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ENZYMATIC DEGRADATION OF CHLOROPHYLL *a* BY MARINE PHYTOPLANKTON *IN VITRO**

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Key Word Index—Chlorophyll; chlorophyllase; diatoms; phaeopigments; phytoplankton.

Abstract—The enzymatic degradation of chlorophyll *a* and the formation of chlorophyllide *a*, phaeophytin *a*, and phaeophorbide *a* were detected *in vitro* in several species of marine phytoplankton. Loss of phytol and Mg^{2+} were found to be catalysed by chlorophyllase and a magnesium-releasing enzyme, respectively. The activities of the two enzymes could be distinguished from each other by inhibiting with Mg^{2+} and/or *p*-chloromercurobenzoate. Both enzymes are activated by cell disintegration. Degradation products were not detected spectrophotometrically *in vivo*. Additionally, in some species, chlorophyll *a* was degraded to products which do not absorb visible light.

INTRODUCTION§

The distribution of chlorophyll degradation products in aquatic environments has been variously interpreted as reflecting phytoplankton physiological state or herbivore grazing pressure, yet the mechanisms responsible for the formation of the major degradation products are poorly understood. Yentsch [1] and Lorenzen [2] suggested that phaeopigment formation could result from either prolonged darkness or passage of cells through herbivore guts. Currie [3] and Jeffrey [4] suggested that phaeopigments *in situ* are mainly associated with copepod faecal pellets. The occurrence of phaeopigments or other products of chl catabolism in intact phytoplankton cells has not been conclusively demonstrated.

The three major degradation products of chl *a*, namely chlide *a*, phaeo *a*, and phbide *a*, result from the loss of phytol, Mg^{2+}, and both phytol and Mg^{2+} respectively. Of these phbide *a* is usually the most abundant degradation product in aquatic environments. The hydrolysis of phytol is catalysed by the glycoprotein [5] chlase (chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14), which has high activity in some marine phytoplankton [6–8]. The loss of Mg^{2+} can occur *in vitro* by the displacement of Mg^{2+} with two H^+ under acidic conditions.

This study presents evidence for the existence of an enzyme capable of catalysing the removal of Mg^{2+} from chl or chlide *a*, leading to the formation of phaeopigments. In conjunction with chlase, magnesium-releasing enzyme (MRE), allows for the wholly enzymatic formation of all the major chl degradation products.

RESULTS

The enzymatic release of Mg^{2+} and phytol from chl *a* were first inferred from kinetic studies of changes in the visible absorption spectrum of aqueous suspensions of chloroplast membrane preparations of the diatom, *Skeletonema costatum* (Fig. 1). Continuous scanning of visible wavelengths revealed a decrease in the red maximum absorbance which followed first order kinetics. This decrease in absorbance asymptotically approached a non-zero value, generally 20% of the initial absorbance, and was associated with several characteristic peak changes: (a) a shift of the red maximum to longer wavelengths; (b) loss of the chl *c* peaks at 635 and 460 nm; (c) a shift of the maxima in the Soret region to shorter wavelengths and (d) the appearance of a peak at 540 nm. The absorbance due to β-carotene (495 nm) and fuxoxanthin (530 nm) was not affected; decreases in absorbance at these wavelengths are due to loss of the long wavelength tails of chls *a* and *c* in the Soret region. The decreased absorbance in the chl *a* maxima, the blue shifts of the Soret peaks, and the appearance of the peak at 540 nm all imply the formation of Mg^{2+}-less degradation products of chl *a*.

Identically prepared chloroplast membranes from

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§Abbreviations: chl; chlorophyll; chlase; chlorophyllase; chlide; chlorophyllide; MRE; magnesium-releasing enzyme; phaeo; phaeophytin; phbide; phaeophorbide; PCMB; *p*-chloromercurobenzoate; PMSF, phenylmethylsulfonyl fluoride.

the chlorophyte *Duraliella tertiolecta* revealed no detectable absorbance changes. However, membrane preparations from *S. costatum* could degrade pigments in the membrane preparations of *D. tertiolecta*. A membrane preparation from *S. costatum* was allowed to react to completion and divided in half. One half was mixed 1:1 with a fresh membrane preparation from *D. tertiolecta*; the other half was diluted 1:1 with buffer. The resulting difference spectra showed a gradual decrease in the chls *a* and *b* absorbance maxima and the appearance of the maxima associated with phaeo (or phbide) *a* and *b*.

