Separation and quantification of pigments from natural phototrophic microbial populations

(Algal pigments; bacteriochlorophylls; carotenoids; metalimnetic phototrophic populations; anaerobic ecosystem; high-performance liquid chromatography)

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1. SUMMARY

A simple high-performance liquid chromatography (HPLC) method is described for the rapid (approx. 20 min) simultaneous separation and identification of the major chlorophylls and carotenoids from phytoplankton cells and phototrophic sulfur bacteria. Lyophilized samples were extracted with acetone in the dark at room temperature. Pigments were eluted from a silica column with a hexane-acetone mixture (80:20, v/v). About 20 algal and bacterial chlorophyll and carotenoid pigments could be separated in one run. The method allowed for the detection of trace amounts of major carotenoids (> approx. 6 ng·l⁻¹) and of chlorophylls and pheophytins (> approx. 200 ng·l⁻¹) in natural samples. The method has been applied to samples from the metalimnion of Lake Vechten (The Netherlands) and has proved very useful in estimating algal and bacterial pigments simultaneously with respect to depth distribution and biomass changes of the microbial populations.

2. INTRODUCTION

The analysis of algal photosynthetic pigments has found wide application as a chemo-taxonomic tool in aquatic ecology. Individual carotenoids, signature pigments, function as indicators of algal biomass and community structure. Pigment analysis is particularly useful in environments where conventional microscopy fails, where groups of organisms are easily overlooked because of their small size, are altered beyond recognition by sample treatment due to their delicacy or are masked by large quantities of detritus. Until very recently, the separation of pigments has been largely restricted to paper and thin layer chromatography [1–5]. From pigment analysis using paper chromatography, we were able to reveal the structure and relative abundance of metalimnetic phototrophic populations [6]. However, paper chromatography requires large concentrations of pigments and long development times. It is therefore unsuitable as a tool in more extensive studies where detailed knowledge of the depth distribution and of biomass changes of the various groups of organisms is needed. Due to its high precision, sensitivity, high resolution capability and very short development times, HPLC has become more and more popular (e.g., [7–9]). Pigment samples from the metalimnion of Lake Vechten form complicated mixtures of fractions originating from prokaryotic as well as from sedimenting and migrating eukaryotic phototrophic populations. We
therefore investigated the scope and limitations of HPLC for the quantitative separation of bacteriochlorophylls and accompanying carotenoids in the presence of algal pigments.

Satisfactory separation of pigments was achieved using a silica column in combination with a simple elution technique [7]. For optimal separation of carotenoids, a hexane-acetone mixture was used [10]. Special care was taken to reduce the risk of breakdown of labile chlorophylls during drying and preconcentration of samples. The method was calibrated with pure cultures and several natural samples as sources of standard pigments, and tested using samples from Lake Vechten. The conditions chosen were optimized not for the separation of particular compounds, but rather for the best overall resolution. As the method is very sensitive (detection limit for bacteriochlorophylls approx. 300 ng·1⁻¹ in natural water), it also shows promise for the assay of pigments in sediments where small sample volumes are often required. By the use of appropriate conversion factors, amounts of pigment can be expressed as carbon biomass of the respective groups [6].

3. MATERIALS AND METHODS

3.1. Chemicals

Solvents for extraction (acetone 99%) and for chromatography (n-hexane, acetone and methanol) were Baker HPLC grade (Baker Chemicals, Deventer, The Netherlands). The first eluent consisted of hexane and acetone (80:20, v/v). The hexane contained 0.1% methanol as a stabilizer. When more polar components were present (e.g., pheophorbide and chlorophyll c) a second, more polar eluent, consisting of hexane, acetone and methanol (60:20:20, v/v/v), was employed.

3.2. Standards

Various classes of algae and phototrophic bacteria with a well-documented chlorophyll and carotenoid composition were chosen for study. Synechococcus-type cells (Cyanobacteria), Chlorella vulgaris (Chlorophyceae) and Stephanodiscus hantzschii (Bacillariophyceae) were isolated in monoculture from Lake Vechten. They were cultivated semi-continuously at a temperature of 15°C and a light intensity of 10 μEinstein·m⁻²·s⁻¹ in a 12-h light-dark regime (LD: 12/12) with continuous dilution during the light period using a peristaltic pump (LKB, varioperpex II, 2120) at a dilution rate of 0.005 h⁻¹ [11]. Pigments were also obtained from a strain of Rhodomonas (Cryptophyceae) and from Ceratium hirundinella (Dinophyceae) obtained from net plankton samples from Lake Vechten taken during the summer, when this organism dominated the phytoplankton community very strongly.

