The South African SIBEX I Cruise to the Prydz Bay region, 1984: Determination of photosynthetic pigments and their breakdown products by High Performance Liquid Chromatography

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The photosynthetic pigment data obtained in the course of the South African SIBEX I Cruise to the Prydz Bay region is discussed with relevance to sampling and analytical problems and its ecological interpretation. The pigments were separated and quantified using reverse phase High Performance Liquid Chromatography (HPLC) and the results showed that the pigment structure of both water column and ice algae were typical of Bacillariophycae. The results were compared with chlorophyll a determinations using the SCOR/UNESCO method but some technical differences made these critical comparisons difficult. Finally an optimized methodology is discussed for future work of this nature.

Gegewens oor fotosintetiese pigmente wat tydens die Suid-Afrikaanse SIBEX I-vaart na die Prydzbaai-gebied verkry is, word bespreek met verwysing na monsternemings- en analitiese probleme en na 'n ekologiese interpretasie. Die pigmente is geskei en kwantitatief bepaal deur hoëdrukvloeistofchromatografie (HDVC) met omgekeerde fase te gebruik. Daar is gevind dat die pigmentstruktuur van sowel waterkolom- as ysalgmonsters kenmerkend van die Bacillariophycae was. Die resultate is vergelyk met chlorofil a-bepalings met behulp van die SCOR/UNESCO-metode, maar sekere tegniese verskille het hierdie kritiese vergelykings bemoeilik. Ten slotte word 'n geoptimaliseerde metodologie vir toekomstige werk van hierdie aard bespreek.

Introduction

Chlorophyll *a* has traditionally been used as a measure of algal biomass in natural waters and together with chlorophylls *b* and *c*, xanthophylls and carotenes (Liaaen-Jensen 1977) comprises the different classes of photosynthetic pigments (Gibbs 1979).

Associated with these pigments are a series of breakdown products which can interfere with the spectrophotometric determination of chlorophyll *a* (Mantoura & Llewellyn 1983). These breakdown products, which include pheophytins, pheophorbides and chlorophyllides, are formed as a result of certain biochemical transformation processes (Barret & Jeffrey 1964).

The most convenient method used up to now has been the SCOR/UNESCO method, using trichromatic equations (Lorenzen 1967, Strickland & Parsons 1972). This method, in waters of high production, can result in significant overestimation of the biomass as a result of high concentration of breakdown products which are spectroscopically indistinguishable from the parent molecule.

As a result of the above potential problems, a chromatographic method using reverse phase High Performance Liquid Chromatography was developed to separate out all the pigment components and quantify them individually (Mantoura & Llewellyn 1983, Wright & Shearer 1984).

This methodology allows a rapid separation of all the pigments and their detection either by absorbance at a wavelength of 440 nm with a sensitivity of 10^{-8}M or by spectrofluorescence with a sensitivity of 10^{-11}M for the chlorophylls and their breakdown products only (Bidigare *et al.* 1985).

Once the parent molecules and their breakdown components are separated and quantified, they can be used to infer a number of conclusions of the processes transforming phytoplankton carbon (Jensen & Sakshaug 1973). Thus for example: 1) chlorophyllide *a*, the dephytolated product of chlorophyll *a* appears as a result of the action of the enzyme chlorophyllase; 2) pheophorbide *a*, the protonated product of chlorophyllide *a*, occurs as a result of the displacement of magnesium by the acidic stomach condition of grazers. Thus conclusions may be derived not only relative to the physiology and taxonomy of the algae, but on the biogeochemistry of the pigments as well.

In this study, a methodology based on the published method of Mantoura & Llewellyn (1983) was developed to separate photosynthetic pigments, with the objective of determining the pigment structure of Antarctic algal samples. The data were collected in the course of SIBEX I Cruise of the *SA Agulhas* in the vicinity of Prydz Bay during April 1984 (Allanson 1985).

