Analytical methods used in the GeP&CO experiment

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I.- Introduction

The Geochemistry, Phytoplankton and Color of the Ocean program (GeP&CO) is a 3 year long program that aims to describe and understand the variability of phytoplankton populations in the ocean, and their consequences on the oceans geochemistry. GeP&CO is part of the French program Processus Océaniques et Flux (PROOF) supported by several French institutes : INSU/CNRS, IRD, CNES, and IFREMER. The observations are made quarterly across the North Atlantic, along the eastern coast of the United states, through the Caribbean Sea to Panama, and across the equatorial and tropical South Pacific to New Zealand and New Caledonia. GeP&CO uses the commercial trips of merchant ship Contship London, owned by MS "Alexandra Rickmers" Schiffsbeteiligungsgesellschaft mbH & Co in Hamburg. The first cruise took place in November-December 1999, and the last one is scheduled to start in June of 2002.

The observations correspond to three main categories of objectives. The characterization of phytoplankton populations uses mainly photosynthetic pigments determinations, and also cells counts made either automatically by flow cytometry, or using a microscope. Geochemical measurements consist of determinations of nutrients and of total CO_2 and alkalinity that are subject to changes caused by biological activity. Optical measurements are also made (surface ocean reflectance, light absorption by particles and by dissolved organic matter) in order to build a database that will be used to investigate the relationships between ocean properties and satellite detected ocean color.

We describe here the protocols and analytical procedures that are used to produce the GeP&CO data. A method that aims to measure the concentration of phycoerythrin is still being developed and is only briefly mentioned : it uses fluorescence excitation- emission spectra of seawater volumes concentrated on membrane filter.

II.- Sampling conditions at sea

A cabin on the main deck has been kindly lent to the GeP&CO experiment, for the purposes of seawater filtration, samples conditioning and storage, and also for the

spectrophotometric measurement of spectra of light absorption by colored dissolved organic matter. Seawater samples are taken every 4 hours (i. e. 6:00, 10:00, 14:00, 18:00, and 22:00, local time ; sampling at 2:00 is skipped) in the engine room, at the intake of the cooling system, at about 5 m depth, where a thermosalinograph has been installed (Delcroix *et al*., 1998). The duration of the trip is about 38 days. Then, it takes another 38 days until the ship calls again in Le Havre, and from one week to two months before measurements are made. Thus, storage is generally between 50 and 140 days long. All samples (except the flasks for total CO_2 and alkalinity measurements) are stored at -80°C, in order to prevent degradation of biological and optical properties (Sosik, 1999).

III.- Nutrients

Seawater samples (20 ml, in polyethylene tubes, poisoned with mercury chloride and kept frozen at -80°C until analysis) are processed on a 4 channels Technicon AAII analyzer.

Phosphates react with ammonium molybdate and antimony oxytartrate to form a yellow phosphomolybdic complex. The later is then reduced by ascorbic acid giving a blue coloration that is proportional to phosphate concentration.

Colorimetry is also used for the determination of nitrite that gives a red compound with sulfanilamide and ethylenediamine naphtyl. Nitrate concentration is the difference between nitrate + nitrite (after nitrate has been reduced into nitrite by ammonium chloride in presence of copper treated cadmium) and nitrite alone.

Silicates (in practice, mostly orthosilicic acid) react with ammonium molybdate giving a yellow silicomolybdic complex. Oxalic acid is used to remove phosphate and AsO_4 from this complex. Finally, after reduction using metol (??), a blue coloration appears that is used to estimate silicate concentration.

Final concentrations (µg/l) are obtained by comparison with standards of NO_2, NO_3, PO_4 and $Si(OH)_4$ processed in the same way.

IV.- Total CO_2 and alkalinity

Seawater is taken directly into 0.5 L glass bottles in which it is stored until analysis in the laboratory. Seawater is allowed to overflow for about one minute, avoiding air bubbles, and 1 ml of water saturated with mercury chloride is added for preservation. The bottle is then stopped with a glass stopper smeared with Apiezon® grease, leaving one ml of air inside to prevent against increase of volume sample with temperature. These bottles are stored at room temperature until taken to the Laboratoire de Biogéochimie et de Chimie Marines (LBCM). There, they are analysed using addition of acid and potentiometry, according to Goyet et al. (1991).

For cruises GeP&CO_A to _C, correction for salinity was made using salinity data recorded by a thermosalinograph onboard of Contship London (http://www.ird.nc/ECOP/siteecopfr/cadres.htm). For the other cruises, seawater samples were taken for salinity measurements in the same time as those for total carbonate and alkalinity, and these measurements are used to account for the effect of salinity.

