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# The relative abundance of pheophorbide *a* and pheophytin *a* in temperate marine waters[1]

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*Abstract*

Pheophytin *a* and pheophorbide *a* were measured with reverse-phase, high-performance liquid chromatography (HPLC) in samples from Dabob Bay, a temperate fjord in Puget Sound, Washington. Pheopigment diversity was higher than expected in all samples analyzed (water column, sediment trap material, and fecal pellets of *Calanus pacificus*): two major forms of pheophytin *a* and three major forms of pheophorbide *a* were separated and quantified. These pigments were labeled by their relative polarity *a*1 and *a*2 in the case of pheophytin and *a*1, *a*2, and *a*3 in the case of pheophorbide, with *a*1 being the most polar. Pheophytin *a*1 and pheophorbide *a*2 had the same chromatographic characteristics as the in vitro forms obtained by acidification of Chl *a* in acetone and chlorophyllide *a* in methanol. Pheophorbide *a*1 was present only in the euphotic zone. Pheophorbide *a*2 showed a maximum at the depth of the Chl *a* maximum and was least abundant in sediment traps. Pheophorbide *a*3 was most abundant below the euphotic zone, in sediment traps, and in *C. pacificus* fecal pellets. Pheophytin forms showed no clear pattern, although pheophytin *a*2 was more abundant in laboratory experiments than in field samples. The total mass concentration of pheophorbide forms was four times higher, on average, than that of pheophytin forms ($n = 64$). Because of their field distributions, the more polar may be the more degraded forms of the chlorophyll molecule.

The methods most often used to estimate pheopigments of chlorophyll *a*-type by fluorometric and spectrophotometric techniques assume that pheophytin *a* is the main degradation product of Chl *a* in natural waters (Yentsch and Menzel 1963; Holm-Hansen et al. 1965; Lorenzen 1967*a*; Moss 1967). However, recent analyses of field samples indicate that pheophorbide *a* is also present and, in some cases, may be more abundant than pheophytin *a* (Shuman and Lorenzen 1975; SooHoo and Kiefer 1982). Pheophorbide *a* and pheophytin *a* both lack the Mg atom that is present in the tetrapyrrole ring of the chlorophyll molecule. In addition, pheophorbide *a* lacks the phytol chain which in Chl *a* is attached to the ring. Zooplankton grazing is considered to be the major source of these pigments in the marine environment (Currie 1962). No systematic pattern has emerged about relative abundance of each pigment, although it may be related to the type of grazer. Light may degrade pheopigments further to colorless compounds that are lost to standard analytical techniques (Lorenzen 1967*b*; Moreth and Yentsch 1970).

In this study the relative abundance of pheophytin *a* and pheophorbide *a* in a temperate coastal environment was determined, together with the multiplicity of pheopigment forms. Various sources of pheopigments in the water column and in macrozooplankton fecal pellets produced in the laboratory were investigated to document the degradation of Chl *a* in the marine environment and to assess the accuracy of estimating pheopigments by the fluorometric technique.

## *Methods*

Samples for pheopigment analysis were taken in Dabob Bay, a temperate fjord in Puget Sound, Washington (47°50′N, 122°55′W). Suspended material was sampled from the water column with Niskin bottles. Rapidly sinking fecal pellets (around 100 m $d^{-1}$: Lorenzen and Welschmeyer

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Table 1. Chromatographic and spectral characteristics of Chl *a*, pheophorbide *a*, and pheophytin *a*. Pheopigments were obtained by acidifying chlorophyllide *a* in methanol and chlorophyll *a* in acetone.

| | Chl *a* | Pheophorbide *a* | Pheophytin *a* |
|---|---|---|---|
| Absorbance max (nm) | 432, 663 (acetone) | 410, 668 (methanol) | 410, 667 (acetone) |
| Mole wt | 893.5 | 590.0 | 869.2 |
| Specific absorption coefficient (Lorenzen and Jeffrey 1980) | 87.67 | 47.2 | 51.5 |
| Elution solvent (% MeOH) | 100 | 90 | 100 |
| Retention time (min) | 53.68±0.05 | 8.40±0.16 | 71.25±2.37 |
| Column calibration peak | ht | area | area |
| *a* (relative absorb.) | −0.1107 | 0.0029 | 0.0029 |
| $b\left(\frac{\text{relative absorb.}}{\mu\text{g pigment}}\right)$ | 38.129 | 1.180 | 1.576 |
| $r^2$ | 0.99 | 0.87 | 0.99 |
| Detected at (nm) | 440 | 410 | 410 |