Heat treatment of membrane preparations from *S. costatum* at 90° for 1 min followed by rapid cooling to 0° completely inhibited any absorbance changes. Heat-treated extracts from *S. costatum* had no effect on *D. tertiolecta* membrane preparations. When the heat-treated extracts were mixed with untreated extracts from *S. costatum*, in which the absorbance decrease was allowed to go to completion, absorbance decreases in the heat-treated extracts were observed.

The effect of temperature on MRE activity was examined in extracts of *S. costatum* and *Phaeodactylum tricornutum*. In both species MRE activation energy was 12.5 kcal/mol between 4 and 30° and exhibited a temperature maximum at 30°. In *S. costatum* and *P. tricornutum* extracts, MRE activity exhibited a broad pH maximum between pH 7.4 and 8.2. Below pH 6.8 the non-enzymatic reaction became important and above pH 9.5 enzyme activity decreased rapidly.

Whole cell absorption and derivative spectra of axenic cultures of marine phytoplankton do not indicate the presence of detectable phaeo *a* and/or phbide *a* *in vivo*. Rapid extraction of frozen cells (or frozen cell-free extract) in dry acetone at −15° was used to determine the actual *in vivo* concentrations of chl *a* and its degradation products. The results of such analyses with 12 species of marine phytoplankton are presented in Table 1. Chl *a* breakdown products are detectable in only three diatom species. In all other species tested, chl *a* represents 100% of the total *a* pigment (total *a* = chl *a* + chlide *a* + phaeo *a* + phbide *a*). For comparison, an aliquot of each species was also extracted by routine procedures in 90% acetone. In four diatom species, detectable loss of Mg^{2+} and of total *a* pigment was observed. Dephytolization, resulting from the 90% acetone extraction, was observed in all species.

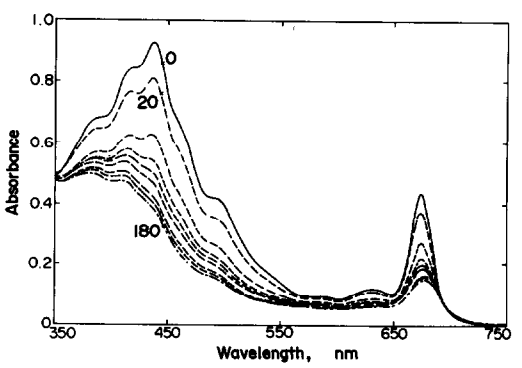

Fig. 1. Observed absorbance decreases in cell-free extracts of *S. costatum* at 20°. Extract was prepared in 50 mM Tris–HCl, pH 8.0 at 0–2° and stored in the dark between scans. —Initial spectrum at 20°; -- 20 min, -·-·- 180 min.

The loss of phytol, Mg^{2+}, and total *a* pigment in the preparation and incubation of cell-free extracts of the same 12 phytoplankton species is shown in Table 2. The data are corrected for pigment loss associated with unbroken cells and larger membrane fragments removed from the cell-free extracts. Measurable loss of phytol and Mg^{2+} was observed in all species except *D. tertiolecta* after 60 min incubation at 25°. Significant losses of total *a* pigment occurred during the 10 min preparatory period at 0° in three diatom species.

A quantitative study of the transformation of chl *a* to its various degradation products and the loss of total *a* and chlide *c* in a cell-free extract of *S. costatum* is shown in Fig. 2. The initial determination indicates the distribution of pigments in whole cells prior to the preparation of cell-free membrane preparations. The next determination immediately followed cell disruption before centrifugation. At time zero, the cell-free extract was brought to 20° and aliquots were removed for spectrophotometric and quantitative pigment analysis. The data in Fig. 2(a) indicate the percent of the total *a* pigment at each sampling time. The loss of total *a* and chl *c* during the course of the experiment is shown in Fig. 2(b). During preparation of the cell-free extract, > 50% chl *a* was converted to roughly equal amounts of chlide *a*, phaeo *a*, and phbide *a* with some loss of total *a* and chl *c*. All pigments except phbide *a* declined slowly during the 20° incubation accompanied by steady losses of total *a* and chl *c*. After 3 hr, only 5% of the initial chl *a* remained intact; 50% was converted to phbide *a*, 5% to phaeo *a*, and 40% was converted to non-visible light absorbing forms.

