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NOTE

# Chlorophylls *a* and *b* and divinyl chlorophylls *a* and *b* in the open subtropical North Atlantic Ocean

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ABSTRACT: Divinyl chlorophyll *a* (chl $a_2$) and divinyl chlorophyll *b* (chl $b_2$) are chemotaxonomic tracers for the marine photooxytrophic procaryote *Prochlorococcus marinus*. Here we report the complete separation of chlorophyll *a* (chl $a_1$) and chl $a_2$ on a reverse-phase high-pressure liquid chromatography system that also achieves good separation of most other chemotaxonomically important pigments. Chlorophyll *b* (chl $b_1$) and chl $b_2$ are partially resolved, and their relative abundances are estimated with an on-line spectrophotometric method. Using these methods, we determined that chl $a_2$ and chl $b_2$ contributed up to 40% to total chl *a* (the sum of chl $a_1$ and $a_2$) and up to 95% to total chl *b*, respectively, in samples from the subtropical North Atlantic. The results suggest that *Prochlorococcus* represented a significant fraction of the total phytoplanktonic biomass. A comparison of chl *b/a* ratios observed in the field and chl *b/a* ratios measured in cultures of *P. marinus* suggests the presence of 2 strains of this organism in the subtropical North Atlantic. The spectroscopic differences between chl $a_1$ and chl $a_2$ would have led to small underestimates of total chl *a* in these samples had these been analyzed by spectrophotometric methods. However, the standard fluorometric method would have underestimated total chl *a* on the average by 8% with maximum values of 20%.

The newly discovered and described photooxytrophic procaryote *Prochlorococcus marinus* (Chisholm et al. 1988, 1992) has been detected by flow cytometry in most subtropical and tropical oceans (Chisholm et al. 1988, Li & Wood 1988, Olson et al. 1990, Vaulot et al. 1990). Its numerical abundance is usually very high relative to other phytoplankton. However, the contribution of this very small organism (0.6 to 0.8 µm) to phytoplankton biomass has been difficult to estimate due to its unknown cellular carbon concentration. The pigment complement of *P. marinus* is unusual, because it has divinyl chlorophyll *a* (chl $a_2$) instead of chlorophyll *a* (chl $a_1$) as its major photosynthetic pigment. In addition, it has divinyl chlorophyll *b* (chl $b_2$), possibly 2,4-Mg divinylpheoporphyrin $a_5$ (Goericke & Repeta 1992) and, in at least one strain, chlorophyll *b* (chl $b_1$) when growth is light saturated (>40 µEinst $m^{-2}$ $s^{-1}$; Goericke & Moore unpubl. data). Because no other wild-type photooxytroph is known which has this pigment (Goericke & Repeta 1992), chl $a_2$ is a chemotaxonomic marker for *Prochlorococcus* which can be used to study its distribution in the field.

Chl $a_2$ and chl $b_2$ are characterized spectrally by an 8 to 10 nm bathochromic shift in the Soret band when compared to chl $a_1$ and chl $b_1$, respectively (Goericke & Repeta 1992). These spectral characteristics may lead to biased estimates of total chl *a* (i.e. the sum of chl $a_1$ and $a_2$) in natural samples when spectrophotometric (Jeffrey & Humphrey 1975) or fluorometric (Holm-Hansen et al. 1965) methods are used to measure total chl *a*. The complete separation of chl $a_1$ and $a_2$ has only been achieved by normal-phase high-pressure liquid chromatography (Gieskes & Kraay 1983). However, normal-phase systems are not well suited for the routine analysis of methanol or acetone extracts of natural samples. Complete separation by C-18 column-based reverse-phase high-pressure liquid chromatography (RP-HPLC), which is commonly used for the analysis of phytoplankton pigments in seawater, has not been reported (Veldhuis & Kraay 1990).

We used a C-8 column-based RP-HPLC system, as suggested by Paerl & Millie (1991), to achieve a complete separation of chl $a_1$ and chl $a_2$. Chl $b_1$ and $b_2$ are partially separated on this system; therefore we quantified these pigments with an on-line spectrophotometric method. These methods were used to determine the contribution of *Prochlorococcus* to total chl *a* and

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total chl *b* in samples from the subtropical North Atlantic.