1983) were collected with sediment traps (Lorenzen et al. 1981) suspended for 1- or 30-d intervals at midwater depths (55 m). Fecal pellets were obtained from copepodite V and females of *Calanus pacificus* previously isolated from mixed zooplankton collections and maintained in the dark at 8° or 12°C. They were fed either the diatom *Thalassiosira weissflogii* or the flagellate *Dunaliella tertiolecta* grown in f/2 medium (Guillard and Ryther 1962). Fecal pellets were washed with filtered seawater to avoid contamination with living phytoplankton cells.

The sampling schedule was as follows: 30-d sediment trap samples were collected from May 1982 through March 1983; water column samples in August, September, and October 1982; overnight sediment trap samples and fecal pellet experiments in the laboratory from August 1982 to April 1983.

The samples were concentrated by filtering through Gelman A/E or Whatman GF/F filters under a pressure differential of 7.5 mm of Hg. When at sea, samples were stored in liquid nitrogen and held for periods of up to 2 weeks before analysis. Pigments were extracted with 90% acetone; cells were either ground with a tissue grinder or disrupted with ultrasound before analysis. The extract was centrifuged until clear. Pigment concentrations were estimated by the fluorometric technique with a filter fluorometer (Turner 111 or Sequoia-Turner 112) calibrated with *Isochrysis galbana* culture (Lorenzen 1966).

For HPLC analysis, replicate samples of the 90% acetone extract were injected onto a reverse-phase microparticulate Zorbax C-18 ODS column through a 50-μl sampling loop. A stepwise solvent elution program consisting of 90% methanol (MeOH) in distilled water, 100% MeOH, and 100% ethanol (EtOH) was used at a rate of 1 ml $min^{-1}$. The pigments were detected by absorption with a UV-visible spectrophotometer (LDC-SpectroMonitor I) attached to the column outflow. Pheophytin *a* and pheophorbide *a* peaks were monitored at 410 nm and Chl *a* at 440 nm (consecutive runs). Pigment concentration was estimated manually by either peak area (pheopigments) or peak height (Chl *a*) (Snyder and Kirkland 1974). The column was calibrated with pure pigments by a linear regression model relating absorbance to pigment concentration. Pheophytin *a* and pheophorbide *a* were obtained by acidifying Chl *a* (in acetone) and chlorophyllide *a* (in methanol). Table 1 summarizes the chromatographic and spectral characteristics of the pigments calibrated.

## *Results*

*Pheopigment diversity*—Analyses of field samples showed that pheophorbide *a*, as obtained in vitro after acidification of chlorophyllide *a*, was only one of various pheo-