V.- Photosynthetic pigments measurements using HPLC

Filtration of seawater samples is made on Whatman GF/F filters, 25 mm in diameter, using vacuum maintained below 0.25 atm. Filtration is stopped and filtered volume is measured after one hour filtration time, in order to avoid damage to the pigments that may occur when the filters are clogged and filtration takes too long. The volume that is filtered

under such conditions is usually about 2.5 or 3 liters in clear waters, and sometimes only 1.5 liter in rich coastal waters. Filters are then folded with the phytoplankton cells inside, inserted in a numbered plastic envelope, and stored at -80°C. They are recovered at the next call in Le Havre, transported to LODyC in Paris, and later to the Station Marine d'Arcachon using containers with dry ice.

Measurements are made in Arcachon using the high performance liquid chromatography equipment of the Laboratoire d'Océanographie Biologique. This equipment, a Thermo Separations HPLC system, has a binary pump, a 3 µm pore size Licospher (endcapped) C-8, column, 250 x 4 mm, maintained at 30°C, and a Thermo Separations UV LP 6000 diode array detector. A refrigerated automat can handle several samples allowing measurements at night.

The method is adapted from Goericke and repeat (1993). Filters are extracted in 2 ml of 100% methanol during 1 hour at 4°C in obscurity after ultrasonication (15 s). Extracts are filtered through 0.2µ filters to remove all solid particles, and then loaded into the autosampler which is capable of cooling pigments extracts to 2°C and adding 1 M ammonium acetate (2:1, v/v) prior to injection. Pigments are separated at a flow rate of 0.6 ml/min. The proportion of solvents varies linearly along the separation, programmed as follows (minute; % solvent A; % solvent B) : (0;75;25), (1;50;50), (20;30;70), 25;0;100), (35;0;100), (40;0;75). Solvent A is 70:30 (v/v) methanol: 1 M ammonium acetate and solvent B is 100% methanol. The column is then restored to original conditions during 10 min. Pigments are detected using absorption at 440 nm.

Numeric chromatograms are recorded and processed using the PC1000 Thermo Separations software. Calibration is made using pigments standards purchased from DHI Water and Environment, Horsholm, Denmark.

VI.- Spectrofluorometric measurements of chlorophyllous pigments

Filtration and filters storage are made in the same way as above for the HPLC measurements, the main difference being that the filtered volume is only half a liter, which takes usually less than 10 minutes and exerts minimal damages to the cells. Measurements are made at LODyC in Paris, using a F-4500 Hitachi spectrofluorometer. Filters are first ground in 10 ml glass tubes with 6 ml of 90% acetone, using a scratched glass rod, the tubes are stopped and extraction is allowed overnight, at darkness, in a refrigerator. After extraction, the content of each tube is centrifuged, one ml is transferred in a square 1 cm x 1 cm quartz cell, and fluorescence emission excitation spectra are measured in the spectrofluorometer, operating in ratio mode. Measuring parameters are as follows : excitation wavelength varies from 390 to 480 nm, with a 3 nm step, and excitation slit adjusted to 5 nm, and emission is measured from 620 to 720 nm at a 4 nm step, with emission slit adjusted to 10 nm. This yields 31 x 26 = 806 fluorescence measurements for each sample.

Computation of the concentration of chlorophyllous pigments is then made according to Neveux and lantoine (1993), assuming that each one of these 806 fluorescence values is the sum of the contribution of n distinct pigments, with concentrations C_1, $C_2 \ldots C_n$. The $C_i s$ are then estimated as the values that minimize :

$$Q^2 = \sum_{\lambda_{exc}=390}^{480} \sum_{\lambda_{em}=620}^{720} \left(F_{\lambda_{exc},\lambda_{em}} - \sum_{i=1}^{n} C_i F^*_{i,\lambda_{exc},\lambda_{em}} \right)^2 \qquad (1)$$

where F is the fluorescence of the sample at wavelengths λ_{exc} and λ_{em}, and F_i^* is the fluorescence of unit concentration of pigment i at the same wavelengths determined on pure pigments to calibrate the instrument (figure 1). The main improvement to the method first described by Neveux and Lantoine (1993) is the number of fluorescence measurements which

is now 806 instead of 24, thus allowing a better discrimination of pigments whose fluorescence properties are only slightly different.