Seawater samples (4.4 l) were collected 200 miles south of Bermuda (27° 1' N, 68° 0' W) in January 1992 using a Niskin bottle rosette sampler. Seawater was filtered through 25 mm Whatman GF/F filters using a vacuum of less than 12 mm Hg. Filters were stored in liquid nitrogen and extracted in 1.5 ml 100% acetone for 30 min, ground at high speed with a Teflon pestle and extracted in the grinding tubes for an additional 30 min. Glass fibers and cell debris were separated from the acetone extract by centrifugation. We used a Waters HPLC system (Waters 660 solvent delivery system and Waters 990 diode array detector), a Rainin Dynamax 10 cm 3 µm C-8 column and a linear binary solvent gradient for the analysis of the chlorophylls. Solvents A (MeOH:0.5 N aq. ammonium acetate, 75:25) and B (MeOH) were programmed on the following gradient (minutes; % solvent A, % solvent B): (0; 100, 0), (20; 35, 65), (30; 25, 75), (35; 0, 100), (40; 0, 100), (45; 100, 0). The coefficient of variation for replicate analyses (200 to 450 µl injections) of the same pigment extract is dependent on the concentrations of the individual pigments in the extract; for the major pigments studied here it was typically in the range 2 to 5%. The chromatographic system was calibrated using chlorophylls isolated from the corn mutant ON 8147, the *Prochlorococcus marinus* clone MED4, which does not have any detectable chl $b_1$, and higher plants using the extinction coefficients given in Table 1. Pigments were quantified by integrated absorbance at 440, 468 or 478 nm. Chl $b_1$ and chl $b_2$ were estimated by peak area when the concentrations of the 2 pigments were similar enough that 2 peaks were clearly evident in the chromatogram. This was the case whenever the lesser of the 2 chlorophylls constituted more than 30% of the total. When only a single peak was evident we used an on-line spectrophotometric method in which the area of the total chl *b* peak was measured at 468 and 478 nm, $A_{468}$ and $A_{478}$, respectively, and the concentrations of the 2 chlorophylls were calculated using bichromatic equations:

$$[\text{chl } b_1] = k_1 A_{468} - k_2 A_{478}, \quad [\text{chl } b_2] = k_3 A_{478} - k_4 A_{468}.$$

The coefficients $k_i$ [µg-pigment] were derived from detector calibrations and 468/478 nm on-line absorbance ratios for pure chl $b_1$ and $b_2$, using methods strictly analogous to those that are used to derive bi- or trichromatic equations used in the spectrophotometric analysis of mixtures of chlorophylls *a*, *b* and *c* (cf. Jeffrey & Humphrey 1975); however, instead of extinction coefficients we used detector calibration constants. On the HPLC system described here, chl $b_1$ and chl $b_2$ have 468/478 nm absorbance ratios of 1.229 (standard deviation, SD = ± 0.004, n = 7) and 0.837 (SD = ± 0.005, n = 7), respectively; but it is expected that these are instrument dependent.

The C-8 column-based RP-HPLC system described here achieves a baseline separation of chl $a_1$ and chl $a_2$ (Fig. 1). Chl $b_1$ and chl $b_2$ are partially separated (Fig. 1 insert). Although it is possible to quantify the 2 pigments by peak area when both are present at similar concentrations, the on-line spectroscopic method will yield more objective results when either

