

## Distribution and sources of cyclic pheophorbides in the marine environment

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### Abstract

The labile chlorophyll *a* (Chl  $a_1$ ) degradation product, 13<sup>2</sup>, 17<sup>3</sup>-cyclopheophorbide *a* enol (CPP516), was only recently found in marine sediments, where it can contribute a significant fraction to solvent-extractable sedimentary chlorins. We developed a new chromatographic method for the analysis of CPP516 to study its distribution and sources in the marine environment. We found high concentrations of CPP516 in surficial sediments and particulate matter collected in sediment traps. It contributed 40 to 70% to the sum of all Chl  $a_1$  degradation products in fecal material from herbivorous microzooplankton and macrozooplankton. We did not find CPP516 in exponentially growing or stationary phase algal cultures. These data suggest that a major source of CPP516 is herbivores feeding on phytoplankton. We also found a series of compounds similar to CPP516 in sediments and sediment-trap samples. Mass-spectrometric and ultraviolet(UV)/visible-spectrometric analyses suggest that these are CPP516 analogs of divinyl-Chl *a* and Chl *bs* and Chl *cs*, conclusions that have to be confirmed by synthesis. CPP516, dissolved in organic solvents, easily degrades to 13<sup>2</sup>-oxopyropheophorbide *a* and chlorophyllone *a* under oxic conditions. When associated with particles, CPP516 is stable in oxic aquatic environments on a timescale of days, possibly stabilized by the chelation of metal cations. The chromophore of CPP516 differs substantially from that of other Chl  $a_1$  degradation products; it has absorption maxima at 360, 426, and 688 nm. CPP516 does not fluoresce as other Chl  $a_1$  degradation products. As a consequence, it is invisible to fluorescence-based analyses of "pheopigments." Thus, rates of grazing and growth derived from such measurements, as used in conjunction with the gut fluorescence and the Chl-budget method, will be underestimates of true rates when CPP516 contributes significantly to total chlorins.

Chlorophyll *a* (Chl  $a_1$ ; Fig. 1, structure 1.1) and its degradation products have been used extensively in the past as tracers for carbon associated with phytoplankton. The fate of Chl  $a_1$  during herbivory has been studied in particular because the nature and concentrations of Chl  $a_1$  degradation products may reflect the activity of herbivores. Initially, it was thought that grazing quantitatively degrades Chl  $a_1$  to "pheopigments" (Shuman and Lorenzen 1975), i.e., to compounds that have a pheophorbide *a*-pheophytin *a* chromo-

phore (Fig. 1, 5.1) with its associated fluorescence. Making this assumption, methods were proposed to determine grazing rates of herbivores from concentrations of pheopigments in the guts of macrozooplankton (Mackas and Bohrer 1976) or to determine in situ growth and loss rates of phytoplankton (Welschmeyer and Lorenzen 1985). However, results from these methods were questioned once it was realized that Chl  $a_1$  is not quantitatively degraded to pheopigments but is partially degraded to products that do not fluoresce or absorb light in the visible spectrum (Conover et al. 1986). The observed loss of pheopigments during grazing was highly variable among different studies, with values ranging from 5 to 99% (Dagg and Walser 1987; Lopez et al. 1988; Downs 1989; Penry and Frost 1991; Strom 1993; Peterson and Dam 1996).

In parallel to the work by biological oceanographers, natural product chemists have studied the antioxidative properties of the lipophilic extracts of marine benthic organisms. This work revealed a range of antioxidants that, based on structural similarities, are thought to be derived from the Chl  $a_1$  of marine algae. These compounds were not only pheophorbide *a* (5.1b), pheophytin *a* (5.1a), and their "pyro" analogs, i.e., their 13<sup>2</sup>-desmethoxycarbonyl derivatives pyropheophytin *a* (5.2a) and pyropheophorbide *a* (5.2b), but also compounds with a seven-membered ring fused to the isocyclic ring. Of the latter compounds, chlorophyllone *a* (Chlone  $a_1$ ; 3.1a,b), its methyl esters, and 13<sup>2</sup>-oxopyropheo-

### Acknowledgments

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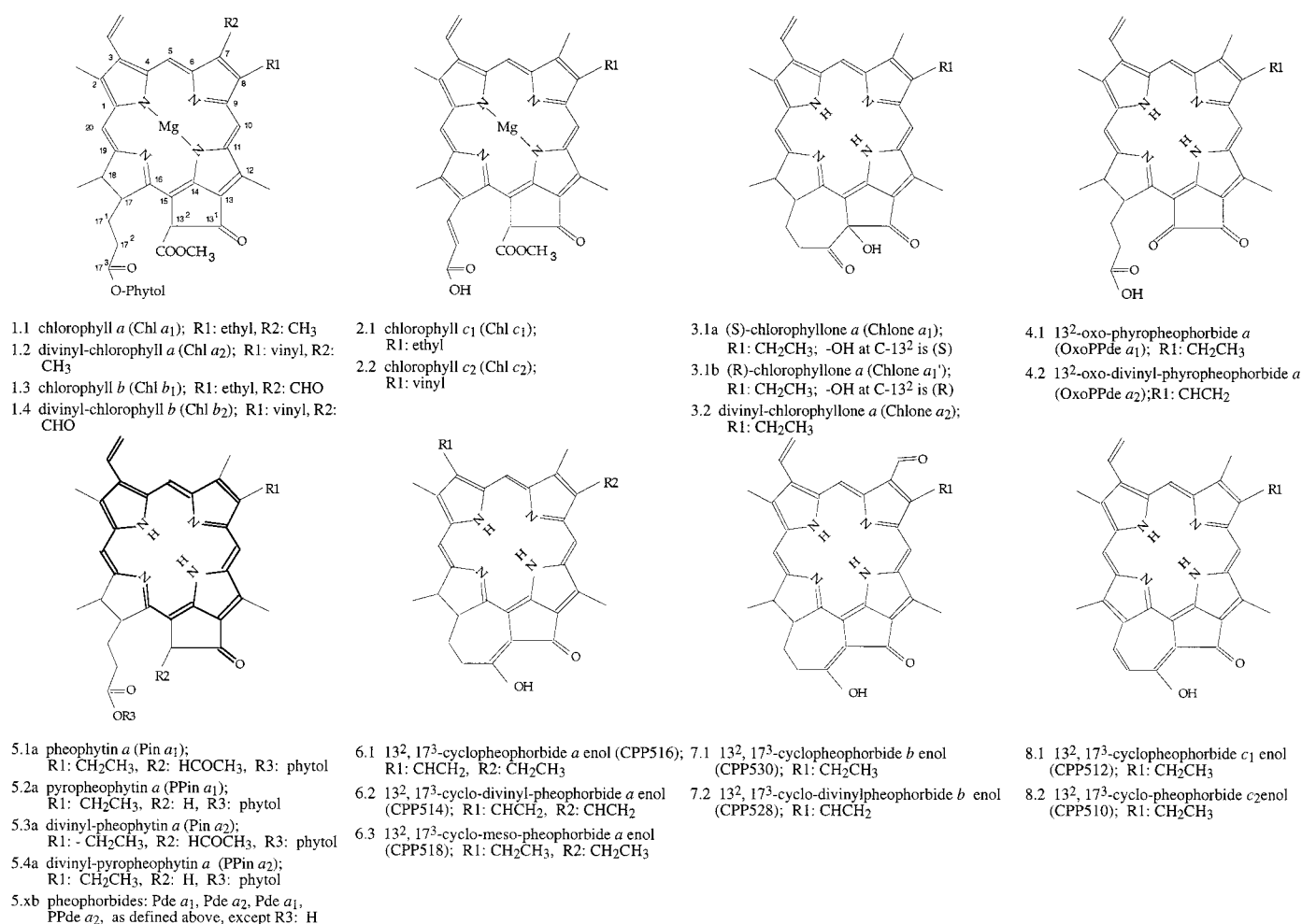


Fig. 1. Structures of the main compounds referred to in the text. Panel 1 shows the carbon numbering system used in the text (IUPAC tetrapyrrole nomenclature convention, Moss 1987). The pheopigment-chromophore, i.e., the system of conjugated double bonds, is shown in bold in panel 5. Chemical changes to the chromophore will affect the UV/visible spectral properties significantly, e.g., the substitution of the 8-ethyl with a vinyl group as seen in Chl *a*<sub>2</sub> (structure 1.2). Other changes, e.g., removal of the 13<sup>2</sup>-carbomethoxy to form pyropheophorbide *a* (5.1b) have no effect.

phorbide *a* (OxoPPde *a*<sub>1</sub>; 4.1) were found in benthic suspension feeders (Sakata et al. 1990; Yamamoto et al. 1992; Watanabe et al. 1993). These compounds are thought to be derived from 17<sup>3</sup>, 13<sup>2</sup>-cyclopheophorbide *a* enol (a pheophorbide derivative with a molecular weight of 516 dalton, i.e., CPP516; 6.1), which has been isolated from a sponge (Karuso et al. 1986). Of these compounds, CPP516 is particularly interesting because its chromophore differs significantly from the pheopigment chromophore. Its ultraviolet (UV)/visible absorption maxima are at 363, 429, and 690 nm, it has no or negligible fluorescence, easily degrades in solutions of organic solvents, and does not separate well on traditional chromatographic media (Falk et al. 1975; Ocampo et al. 1999).