For the purposes of this report, only results of the major components chlorophyll *a* and chlorophyllide *a* were interpreted. In conclusion some recommendations are given towards the improvement of future exercises of this nature.

Methodology

The separation was obtained using a 15 cm (5 µm particle size) Altex-ODS reverse phase column and a Beckman high pressure system with a Beckman 165 twin channel UV/VIS absorbance detector. Calibration of the chlorophyll *a* and *b* was carried out using standards from the Sigma Chemical Co. As chlorophyllide *a* has identical spectral characteristics to its parent molecule, it was quantified using the same calibration curve. The composition of the two mobile phase

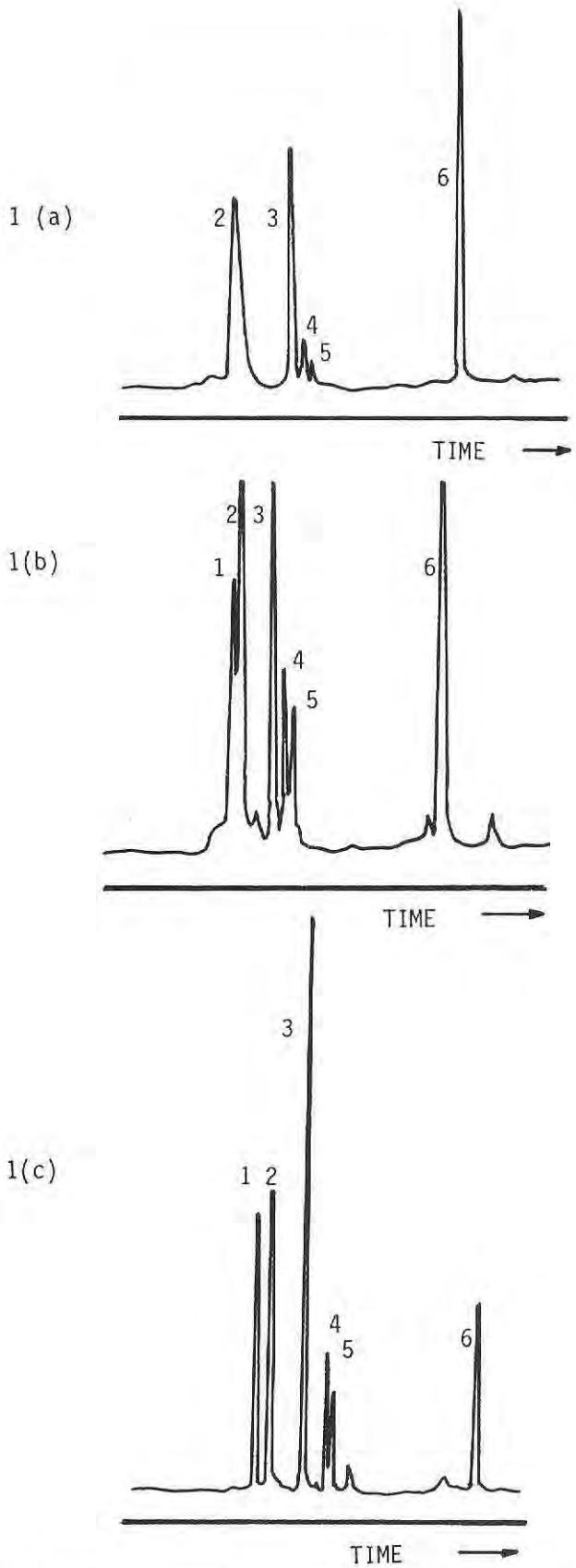

Fig. 1. The chromatograms represent the pigment structure from two samples collected during the SIBEX I Cruise of the *SA Agulhas* to Prydz Bay in April 1984 (Fig. 1a & b) and a pure culture of *Phaeodactylum tricornutum* (Fig. 1c). The samples were collected from molten sea-ice (1a) and surrounding water (1b) and show by the presence of fucoxanthin and chlorophyll *c* that the algae are dominated by diatoms. The peak identification is as follows: 1) chlorophyllide *a*; 2) chlorophyll *c*; 3) fucoxanthin; 4) diatoxanthin; 5) diadionoxanthin; 6) chlorophyll *a*.

components was as described in Mantoura & Llewellyn (1983) but the gradient profiles used for the separations were changed according to column type.