Table 1. Spectral and chromatographic characteristics of the major pigments present in samples from the Sargasso Sea. α = weight-specific absorption coefficient (l $g^{-1}$ $cm^{-1}$) in acetone; $\lambda_{max}$ = published major wavelength maximum (nm); $\lambda_{on\text{-}line} = \lambda_{max}$ in the eluent measured on-line (nm); $t_r$: retention time (min) on our C-8 RP-HPLC system (cf. Fig. 1)

| # | Pigment | α | $\lambda_{max}$ | Source[a] | $\lambda_{on\text{-}line}$ | $t_r$ |
|---|---|---|---|---|---|---|
| 1. | Chlorophyll $c_3$ | nd | – | – | 458, 590 | 2.7 |
| 2. | Chlorophyll $c_{1,2}$ | 38.2 | 629 | (1) | 446, 584, 634 | 4.7 |
| 3. | Peridinin | 134 | 466 | (2) | 475 | 10.3 |
| 4. | 19'-Butanoyl-oxyfucoxanthin | 145 | 449 | (3) | 446, 468 | 13.4 |
| 5. | Fucoxanthin | 160 | 449 | (2) | 452, (468) | 14.3 |
| 6. | 19'-Hexanoyl-oxyfucoxanthin | 151 | 449 | (4) | 446, 470 | 16.2 |
| 7. | Diadinoxanthin | 225 | 448 | (5) | 442, 480 | 18.1 |
| 8. | Alloxanthin | 227 | 454 | (4) | 450, 480 | 20.1 |
| 9. | Zeaxanthin (lutein) | 234 | 452 | (2) | 450, 476 | 21.6 |
| 10. | Chlorophyll $b_2$ | 51.5 | 647 | (3) | 478, 606, 658 | 28.2 |
| 11. | Chlorophyll $b_1$ | 51.4 | 647 | (6) | 468, 602, 654 | 28.4 |
| 12. | Chlorophyll $a_2$ | 87.9 | 664 | (3) | 442, 622, 668 | 33.2 |
| 13. | Chlorophyll $a_1$ | 87.7 | 664 | (6) | 430, 620, 666 | 33.8 |
| 14. | α/β-Carotene | – | – | – | 444, 472 | 38.3 |

[a]References for α and $\lambda_{max}$: (1) Jeffrey (1972); (2) Jensen (1978); (3) We assumed that 19'-butanoyl-oxyfucoxanthin and 19'-hexanoyl-oxyfucoxanthin, chl $a_1$ and chl $a_2$, and chl $b_1$ and chl $b_2$ have equal mol-specific extinction coefficients; (4) Goericke & Welschmeyer (1993a); (5) Johansen et al. (1974); (6) Jeffrey & Humphrey (1975)

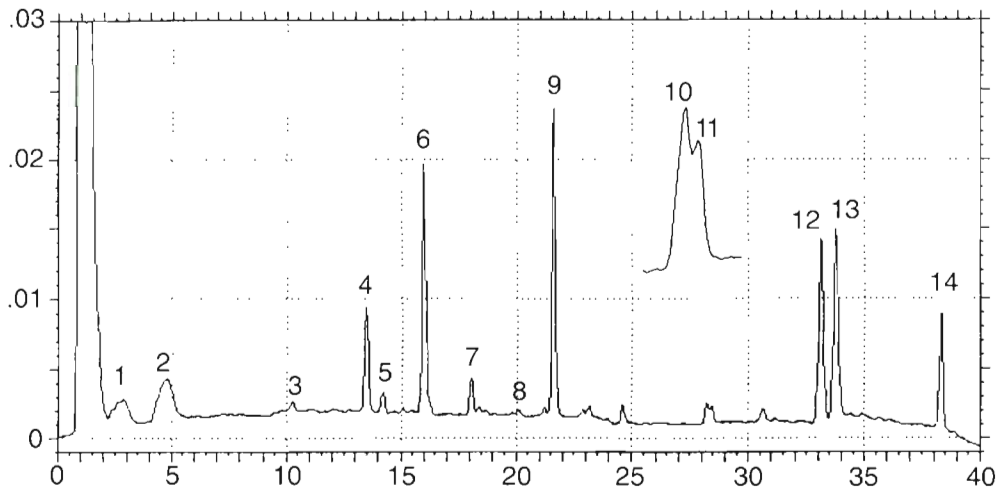


Fig. 1. Chromatogram (absorbance at 440 nm) of a sample from 98 m in the Sargasso Sea. Note that chl $a_1$ and chl $a_2$ (peaks 12 and 13) are completely separated. The insert (absorbance at 473 nm) shows the partial separation of chl $b_1$ and chl $b_2$ which elute at 28.3 min. Pigments were identified based on on-line absorbance spectra, and peak numbers correspond to numbers in Table 1. Note that it is possible to improve the separation of polar chlorophylls (chl $c_3$ and chl $c_{1,2}$) by increasing the water content of solvent A