Recently, Chlone *a*<sub>1</sub> and CPP516 have been found in marine and lacustrine sediments (Chillier et al. 1993; Harris et al. 1995; Ocampo et al. 1999). CPP516, in particular, was found to be the major Chl *a* degradation product in sediments from the Black Sea and the Peru margin and was found in particulate organic matter (POM) collected in sed-

iment traps (Ocampo et al. 1999). Sources of CPP516 found in recent marine sediments are not known; enzymatically mediated synthesis from Chl *a*<sub>1</sub> degradation products in sediments or in the intestines of grazers feeding on phytoplankton are most likely (Ocampo et al. 1999). The latter possibility is particularly interesting as it would represent one means of removing Chl *a* degradation products from the analytical window of fluorescence-based analyses. To address this question, we developed a chromatographic system for the analysis of CPP516 and related compounds and studied the distribution of these compounds in microzooplankton and macrozooplankton fecal material, in POM collected in floating sediment traps, and in marine sediments. We report here that CPP516 and related compounds contribute significantly to chlorophyll degradation products in marine herbivore fecal matter. Our results suggest that the destruction of Chl *a*<sub>1</sub> to colorless residues by herbivores feeding on phytoplankton may have been overestimated in the past as herbivores degrade a large fraction of the ingested Chl *a*<sub>1</sub> to CPP516.

## Methods

**High-pressure liquid chromatography (HPLC)**—C-18 column-based reverse-phase HPLC (rp-HPLC) was carried out on an Alltech C-18 HS Adsorbosphere column. The C-18 rp-HPLC system is as follows. The column dimensions are 3  $\mu\text{m}$ , 4.6 mm, and 10 cm. Solvents are (A) methanol:aqueous 0.5 N ammonium acetate (75:25 v/v) and (B) methanol:acetone (20:80). The gradient is (time, A%) 0 min, 80%; 27 min, 5%; 34 min, 5%; and 36 min, 80%. The flow rate is 1.5 ml  $\text{min}^{-1}$ . A chromatographic procedure was developed for the analysis of CPP516 and related compounds based on the YMC C-30 Carotenoid column. The C-30 rp-HPLC system is as follows. The column dimensions are 3  $\mu\text{m}$ , 4.6 mm, and 15 cm. Solvents are (A) methanol:aqueous 0.5 N ammonium acetate (75:25 v/v) and (B) chloroform:ethyl acetate:methanol:water (50:35:14:1 v/v). The gradient is (time, B%) 0 min, 15%; 24 min, 100%; 38 min, 100%; and 40 min, 15%. The flow rate is 1.5 ml  $\text{min}^{-1}$ . Water and methanol were added to solvent B to ensure that solvents A and B are miscible and to improve sensitivity during liquid-chromatography/mass-spectroscopy (LC/MS). The chromatographic systems were calibrated with pigment standards (*see below*) using published extinction coefficients (Ma and Dolphin 1996; Jeffrey et al. 1997). CPP514 (*see below*, 6.2) was quantified assuming that its extinction coefficient at 690 nm in  $\text{CHCl}_3$  is identical to the coefficient of CPP516, a probable assumption considering the effect of the additional vinyl group on the extinction coefficient of divinyl-chlorophyll *a* (Chl  $a_2$ , 1.2, Goericke and Repeta 1993) when compared to Chl  $a_1$ .

**Pigment standards**—Chl  $a_1$  was isolated and pheophorbide  $a_1$ , pyropheophorbide  $a_1$ , pheophytin  $a_1$ , and pyropheophytin  $a_1$  were synthesized as previously described (Goericke et al. 1999). CPP518, 516, 514, and 512 (i.e., cyclic pheophorbides, CycPdes) were isolated from Peru margin sediments. The sediment (~40 g wet) was extracted twice in 80-ml MeOH and twice in 80-ml  $\text{MeCl}_2$  under  $\text{N}_2$ . The first MeOH extract was discarded, and the second MeOH and the  $\text{MeCl}_2$  extracts were combined and evaporated under reduced pressure in the presence of benzene. The solid was taken up with benzene and filtered through cotton, and half was subjected to Si-flash chromatography (2  $\times$  6 cm column). Hydrocarbons were eluted from the column with hexane and CycPdes with hexane:acetone (24:1). The CycPde fraction was chromatographed on the C-30 rp-HPLC system and CPP 518, 516, 514, and 512 were collected. These fractions were evaporated and taken up with chloroform for the determination of their UV/visible (Shimadzu 1601, 0.5 nm resolution) and mass spectra (*see below*). Small aliquots of the CPP516 and CPP514 fractions were analyzed on the C-30 rp-HPLC system to assess the purity of the preparations, which ranged from 94 to 99.5%.

**Mass spectroscopy**—MS analyses were carried out on a Finnigan MAT LCQ in positive-ion mode using atmospheric pressure electrospray ionization (ESI) as previously described (Goericke et al. 1999). The instrument has an ion trap that can be used to store specific ions. These ions can

be fragmented in the trap and the fragments can be analyzed ( $\text{MS}^2$  experiment). Fragments generated during  $\text{MS}^2$  experiments can also be stored and fragmented ( $\text{MS}^3$  experiments). These fragments of fragments can also be fragmented, etc., thus performing  $\text{MS}^n$  experiments. Compounds were introduced into the MS either via infusion or liquid chromatography. Compounds dissolved in acetone (0.05 to 1  $\mu\text{g ml}^{-1}$ ) were infused at 3 to 25  $\mu\text{L min}^{-1}$  and analyzed using MS and  $\text{MS}^n$  experiments. For standard LC/MS we diverted 6% of the HPLC eluent into the ionization source via a flow splitter. Eluting compounds were characterized by alternating approximately every second between a full MS scan and an  $\text{MS}^2$  scan of the most intense ion detected during the previous full MS scan.

**Sediments**—Surficial sediments were collected from the Peru Margin (12°40.3'S, 77°38.5'W, 1210 m, 1998 January) and the Santa Barbara Basin (34°20.4'N, 120°0.5'W, 538 m, 1996 September) by multicorer and stored at 4°C in airtight 250- or 1000-ml containers until extraction. One to ten grams of sediment slurry were extracted twice in 10 volumes of degassed acetone under  $\text{N}_2$  using sonication (3  $\times$  1 min) to facilitate extraction. The extracts were combined and directly analyzed by rp-HPLC (*see below*). Further extraction in degassed  $\text{MeCl}_2$  gave higher yields of nonpolar compounds (3 to 5% for some compounds) but did not yield any new compounds. Consequently we did not carry out this additional extraction step to minimize sample handling and thus the degradation of oxygen sensitive pigments such as cyclic pheophorbides.

**Sediment-trap material**—A floating 1 m<sup>2</sup> conical sediment trap (Kiel design) was deployed for 48 h in the Eastern Tropical North Pacific (ETNP, 17°40.9'N, 107°45.0'W, 1997 May) off Mexico at a depth of 120 m. The collecting cup was filled with brine (1% NaCl) prior to deployment. The material found in the collecting cup after retrieval consisted primarily of small compact fecal pellets (cylindrical 0.5  $\times$  3 mm), most likely derived from the red pelagic crab *Pleuroniodes planipes*. These crabs were observed in the water during trap deployment and produce such fecal pellets when grazing on diatoms (Goericke, unpubl. data from the California current). The walls of the sediment trap, and in particular the grid covering it, were coated with stringy, brownish, gelatinous material that strongly resembled salp fecal material. Salps had been abundant in the water column during trap deployment. This material was washed off the trap with seawater after the trap had been drained and was recovered from the wash with a 202- $\mu\text{m}$  Nitex screen. Materials from the cup and the 202- $\mu\text{m}$  rinse were isolated on separate glass-fiber filters and stored in liquid  $\text{N}_2$ . In the laboratory the material was extracted in acetone as described above, except that samples were not sonicated but ground under  $\text{N}_2$  in a glass grinding tube with a Teflon pestle. Samples were kept on ice during grinding to prevent heating. The two acetone fractions were combined, filtered through a cotton-plugged pasteur pipette, purged with  $\text{N}_2$ , and stored under  $\text{N}_2$  at -18°C until analysis within hours of extraction. Some samples were further extracted with  $\text{MeCl}_2$ . These samples were also filtered, then the  $\text{MeCl}_2$  extract was evap-

orated under  $N_2$ , taken up with acetone, and analyzed by C-30 rp-HPLC. Similar to the results with sediments, the  $MeCl_2$  extract did contain more nonpolar pigments,  $\sim 5\%$  of those extracted with acetone, but no new compounds.