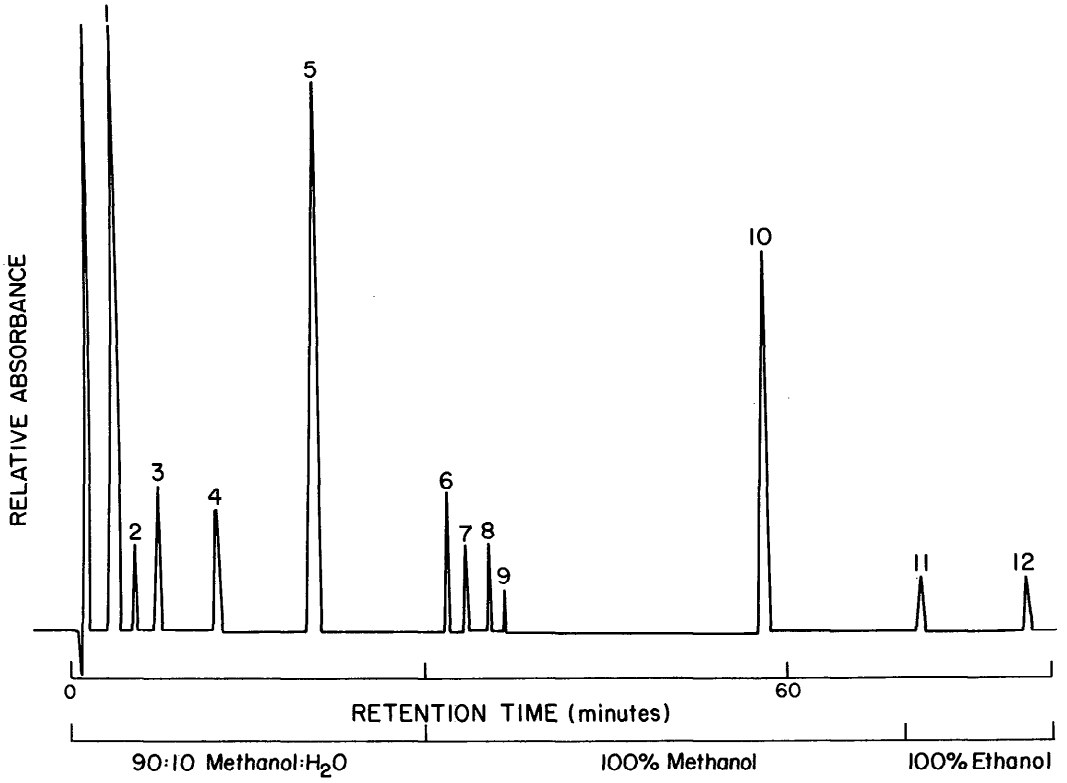


Fig. 1. Separation of pigments and pheopigments by HPLC from water column sample (12 m), Dabob Bay, August 1982. Time from left to right. Solvents on *x*-axis and detector response on *y*-axis. Peak identities: 1—chlorophyll *c*; 2—pheophorbide *a*1; 3—pheophorbide *a*2; 4—pheophorbide *a*3; 5—fucoxanthin; 6–9—xanthophylls; 10—chlorophyll *a*; 11—pheophytin *a*1; 12—pheophytin *a*2. Pigments monitored at 410 nm.

phorbides observed. Three major forms were found: pheophorbide *a*1, the most polar, was the first to elute in the chromatogram (retention time 6 min); pheophorbide *a*2, which had the same retention time as the pheophorbide *a* obtained in vitro, was eluted second (8.4 min); pheophorbide *a*3, the least polar, was eluted at 15 min (Fig. 1). All three peaks were identified as pheophorbides because of their acid ratio (fluorescence before and after acidification), their chromatographic behavior, and their absorbance at 410 and 666 nm as they elute from the column (Table 2). No absorbance spectra are available for forms *a*1 and *a*3 because of scarcity of material. These pigments were quantitatively estimated with the calibration for the in vitro pheophorbide *a* (Table 1). This procedure assumes similar molecular weights and similar weight-specific absorption coefficients for the three compounds.

All three pheophorbides were found in the water column samples (Fig. 2A). Pheophorbide *a*1 was the least abundant and found only in the euphotic zone. Pheophorbide *a*2 was most abundant in the euphotic zone, with a subsurface maximum that coincided in depth with the Chl *a* maximum (12 m). Measurable amounts were also found below the euphotic zone (40–80 m). Pheophorbide *a*3 was not detected at the surface, but increased in concentration at the depth of the Chl *a* maximum and had a maximum concentration below the euphotic zone.

Only pheophorbides *a*2 and *a*3 were measured in the 30-d deployment of sediment traps (55 m) (Fig. 2B). Pheophorbide *a*3 was the most abundant of the two, and it was

Table 2. Chromatographic and fluorometric characteristics of the major pheopigments found in field samples (sediment traps and water column) and in fecal pellets of *Calanus pacificus* fed on *Thalassiosira weissflogii* (diatom). Pigments were collected from the column after elution. $F_o/F_a$—Fluorescence before and after acidification.

| | Retention time (min) | $F_o/F_a$ |
|---|---|---|
| Pheophorbide *a*1 | 6.3±1.0 | 1.26 (90% MeOH) |
| Pheophorbide *a*2 | 8.4±0.4 | 1.09 (90% MeOH) |
| Pheophorbide *a*3 | 14.8±1.7 | 1.21 (90% MeOH) |
| Pheophytin *a*1 | 75.2±2.4 | 1.13 (100% EtOH) |
| Pheophytin *a*2 | 81.0±1.9 | 1.40 (100% EtOH) |

associated with the seasonal variation of particulate flux out of the euphotic zone. Pheophorbide *a*3 was the only form observed in overnight sediment trap deployments and in *C. pacificus* fecal pellets.