of the 2 pigments dominates total chl *b*. We tested this method by analyzing mixtures of authentic chl $b_1$ and chl $b_2$ and comparing actual ratios of the 2 pigments with measured ratios (Fig. 2). The slope of a line fit to the data is 0.993 (SD = ± 0.013) and the intercept of the line is 0.006 (SD = ± 0.008). These results demonstrate that values of the ratio were measured in this case with a precision of SD = ± 0.016. However, this precision is dependent on the amount of total chl *b* present; we estimate that the precision ranges from SD = ± 0.02 to ± 0.10 in the case of our field samples (see below). Separations of most other major chlorophylls and xanthophylls by our method are similar compared to commonly used C-18 column-based systems (Mantoura & Llewellyn 1983, Wright et al. 1991). However, the present C-8 system using our standard solvent gradient, or the solvent system of Wright et al. (1991), failed to resolve zeaxanthin and lutein and α- and β-carotene.

We used this chromatographic system to analyze pigment samples from the southern Sargasso Sea. The water column was stratified at the time of sampling, and the *in vivo* fluorescence profile showed a pronounced maximum below 100 m (Fig. 3). Chl $a_1$ and chl $a_2$ were present from the surface to 200 m (Fig. 4A), but were undetectable at 250 m. Their concentration maximum, i.e. the subsurface chlorophyll maximum (SCM), was located at 116 m (Fig. 4A). The ratio of chl $a_2$ to total chl *a* ranged from about 0.25 in the surface layer to 0.4 at the SCM (Fig. 4B). Chl $b_1$ had a distribution similar to chl $a_1$ (Fig. 4C). The ratio of chl $b_1$ to chl $a_1$ was about 0.08 and did not vary systematically as a function of depth (data not shown). The concentration of chl $b_2$ varied by almost 2 orders of magnitude as a function of depth (Fig. 4C). The ratio of chl $b_2$ to total chl *b* ranged from 0.35 in the surface layer to 0.95 at and below the SCM (Fig. 4B).

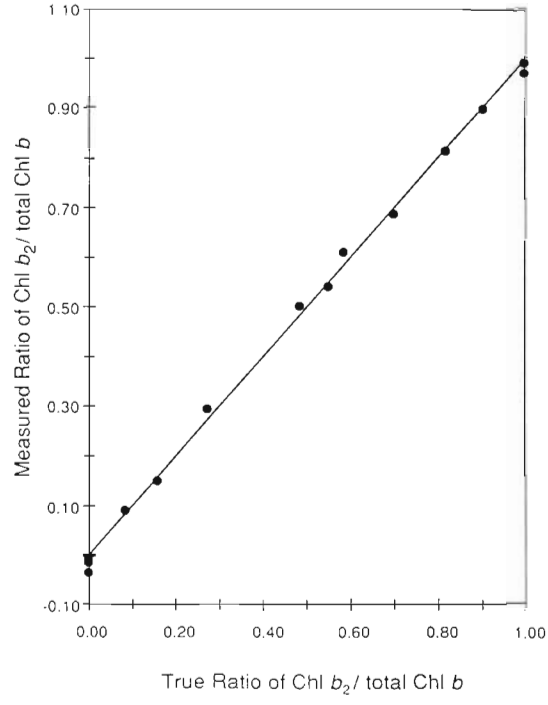


Fig. 2. True and measured values of the ratio of chl $b_2$ to total chl *b*. The measured ratio was determined from integrated total chl *b* absorbances at 468 and 478 nm, as described in the text