*Microzooplankton fecal material*—Unialgal stock cultures were maintained in f/2 medium without added silicate (Guillard and Ryther 1962). Protozoa, isolated from northern Puget Sound waters, were maintained on algal mixtures in a trace metal-enriched seawater medium (Gifford 1985). Algae and protozoans were added to filtered seawater ( $0.2 \mu m$ ) in four replicate 23-liter polycarbonate bottles and incubated ( $13^\circ C$ ,  $< 1 \mu mol$  photons  $m^{-2} s^{-1}$ , 12:12 light:dark). See Strom and Morello (1998) for details. The following combinations of protozoan grazers and algae were used: *Amphidinium* sp. (a heterotrophic dinoflagellate) fed *Isochrysis galbana* and *Rhodomonas* (formerly *Pyrenomonas*) *salina*; *Strombidinopsis acuminatum* (an oligotrich ciliate) fed *Rhodomonas salina*, *Gymnodinium simplex*, and *Heterocapsa triquetra*; and *Noctiluca scintillans* (a heterotrophic dinoflagellate) fed *Prorocentrum micans*. After most of the algae had been consumed (7 to 15 d), the entire contents of the bottles were filtered through 47-mm glass-fiber filters (Poretics GF-75) and stored in liquid  $N_2$  or, for short periods of time, on dry ice.

*Macrozooplankton feeding experiments*—Copepods were collected off La Jolla in Scipps Canyon from a depth of 80 m with a 350- $\mu m$  net in the early morning. The animals, primarily *Calanus pacificus*, were brought into the lab. The animals were given 6 h to empty their guts, and their fecal pellets were collected by siphoning water off the bottom of the experimental vessels (12 L carboys) and straining this water through 160- and 25- $\mu m$  Nitex screens. Fecal pellets were collected off the 25- $\mu m$  screen. The diatom *Thalassiosira weissflogii* was added to the carboy ( $\sim 10 \mu g$ -Chl  $a_1 L^{-1}$  final concentration) after the copepods had purged their guts. After 24 h, fecal pellets were recovered from the carboy as described above. These pellets (readily identifiable under a microscope) were filtered on a 25-mm glass-fiber filter and stored in liquid  $N_2$  until analysis. The filters were extracted as described above and analyzed on the C-30 rp-HPLC system. The euphausiid, *Euphausia* sp., was maintained in a tank in filtered seawater. It fed on the diatom, *Thalassiosira weissflogii*, that was added to the tank daily. Fecal pellets were collected from the bottom of the tank and extracted as described above.

*CPP516 degradation experiments*—We performed degradation experiments on CPP516 dissolved in organic solvents and associated with sediments suspended in oxygenated and deoxygenated seawater at room temperature. We used for these experiments sediments from the Peru margin. We extracted sediment under  $N_2$  as described above and incubated the acetone extract in a test tube. After taking an initial sample we slowly bubbled air through the solution and exposed the test tube to an irradiance of  $15 \mu E_i m^{-2} s^{-1}$ . Samples were taken approximately every hour after adjusting the volume of acetone for evaporation. Samples were immediately analyzed on the C-30 rp-HPLC system. The

degradation experiment with pure CPP516 was performed as described above, except that a solution of purified CPP516 was used. A degradation experiment with suspended sediments was performed in the dark. For this experiment 130-ml seawater in a glass flask was either bubbled for 1 h with  $N_2$  or with air. Approximately 20 g of anoxic sediments were introduced into the flask and held in suspension with a magnetic stirring bar. The experiments were sampled by removing 2 ml of the slurry from the flask, filtering the slurry on a glass-fiber filter, and storing this filter immediately under liquid  $N_2$ . These samples were extracted and analyzed as described above for sediments. Degradation constants ( $k$ ) were determined by fitting pigment concentrations ( $C$ ) to the equation:  $C(t) = C(t_0) \exp(-k \text{ time})$ .

## Results

*Characterization and analysis of cyclic pheophorbides*—We found high concentrations of 13<sup>2</sup>, 17<sup>3</sup>-cyclophorbide *a* enol (CPP516; Fig. 1, structure 6.1) in extracts of sediments from the Peru margin, consistent with the results of Ocampo et al. (1999). CPP516 eluted on our C-18 rp-HPLC system as a tailing peak (Fig. 2) slightly after Chl *a*. We identified CPP516 in these samples based on a comparison of its on-line UV/visible and mass-spectroscopic characteristics (UV/visible: 358, 424, 629, 682 nm, Fig. 3; mass spectroscopic:  $m/z$  517,  $MH^+$ , Fig. 4D) with published data (Ma and Dolphin 1996; Ocampo et al. 1999).

CPP516 elutes on most C-18 rp-HPLC systems in use in our laboratory (nonendcapped columns or new 15-cm columns) as a broad peak that can often not be distinguished from the baseline. The tailing can be reduced when older and shorter columns are used in conjunction with shorter gradients. The chromatogram shown in Fig. 2 is one such example. However, the chromatography of CPP516 on this C-18 rp-HPLC system is unsatisfactory. To overcome these problems, a chromatographic system based on a C-30 column was developed. On this C-30 rp-HPLC system, CPP516 elutes at 22 min (Fig. 3), after a cluster of sterol chlorin esters (SCEs) and after the baseline hump that is usually associated with the chromatography of sediment extracts. No effort was made to optimize this system for the resolution of other pigments found in sediments.

We used the C-18 rp-HPLC system to isolate CycPdes from Peru margin sediments for off-line characterization. The UV/visible spectrum of CPP516 in chloroform (Table 1) was virtually identical to the spectrum of synthetic CPP516 (Ma and Dolphin 1996:  $\lambda_{max} [CHCl_3] = 364, 430, 456, 630, 690$ ). Its mass spectrum had a molecular ion at  $m/z$  517 ( $MH^+$ ), as well as ions at  $m/z$  1033 ( $2MH^+$ ) and 1549 ( $3MH^+$ ). The  $MS^2$  spectrum of  $m/z$  517 gave ions at  $m/z$  502 ( $CH_3$ , 17%), 499 ( $H_2O$ , 10%), 489 (C-13<sup>18</sup>O or C-17<sup>18</sup>O, 56%), 488 ( $C_2H_5$  from C-8, 100%), and 461 (56, i.e., C-17<sup>18</sup>H<sub>2</sub>C-17<sup>18</sup>HC-17<sup>3</sup>HO, 9%), consistent with the structure of CPP516.

On-line (C-30 rp-HPLC system) UV/visible and mass-spectroscopic analyses of Peru margin sediments and ETNP sediment-trap samples revealed a series of other compounds with properties similar to those of CPP516, i.e., strong ab-

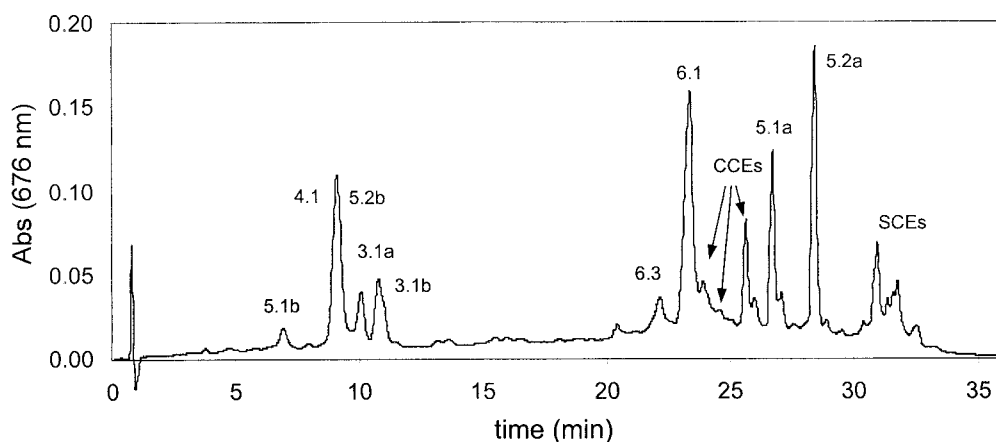


Fig. 2. C-18 rp-HPLC system chromatogram (410 nm) of a pigment extract of Peru Margin surficial sediments (we routinely achieve much better separations of other chlorins on new 15-cm C-18 columns). The peak numbers correspond to the compound numbers in Table 1. SCE: sterol chlorin esters (cf. King and Repeta 1991); CCE: carotenol chlorin esters (cf. Goericke et al. 1999).