The sampling and analytical procedures used in the course of the study are described below but further useful improvements are recommended in the discussion section.

Sample volumes of 2 000 ml were collected from different depths using 8 litre Niskin sampling bottles, filtered through 47 mm Whatman GF/F glass fibre filters which were wrapped in aluminium foil and stored in liquid nitrogen for the duration of the cruise.

The sample filters were extracted through 30 min incubations in 90 % acetone in an ultra sonic bath (Bransonic). As will be seen later this is not an entirely satisfactory method as it results in some breakdown of the chlorophyll *a* molecules. The extract was then filtered using Millex- HV (Millipore Co.) and further concentrated using SEPAK (Millipore Co.) disposable reverse phase columns. A concentration factor of 5 was attained by eluting the pigments from the SEPAK columns using 1 ml of 90 % acetone.

The separation and quantification of the pigments was carried out on 100 µl injections of sample through a gradient using methanol, water and acetone (Mantoura & Llewellyn 1983). Separation times of 18 min were obtained using 15 cm columns and quantification was obtained by use of an external standard of chlorophyll *a*.

Results and discussion

The chromatograms of the samples obtained from the work area show that the algal pigment structure is dominated by chlorophyll *c*, fucoxanthin and chlorophyll *a* (Fig. 1a & b). This pigment structure is typical of bacillariophycae especially if fucoxanthin is the dominant accessory pigment. This is seen by comparing the chromatograms of samples from the ice edge (Fig. 1a & b) with that of the diatom *Phaeodactylum tricornutum* (Fig. 1c).

Except for differences in the concentrations of these pigments and breakdown products, all samples had similar structures thus allowing an initial conclusion to be made that diatoms completely dominated the primary producers in this region at that time. These results may be usefully compared to those also obtained in the Prydz Bay region (Wright & Shearer 1984) where it was shown that the samples had identical pigment structures but with lower concentrations of chlorophyllide *a*. This discrepancy in relative concentrations of this breakdown product could be accounted for by the period at which sampling was carried out. The declining productivity rates in the late summer will yield higher concentrations of chlorophyllide *a*. The concentrations of chlorophyll *a* in the pelagic environment was very low <100 µM and to obtain the required sensitivity one of the following approaches had to be adopted:

1. use a fluorescence detector with which much greater sensitivities can be obtained (10^{-12M}).
2. use a preconcentration step where disposable C18 cartridges (SEPAK, Millipore Co.; ChromPac, Hamilton) can achieve concentration factors of up to 50 (Mantoura & Llewellyn 1984).
3. filter larger volumes of water.

Quite clearly the last option is logistically the least favoured as there is usually a shortage of water in the course of sampling thus an option has to be made between enhanced detection and pre-concentration. The use of fluorescence is favoured for the quantification of chlorophyll type

molecules however if the detection of non fluorescing components such as xanthophylls is required then the samples will have to be pre-concentrated prior to detection by absorbance at 440 nm.

The breakdown product chlorophyllide *a* was found to be a dominant component of the samples (Fig. 1a & c) and it is hypothesized that as it arises from the activity of the enzyme chlorophyllase on chlorophyll *a* that it may be a useful indicator of the degree of senescence in a sample. The activity of chlorophyllase is especially high in diatoms (Barret & Jeffrey 1964) thus some care has to be exercised to minimise the extraction time of a sample to reduce the likelyhood of error at the analytical stage. This problem has led to the extraction method being changed from an ultra sonic bath incubation to the use of a homogenizer to grind the sample and filter in 90 % acetone.