We presently use $n = 13$ pigments : chlorophyll *a*, chlorophyll *b*, chlorophyll c_2 , chlorophyll c_3, pheophytin *a*, pheophytin *b*, pheophytin c_2, pheophytin c_3, divinyl-chlorophyll *a*, divinyl-chlorophyll *b*, divinyl-pheophytin *a*, divinyl-pheophytin *b*, and a fictitious pigment with a flat fluorescence excitation-emission spectrum that accounts for background signal and extract turbidity. Concentrations are presented in this order in the files of results that can be accessed to on this website ; the concentration for the fictitious pigment is not given. New pigments can be introduced into the numerical analysis, if standards become available. Numerically minimizing Q^2 sometimes gives small negative concentrations for some pigments that are at very low concentration. In such cases, the computation is made again without these pigments, until only positive concentrations are found. Concentrations are given in milligrams per cubic meter.

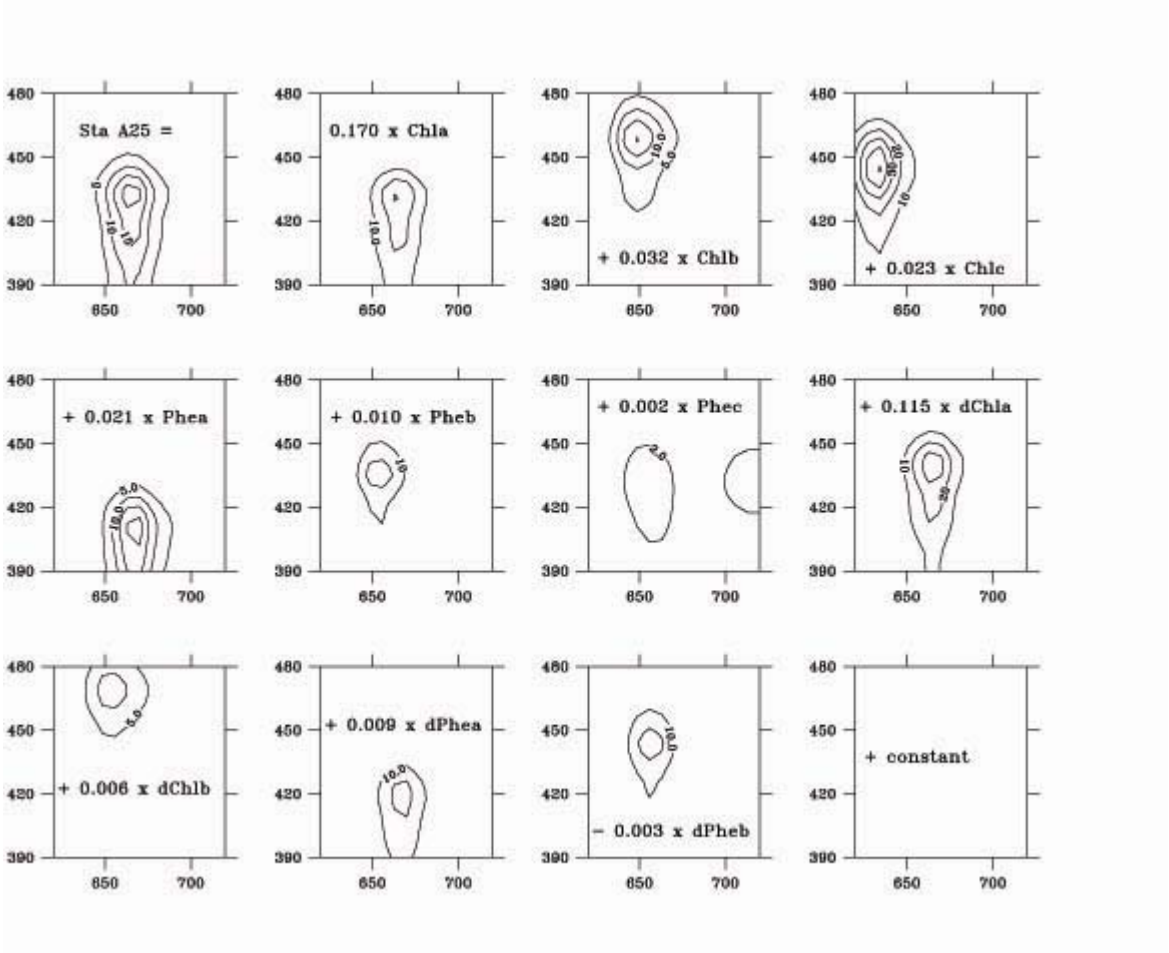

Figure 1 : principle of the numerical determinations of chlorophyll by spectrofluorometry.

