., Longmore, A. R., Flett, D. J. (1986). Determination of chlorophyll in marine waters: intercomparison of a rapid HPLC method with full HPLC, spectrophotometric and fluorometric methods. Mar. Chem. 19: 211–227

Nichols, W. H. (1973). Growth media-freshwater. In: Handbook of phycological methods – culture methods and growth measurements. In: Stein, J. R. (ed.) Cambridge University Press, Cambridge, p. 7–24

Owens, T. G., Falkowski, P. G. (1982). Enzymatic degradation of chlorophyll *a* by marine phytoplankton *in vitro*. Phytochemistry 21: 979–984

Parsons, T. R. (1963). A new method for the microdetermination of chlorophyll *c* in sea water. J. mar. Res. 21: 164–171

Parsons, T. R., Maita, Y., Lalli, C. M. (1984). A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford

Patterson, J., Parsons, T. R. (1963). Distribution of chlorophyll *a* and degradation products in various marine materials. Limnol. Oceanogr. 8: 355–356

Repeta, D. G., Gagosian, R. B. (1982). Carotenoid transformations in coastal marine waters. Nature, Lond. 295: 51–53

Saijo, Y., Kamiya, T. (1973). Occurrence of chlorophyllide *a* in the sea. In: Takenouti, A. Y., et al. (ed.) Biological oceanography of the northern Pacific Ocean. Idemitsu Shoten, Tokyo, p. 191–197

SCOR-UNESCO (1966). Determination of photosynthetic pigments in seawater. Monographs on Oceanographic Methodology, I. UNESCO, Paris, p. 11–18

Shuman, F. R., Lorenzen, C. J. (1975). Quantitative degra-

dation of chlorophyll by a marine herbivore. Limnol. Oceanogr. 20: 580–586

Stauber, J. L. (1984). Photosynthetic pigments in marine diatoms. M.Sc. thesis, University of Sydney

Strickland, J. D. H., Parsons, T. R. (1972). A practical handbook of seawater analysis. Bull. Fish. Res. Bd Can. 167

Suzuki, R., Fujita, Y. (1986). Chlorophyll decomposition in *Skeletonema costatum*: a problem in chlorophyll determination of water samples. Mar. Ecol. Prog. Ser. 28: 81–85

Terpstra, W. (1976). Chlorophyllase and lamellar structure in *Phaeodactylum tricornutum*. III. Situation of chlorophyllase in pigmented membranes. Z. PflPhysiol. 80: 177–188

Terpstra, W. (1977). A study of properties and activity of chlorophyllase in photosynthetic membranes. Z. PflPhysiol. 85: 139–146

Terpstra, W., Goedheer, J. C. (1975). Chlorophyllase and lamellar structure in *Phaeodactylum tricornutum*. I. Chlorophyll-chlorophyllide conversion within the lamellae. Z. PflPhysiol. 75: 118–130

Whitney, D. E., Darley, W. M. (1979). A method for the determination of chlorophyll *a* in samples containing degradation products. Limnol. Oceanogr. 24: 183–186

Wright, S. W., Shearer, G. D. (1984). Rapid extraction and high-performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton. J. Chromatogr. 294: 281–295

This article was submitted to the editor; it was accepted for printing on November 14, 1986