Although chlorophyllide *a* was found at almost all samples collected in the cruise, the samples of ice algae (Fig. 1b) showed a complete absence of this breakdown product. This supports the hypothesis that algae in a high state of activity will have minimal concentrations of chlorophyllide *a*. These high levels of activity of ice algae in the late summer season may play an important role as an energy source for the krill over the winter season when pelagic activity is at a minimum.

To test the usefulness of chlorophyllide *a* as an indicator of the viability of the algal population a relationship was set up as follows:

$$\text{Viability Ratio} = \frac{[\text{chlorophyll } a]}{[\text{chlorophyll } a] + [\text{chlorophyllide } a]}$$

where pigment concentrations are expressed as mass units and the viability is defined as the proportion of chlorophyll *a* in total chlorophyll type components in the sample (see table 1).

Chlorophyllide has identical spectral properties to the parent molecule and is therefore indistinguishable from it by any non-chromatographic technique such as the SCOR/UNESCO method. This can therefore result in significant overestimation of active chlorophyll *a* concentration by 75 % in pelagic environments and up to 400 % in benthic samples (Mantoura & Llewellyn 1983).

A plot of the viability ratio with depth (Fig. 2) shows that activity maxima fall between 35 and 50 m depth and although these results may be qualitatively compared with the data obtained used the SCOR/UNESCO method, a quantitative comparison is precluded as a result of the samples having been taken with too large a time interval. Due to the fact that this study was at its initial stages at the time of the cruise, it is difficult to draw any concrete conclusions of ecological significance with the data. It is hoped however that future studies will confirm the usefulness of the viability ratio as an indicator of algal activity as well as defining to what extent the two methods of chlorophyll determination agree in Antarctic environments.

There have been extensive improvements in the methodology of this technique and perhaps the most significant one in an environmental context is the shortening of analysis time from 18 min to 8.5 min thus doubling the sample turnover rate. This short analysis time was made possible by the use of a 7 cm Altex XL ODS column which was still able to maintain a high resolution typical of the longer columns.

With the methodology for pigment separation being now well established, the most pressing need is for an increase in our understanding of the ecological significance of the presence and distribution of these pigments and their breakdown products. The pigment pheophorbide *a* has been linked to the grazing of phytoplankton by zooplankton but no quantitative evaluation of this model has been carried out to date.

Table 1

This table gives the chlorophyll *a* and chlorophyllide *a* concentrations with depth at stations 34 and 40 in the SIBEX I grid. The concentrations are expressed as mass concentrations and the third column refers to the viability ratio defined in the text. The maxima of the viability ratio are hypothesized to refer to maximum algal activity.

Station	Depth	Chloro-phyll *a*	Chloro-phyllide* *a*	Viability[a] ratio
34	0	0.175	0.092	0.66
	20	0.078	0.105	0.43
	30	0.158	0.104	0.60
	60	0.090	0.110	0.45
	75	0.073	0.202	0.27
	100	0.096	0.317	0.23
40	0	0.044	0.123	0.26
	20			
	30	0.057	0.129	0.31
	50	0.090	0.088	0.51
	75	0.045	0.135	0.25
	100	0.031	0.096	0.24

* pigment concentrations expressed as mg/m^3

[a] viability ratio = $\frac{(\text{chlorophyll } a)}{(\text{chlorophyll } a) + (\text{chlorophyllide } a)}$