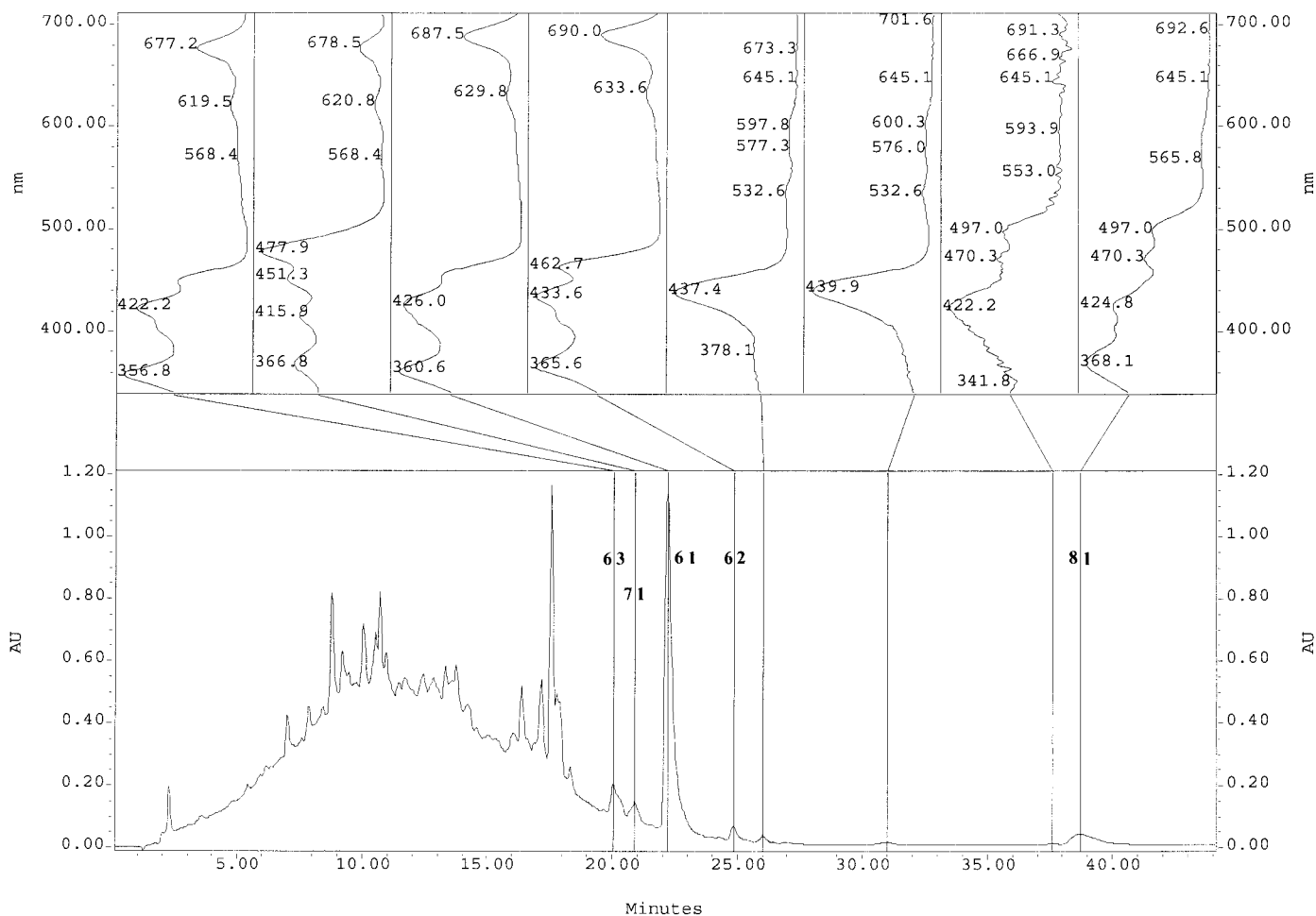


Fig. 3. C-30 rp-HPLC chromatogram (visible at 676 nm) of a pigment extract of Peru Margin surficial sediments. Spectra were collected on-line, i.e., in the eluent. The peak numbers correspond to the number of the compounds in Fig. 1. The spectrum of CPP530 is contaminated by coeluting SCEs. Clean CPP530 spectra (Table 1) were collected during a C-30 rp-HPLC run with increased concentrations of ethyl acetate, which shift CycPdes even further away from pheophytins and sterol chlorin esters but does not elute CPP512.

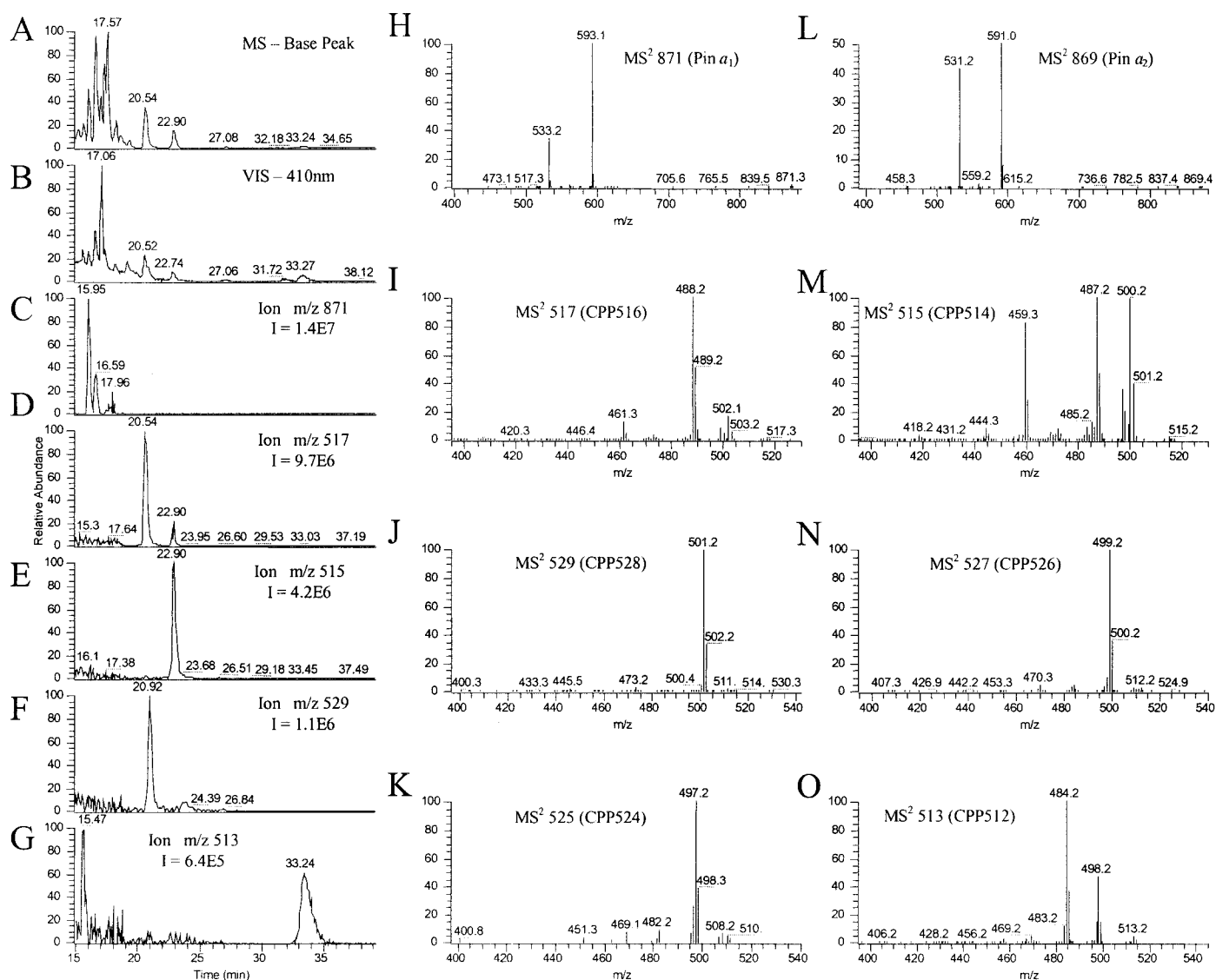


Fig. 4. HPLC/ESI-MS data (C-30 rp-HPLC, partial chromatogram 16–35 min) for pigments extracted from particulate matter collected in sediment traps deployed in the ETNP. Elution times between Figs. 3 and 4 differ because different HPLC hardware with different dead volumes was used. (A) base-peak ion chromatogram, i.e., the intensity of the most intense ion of each scan is plotted against time. (B) Absorbance at 410 nm. (C–G) Ion-chromatograms for  $m/z$  871, 517, 515, 529, and 513. The ion intensity ( $I = x.x$ ) of the major peak is given in arbitrary units. (H–O)  $MS^2$  spectra of pheophytin  $a_1$  and  $a_2$  and of the major CycPdes.

sorption maxima at 350–380, 420–480, and 675–690 nm (Fig. 3) and the characteristic fragmentation pattern of the molecular ion with losses of 15, 18, 28, (29), and 56 or 57 daltons (Fig. 4). These compounds eluted slightly before or after CPP516 (Figs. 3, 5, Table 1). So far we have found compounds with the characteristics of CycPdes with molecular ions at  $m/z$  519, 515, 513, 511 and  $m/z$  531, 529, 527, and 525 (Table 2). Three types of on-line UV/visible spectra were discernible, and these are classified based on similarities of the spectra with those of chlorophylls  $a$ ,  $b$ , and  $c$ : The A type, e.g., CPP516 (Fig. 3, 6.1), CPP518, and CPPA514, has one peak in the UV at  $\sim 360$  nm, a triplet of peaks in the blue green between 410 and 490 nm with an intensity similar to the UV peak, and a peak in the red between 670 and 690 nm with an intensity about one half of

the UV peak. The B type, e.g., CPP530 (Fig. 3, 7.1, *but see note in the figure legend*) and CPP528, is similar to the A type except that the blue green triplet is more intense than the UV peak, and the intensity of the red peak is one-third of the UV peak. The C type, e.g., CPP512 (Fig. 3, 8.1) and CPP510, lacks a major peak in the red, and the intensity of its UV peak is  $\sim 25\%$  larger than the intensity of its blue green triplet.