The phototrophic sulfur bacteria were represented by Chlorobium phaeobacterioides (brown-colored Chlorobiaceae) and Thiocapsa roseopersicina (Chromatiaceae). They were grown with sulfide in batch culture as described in [12]. In addition, representatives of the Chromatiaceae and green-colored Chlorobiaceae were obtained from a shallow, hypereutrophic pond situated in the garden of the institute, and from Lake Vechten.

Pheophytins were obtained by mixing 2–3 drops of 1 M HCL with the respective chlorophyll fractions in the first eluent. Pheophytin was recovered in the hexane phase for identification and quantification.

3.3. Apparatus

The Pye Unicam HPLC system consisted of a constant pressure LC-XPS pump and LC-UV variable wavelength detector (380–600 nm) with an absorbance range from 0.005–1.28. The samples were introduced to the column by means of a Rheodyne syringe-loading system (model 7125) fitted with a calibrated 50-μl sample loop. Chromatographic separation of pigments was performed at 1200 p.s.i. on a 25 × 0.62 cm semi-preparative column (Chrompack), constructed of stainless steel tubing and packing with 5-μm silica particles (Spherisorb SW 5, N = 16000). The effluent from the column was fed to an 8-μl flow cell with a 10-mm light path in the detector, operated at 440 nm. The absorbance signal from the detector was recorded by a chart recorder (Tarkan W&W) operated at a chart speed of 1 cm·min⁻¹. To obtain visible light spectra for identification and for quantification measurements of pigment
fractions, a Pye Unicam SP 8–500 UV/VIS double-beam scanning spectrophotometer (190–900 nm) was used, in combination with 0.4-ml microcuvettes and a 10-mm light path.

3.4. Procedure

All manipulations were performed at room temperature in very dim light. Known sample volumes, i.e., 1000 ml lake water or 25 ml culture solution, were filtered through 47-mm Whatmann GF/F (0.7 μm pore size) under low vacuum (0.3 bar). If necessary, the filters can be stored under nitrogen at a temperature of −20°C for several weeks. The filters were freeze-dried for 2 h at −60°C in pre-frozen dark vacuum bottles at a vacuum of 0.035 bar. Pigments were extracted from the dried

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Retention time (m, s)</th>
<th>Absorption maxima (nm)</th>
<th>( E_{1\text{cm}}^{1%} )</th>
<th>Solvent used</th>
<th>Detection limits</th>
<th>Source</th>
<th>Ref.</th>
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<tr>
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<td>2592</td>
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<td>5 Okenon</td>
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<td>664</td>
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<td>acetone</td>
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<tr>
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<tr>
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<tr>
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<td>662</td>
<td>911</td>
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<tr>
<td>10 Chlorophyll a</td>
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<td>911</td>
<td>acetone</td>
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<td>11 Bacteriochlorophyll e</td>
<td>5,6</td>
<td>442</td>
<td>658</td>
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<tr>
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<td>1400</td>
<td>acetone</td>
<td>3</td>
<td>60</td>
</tr>
</tbody>
</table>

\( a \) \( E_{1\text{cm}}^{1\%} \) specific extinction coefficient in g/100 ml\(^{-1}\) for 10-mm light path.

\( b \) Detection limit A refers to ng per 50 μl injections (S: N = 2) and B to ng\(-1\) in natural water.

\( c \) Source codes are: (1) Chlorella vulgaris; (2) Stephanodiscus hantzschii; (3) Rhodomonas sp.; (4) Synechococcus-type cells; (5) Chlorobium phaeobacteroides; (6) Thiothrix roseopersicina; (7) Ceratium hirundinella; (8) and (9) natural samples from the institute's pond and Lake Vechten, respectively.

\( d \) Petroleum ether.

\( e \) Calculated using acid ratio Chl a:phosphorhodine a = 2; see [4].
filters by intermittent pulverization and ultrasonication in 6 ml acetone under nitrogen at room temperature (20 min). The mixtures were centrifuged and the pigment extracts were decanted from the particulate residue into calibrated test tubes.

The extracts were concentrated 6 times and transferred immediately into the first HPLC eluent, e.g., the original 6-ml extract was evaporated under nitrogen to 0.2 ml and made up to 1 ml with hexane. 50-μl Aliquots were injected via the sampling valve. Prior to injection, extracts were kept on ice in the dark under nitrogen.

