PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **14**: 245–250 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002/pca.711

Seasonal Variability of Phytoplankton at Varna Bay (Black Sea)

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Intrinsic fluorescence and SDS-PAGE analysis were employed to study the seasonal qualitative and quantitative changes of phytoplankton composition at Varna Bay (Black Sea). Variation in the maximum emission wavelength (λ_{max}) of the phytoplankton proteins (398 nm in the summer and 340 nm in the spring) was observed. In addition, a decrease in fluorescence intensity, and a shift in λ_{max} as a result of changes in phytoplankton protein stability, according to the season, was noted. Similarly, SDS-PAGE analysis showed different protein patterns for each season, for example in summertime the major protein constituents were of 14, 37, 48 and 70 kDa, while in the springtime the sizes ranged between 38 and 48 kDa. In general, higher carbohydrate and protein contents correlated with larger phytoplankton biomass found during the summer. The dominant species, the Bacillariophyceae and Dinophyceae, were found to be present in the water body in an alternate pattern. All of these changes could be accounted for by the adaptation of the organism to seasonal variations that modify the sea environment at Varna Bay. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Electrophoresis; fluorescence; carbohydrates; proteins; phytoplankton; seasonal changes.

INTRODUCTION

Phytoplankton has all of the necessary components required to survive and proliferate under a normal marine environment, i.e. proteins, amino acids, lipids, carbohydrates (mostly polysaccharides), minerals and vitamins (Lardinois *et al.*, 1995; Sreepada *et al.*, 1996). Mixing with deeper water layers, and exposure to sunlight, may lead to an adequate supply of the phytoplankton's nutrient requirements inducing, therefore, a rapid growth. Yet, such favourable conditions are not permanent and vary seasonally (Lardinois *et al.*, 1995; Morrison *et al.*, 2001), thus determining the development and composition of the phytoplankton.

The response of phytoplankton to environmental factors affecting the Bulgarian Black Sea coastal zone is well documented (Moncheva *et al.*, 1995; Shtereva *et al.*, 1998). Changes in fluorescence properties of phytoplankton components have been reported (Churilova, 2001; Laney *et al.*, 2001; Rochelle-Newall and Fisher, 2002), suggesting that they may be important markers of the seasonal changes occurring in the water body. Since information about the protein of phytoplankton (i.e. separation, identification and properties) is scarce, we have applied fluorescence and electrophoretic methods to study the variation in protein composition of phytoplankton at Varna Bay (Black Sea) during spring and summer seasons, and we have correlated the results with carbohydrate, cell biomass and dominant phytoplankton species found in the water body. It may be concluded that, at Varna Bay, different environmental conditions during the spring and summer seasons determine the variation in the composition of the protein of phytoplankton that can be analysed by spectrophotometric and/or electrophoretic methods.

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EXPERIMENTAL

Materials. Phytoplankton samples were collected at Varna Bay (Black Sea) over four consecutive years (1994–1998) during the spring and summer seasons using a CTD-rozete system attached to 10 L bottles. The study of the phytoplankton communities was performed according to a protocol previously described (Shtereva *et al.*, 1998). All chemicals were reagent grade from Sigma (St Louis, MO, USA) and were used without further purification. Deionised distilled water was used throughout.

Sample preparation for protein analysis. Volumes of collected water samples (about 8–10 L in each case) were filtered through GF/F glass fibre filters (Whatman, Maidstone, UK), and the collected phytoplankton material was washed with phosphate buffer. Biomass was determined for each sample and extractions were carried out using a similar biomass. Particulate carbohydrates and proteins were collected from water samples using the same volume. The chemical compositions of the various samples were determined by elemental analysis.