CPP516 (6.1) is a derivative of Chl  $a_1$  (Ma and Dolphin 1996). CPP514 has a similar UV/visible spectrum, i.e., its  $\lambda_{max}$  in the red is almost identical to the  $\lambda_{max}$  of CPP516, and its  $\lambda_{max}$  values in the UV and blue green are redshifted by  $\sim 10$  nm. These spectral changes are consistent with those observed when the 8-ethyl group of chlorins is substituted with a vinyl group, as, for example, in divinyl-Chl  $a$  (Chl

Table 1. Physical characteristics of the major pigments discussed. Given are the number of the pigment's structure (No., cf. Fig. 1), the abbreviation of the pigment's name (Name), the elution times in minutes of the pigment on the 36 min C-18 rp-HPLC system ( $tr_{C-18}$ ) and the 45 min C-30 rp-HPLC system ( $tr_{C-30}$ ), its absorption maxima in (1) acetone or (2) chloroform ( $\lambda_{max}$ ) and its on-line absorption maxima ( $\lambda_{PDA}$ , on a Waters 991 PDA with 1.3-nm resolution). On-line data for CycPdes are for the 45 min C-30 rp-HPLC system; for all others on-line data are for the 36 min C-18 rp-HPLC system. Structural assignments for cyclic pheophorbides except CPP516 are tentative until these are synthesized from the putative parent chlorophylls.

No.	Name	$tr_{C-18}$	$tr_{C-30}$	$\lambda_{max}$	$\lambda_{PDA}$
4.1	OxoPPde $a_1$	8.9	7.9	<u>388</u> , 419, 519, 622, <u>677</u> <sup>2</sup>	<u>386</u> , (412), 515, 620, <u>676</u>
4.2	OxoPPde $a_2$	10.4	8.7	n.d.	n.d.
3.1a	Chlone $a_1$	10.1	8.8	<u>416</u> , 506, 538, 611, <u>669</u> <sup>2</sup>	<u>410</u> , 504, 535, 614, <u>666</u>
3.1b	Chlone $a_1'$	10.8	9.5	<u>415</u> , 507, 537, 615, <u>673</u> <sup>2</sup>	<u>408</u> , 504, 535, 614, <u>668</u>
3.2a	Chlone $a_2$	10.9	9.6	n.d.	n.d.
3.2b	Chlone $a_2'$	12.0	10.4	n.d.	417, 507, 612, <u>670</u>
5.1a	Pheophytin $a_1$	26.7	15.9	<u>410</u> , 506, 535, 608, <u>666</u> <sup>1</sup>	<u>411</u> , 537, 609, <u>668</u>
5.3a	Pheophytin $a_2$	27.2	16.6	<u>416</u> , 511, 538, 611, <u>667</u> <sup>1</sup>	<u>417</u> , 515, 611, <u>670</u>
6.1	CPPA516	23.4	20.5	<u>363</u> , <u>429</u> , 456, 632, <u>690</u> <sup>2</sup>	<u>358</u> , 426, 453, <u>627</u> , <u>688</u>
6.2	CPPA514	n.d.	22.9	<u>369</u> , <u>437</u> , 465, 633, <u>691</u> <sup>2</sup>	<u>366</u> , 434, 462, 630, <u>689</u>
6.3	CPPA518	22.4	18.5	<u>360</u> , <u>425</u> , 451, 621, <u>679</u> <sup>2</sup>	<u>357</u> , 422, 448, 620, <u>676</u>
8.1	CPPA512	n.d.	33.2	<u>373</u> , <u>428</u> , 474, 503, <u>570</u> <sup>2</sup>	<u>368</u> , 426, 472, 495
7.1	CPPA530	22.1	19.2	n.d.	<u>366</u> , 446, 474, 620, <u>679</u>
7.2	CPPA528	n.d.	20.9	n.d.	371, <u>458</u> , 484, 622, <u>684</u>
?	CPPA526	n.d.	27.1	n.d.	n.d.
?	CPPA524	n.d.	31.7	n.d.	<u>356</u> , 421, 449, 620, <u>676</u>

$a_2$ , 1.2). The mass of CPP514 is consistent with one additional unsaturation, and its MS<sup>2</sup> spectrum (Fig. 4M) displays the expected absence, compared to CPP516, of the strong MH<sup>+</sup> - 29 dalton fragment due to the loss of the 8-ethyl group (cf. Fig. 4I). Otherwise the MS<sup>2</sup> spectrum and MS<sup>n</sup>

fragmentation patterns of CPP514 are almost identical to the spectra of CPP516. We regard it as likely that CPP514 (6.2) is the Chl  $a_2$  analog of CPP516, a suggestion that has to be proven by synthesis.

The UV/visible spectrum of CPP518 differs from the spectrum of CPP516 by a 3-nm blueshifted UV peak and a 10-nm blueshifted red peak, spectral changes expected from the saturation of the 3-vinyl group (Belanger et al. 1982).

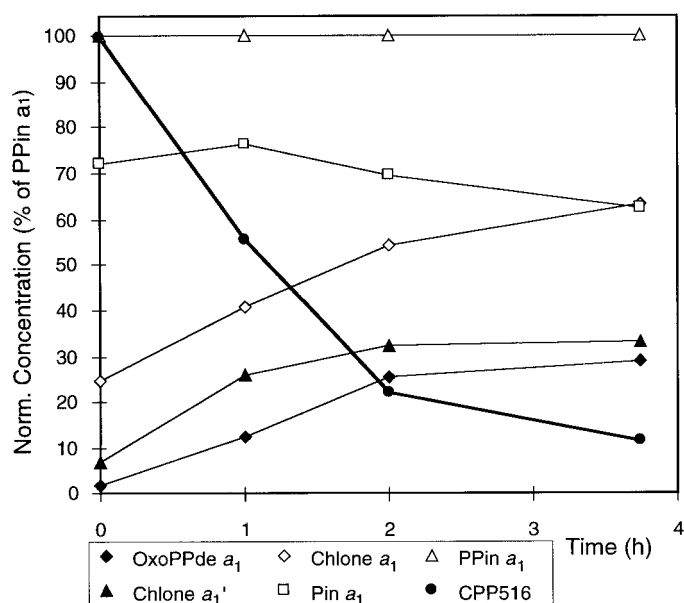


Fig. 5. Acetone extract of Peru margin sediments was exposed to air and light for 3.5 h. Concentrations of the Chl  $a_1$  degradation products OxoPPde  $a_1$ , Chlone  $a_1$ ,  $a_1'$ , pheophytin  $a_1$  (Pin  $a_1$ ), and pyropheophytin  $a_1$  (PPin  $a_1$ ), normalized to the concentration of pyropheophytin  $a_1$ , are plotted against time. The concentration of CPP516 was scaled down by a factor of 2.

Table 2. MS and MS<sup>2</sup> data acquired on-line (C-30 rp-HPLC system). Listed are the abbreviation of the pigment's name, its molecular ion (MH<sup>+</sup>), its major or characteristic MS<sup>2</sup> fragments (MS<sup>2</sup>), and the corresponding mass loss in parentheses. Major fragments (50 to 100% intensity) are underlined. Symbols used: ph, phytol, i.e., loss of 278 daltons; n.d., no data; w, weak MS<sup>2</sup> signal.