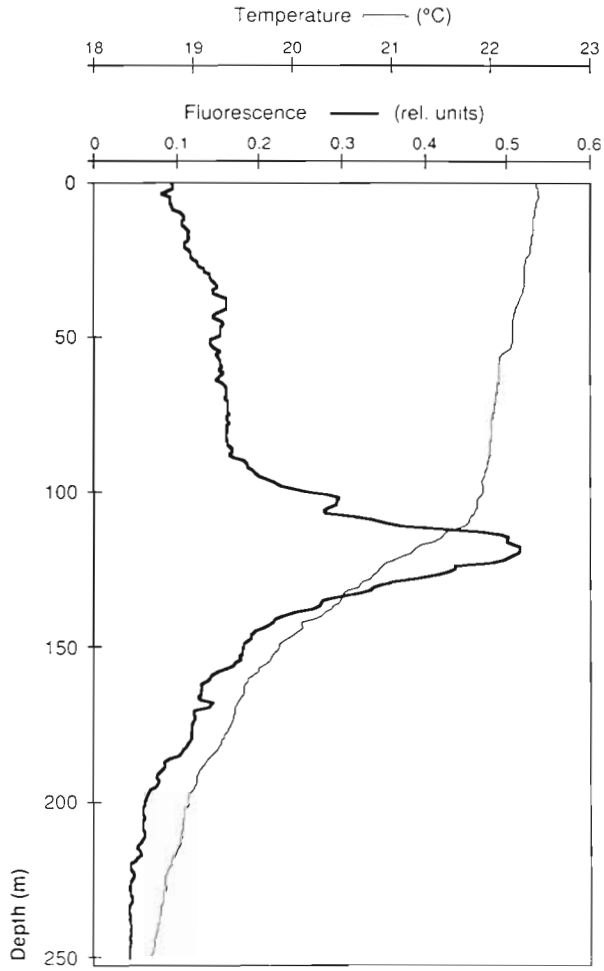


Fig. 3. Depth profiles of temperature and induced *in situ* fluorescence at a station (EN232C02) 300 km south of Bermuda

These results clearly illustrate the importance of *Prochlorococcus* in the subtropical open ocean. Assuming that carbon to chl ratios were similar for *Prochlorococcus* and other phytoplankton (i.e. cyanobacteria and eucaryotic microalgae), these data suggest that the contribution of *Prochlorococcus* to phytoplankton biomass ranged from ca 25% of the total in the surface layer to ca 40% of the total at the SCM. These data corroborate the results of Goericke & Welschmeyer (1993b) who reported that *Prochlorococcus* was present at a station southeast of Bermuda throughout the year with the exception of one observation in February 1987. They calculated values of relative *Prochlorococcus* pigment-biomass (i.e. chl $a_2$ as a % of total chl *a*) ranging from less than 10% to 75% of the total. These results also lend support to the conclusions of Olson et al. (1990), Vaulot et al. (1990), and Veldhuis & Kraay (1990) that *Prochlorococcus* is ubiquitously present in the tropical and subtropical ocean, often representing a significant fraction of phytoplankton biomass. Our results suggest that *Prochlorococcus* pigment-biomass can be similarly important in the surface layer and at the SCM, consistent with reports by Olson et al. (1990) of relatively high concentrations of *Prochlorococcus* in the upper euphotic zone of the Sargasso Sea in late fall and winter.

Our data (Fig 4B) demonstrate as well that *Prochlorococcus* can be the major contributor to total chl *b* in the subtropical open ocean, particularly at the SCM. These results question the conclusions of others who have ascribed the total chl *b* signal in subtropical areas entirely to green algae (e.g. Jeffrey 1976). Attribution of chl $b_1$ to specific groups of phytoplankters is difficult because chl $b_1$ is not only found in chlorophytes, prasinophytes and euglenophytes but also in high light grown cultures of the *P. marinus* clone SS120 (Goericke & Moore unpubl.). Thus, our data suggest that eucaryotes containing chl $b_1$, which are characterized by chl $b_1/a_1$ ratios ranging from 0.05 to 0.5 (Wood 1979), represented a small but significant fraction of the total pigment-biomass at and below the SCM. Chl $b_1$ present above the SCM may have been associated with eucaryotes and *Prochlorococcus*.