The chromatogram was developed with the first eluent at a flow rate of 3 ml·min⁻¹. To elute pheophorbide and chlorophyll c, a 3-way valve was switched to the intake tube of the second eluent, and development was continued till the elution of the pigment fractions was complete (approx. 36 min).

Before use, the HPLC column was equilibrated by recycling the first eluent at a flow rate of 1 ml·min⁻¹. The column was stored in the first eluent. Reconditioning of the column between runs was not necessary to obtain reproducible results.

3.5. Quantification

To prepare standards, the pigments extracted from the various algal and bacterial classes (see 3.2.) were separated by semi-preparative HPLC and collected using a fraction collector. (Frac. 100, Pharmacia). Their identity and purity were confirmed by their absorption spectra and retention times, using data from [4,6,13–20].

Pigments extracted from natural samples were identified by comparing the retention times of their peaks with those of the standards, and by their spectral characteristics.

The weight of the pigments identified was estimated from calibrations based on the available standards. Standards were quantified spectrophotometrically from their absorption maxima in the appropriate solvent, using the corresponding specific extinction coefficients, as reported in the literature (see Table 1). Calibration curves were made by serial dilution of the standards. The photometrically estimated amounts of particular pigments were plotted against the corresponding peak areas on the chromatogram (see Fig. 2). Peak areas (cm²) were estimated by hand, taking peak heights and widths at half-height relative to absorbance setting and recorder speed.

4. RESULTS AND DISCUSSION

4.1. Development of the method

Our choice for normal phase HPLC on silica was incompatible with aqueous samples. We compared several alternative procedures for drying the samples. In all cases, drying of the samples holds the risk of the breakdown of labile chlorophylls and the formation of alteration products.

For this reason, extracts should be kept on ice in the dark under nitrogen. Nevertheless, chlorophyll a', an isomeric product of chlorophyll a [16] was found in most cases on our chromatograms of algal samples (e.g., Fig. 1D). It is not yet clear to what extent chlorophyll a' represents a natural product from moribund algae. In the course of our work it became clear that the formation of both chlorophyll a' and pheophytin a could occur during sample processing, particularly in the procedure of drying the ultimate extract either by evaporation or by decanting it through a layer of anhydrous sodium sulfate or calcium chloride [7]. We adopted the method of freeze-drying of the filters prior to the extraction procedure. Apart from its speed and simplicity, this drying procedure reduces the formation of isomeric chlorophyll a' and pheophytin a from healthy algal cultures to negligible amounts. The drying of ultimate extracts in HPLC should be avoided whenever possible [9].

Mixtures of hexane and acetone were selected for both the first and the second mobile phases because of their good optical properties and selectivity, particularly with regard to carotenoids. Acetone is very suitable as a modifier to adjust the elution strength for particular samples, e.g., in the case of co-elution of important pigment fractions. Hexane–acetone mixtures were successfully used in HPLC gradient elution systems [10].

Clearly, the choice of a particular eluent forms a compromise between various elution patterns enabling maximum separation quality for the
specific signature pigments. Co-elution of fractions cannot be avoided in all cases when using an eluent of constant strength. Generally, it can be overcome in gradient elution systems, or by using additional detection for chlorophylls at the long wavelength absorption maximum.

Carotene species (α, β and γ) could not be separated in our system. Furthermore, the resolution of Bchl d and zeaxanthin was poor, i.e., the Bchl d peak was partially masked by the zeaxanthin peak (Fig. 3A). Both pigments were found in samples from the metalimnion of Lake Vechten, and constitute important signature pigments. It was important to be able to quantify both fractions. To avoid the interference with zeaxanthin, Bchl d was quantified off-line by spectrophotometry at the absorption maximum of 650 nm. Later, Bchl d and zeaxanthin were estimated directly from the chromatograms, applying a correction for peak overlap. For this, the 2 fractions were obtained using paper chromatography [6]. They were further purified by HPLC. A correction for peak overlap was obtained by comparing the chromatograms of the pure pigments with various mixtures of both pigments. In the calibration curves, peak areas of pure Bchl d and zeaxanthin were plotted against the spectrophotometrically estimated amounts (Fig. 2B,C).

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The chlorophylls from Chlorobiaceae consist of a series of naturally occurring homologues [14]. On separation using HPLC, Bchl c and d display a very characteristic elution pattern, consisting of a broad band made up of several peaks (Fig. 3D). The elution pattern of Bchl e consists of 3 separate peaks (Fig. 1E). The absorption spectra of all 3 fractions were identical. In the calibration curve for Bchl e, the total peak areas of the 3 homo-
logues were plotted against the spectrophotometrically estimated total amounts. For comparative purposes, the calibration curve of the central peak (Bchl e2) is also given (Fig. 2C, arrow).