Name	MH <sup>+</sup>	MS <sup>2</sup> fragmentation pattern
OxoPPde $a_1$	549	<u>531</u> (18), <u>521</u> (28), <u>503</u> (18 28), 476 (73)
OxoPPde $a_2$	547	529 (18), <u>519</u> (28), <u>501</u> (18 28), 474 (73)
Chlone $a_1$	533	518 (15), <u>515</u> (18), <u>505</u> (28), 489 (18+28)
Chlone $a_2$	531	516 (15), <u>513</u> (18), 503 (28), 487 (18+28)
Pheophytin $a_1$	871	839 (32), <u>593</u> (ph), 533 (ph 60)
Pheophytin $a_2$	869	837 (32), <u>591</u> (ph), 531 (ph 60)
CPPA518	519	504 (15), <u>501</u> (18), 490 (29), 463 (56)
CPPA516	517	502 (15), 499 (18), <u>488</u> (29), 461 (56)
CPPA514	515	500 (15), 497 (18), <u>487</u> (28), 459 (56)
CPPA512	513	<u>498</u> (15), 495 (18), <u>484</u> (29), 469 (44), <u>467</u> (46), 457 (56)
29.1 min	511 <sup>w</sup>	496 (15), 493 (18), 482 (29)
CPPA530	531	<u>513</u> (18), <u>503</u> (28), 474 (57)
CPPA528	529	514 (15), <u>511</u> (18), <u>501</u> (28), 473 (56)
23.5 min	529	514 (15), 511 (18), <u>501</u> (28), 499 (30)
CPPA526	527 <sup>w</sup>	512 (15), <u>509</u> (18), 499 (28), <u>470</u> (57)
CPPA524	525 <sup>w</sup>	510 (15), 508-509 (18?), <u>497</u> (28), 469 (56)

The mass difference between CPP516 and CPP518 is consistent with this suggestion, as is the MS<sup>2</sup> spectrum, which has a stronger MH<sup>+</sup> – 29 dalton fragment than CPP516. Thus we suggest that CPP518 (6.3) is the 3-desvinyl, 3-ethyl Chl *a* analog of CPP516.

The UV/visible spectrum of CPP530 (Table 1) differs from the spectrum of CPP516 as the spectrum of Chl *a*<sub>1</sub> differs from the spectrum of Chl *b*<sub>1</sub> (1.3), i.e., the UV peak and the blue green triplet are 10 to 20 nm redshifted and the red peak is 10 nm blueshifted. The mass difference between CPP516 and CPP530 is consistent with this suggestion, i.e., the substitution of the 7-methyl with a formyl group that is 14 daltons heavier. The MS<sup>2</sup> spectrum is missing the expected MH<sup>+</sup> – 29 fragment due to the 8-ethyl group. MS<sup>n</sup> experiments, which could be used to demonstrate the presence of this group, have not been carried out on CPP530 because we have not been able to isolate it in sufficient quantities. The UV/visible spectrum of CPP528 differs from the spectrum of CPP530 as the spectra of CPP516 and CPP514 differ, but for a 5-nm redshifted red peak. Based on the UV/visible spectra and the observed mass differences, we suggest that CPP530 (7.1) and CPP528 (7.2) are the Chl *b*<sub>1</sub> and Chl *b*<sub>2</sub> analogs of CPP516.

The UV/visible spectrum of CPP512 differs from the spectrum of CPP516 as the spectra of Chl *a*<sub>1</sub> and chlorophyll *c*<sub>1</sub> (Chl *c*<sub>1</sub>, 2.1) differ, i.e., redshifted UV and blue green peaks and the absence of strong peaks in the red, as is characteristic of porphyrins. The MS<sup>2</sup> spectrum is similar to the spectrum of CPP516, with the expected MH<sup>+</sup> – 29 fragment due to the loss of the 8-ethyl group (Fig. 4O). These data support the suggestion that CPP512 (8.1) is the Chl *c*<sub>1</sub> analog of CPP516. We observed a weak ion at m/z 511 in some samples but have not been able to record its UV/visible spectrum. It is possible that this ion represents the Chl *c*<sub>2</sub> analog of CPP512 (7.2). Other compounds, with UV/visible and mass-spectral properties characteristic of CycPdes, were observed in the sample from the ETNP, eluting after CPP516 (Tables 1, 2, Figs. 4K, 4N).

We observed a large number of chlorophyll degradation products in extracts of fecal matter collected in sediment traps deployed in the ETNP. The chlorophyll degradation products were primarily derived from Chl *a*<sub>1</sub> and Chl *a*<sub>2</sub>. Degradation products of Chl *a*<sub>2</sub> are characterized by redshifted Soret peaks and a 2-dalton mass loss relative to the Chl *a*<sub>1</sub> analog. Based on these diagnostics we found evidence for the presence of pheophytin *a*<sub>2</sub> (Fig. 4L), pyropheophytin *a*<sub>2</sub>, pheophorbide *a*<sub>2</sub>, pyropheophorbide *a*<sub>2</sub>, OxoPPde *a*<sub>2</sub>, and Chlone *a*<sub>2</sub> in our sample, results to be presented and discussed in detail elsewhere.

*Sources of cyclic pheophorbides in the marine environment*—We found high concentrations of CycPdes in POM collected in sediment traps deployed in the ETNP (Fig. 4), contributing significantly to solvent-extractable chlorins (Table 3). These results suggest that one source of CycPdes in the marine environment are herbivorous macrozooplankton, major contributors to POM fluxes into sediment traps. To study specific sources of CycPdes, we analyzed extracts of macrozooplankton and microzooplankton fecal matter and algal cultures. We did not detect any CPP516 or related com-

Table 3. Contribution of CycPdes derived from Chl *a*<sub>1</sub> and Chl *a*<sub>2</sub> to solvent extractable Chl *a*<sub>1</sub> degradation products in particulate matter from a variety of sources. Included in the category pheophytins (Pin *as*) are sterol and carotenol chlorin esters, and in the category pheophorbides (Pde *as*) are Chlone *a* and OxoPPde *a*. Percentages were calculated on a molar basis. Data for ETNP sediment traps include CPP516 and CPP514, but not CPP530 and CPP528, because we believe these to be derived from Chl *b*<sub>1</sub> and *b*<sub>2</sub>, respectively.

	Pde <i>as</i> (% total)	Pin <i>as</i> (% total)	CycPdes (% total)
Peru Margin sediments	22	10	69
Santa Barbara Basin sediments	19	47	34
Sediment trap POM	26	15	59
Euphausiid fecal matter	0	42	58
Copepod fecal matter	17	29	54
<i>Amphidinium</i> sp. fecal matter	16	42	42
<i>S. acuminatum</i> fecal matter	45	15	40
<i>N. scintillans</i> fecal matter	24	5	71

pounds in exponentially growing and stationary phase cultures of the diatom *Thalassiosira weissflogii*. Relative to other Chl *a*<sub>1</sub> degradation products, we found large amounts of CPP516 in fecal matter from the euphausiid *Euphausia* sp. and the copepod *Calanus pacificus* that had been fed *T. weissflogii*. The only Chl *a*<sub>1</sub> degradation products associated with *Euphausia* sp. fecal pellets were pheophytin *a*<sub>1</sub>, pyropheophytin *a*<sub>1</sub>, and CPP516. The latter contributed 58% of all solvent-extractable Chl *a*<sub>1</sub> degradation products (Table 3). The complement of Chl *a*<sub>1</sub> degradation products in *C. pacificus* fecal material was much more diverse, i.e., derivatives of pheophorbide *a*<sub>1</sub> and pheophytin *a*<sub>1</sub> and carotenol and sterol chlorin esters (cf. King and Repeta 1991 and Goericke et al. 1999). CPP516 contributed 54% of all solvent-extractable Chl *a*<sub>1</sub> degradation products (Table 3).

Grazing experiments were also performed with three protozoan heterotrophs: an oligotrich ciliate *Strombidinopsis acuminatum* and the heterotrophic dinoflagellates *Amphidinium* sp. and *Noctiluca scintillans*. CPP516 was identified in the fecal material of all three protozoans based on its elution time and on-line UV/visible and mass spectra. It represented in all cases a large proportion of the total solvent-extractable, identifiable Chl *a* degradation products, ranging from 40 to 71% of the total (Table 3). We did not detect any other CycPdes in these extracts.

*Degradation experiments with cyclic pheophorbides*—We studied the degradation of CPP516 dissolved in organic solvents and associated with sedimentary particles suspended in seawater. An acetone extract of Peru margin sediments was exposed to air and light. Changes in the concentrations of CPP516 and other chlorophyll degradation products were followed over 4 h (Fig. 5). Concentrations of pheophytin *a*<sub>1</sub> and pyropheophytin *a*<sub>1</sub> in the extract declined slowly with time (degradation constants, *k*, of 0.05 and 0.03 h<sup>-1</sup>, respectively), as expected for chlorins under those conditions. Concentrations of CPP516 declined almost an order of magnitude more rapidly (*k* = 0.28 h<sup>-1</sup>) than those of pheophytin *a*<sub>1</sub>, pyropheophytin *a*<sub>1</sub>, and other, minor, chlorins. Concom-

itant with the decline of CPP516, we observed an increase in the concentrations of OxoPPde  $a_1$  and Chlone  $a_1$  and  $a'_1$ . Structural considerations suggest that CPP516 can degrade to OxoPPde  $a_1$  and Chlone  $a_1$  and  $a'_1$  (see below). To test this hypothesis we performed the same experiment with purified CPP516 dissolved in acetone:water (9:1). The degradation constant,  $k$ , of CPP516 was  $0.14 \text{ h}^{-1}$ . OxoPPde  $a_1$ , Chlone  $a_1$ , and  $a'_1$  produced from CPP516 during the experiment were identified based on their on-line UV/visible and MS spectra.