Pheophytin *a* eluted as two main peaks and several minor ones (Fig. 1). They eluted between 71 and 85 min. Of the two principal peaks, pheophytin *a*1, the most polar, had a retention time of 75.2 min (Table 2), similar to the in vitro pheophytin *a* obtained by acidification of Chl *a* in acetone (Table 1). Pheophytin *a*2 was less polar and eluted later (81.0 min). Both peaks have similar acid ratios, chromatographic behavior (Table 2), and absorbance at 410 and 666 nm. Similar to the procedure used for the various pheophorbides, both pheophytin peaks were estimated with the calibration for in vitro pheophytin *a* (Table 1).

Both pheophytin forms were observed in all samples analyzed. In the water column (Fig. 3A), pheophytin *a*1 was most abundant in the photic zone with a subsurface maximum in concentration (8–18 m) at and below the Chl *a* maximum. Pheophytin *a*2 also decreased with depth but had a relatively high concentration below the euphotic zone (25–80 m). No difference in the relative abundance of both pheophytins was observed in the 30-d (Fig. 3B) and overnight sediment trap samples, but pheophytin *a*2 was always more abundant in *C. pacificus* fecal pellets.

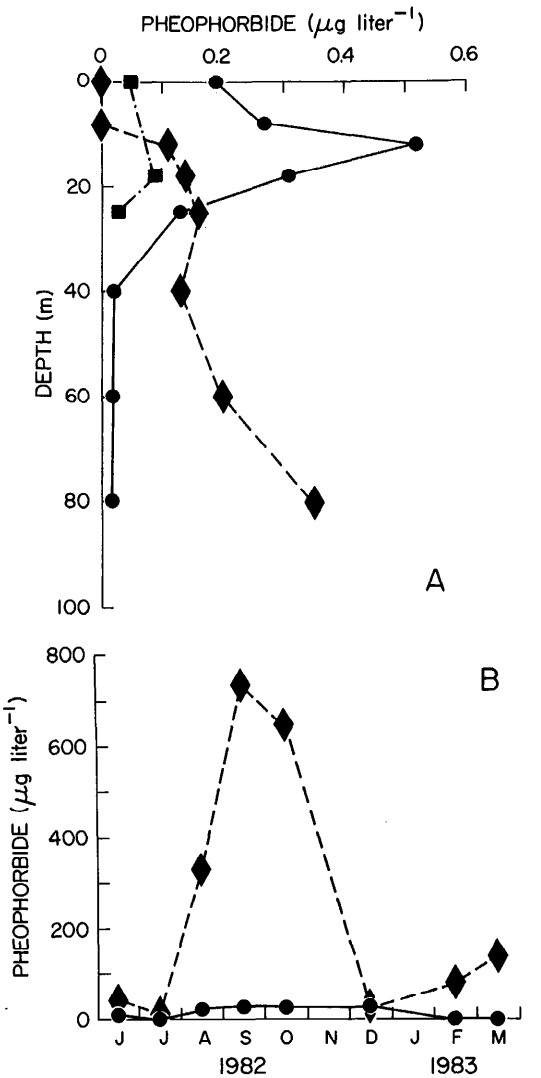


Fig. 2. Distribution of pheophorbidelike molecules in Dabob Bay, August 1982, as measured by HPLC. A. Pheophorbide concentration in the water column: pheophorbide *a*1 (■); pheophorbide *a*2 (●); pheophorbide *a*3 (◆). B. Symbols as in panel A, but of pheophorbide concentration in 30-d deployments of sediment traps. Pigment characteristics given in Table 2.

*Comparison of pheopigment estimation by HPLC and by fluorometry*—The combined pheopigment concentrations estimated by HPLC (three pheophorbides and two pheophytins) were added and compared to