These measurements are calibrated versus pigments standards purchased from DHI Water and Environment, Horsholm, Denmark. Divinyl chlorophyll *b* has been prepared, isolated and quantified by Jacques Neveux. The evolution of the calibration through the GeP&CO experiment is presented and discussed in derivefluo.pdf

VII.- Spectrofluorometric measurements of phycoerythrin

Phycoerythrin is not soluble in acetone or alcohol. The common way to measure its concentration in the phytoplankton is to extract it a glycerol-phosphate buffer (Lantoine and Neveux, 1997). This procedure however requires much time and large volumes of seawater, and its extraction rate is not known, possibly being around 80%, and difficult to control. We have thus developed a new method without extraction in which the fluorescence of phycoerythrin is measured directly on the surface of filters. We use a Hitachi F-4500 spectrofluorometer operating in ratio mode, and fluorescence is measured, and recorded, with wavelength of excitation light varying from 384 nm to 576 nm with a 8 nm step, and emission from 500 to 730 nm, with a 10 nm step. We use black nitrocellulose Millipore HA filters, 13 mm in diameter, and filtration is made with Millipore Swinnex type filter holders using a syringe. Filtered volume is 100 ml. Filters are stored at -80°C in biopsy boxes until analysis in the laboratory, where they are placed between two prisms, the two parts close together making a volume with the same shape and size as a 1 cm x 1 cm spectrophotometer cell. This assemblage is then placed in the spectrofluorometer in the same way as an ordinary cell, with the filter at a 45° angle of the light path (Figure 2).

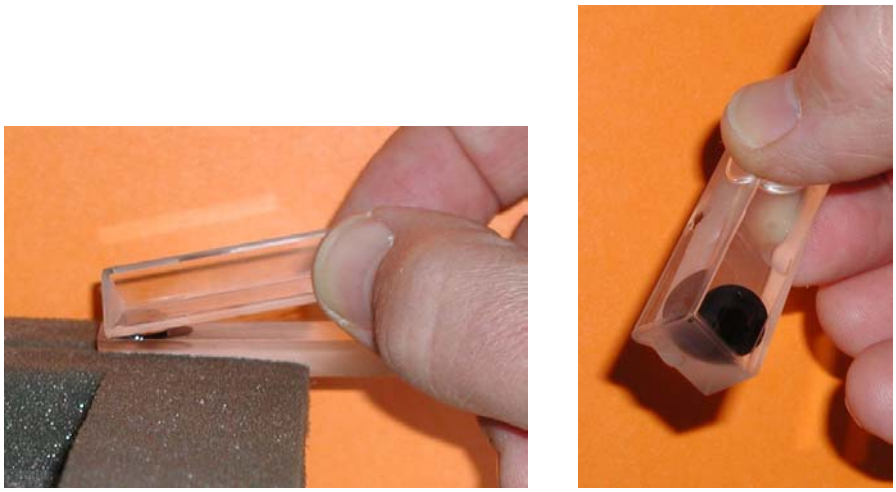

Figure 2 : handling the 13 mm HABG Millipore filters for the determination of excitation – emission phycoerythrin fluorescence spectra.

Baseline correction

There is a wavelength domain, from 500 to 576 nm, where the excitation light diffused by the filter is "seen" by the photomultiplier, giving very high instrument response. Even at excitation and emission wavelengths that differ by only a few nanometers (up to 15 nm), the overlap between excitation and emission monochromators allows some diffused light to reach to photomultiplier. We choose black filters that absorb light to reduce diffusion, but the response of a Millipore HA filter with no algae placed in the spectrofluorometer as explained is still high when emission and excitation wavelengths differ by less than 80 nm. The chromophores of phycoerythrin are fluorescent typically when excited at 488 to 544 nm, and emission is measured at 510 or 560 nm, for phycoerythrobilin (PEB) and phycourobilin (PUB) respectively.

Thus, the fluorescence we want to measure is superimposed to a strong background caused by the diffusion light. Further, this background cannot be considered as constant, for several reasons, an obvious one being that in cases where the filter is completely covered by algae, light diffusion will decrease because some light, at some wavelength (typically : around

430 nm), is absorbed instead of being diffused. Thus, subtracting a constant baseline (for instance, provided by a filter with no algae) cannot fit.