To study the stability of CPP516 in oxic aqueous systems, we suspended Peru margin sediments in the dark in aerated and oxygen-depleted seawater and followed concentrations of CPP516 and other Chl  $a_1$  degradation products over time. Concentrations of all Chl  $a_1$  degradation products were somewhat variable (e.g., Fig. 6A), most likely due to the difficulty of mixing and sampling a slurry accurately. To compensate for such sampling errors, we normalized the concentrations of all major Chl  $a_1$  degradation products to the concentration of pheophytin  $a_1$  (cf. Fig. 6B). We did not observe any systematic variations in the normalized concentrations of any pigment in the deoxygenated treatment (Fig. 6C). Under aerated conditions (Fig. 6B), concentrations of neither CPP516 nor OxoPPde  $a_1$  changed systematically. However, concentrations of Chlone  $a_1$  and  $a'_1$  increased significantly, by a factor of 3 to 4. Because of this unexpected result, i.e., the production of Chlone  $a_1$  without a decrease of CPP516, we repeated the experiment with almost identical results for both the aerated (Fig. 6D) and the deoxygenated (data not shown) treatment. Concentrations of other CycPdes, as well as pheophorbides and other pheophytins (data not shown), were low and remained low over the course of both experiments.

## Discussion

Ocampo et al. (1999) discovered high concentrations of CPP516 in marine sediments. This discovery suggests that a major degradation pathway of Chl  $a_1$  in the marine environment proceeds through pyropheophytin  $a_1$  or pyropheophorbide  $a_1$ , with subsequent closure of a ring between C-17<sup>3</sup> and C-13<sup>2</sup>. Our measurements corroborate these observations; we found that CycPdes represented up to 70% of all solvent-extractable Chl  $a_1$  degradation products in marine sediments. Using a newly developed rp-HPLC system, we found a large number of other CycPdes in marine sediments and water-column detrital matter. A consideration of their UV/visible and mass-spectral properties suggests that these are derived from other chlorophylls, i.e., Chls  $a_2$ ,  $b_1$ ,  $b_2$ ,  $c_1$ , and  $c_2$ . Structural considerations suggest that it is less likely that these other CycPdes arose through modifications to Chl  $a_1$  and its degradation products. An exception is CPP518, which we believe to be the 3-desvinyl, 3-ethyl derivative of CPP516, which may be produced from CPP516 as this undergoes diagenesis. These suggestions will have to be backed up by further spectral studies and synthesis of these new compounds from their presumptive parent chlorophylls.

*Sources of cyclic pheophorbides in the marine environment*—Ocampo et al. (1999) suggested that CPP516 found in marine sediments might be produced by zooplankton as these feed on phytoplankton. This suggestion is corroborated by some prior reports and the results reported here. CPP516 was first extracted from a marine sponge (Karuso et al. 1986). Chlorins thought to be structurally derived from CPP516, Chlone  $a_1$ , OxoPPde  $a_1$ , and related compounds, were found in the viscera of benthic bivalves (Sakata et al. 1990; Yamamoto et al. 1992; Watanabe et al. 1993) and in extracts of diatoms attached to these. However, the inference that these compounds are synthesized by diatoms (Watanabe et al. 1993) is questionable as it may have been difficult to separate the attached diatoms from other detrital matter containing CPP516. Neveux (1985) described an unidentified pigment, with a UV/visible spectrum very similar to CPP516, that he extracted from a surface bloom of the heterotrophic dinoflagellate *Noctiluca miliaris*. Vernet et al. (1996) observed an absorption maximum at 708 to 712 nm in the particulate-absorption spectra of fecal matter derived from macrocrustaceans grazing on *Phaeocystis* c.f. *pouchettii*. Even though Vernet et al. were not able to detect a corresponding spectral signature in solvent extracts or find a chromatographic peak with similar spectral characteristics, it is likely that this in vivo absorption maximum was due to CPP516. The large amounts of CycPdes in particulate matter collected in sediment traps deployed off Peru (Ocampo et al. 1999) and in the ETNP (this report) demonstrate that large zooplankton, most likely macrocrustaceans off Peru and Salps in the ETNP, can be an important source of these pigments. Resuspended sediments are an unlikely source of POM collected in the traps deployed in the ETNP as the trap station was in deep water, more than 350 km offshore.

The absence of previous reports of CPP516 associated with algal cultures and our inability to detect it in algae suggests that CPP516 is not synthesized by algae or produced during their senescence. We have shown that herbivorous macrozooplankton and microzooplankton convert a significant fraction of the ingested Chl  $a_1$  to CPP516; CPP516 contributed 55% to the Chl  $a_1$  degradation products found in macrozooplankton fecal matter, and it contributed 40 to 70% to the Chl  $a_1$  degradation products found in microzooplankton fecal matter. These results, and the prior reports of CPP516 or pigments with similar characteristics in herbivore fecal matter, suggest that CPP516 is universally produced when marine herbivores feed on phytoplankton. It is impossible at the present time to rule out the production of CycPdes from other chlorophyll degradation products in marine sediments. However, as the contributions of CPP516 to total extractable chlorins are similar in fecal material and sediments, such production is unlikely to be a major source.

In the ETNP we found high concentrations and large numbers of CycPdes in particulate matter derived from salps and collected in sediment traps. The series of CycPdes found in these samples are likely derived from Chl  $a_1$  and  $a_2$  and Chl  $b_1$  and  $b_2$ . Degradation products of Chl  $a_2$  and Chl  $b_2$  are expected in this environment because the cyanobacterium *Prochlorococcus* sp. (Chisholm et al. 1988), whose primary chlorophylls are Chl  $a_2$  and Chl  $b_2$  rather than Chl  $a_1$  and Chl  $b_1$  (Goericke and Repeta 1992), contributed significantly