The ratio of chl $b_2$ to chl $a_2$ ranged from ca 0.15 in the surface layer to 2.9 below the SCM, and the ratio of total chl *b* to chl $a_2$ ranged from 0.3 in the surface layer to 3.0 below the SCM (Fig. 5); a range of chl *b/a* values not observed in any culture study with eucaryotes (Wood 1979) or *Prochlorococcus marinus* (Partensky et al. 1993, Moore et al. unpubl.). The large variation of the $b_2/a_2$ ratio in our field samples indicates either that the observed population of *Prochlorococcus* has an unusual capacity for physiological adaptation or that different species or strains of *Prochlorococcus*, with differing $b_2/a_2$ ratios, were present. Culture studies point to the latter possibility, since *b/a* ratios differed in cultures of *P. marinus* clones SS120 (isolated from the SCM of the Sargasso Sea) and MED4 (isolated from the surface layer of the Mediterranean) by a factor of 8 (Moore et al. unpubl.). Values of the chl *b/a* ratio for the *P. marinus* clone SS120 (solid bold line in Fig. 5) were similar to values measured in the field at and below the SCM, whereas values of the chl *b/a* ratio for the clone MED4 (broken bold line in Fig. 5) were slightly less than values of the $b_2/a_2$ ratio measured in the surface layer. These results suggest that 2 different strains or species of *Prochlorococcus* were present in the Sargasso Sea; a suggestion which can be corroborated using molecular probes.

The application of spectrophotometric or fluorometric methods for the measurement of total chl *a* may have resulted in biased estimates of total chl *a* for these samples due to the spectral differences between chl $a_1$ and chl $a_2$. The weight-specific absorption coefficient of chl $a_2$ in 80% acetone at 664 nm was measured by

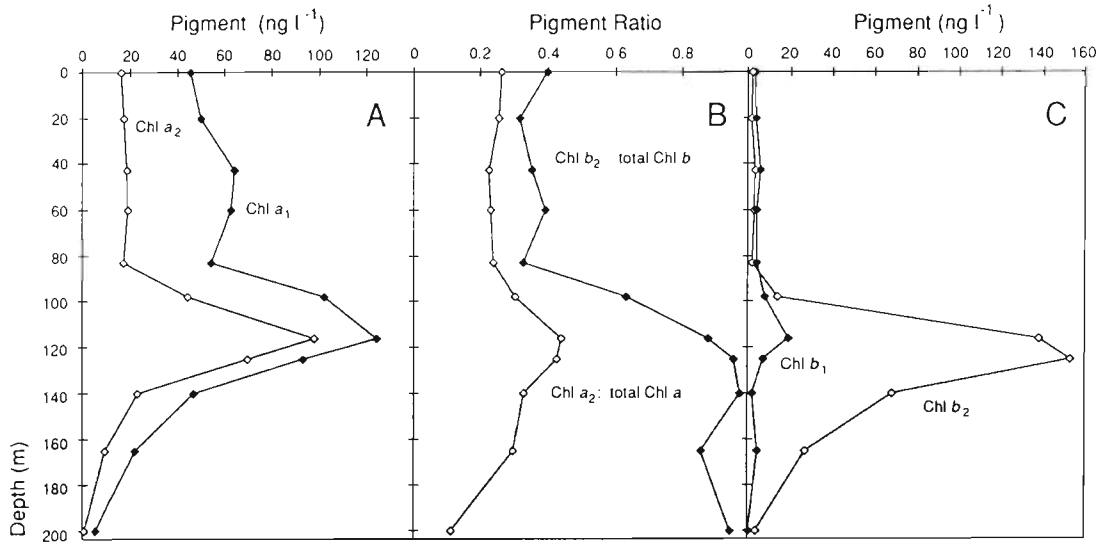


Fig. 4. Depth profiles of (A) concentrations of chl $a_1$ (◆) and chl $a_2$ (◇), (B) the ratios chl $b_2$: total chl $b$ (◆) and chl $a_2$: total chl $a$ (◇), and (C) concentrations of chl $b_1$ (◆) and chl $b_2$ (◇)