Particulate protein and carbohydrate contents. Protein content was measured by the method of Lowry *et al.* (1951), and also by absorbance at 280 nm, using bovine

Received 30 January 2002
Revised 17 July 2002
Accepted 8 August 2002

serum albumin (BSA) as standard, with an Uvikon 930 UV spectrophotometer (Kontron, Zürich, Switzerland). Carbohydrate concentration was determined spectrophotometrically using l-tryptophan reagent according to the method described by Romankevich (1990). Boric acid (25 g) and 1-tryptophan (5 g) were dissolved separately each in a small volume of concentrated sulphuric acid, the solutions were mixed and the volume adjusted to 1 L with concentrated sulphuric acid. The test sample (0.1–0.5 mL) was adjusted to 2 ml with distilled water and 3 mL of the 1-tryptophan reagent was added: the mixture was held in the dark at 100°C for 15 min and the complex so formed assayed at 540 nm against a blank. The assay was calibrated using glucose solution (10 mg/mL) as standard.

Protein extraction. The filter with the phytoplankton sample was homogenised with 20% (w/v) trichloroacetic acid in acetone containing 0.1% 2-mercaptoethanol and 1% sodium dodecyl sulphate (SDS). Extraction with the same solvent was repeated three times, after which the remaining pellet was lyophilised. The sample was dissolved in phosphate buffer before the analysis. Various extraction schemes were evaluated in order to determine differences in protein extraction (Tanoue, 1995, 1996; Nguyen and Harvey, 1997). For this purpose, proteins were also extracted using a modified technique (Nguyen and Harvey, 2001) in which the filter was homogenised in 0.1 M sodium hydroxide for 1 h at 25°C prior to the trichloroacetic acid: acetone extractions as described above, followed by solubilisation in 8 M urea in 50 mM ammonium bicarbonate.

Fluorescence spectra. Fluorescence measurements were performed on the lyophilised protein material obtained from phytoplankton cells using a Jasco (Hachioji City, Japan) model FP-770 spectrofluorometer. The protein concentration was adjusted to 0.15 mg/mL with 0.01 M phosphate buffer (pH 7.2) giving an absorbance of less than 0.1 in a 1 cm path length cell in order to guarantee a linear increase in the relative fluorescence intensity. The sample temperature was maintained at 30°C with a thermostatically controlled circulating water bath. Fluorescence emission spectra were taken at excitation wavelengths of 274 and 295 nm and recorded over the frequency range from the excitation wavelength to 500 nm (Khan *et al*., 1980). The magnitude of protein stability was calculated using the expression:

$$\text{percentage stability (\%C)} = [(I_0 - I_1)/I_0] \times 100$$

where I_0 and I_1 are the fluorescence intensities of protein in summer and spring, respectively.

Electrophoretic separations. SDS-PAGE was carried out according to Laemmli (1970), as modified by Gorinstein *et al.* (1996), on 1.5 mm thick gels prepared with acrylamide ($T = 18\%$) and bisacrylamide ($C = 2.7\%$). The gels consisted of a 2 cm stacking gel and an 8 cm running gel. The protein sample was dissolved in buffer containing 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS in 0.0.0625 M Tris–hydrochloride (pH 6.8), boiled for 5 min and loaded onto the gel [ca. 10–15 μg of protein solution (10 μL) per slot]. Electrophoresis was performed at 75 mA for 95 min after which the gels stained were with 0.25% Coomassie Brilliant Blue R-250 in methanol:water:acetic acid (5:5:1, by volume), and de-stained with the same solvent. The molecular weight markers α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), egg albumin (45.0 kDa), and bovine serum albumin (66.0 kDa) were used.

Statistic analysis. The statistical significance of a studied parameter was determined from five replicates of each measurement using Student's test; a p-value of < 0.05 was adopted as statistically significant.

RESULTS AND DISCUSSION

Various physical, chemical and biological variables, namely, protein, carbohydrate, ambient average temperature, phytoplankton biomass and type of most abundant phytoplankton cells, were determined by sampling the water column at Varna Bay (Black Sea) over a four year period.