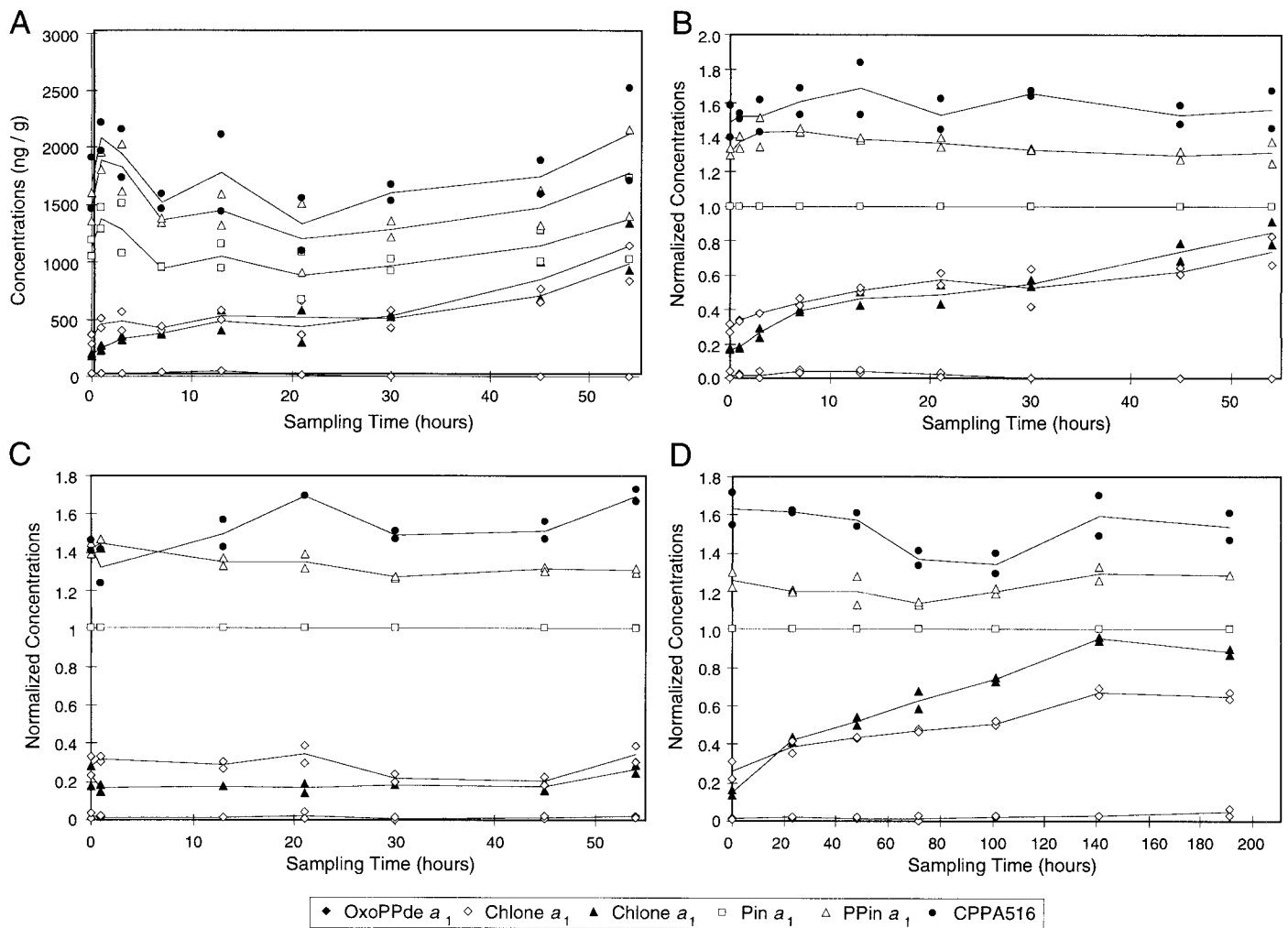


Fig. 6. Chlorin degradation experiments with sedimentary particles suspended in seawater. Concentrations and normalized concentrations of CPP516 were scaled down by a factor of 2. (A) Concentrations of the major Chl *a* degradation products (abbreviated as in Fig. 5) extracted from sediments incubated under oxic conditions plotted against time. Concentrations are variable, either due to changes in the extractability of the pigments, degradation of some, or most likely, due to the difficulty in mixing and sampling a slurry accurately. (B) The data shown in A were normalized to the concentration ( $\mu\text{g}$ ) of pheophytin *a* to gauge changes of concentrations relative to pheophytin *a*. (C) As in B, but for an incubation under deoxygenated conditions. (D) Data as in B (aerated) but for a second experiment. Results for the experiment 2 deoxygenated treatment (data not shown) are very similar to those shown in C.

to phytoplankton pigment-biomass (35% of the total, Goericke et al. 2000). Other phytoplankters present were *Synechococcus* sp. and eucaryotic prymnesiophytes and pelagophytes. However, the high proportion of Chl *a*<sub>2</sub>-derived degradation products in salp fecal matter, with a CPP516: CPP514 molar ratio of 2:1, is surprising because it is thought that picoplankton, particularly the very small *Prochlorococcus* (0.6–0.8- $\mu\text{m}$  diameter, Chisholm et al. 1992), do not contribute significantly to export production. Thus, our observation suggests that at least salps can feed effectively on the very small *Prochlorococcus* sp. Indeed, salps have been shown to feed on very small phytoplankters and on organisms as small as bacteria (Harbison and McAlister 1979; Mullin 1983), consistent with our observation. These results suggest that CycPdes can easily be used as tracers of pigment flux in the marine environment and, by implication, carbon flux.

**Degradation of CPP516**—We have shown that CPP516 quickly degrades in organic solvents when these are handled carelessly. In one experiment (Fig. 5) 50% of the CPP516 present degraded within 90 min; the degradation of other chlorins proceeded at rates an order of magnitude slower (Fig. 5). Similar results were obtained by Falk et al. (1975), who observed that CPP516 is extremely unstable in organic solvents in the presence of air. Sachs (1997) and Ocampo et al. (1999) also observed that CPP516 degrades rapidly under a variety of conditions. Once care is taken to exclude oxygen from extracts, this degradation can be minimized. The degradation products of CPP516 were OxoPPde *a*<sub>1</sub>, Chlone *a*<sub>1</sub>, and *a*<sub>1</sub>'. Structural considerations suggest that the degradation of CPP516 to Chlone will result in approximately equal amounts of the two stereo isomers of Chlone (i.e., *a*<sub>1</sub> and *a*<sub>1</sub>'), as was observed (Fig. 5), because a chiral center is formed at the C-13<sup>2</sup> during the reaction. This degradation

clearly demonstrates that Chlone can be formed as an artifact in sediment extracts.

The instability of CPP516 in organic solvents is in stark contrast with its omnipresence in the marine environment, not only in anoxic sediments but also in fecal matter produced under oxic conditions. If CPP516 were as labile in nature as it is in solutions of some organic solvents, its abundance in nature would be very low. Yet, the contribution of CPP516 to solvent-extractable Chl  $a_1$  degradation products was similar in sediments and in fecal material (Table 3), suggesting that CPP516 associated with particles is at least as stable as other Chl  $a_1$  degradation products. Results of our degradation experiments with sediments suggest that solvent-extractable CPP516 is stable at least on a timescale of days in aqueous systems when associated with particles. Taking these results at face value, we would not expect significant degradation of CypPdes associated with fecal pellets while these sink at a speed of about 100 m per day (Lorenzen et al. 1983) to the bottom of the ocean. It is possible that metal ions chelated to the keto-enol system of CypPdes stabilize these compounds in nature, as such systems are quite susceptible to oxidation in their free form (Saito and Matsuura 1979).

The surprising result of our degradation experiments with sediments suspended in oxic seawater was that even though concentrations of CPP516 were constant and concentrations of OxoPPde  $a_1$  were at the limits of our detection, concentrations of Chlone  $a_1$  and  $a'_1$  increased by factors of 3 to 4. The readily extractable chlorins cannot be a source for these, as no other Chl  $a_1$  degradation product showed a concomitant decrease. Such a source would have to be a pool of chlorins tightly bound to sedimentary particles that are released from the particles under the influence of oxygen. If this source is a pool of CPP516, it would have to be converted to Chlone as it desorbs from the particles under the influence of oxygen. A second possibility is that a tightly bound pool of Chlone  $a_1$  exists in anoxic sediments that desorbs in the presence of oxygen. It is possible that CPP516 or Chlone  $a_1$  are sulfur bound into nonextractable matter at the 13<sup>2</sup>, 17<sup>3</sup>-double bond and released as this bond is oxidized in the presence of O<sub>2</sub> (cf. Schaeffer et al. 1994).

*Implications for biological oceanography*—The chromophore of CPP516 differs significantly from the chromophore of pheophorbide  $a_1$  in that CPP516 has strong absorption maxima in the UV and the blue and a red peak shifted by 20 nm. Even more striking is the absence of fluorescence (Falk et al. 1975, and our unpubl. data). Interestingly, CPP516 in organic solvents easily degrades to Chlone  $a_1$ , which has a pheophorbide  $a_1$  chromophore, i.e., it fluoresces (unpubl. data). This implies that CPP516 will not be detected with the standard fluorometric methods that are commonly used to measure concentrations of pheopigments (Yentsch and Menzel 1963) in biological samples. Such measurements are made to determine grazing rates of zooplankton from the amount of pheopigments found in the guts of macrozooplankton, i.e., the gut-fluorescence method of Mackas and Bohrer (1976), or to determine rates of phytoplankton growth and loss from concentrations of pheopigments in the water column and in sediment traps, i.e., the Chl budget of

Welschmeyer and Lorenzen (1985). Rates calculated with these methods will be underestimates of true rates if concentrations of Chl  $a_1$  degradation products are underestimated, as will be the case when CPP516 is present, or if Chl  $a_1$  is completely degraded. Our data show that CPP516 contributes 40 to 70% to Chl  $a_1$ -derived chlorins in herbivore fecal matter. Initially, i.e., when organic solvent extracts are analyzed shortly after extraction, CPP516 will not be detected fluorometrically. However, if these extracts are in contact with oxygen for longer periods of time, CPP516 may partially degrade to Chlone  $a_1$  or OxoPPde  $a_1$ . The newly formed Chlone  $a_1$  will fluoresce as if it were a normal pheopigment, i.e., a compound with a pheophorbide  $a_1$  chromophore. Considering the absorption spectrum of OxoPPde  $a_1$ , we expect its fluorescence yield to be less than that of pheophorbide  $a_1$  or Chlone  $a_1$  under standard conditions (cf. Yentsch and Menzel 1963). Thus, the presence of CPP516 and, depending on extraction protocol, variable degradation rates of CPP516 to Chlone  $a_1$  or OxoPPde  $a_1$  may have contributed to the observed variability of the conversion efficiency of Chl  $a_1$  to pheopigments by grazers (Dagg and Walser 1987; Lopez et al. 1988; Downs 1989; Penry and Frost 1991; Strom 1993; Peterson and Dam 1996).

To summarize, Herbivores do not only degrade Chl  $a_1$  to compounds that have a pheophorbide  $a$  chromophore, i.e., to pheopigments that can be detected fluorometrically as pheophorbide  $a$  mol equivalents, but also to cyclic pheophorbides with differing spectral and fluorometric properties. This observation implies that the analytically defined concept of pheopigments is no longer adequate to encompass all the Chl  $a_1$  degradation products found in the fecal matter of marine herbivores. It is likely that the presence of CPP516 in the fecal matter of grazers, and the subsequent conversion of CPP516 to chlorophyllone  $a_1$  and 13<sup>2</sup>-oxopyropheophorbide  $a_1$ , is one of the factors contributing to low and variable Chl  $a_1$ -pheopigment conversion efficiencies. Such experiments should be repeated in conjunction with analytical methods capable of quantifying cyclic pheophorbides. If it can be shown that Chl  $a_1$  is not partially degraded to colorless compounds when herbivores digest phytoplankton, but instead to CPP516, these analytical methods could be used to provide better measurements of Chl  $a_1$  degradation products in fecal matter. Such measurements would lead to more accurate estimates of zooplankton grazing rates in the marine environment.

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