Shedbalkar & Rebeiz (1992) as 77.7 l g$^{-1}$ cm$^{-1}$, a value which is probably not significantly different from the extinction coefficient of chl $a_1$ in 80% acetone (89.2 l g$^{-1}$ cm$^{-1}$; Jeffrey et al. 1974) because the chl $a_2$ preparation used for the determination of the extinction coefficient by Shedbalkar & Rebeiz was slightly (ca 5% of the pigment) contaminated with lipids. However, our data show that the extinction coefficients at the Soret maxima differ, since the ratios of the absorbances at the Soret and red maxima (S/R ratios) are significantly different ($p < 0.0001$) for the 2 chlorophylls (S/R ratio for chl $a_1$: 1.24 ± 0.02 (95% CI) and chl $a_2$: 1.41 ± 0.004). These results imply that standard spectrophotometric methods for the measurement of total chl $a$ (e.g. Jeffrey & Humphrey 1975) are biased negligibly (<9%) when equal amounts of chl $a_1$ and chl $a_2$ are present because these methods rely on absorbance measurements in the red. However, measurements of total chl $a$ by

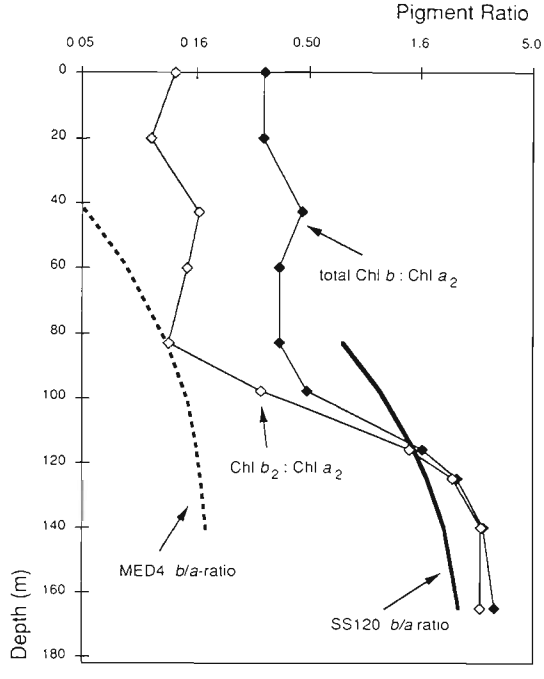


Fig. 5. Depth profiles of the ratios chl $b_2$: chl $a_2$ (◇) and total chl $b$: chl $a_2$ (◆). Note that the x-axis is scaled logarithmically. Variations of total chl $b$: chl $a_2$ ratios with irradiance for *Prochlorococcus marinus* clones SS120 (solid line) and MED4 (broken line) as determined by Moore et al. (unpubl.) are included. The length of these lines corresponds to the range of light intensities at which these strains were grown successfully

Table 2. Fluorescence response factors ($K_{fl}$) and acid ratios (τ) for pigments analyzed on a Turner Designs Model 10 fluorometer equipped for chlorophyll analysis (Holm-Hansen et al. 1965). $K_{fl}$ is the fluorescence of a solution of the pigment normalized by its concentration (µg ml$^{-1}$). The acid ratio is the ratio of the fluorescence before and after acidification of the sample with 2 drops of 0.1 N HCl. The chlorophyll $a$ ($chl_{fluor}$) and pheopigment ($pheo_{fluor}$) equivalents of each pigment are calculated assuming the fluorometer was calibrated with pure chl $a_1$. Equivalents are given as g chl $a_1$ and g 'pheopigment' simulated by 1 g of the pigment

| Pigment | $K_{fl}$ | τ | $chl_{fluor}$ | $pheo_{fluor}$ |
|---|---|---|---|---|
| Chl $a_1$ | 19.8 | 2.10 | 1.00 | 0.00 |
| Chl $a_2$ | 22.9 | 1.89 | 1.04 | 0.24 |
| Chl $b_1$ | 5.0 | 0.63 | −0.28 | 1.13 |
| Chl $b_2$ | 4.7 | 0.58 | −0.33 | 1.20 |