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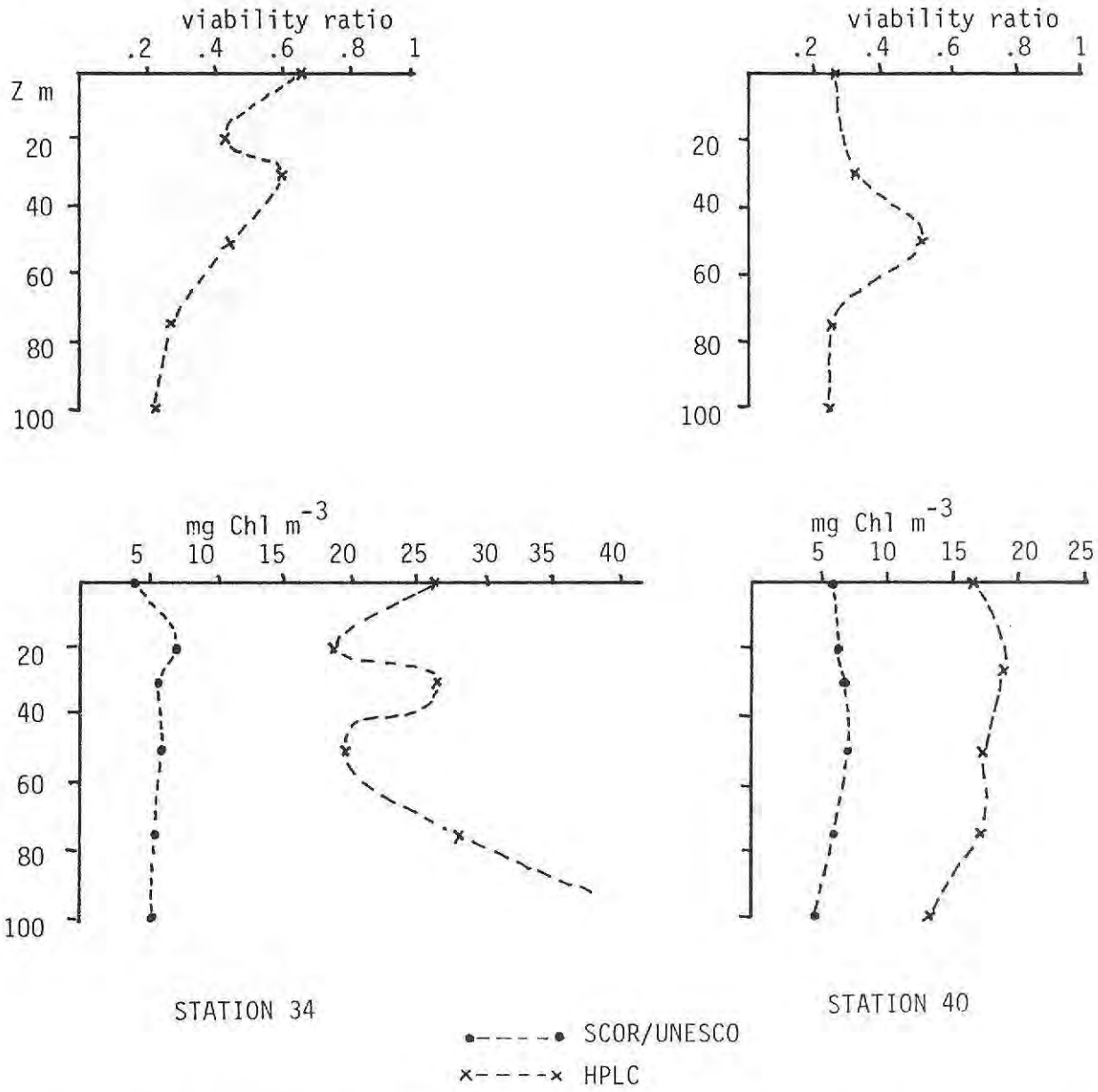

Fig. 2. Diagram showing the vertical distribution of the viability ratio (see text), SCOR/UNESCO chlorophyll *a* and total chlorophyll as measured by HPLC. The total HPLC chlorophyll is the sum of chlorophyll *a* and chlorophyllide *a*. The samples were collected at stations 34 and 40 in the SIBEX I grid in the vicinity of Prydz Bay in April 1984.