It should be noted that the wavelength setting of the detector at 440 nm does not correspond with the absorbance maximum of the respective pigment fractions. This value is a compromise enabling reasonable sensitivity to be achieved for both chlorophylls and carotenoids, as can be seen from the respective absorbance maxima in Table 1. As the absorbance of Bchl a is minimal at 440 nm (Table 1), this compound could easily be overlooked in our HPLC method unless very large amounts from a pure culture were present (Fig. 1F). Bchl a can be measured selectively at the detector setting of 380 nm (Fig. 1F). Routinely, amounts of Bchl a were estimated spectrophotometrically from its red absorption maximum (768 nm) in the crude acetone extract. The elution pattern of Bchl a on HPLC and detection at 380 nm (Fig. 1F) showed a clearly outlined peak and shoulder accompanied on both sides by 2 minor peaks whose peak areas comprised about 2% of the peak area of the main component. The compounds corresponding with the minor peaks were collected and analyzed spectrophotometrically. Their absorption spectra were similar to that of the major compound, which was identified as Bchl a. The 2 compounds seemed closely related to Bchl a, but as they constituted only a minor fraction, further identification was omitted.

4.2. Analysis of organisms and calibration

Fig. 1 shows examples of the separation and identification of chlorophylls and carotenoids from various phototrophic organisms. The chromatographic and spectroscopic properties of major pigments separated by HPLC are summarized in Table 1. The culture of *Rhodomonas* sp. was slightly contaminated with flagellated cells of *Isochrysis galbana* (Chrysophyceae), so besides alloxanthin, which is a specific carotenoid for Cryptophyceae, the Chrysophycean carotenoids fucoxanthin, diadinoxanthin and echinenon were also found (Fig. 1D). Minor pigments detected by HPLC but not listed in Table 1 were violaxanthin in *Ch. vulgaris* (Fig. 1A); echinenon in *Synechococcus*-type cells (Fig. 1B) and in the *Rhodomonas* sp. culture (Fig. 1D); allomeric chlorophyll a in *Rhodomonas* sp. and *S. hantzschii* (Fig. 1D, C) and bacteriochlorophyll a in *T. roseopersicina*, which was detected at the wavelength of 380 nm (Fig. 1F). *S. hantzschii* contained pheophorbide a and chlorophyll c. Both pigments were eluted using the second eluent (Fig. 1C).

The high sensitivity and close linearity in determination of pigments by HPLC can be judged from the respective calibration curves (Fig. 2). Although the sensitivity for the assay of pigments varies greatly among respective pigments, it was satisfactory in all cases. The sensitivity of pigment detection depends greatly on the extent to which the absorbance maximum of the pigment and the detection wavelength are in accordance. Furthermore, the effects of peak broadening during elution play a role. Generally, sensitivity decreases as retention times increase (Table 1). Detection limits of the method were defined as peak heights twice
the background noise (S:N = 2). They are listed in Table 1 in ng per 50 μl injections. Multiplied by the routinely used concentration factor of natural samples of 20 they yield the detection limits for natural water samples in ng·l⁻¹.

The system showed greatest sensitivity (6–10 ng·l⁻¹) for the specific carotenoids of anoxygenic phototrophic bacteria, i.e., chlorobactene, isorenieratene, spirilloxanthin and okenon (Fig. 2D), which may be very useful by the study of e.g., anaerobic ecosystems where narrow bands of organisms alternate and sample size is limited.

Specific carotenoids originating from oxygenic phototrophs were also detected easily (10–20 ng·l⁻¹), with lowest sensitivity for fucoxanthin and peridinin (about 40 ng·l⁻¹) (Table 1). The sensitivity for breakdown products, such as pheophytin a and pheophorbide a, was slightly better than that for the bacteriochlorophylls d and e, i.e., 100–200 ng·l⁻¹ vs. 300–400 ng·l⁻¹ (Table 1).

Retention times for the elution of pigment compounds on HPLC ranged from approx. 2–20 min for the first eluent (Table 1). They were found to vary within the range of 1–2%. The accuracy upon multiplicate injections was within 2%. The overall precision of the method, as tested for algal cultures, was within 5%.

It is clear from the results that the HPLC method presented here allows separation and quantification of the major class-specific pigments of algae and phototrophic bacteria.