HPLC can be severely biased when chl $a_1$ and $a_2$ are not separated and quantified by absorbance at 440 nm (Bidigare 1991). On our C-18 RP-HPLC system (Goericke & Repeta 1992) total chl *a* is overestimated by 20% when equal amounts of chl $a_1$ and $a_2$ are present.

We also determined the response of a fluorometer to chlorophylls $a_1$, $a_2$, $b_1$, and $b_2$ in order to estimate the bias due to the spectral differences between the chlorophylls when total chl *a* is measured fluorometrically (Holm-Hansen et al. 1965). The fluorescence response factor of chl $a_2$ was slightly higher than the factor for chl $a_1$ (Table 2), and the acid ratio of chl $a_2$, i.e. the fluorescence before and after acidification with dilute HCl (cf. Holm-Hansen et al. 1965), was 10% lower. Thus, 1 g chl $a_2$ will simulate 1.04 g chl $a_1$ and 0.24 g 'pheopigment' on our fluorometer which was calibrated with chl $a_1$ (Table 2). The response factors and acid ratios of chl $b_1$ and chl $b_2$ were very similar; the presence of 1 g of either pigment simulated about -0.3 g chl $a_1$ and slightly more than 1 g 'pheopigment' (Table 2). In the case of our samples from the Sargasso Sea, the spectral differences between chl $a_1$ and chl $a_2$ and the presence of chl $b_1$ and chl $b_2$ would have led on the average to an 8% underestimation of total chl *a*, with maximal values of 20%. These results show that the spectral differences between chl $a_1$ and chl $a_2$ can bias the fluorometric methods for the measurement of total chl *a*. However, this bias is small when compared to commonly observed differences between fluorometrically determined total chl *a* and total chl *a* determined by HPLC (Trees et al. 1985, Goericke 1990).

A consequence of the very high concentrations of chl $b_1$ and chl $b_2$ in our samples would have been a severe overestimation of 'pheopigment' concentrations by the fluorometric method (Loftus & Carpenter 1971, Vernet & Lorenzen 1987). Whereas we did not observe any significant quantities of pheophorbides or pheophytins (absorbance detection at 668 nm) in these samples, the fluorometric method would have given total chl *a*:pheopigment ratios larger than 1.0 below the SCM. These results corroborate the conclusion of Herbland (1988) that the deep pheopigment maxima usually observed in subtropical and tropical oceans are an 'illusion' rather than 'reality' due to the very high concentrations of total chl *b* at the bottom of the euphotic zone. However, it is likely that estimates of 'pheopigments' in the surface layer of the open subtropical and tropical ocean are significantly affected by the presence of total chl *b* as well, because concentrations of 'pheopigments' are often very small or at least of similar magnitude as concentrations of total chl *b* (Gieskes 1991, Goericke unpubl. obs.).

To conclude, the C-8 column-based RP-HPLC system described here achieves a complete separation of chl $a_1$ and chl $a_2$ and a partial separation of chl $b_1$ and chl $b_2$. The separation of most other chemotaxonomically important pigments is as good as separations achieved on C-18 column based systems. Measurements of total chl *a* and, in particular, 'pheopigment' can be significantly biased when traditional methods are used to determine total chl *a* in samples from the open subtropical and tropical ocean where *Prochlorococcus* is abundant. It is necessary to separate chl $a_1$ and chl $a_2$ chromatographically when concentrations of total chl *a* are to be measured accurately. The application of this method to the analysis of pigment samples from the subtropical and tropical open ocean will lead to a better understanding of the role of *Prochlorococcus* in the marine environment; results of this study, for example, suggest that 2 different strains of *Prochlorococcus* are present in the Sargasso Sea.

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