To solve the problem, we identified a small number (=9) of excitation - emission wavelengths pairs (*Pk*) dispersed in the excitation – emission field, at positions where the fluorescence of photosynthetic pigments is expected to be null. We start from a first guess baseline $b(\lambda ex,\lambda em)$ given by a Millipore HA filter with no algae, taken as a 'blank', that we multiply by a coefficient $c(\lambda ex,\lambda em) = 0.5$. c is then increased step by step by small increments. When b is found equal or slightly greater than the fluorescence f of a given sample at a position $\lambda ex_i,\lambda em_j$, c at *Pk* closest to $\lambda ex_i,\lambda em_j$ is fixed. The process is stopped when c is fixed at all 9 *Pk*. Then, c is objectively interpolated at all $\lambda ex,\lambda em$ pairs, and we estimate the baseline as $c(\lambda ex,\lambda em)$ x $b(\lambda ex,\lambda em)$. This complex procedure in fact builds up a baseline whose shape is approximately similar to the instrument response to a Millipore HA black filter and that is tangent to the sample's fluorescence at places where photosynthetic pigments do not emit fluorescence. This baseline is then subtracted from the sample's fluorescence.

At this stage however, the signal is still affected by the very strong light diffusion at measurement points where excitation and emission wavelengths differ by less than 70 nm. This is the case for the fluorescence of the PEB at 490 (exc) and 560 (em) and of the PUB chromophores at 540 and 580 nm.. To improve the signal identification, we have defined an excitation – emission wavelength domain for each one of the two chromophores. A flat surface is forced to intercept the sample's fluorescence field at the periphery of these domains, and we retain as the useful signal the integrated volume between this flat surface and the sample's fluorescence convex one (Figure 3). These two values are used to estimate the phycoerythrin concentration, and the PUB/PEB ratio. In addition, the fluorescence excitation spectra at emission wavelength = 670 nm are stored in the GeP&CO database.

Voir avec Boping pour le calcul de la concentration en phycoerythrine et du rapport PEB/PUB, et pour la calibration.

VIII.- Flow cytometry counts of picoplankton

1.5 ml seawater samples are placed in cryotubes and poisoned with 1‰ glutaraldhyde, then stored at -80°C until analysis in the laboratory. The flow cytometry measurements are then performed within two hours after de-freezing on 0.1 ml water volumes with a fluorescence activated cell sorter scan (FACScan) flow cytometer (Becton-Dickinson). Bacteria counts were made on a separate subsample, treated with Syber green. Filtered seawater served as sheath fluid, and 2µ beads were used as standards (Blanchot and Rodier, 1996). The data were treated with the Becton-Dickinson LYSYS II software, and analysed with the cytowin software (Vaulot, 1989) for the identification and count of Prochlorococcus spp., Synechococcus spp., picoeucaryotes and bacteria.

IX.- counts of Coccolithophorids

Filtration of 1.5 L of seawater is made onboard on Millipore nitrocellulose AA filters, 25 mm in diameter. The filters are then stored in biopsy boxes and kept dry until identification and counting of Coccolithophorids cells using a microscope, at magnification 1250 (Eynaud et al., 1999).

X.- Light absorption by algae

Seawater samples are filtered (approximate volume 2 L) at sea and stored at -80°C in biopsy boxes until analysis in the laboratory. Filters are Whatman GF/F, 25 mm in diameter. The spectral absorption of light by algae is measured in a Beckman DU 640 spectrophotometer, placing the filter close to the instrument photoreceptor window, as in Dupouy et al. (1997). The measurements are made in two steps : first, absorption of light by the filter is measured from 400 to 800 nm (step 1 nm), then, photosynthetic pigments are removed using hot methanol (Kishino, 1985), the filter is placed back in the spectrophotometer, in the same position, and absorption is measured again. The absorption of light by algae is the difference between the two measurements. In principle, the measurement after pigments removal by methanol represents absorption by particles. However, the filters collected during the GeP&CO cruises had a residual red coloration probably caused by the ship's antifouling painting, and this later measurement will not be included in the GeP&CO database.

XI.- Light absorption by colored dissolved organic matter (CDOM)

The method is adapted from Mitchell et al. (2002). Seawater samples (approximate volume 100 ml) are filtered on Nuclepore filters, 25 mm in diameter, 0.1 micron pore size. The first 2 or 3 ml are used to rinse the filter and are eliminated. The remnant filtered seawater is kept in brown glass bottles kept at ambient temperature at darkness. Maximum storage duration is 16 h, from 6:00 am to 10:00 pm. The samples taken on a given day are all processed at the end of this day. Light absorption spectra are measured from 200 to 800 nm using 10 cm light path cells, in a Beckman DU 640 spectrophotometer. A baseline obtained using Milli-Q water is subtracted. Quality of this reference water is checked versus absorption by air.

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