When the same experiment was repeated in the presence of 100 mM magnesium chloride, the pattern of chl *a* degradation showed significant changes (Fig. 3). During the preparation of the extract, phaeo *a* formation and loss of total *a* and chlide *c* were inhibited. In the subsequent incubation period, phaeo *a* remained low while chlide *a* and phbide *a* increased slowly in approximately equal amounts. Loss of total *a* and chl *c* were also inhibited. After 3 hr, 63% of the Mg^{2+} and 89% of the phytol were displaced from the chl *a* initially present, as compared with 96% Mg^{2+} and 89% phytol losses in the absence of $MgCl_2$.

A third pattern of chl *a* degradation was observed when extracts of *S. costatum* were prepared and incubated with 5 mM PCMB (Fig. 4). On preparation of the extract, only phbide *a* was formed in significant amounts. During the incubation, the major reaction observed was the conversion of chl *a* to phaeo *a*. Chl *c* declined rapidly only in the first 30 min of incubation. After 3 hr, 25% of the original chl *a* remained intact while 71% magnesium and 21% phytol losses occurred.

The effects of *S. costatum* extracts on externally added pigment substrates are similar: magnesium chloride specifically inhibited magnesium-releasing reactions and PCMB inhibited phytol hydrolysis as well as the loss of total *a* pigments. Neither PMSF nor EDTA had any effect on enzyme activity.

Table 1. Distribution of chl *a* and its breakdown products determined under conditions which limit the loss of phytol and Mg^{2+} (dry acetone, −15°) and routine conditions (90% acetone, 20°). Total *a* pigment = chl *a* + chlide *a* + phaeo *a* + phbide *a*. Percent total *a* lost represents the difference in total *a* pigment between the two extraction techniques. Individual values represent the percent of the total *a* pigment in each extract. Absence of data indicates the measured products were less than the estimated 1% error in each determination

Species	Dry acetone extraction (−15°) % Total *a* pigment					90% acetone extraction (+20°) % Total *a* pigment			
	Chl *a*	Chlide *a*	Phaeo *a*	Phbide *a*	% total *a* lost	Chl *a*	Chlide *a*	Phaeo *a*	Phbide *a*
Bacillariophyceae									
Skeletonema costatum	92.4	4.0	1.3	2.2	17.2	14.8	41.1	11.7	32.4
Phaeodactylum tricornutum	96.0	2.2	1.3	0.5	14.4	33.5	38.4	13.1	15.0
Nitzschia closterium	98.3	1.6	—	—	14.7	35.9	38.0	15.6	10.5
Melosira nummubides	100	—	—	—	3.3	83.9	10.0	5.4	—
Thalassiosira pseudonana	100	—	—	—	—	98.4	1.6	—	—
Dinophyceae									
Cashonena nei	100	—	—	—	—	91.6	8.4	—	—
Chrysophyceae									
Isochrysis galbana	100	—	—	—	—	95.1	4.9	—	—
Monochrysis lutherii	100	—	—	—	—	96.6	3.4	—	—
Haptophyceae						—	—	—	—
Coccolithus pelagicus	100	—	—	—	—	97.3	2.7	—	—
Cryptophyceae									
Chroomonas salina	100	—	—	—	—	98.9	6.1	—	—
Chlorophyceae									
Dunaliella tertiolecta	100	—	—	—	—	98.8	1.2	—	—

Table 2. Loss of total *a* pigment, phytol, and Mg^{2+} during the preparation of cell-free extracts (10 min, 0°), and incubation (60 min, 25°). Losses are measured from −15° dry acetone extractions in Table 1. Corrections for pigment which pelleted during extract preparation were made. Absence of data indicates measured losses were less than the estimated error of each determination

Species	10 min 0°			60 min at 25°		
	Total *a*	Phytol	Mg^{2+}	Total *a*	Phytol	Mg^{2+}
Bacillariophyceae						
Skeletonema costatum	12.0	40.3	41.9	33.8	77.0	80.8
Phaeodactylum tricornutum	10.7	32.5	32.7	26.3	64.8	58.1
Nitzschia closterium	11.2	42.2	47.1	28.7	81.2	83.7
Melosira nummuloides	—	13.1	7.6	14.8	47.4	46.1
Thalassiosira pseudonana	—	—	—	7.9	39.8	42.7
Biddulphia mobiliensis	—	8.6	—	6.7	11.6	14.4
Dinophyceae						
Cashonena nei	—	7.3	—	—	8.9	11.4
Chrysophyceae						
Isochrysis galbana	—	5.7	—	—	7.1	11.6
Monochrysis lutherii	—	6.1	—	—	8.3	7.9
Haptophyceae						
Coccolithus pelagicus	—	—	—	—	6.9	9.9
Cryptophyceae						
Chroomonas salina	—	5.9	—	—	6.3	16.7
Chlorophyceae						
Dunaliella tertiolecta	—	—	—	—	—	—