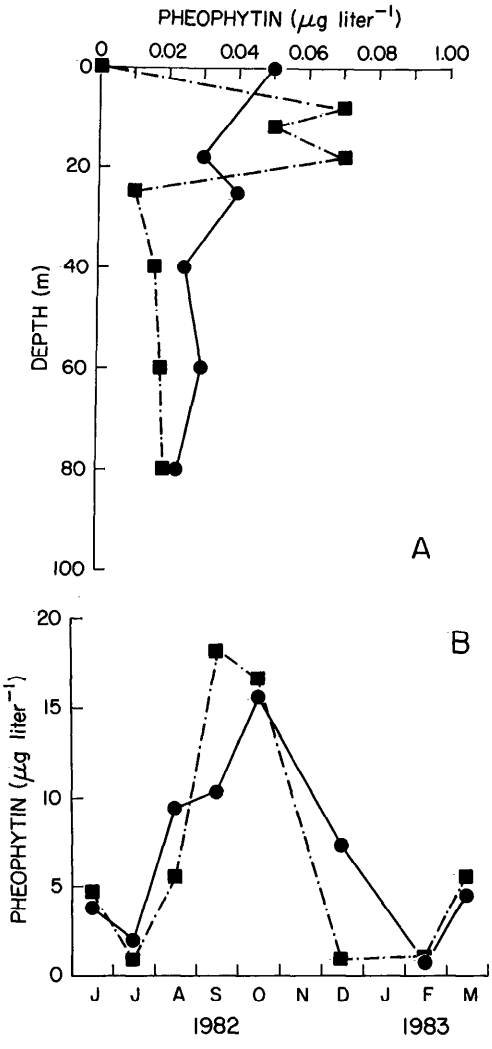


Fig. 3. As Fig. 2, but of pheophytinlike molecules. Symbols: pheophytin *a*1 (■); pheophytin *a*2 (●).

the pheopigment concentration measured by fluorometry. Estimates showed significant correlation and low discrepancy for the samples in the 30-d sediment trap deployment. A linear regression analysis showed that $[\text{pheopigments}]_{\text{HPLC}} = 35.33 + 0.91\ [\text{pheopigments}]_{\text{fluor}}$, where $n = 13$, $r^2 = 0.89$, $b \neq 0$ ($\alpha = 0.05$, $t = 8.92$, df = 11), and $a$ is not significantly different from zero ($\alpha = 0.05$, $t = 0.59$, df = 11).

*Comparison between pheophytin-type and pheophorbide-type pigments*—To compare the relative abundance of pheophytin-type and pheophorbide-type pigments, we added the concentrations of all three pheophorbide and both pheophytin peaks. The frequency distribution of the mass ratio of total pheophytin *a* to total pheophorbide *a* for 63 samples showed a large range of values with a median of 0.2 (Table 3). Few samples showed more than 40% of the total pheopigments as pheophytin *a*. The greatest difference was observed between sediment trap samples and fresh fecal pellets from *C. pacificus* (Table 3). When the median of the ratios of pheopigments for all field samples was compared to the median obtained in the laboratory, the latter showed enrichment in pheophytin *a* forms.

Samples from different environments also showed differences in the ratio of total pheophytin *a* to total pheophorbide *a*. Water column samples had the highest proportion of total pheophytin *a*. Particulates collected during overnight deployments showed less total pheophytin *a*; material collected during 30-d deployments had the highest proportion of pheophorbide *a* (Table 3). When the ratio of total pheophytin *a* to total pheophorbide *a* in a 30-d sediment trap collection is examined in more detail, it appears to follow a seasonal pattern (Fig. 4). Total pheophytin *a* reaches abundances of 25% of total pheophorbide *a* during spring and early summer. Late summer, fall, and winter samples show only 5% of the total pheopigments as pheophytin.

## *Discussion*

The loss of the Mg atom from the tetrapyrrole ring is a chemical reaction that occurs when Chl *a* is exposed to an acidic environment. The rate of conversion is a function of pH as well as the length of exposure for a range of pH from 5 to 7.2 (Daley and Brown 1973). Processes of this kind seem to happen in copepod guts, as the widespread distribution of pheophytin forms seems to indicate. The loss of the phytol chain (dephytolization) occurs chemically under lower pH, or enzymatically by the action of chlorophyllase (Barrett and Jeffrey 1971). It is not known whether this enzyme or some other is responsible for the dephy-

Table 3. Ratio of total pheophytin *a* to total pheophorbide *a* in laboratory experiments and field samples.

| | *C. pacificus* fecal pellets | Sediment traps ($z$ = 55 m) | | Water column ($z$ = 0–80 m) |
|---|---|---|---|---|
| | | 1 d | 30 d | |
| *n* | 11 | 4 | 23 | 25 |
| Median | 0.58 | 0.12 | 0.04 | 0.22 |
| Range | 0.34–1.00 | 0.03–0.24 | 0.02–0.35 | 0.04–0.56 |

tolization of pheophytin and chlorophyll in zooplankton guts. At the same time, very acidic conditions (pH 3) have been observed in guts of crustaceans. So either one or both of these processes may be responsible for the widespread presence of pheophorbide *a*-like pigments in zooplankton fecal pellets found during this and other studies (Lorenzen 1967*b*; Jeffrey 1974; Hallegraeff 1981). Further dephytolization of pheophytin *a* occurs inside fecal pellets after they are released, as suggested by the increasing proportion of total pheophorbide *a* observed from fresh fecal pellets collected during overnight deployments compared to longer term (30-d) sediment trap samples.