4.3. Samples from natural habitats

The pond in the garden of the old country house in which the Limnological Institute is accommodated is surrounded by trees and receives a great amount of leaf litter in the autumn. Regularly during that period, sulfide production is high and phototrophic bacteria develop abundantly, as can be judged from the color of the water. A typical example of a chromatogram run from a pond sample taken in November is shown in Fig. 3D. The abundance of Bchl c in combination with chlorobactene points to the dominant role of representatives of the green-colored Chlorobiaceae belonging to the chlorobactene series [24]. The pond forms an example of a simple phototrophic community which can be characterized and quantified very easily by pigment analysis using HPLC.

A study was made of the decline of metalimnetic phototrophic populations during autumn in the monomictic Lake Vechten [6,25]. Due to autumnal cooling, starting in mid-August, the thermocline in Lake Vechten is progressively destroyed by turbulent entrainment, culminating in complete mixing of the lake in the middle of November. Therefore, during this period, typical summer profiles of both solute matter [26] and oxygenic and anoxygenic photosynthetic activities [27] are gradually destroyed as they come within range of a turbulent aerobic environment. During the present work, water samples were collected weekly in the metalimnion (5–8 m depth stratum) of Lake Vechten at 0.5 m depth intervals, using a Friedinger-type water bottle. To avoid contamination with atmospheric oxygen, samples were transferred through rubber tubing into 1000-ml screw cap serum bottles which had been flushed with oxygen-free nitrogen [6]. The samples were taken to the laboratory and filtered immediately. The filters were usually stored and processed further the next day. Pigment analysis was carried out using HPLC. Chromatograms of metalimnetic samples from selected dates during the turnover period are represented in Fig. 3. The series shows nicely the transition of a very complex ecosystem dominated by prokaryotic photopigments (Bchl d and e, iso-renieratene and okenone, Fig. 3A) into a relatively simple ecosystem dominated largely by Cryptophyceae (i.e., alloxanthin, Fig. 3C). The decline of the cyanobacteria is shown by the decrease of the carotenoid zeaxanthin. The more elaborate results of this study are depicted in the form of profiles of major signature pigments on selected dates (Fig. 4). It was observed that the various groups of organisms were distributed at different levels within the vertical profile (Fig. 4A) [6,28]. In the microaerophilic zone of Lake Vechten, the 5–7 m depth zone [26], algal signature pigments occurred (Fig. 4A), the major ones being peridinin, alloxanthin and fucoxanthin. These carotenoids indicated the presence of migrating
cells of *C. hirundinella* [29], *Cryptomonas* and *Rhodomonas* spp. and *Mallomonas caudata*, respectively, which forms metalimnetic blooms in Lake Vechten [29,30]. Prokaryotic phototrophs peaked at various levels below those of eukaryotes (Fig. 4A). Green-colored Chlorobiaceae, gas vacuole-containing *Chloronema*-type filaments [31], represented by the pigment Bchl $d$, showed mass development at a depth of 6–6.5 m. Motile *Chromatium* spp., represented in the depth profiles by Bchl $a$ and okenon, formed a zone from about 6.5–7.5 m. Finally, non-motile brown-colored Chlorobiaceae, indicated by Bchl $e$, were encountered maximally in the anaerobic zone at a depth of 7.5 m. The vertical distribution pattern of phototrophic sulfur bacteria found in Lake Vechten was consistent with other findings [32] where brown-colored Chlorobiaceae dominate in deep layers while green-colored Chlorobiaceae and Chromatiaceae are abundant in the layers nearer to the surface. It can be seen from Fig. 4 that the *Synechococcus*-type cells (zeaxanthin) showed maximum development at almost the same depth as the *Chloronema*-type filaments.

It can be seen from the profiles presented in Fig. 4 that the decline of the various populations occurred very gradually and at almost similar rates during September and October. Bacterial pigments disappeared completely by the start of November, while the algal pigments decreased to the very low concentrations typical of that period in Lake Vechten, e.g. Chl $a$ concentrations between 3–5 $\mu$g $\cdot$ l$^{-1}$ [33].

HPLC is a sensitive and reproducible method for the separation and qualitative and quantitative determination of photosynthetic pigments [34]. In our opinion, the advantage with regard to ecological research lies in the detection and accurate measurement of trace amounts of pigment (Table 1; Fig. 4D), which facilitates biomass determinations of the phototrophic communities in early developmental stages or during their declines. Its ease and sensitivity will enable us to continue our research on the dynamics of phototrophic microbial populations in stratified systems with emphasis on detritus formation and sinking movement.

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