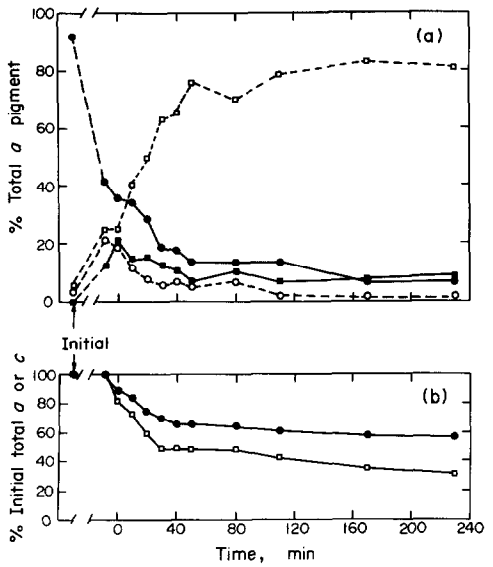

Fig. 2. Quantitative determination of chl *a* degradation and formation of breakdown products in a cell-free extract of *S. costatum*. Initial condition was determined by extraction of frozen whole cells in dry acetone at −15°. The second set of points was measured immediately after cell disintegration. At time zero, the extract was brought to 20°. (a) Percent total *a* pigment present at each determination: (●), chl *a*; (○), chlide *a*; (■), phaeo *a*; (□), phbide *a*. (b) Percent initial total *a* and chl *c* remaining: (●), total *a*; (□), chl *c*.

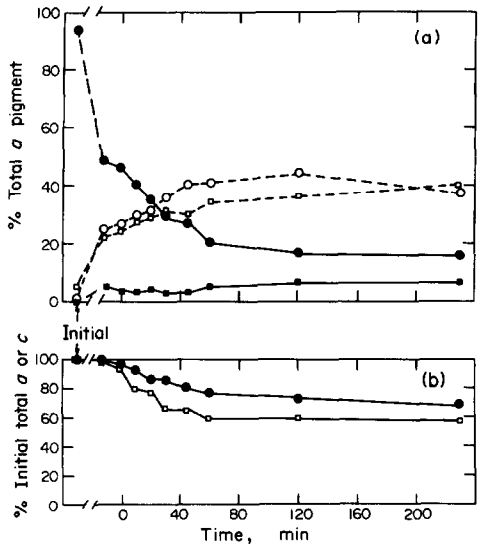

Fig. 3. Quantitative determination of chl *a* and its breakdown products in cell-free extracts of *S. costatum* containing 100 mM $MgCl_2$. Initial condition was determined by extraction of frozen whole cells in dry acetone at −15°. The second set of points was measured immediately after cell disintegration. At time zero, the extract was brought to 20°. (a) Percent total *a* pigment present at each determination: (●), chl *a*; (○), chlide *a*; (■), phaeo *a*; (□), phbide *a*. (b) Percent initial total *a* and chl *c* remaining: (●), total *a*; (□), chl *c*.

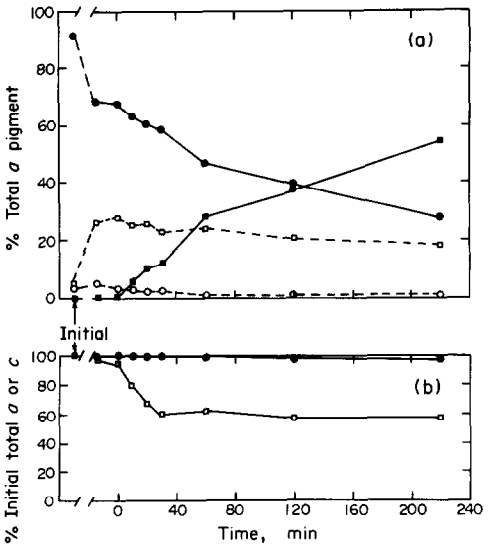

Fig. 4. Quantitative determination of chl *a* and its breakdown products in cell-free extracts of *S. costatum* containing 5 mM PCMB. Initial condition was determined by extraction of frozen whole cells in dry acetone at −15°. The second set of points were measured immediately after cell disintegration. At time zero, the extract was brought to 20°. (a) Percent total *a* pigment present at each determination: (●) chl *a*; (○), chlide *a*; (■), phaeo *a*; (□), phbide *a*. (b) Percent initial total *a* and chl *c* remaining: (●), total *a*; (□), chl *c*.