That different grazers produce preferentially one or another of the pheopigments has been noted in several studies. Pheophytin *a* was observed in clams (Moreth and Yentsch 1970) and salps (Hallegraeff 1981). Crustaceans appear to degrade Chl *a* to pheophorbide *a* (Lorenzen 1966; Jeffrey 1974; Shuman and Lorenzen 1975). In a more detailed study, Daley (1973) found that *Daphnia pulex* fecal pellets contained 60% pheophytin *a* and 40% pheophorbide *a*. Our results with *C. pacificus* coincide with his observations in that both types of pheopigments were produced by a crustacean (with a slightly higher proportion of pheophytin *a*). Compared to fresh fecal pellets produced in the field, the ratio of pheophytin is higher (Table 3). *Calanus pacificus* may produce a higher proportion of pheophytinlike compounds than other macrozooplankton but this conjecture has not been tested. However, this effect may not explain the discrepancy, as the fecal pellets collected during the overnight trap deployments coincided with periods of dominance of *C. pacificus* in Dabob Bay (Runge 1981). Furthermore the higher proportion of total pheophytin *a* in the 30-d deployment of sediment traps from March to July (Fig. 4) could be the result of the presence of other species of grazers in the plankton which were absent during the rest of the year.

The presence of several pheophorbidelike forms may be interpreted as different steps in the degradation of the pheophorbide molecule. Light, and also $O_2$, may be key factors in this process. From the results in the different samples, the degradation seems to have the following direction: pheophorbide *a*3 to *a*2 to *a*1 to colorless compound. Pheophorbide *a*3 is produced by zooplankton (laboratory experiments) and it could sink out of the euphotic zone (overnight traps). If it stays in the euphotic zone, it degrades to *a*2 and then to *a*1 in the presence of light and oxygen. This process could account for the absence of *a*3 in surface layers, coincident with *a*2 and *a*1 maxima. The degradation might also occur in darkness but at a much lower rate—thus the relatively low concentration of *a*2 in the 30-d sediment trap samples.

In conclusion, pheophorbidelike molecules appear to be, on average, the most abundant of the pheopigments in marine temperate waters. Further degradation of the pheophorbide molecule can be observed in the photic zone and to a lesser extent below it. It appears that pheophytin *a* might be an intermediate product of degradation of Chl *a* to pheophorbide *a*. If so, pheophytin-rich samples could be considered less degraded than pheophorbide-rich particles. Reduced degradation, as observed in this study, might be the result of fecal pellets produced at high food concentrations that result in shorter gut passage time (experiments with *C. pacificus*), reduced time of exposure to degradation in the environment (overnight vs. 30-d sediment trap deployments), and the

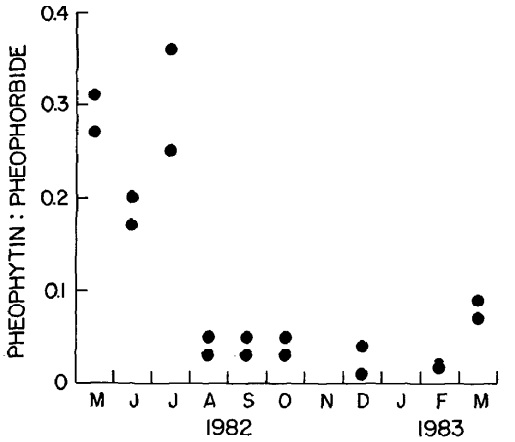


Fig. 4. Distribution of the mass ratio of total pheophytin *a* to total pheophorbide *a*. Seasonal variation of the ratio in 30-d deployment of sediment traps, Dabob Bay.

type of grazers (suspended material in the water column vs. sediment trap samples). From these results and because of the difference in molecular weight between pheophytinlike and pheophorbidelike forms, further research is needed to clarify the difference between specific absorption coefficients of these molecules and whether the dominance of pheophorbide in the acetone extract introduces error in a method developed to estimate pheophytin.

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