DISCUSSION

The data presented here strongly suggest that there are at least two enzymes involved in the degradation of chl *a* in marine phytoplankton. Both enzyme reactions are initiated by cell disruption (physical grinding, hypotonic, or aqueous-acetone treatment) and are associated with pigmented membrane fragments. The formation of chlide *a*, phaeo *a*, and phbide *a* was often accompanied by a loss of total *a* pigment. Chlase and MRE activities are diversely and non-uniformly distributed in the plankton groups examined, the highest activities appear to be associated with diatoms (Table 2).

The hydrolysis of phytol from chl *a* or phaeo *a* is catalysed by chlase. Our results are in general agreement with those of Terpstra [7–9]: (a) Chlase activity is associated with the pigmented membranes; (b) activity is not detectable *in vivo* but is initiated by cell disintegration; (c) the reaction shows a similar time-course of conversion of chl *a* within the membrane fragments; (d) the reaction is slower for the conversion of externally added substrates and (e) enzyme activity is sensitive to sulphydryl-group inhibitors.

The loss of Mg^{2+} also appears to be catalysed by an enzymatic reaction. The evidence that an enzyme is responsible for the release of Mg^{2+} is summarized as follows: (a) unlike the non-enzymatic removal of Mg^{2+} from chl, the reaction described here occurs at neutral and basic pH; (b) the reaction is completely heat labile; (c) at pH > 7.5, high activity preparations from one species (e.g. *S. costatum*) catalyse the

reaction in low activity preparations of another species (e.g. *D. tertiolecta*); (d) the reaction is inhibited by magnesium chloride and (e) activity is only associated with pigmented membranes and no activity is found in 25 000 *g* supernatants.

In addition to the loss of phytol and Mg^{2+}, significant losses of total *a* pigments were observed during the first 30 min of incubation in cell-free extracts of *S. costatum* (Fig. 2b) and other diatoms. One explanation of these results is the degradation of chl *a* to products with low extinction coefficients in the visible spectrum. No additional peaks, which could not be accounted for, were detectable by derivative spectroscopy during the course of an incubation. Formation of 'non-absorbing' products would require disruption of the conjugated bonds in the porphyrin ring. The formation of non-absorbing products appears to require a sulfhydryl group, as loss of total *a* is inhibited by PCMB.

At present very little is known about the catabolism of chls *in vivo* or *in vitro*. It has been shown that in some species of unicellular algae chl *a* turns over *in vivo* relatively rapidly (in the order of 1–10 hr) [10]. Such turn-over times imply highly active catabolic as well as anabolic pathways for chls. The enzymatic hydrolysis of phytol from chl is the best studied of these reactions, yet the role of the enzyme *in vivo* is uncertain (chlase may function in the reverse direction *in vivo*, in the synthesis of chl). The results of this study suggest that the removal of Mg^{2+} can, at least *in vitro*, be catalysed by a MRE associated with the chloroplast membranes. The enzyme appears to be distinct in cofactor requirements from the enzyme involved in Mg^{2+} insertion into protoporphyrin [11]. Finally, the loss of total *a* pigments suggest that at least one or more enzymes, yet to be identified, is involved in the catabolism of chl and these enzymes are highly active in marine diatoms.

EXPERIMENTAL

Culture conditions. Skeletonema costatum (Grev.) Cleve (Woods Hole Clone Skel., Bacillariophyceae) and *Dunaliella tertiolecta* Butcher (Woods Hole Clone Dun., Chlorophyceae) were grown in 4 l. batch cultures at 15° as previously described [12]. All other species were grown in 250 ml flasks under identical conditions. All cells were utilized during exponential growth.

Membrane preparations. Cells were harvested by filtration on 47 mm Gelman type A/E glass fibre filters, and disrupted by 60 sec treatment in 50 mM Tris–HCl, pH 8.0, in a Teflon–glass tissue homogenizer at 0–2°. This brief treatment yielded > 95% broken cells in all species tested. Whole cells and debris were removed by 3 min centrifugation (300 *g*) at 2°. The resulting cell-free extract contained fragments of pigmented membranes accounting for the major fraction of the total pigment (some pigment was lost in the 300 *g* pellet). All pigments in the cell-free extract could be pelleted at 25 000 *g* in 10 min. Chlase and MRE activities were only associated with the pigmented membranes; no activity was found in 25 000 *g* supernatants. Extracts were stored on ice in the dark for less than 10 min before utilization.

Pigment isolation. Isolation and purification of pigments for measurement of enzyme activity with added substrates followed the general procedure of Perkins and Roberts [13]. Purity was verified by TLC [4, 10]. Pigments were purified from 90% Me_2CO extracts of *S. costatum*. Individual pigments were concd in fresh Et_2O and transferred to 50 mM Tris–HCl, pH 8.0, containing 0.01–1.0% Triton X-100. The Et_2O–Triton mixture was equilibrated overnight in the dark before removal of the Et_2O to ensure maximum pigment transfer and solubilization.

Enzyme essays. Qualitative spectrophotometric monitoring of pigment degradation within the lamellar fragments was accomplished using an Aminco DW-2a spectrophotometer equipped with a temp.-controlled cuvette holder and magnetic stirring. Cell-free extracts were thermally equilibrated in the cuvette holder in the dark and then the suspension was repeatedly scanned between 350 and 750 nm against double distilled H_2O using an automatic rescan mode. The appearance, disappearance, or shifting of absorbance maxima were monitored during the course of the reaction. Rates of phaeopigment formation and pigment degradation were estimated by fitting the absorbance at the red chl *a* maximum (*ca* 675 nm) to an exponential decay curve and calculating the initial rate of absorbance change.

For measurement of enzyme activity with added substrate, pigmented membranes were prepared as described, in 50 mM Tris–HCl, pH 8.0, containing 0.01–0.1% Triton X-100. To exhaust internal substrate, extracts were pre-incubated for 2 hr at 20°, after which the extract was divided in half. Triton-solubilized pigments were added to one half to make the extract 20 μM pigment in external substrate, and the second half was given an equal dilution with pigment-free buffer. The disappearance of substrates and formation of degradation products was monitored by differentially scanning the two extracts in the repetitive scan mode of the spectrophotometer.

Both chlase and MRE activites were measured in aq. Me_2CO-extracts of cell suspensions of several species. Cells were harvested by centrifugation and suspended in various concns Me_2CO (generally 30%) mixed with 50 mM KPi buffer, pH 8.0. Progress of the reaction was followed by treating a 1 ml aliquot of the suspension with hexane–Me_2CO–H_2O (3:2:1) and determining pigments.

Pigment analyses. Quantitative analysis of chl *a* and its degradation products in whole cells and cell-free extracts without Triton X-100 was accomplished as follows: whole cells were harvested by filtration as described; cell-free extract (1 ml) was filtered on 25 mm HA Millipore membrane filters. Filters were immediately frozen in liquid N_2 and quickly extracted in 2 ml dry Me_2CO in a Teflon–glass homogenizer at −15° in a freezer. Debris was removed by filtration and was re-extracted with 2 ml dry Me_2CO at − 15°. This gave 100% extraction of all pigments. All subsequent steps were carried out at room temp.

The Me_2CO extract was transferred to a 15 ml glass stoppered centrifuge tube and mixed with 6 ml *n*-hexane, followed by addition of 2 ml double distilled H_2O, and 20 μl 50 mM NaOH. After vigorous mixing, the phases were separated by centrifugation. This gave >98% separation of phytolated from non-phytolated pigments. Each phase was scanned between 350 and 750 nm before (A^0) and after (A^a) vigorous mixing with 50 μl 10% HCl. Chl *a* and its breakdown products were estimated from their red absorbance maxima (*ca* 665 nm) and chlide *c* from its maximum at 630 nm. Phytolated pigments retained in the hexane fraction were calculated from the equations (nmol/sample):

$$\text{chl } a = 35.7\,(A^0_{664} - A^a_{667}) \times \text{vol. hexane fraction},$$

$$\text{phaeo } a = 35.7\,(1.25\,A^a_{667} - A^0_{664}) \times \text{vol. hexane fraction}.$$

Non-phytolated pigments were determined in the aq. Me_2CO phase (*ca* 60% Me_2CO):

$$\text{chlide } a = [18.7(A^0_{664} - A^a_{667}) - 0.645A^0_{630}] \times \text{vol. } Me_2CO \text{ fraction},$$

$$\text{phbide } a = 18.7\,(2.25\,A^a_{667} - A^0_{664}) \times \text{vol. } Me_2CO \text{ fraction},$$

$$\text{chlide } c = 23.9\,(A^0_{630} - 0.15\,A^0_{664}) \times \text{vol. } Me_2CO \text{ fraction}.$$

These equations were derived and checked using solns of pure pigment in 100% Me_2CO which were fractionated using the above procedure, and supercede previously reported equations [10].

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