Intrinsic fluorescence properties of proteins

Some authors have suggested that in the marine and estuarine environments the observed chromophoric dissolved organic matter (CDOM) fluorescence is not due to the phytoplankton itself, but to the transformation of non-fluorescent organic matter of the phytoplankton by bacteria (Schumann and Rentsch, 1998; Kieleck *et al*., 2001; Rochelle-Newall and Fisher, 2002). In spite of this, the seasonal qualitative and quantitative changes of phytoplankton proteins was explored by measuring the intrinsic fluorescence intensity of tryptophan and/or tyrosine residues at an excitation wavelength of 295 nm.

As shown in Fig. 1(A), a fluorescence spectrum could be measured at an excitation wavelength of 295 nm compassing the 346–377.5 nm interval which serves as indication of the presence of tryptophan residues in the protein sample (Khan *et al*., 1980; Determann *et al.*, 1998; Churilova, 2001; Wu and Tanoue, 2001). The highest maximum absorbance was observed at 346 nm with summer samplings [Fig. 1(A)]. These samples also showed a higher intrinsic fluorescence value ($I_0 = 0.6838$) compared to the spring samples ($I_0 = 0.4853$). Interestingly, the maximum emission peak of the spring samples was displaced to a longer wavelength (366 nm) suggesting that, in this case, the tryptophan environment was more polar. This observation indicates that the springtime protein sample was found in a more opened or relaxed state than the equivalent summertime sample. The spectrum of the summer samples showed, in addition, a shoulder at 398 nm, with an $I_0 = 0.6407$, which was missing in the spring samples [Fig. 1(A)]. Thus the relative stability of the summer samples *versus* the spring samples estimated by these methods was about 29%.

The emission spectra of the summer samples revealed also a higher content of phenylalanine and/or tyrosine at an excitation wavelength of 274 nm [Fig. 1(B)]. The shoulders corresponding to tyrosine emission were more pronounced in the summer samples at 452 nm ($I_0 = 0.0610$) and at 450 nm ($I_0 = 0.2975$).

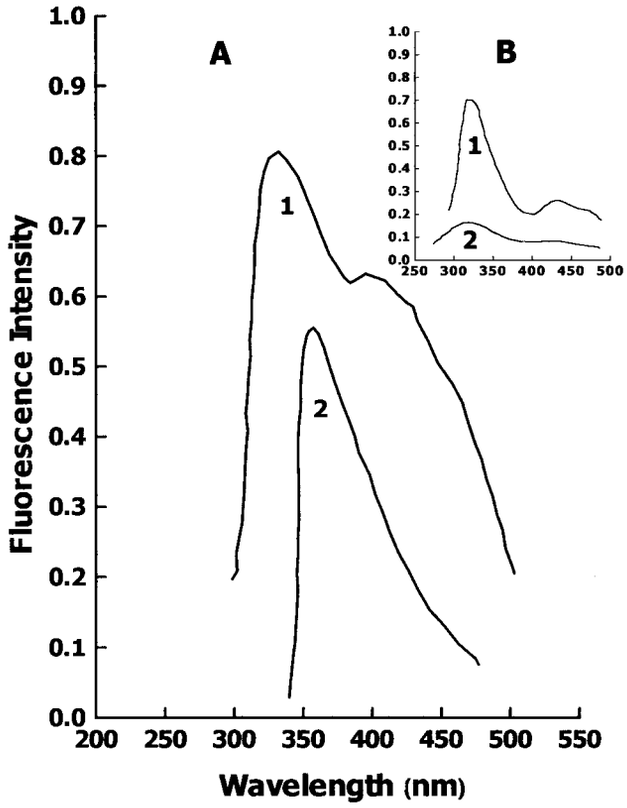

Figure 1. The fluorescence emission spectra (A, excitation at 295 nm; B, excitation at 274 nm) of proteins from phytoplankton samples collected in summer (curves 1) and in spring (curves 2) at Varna Bay.

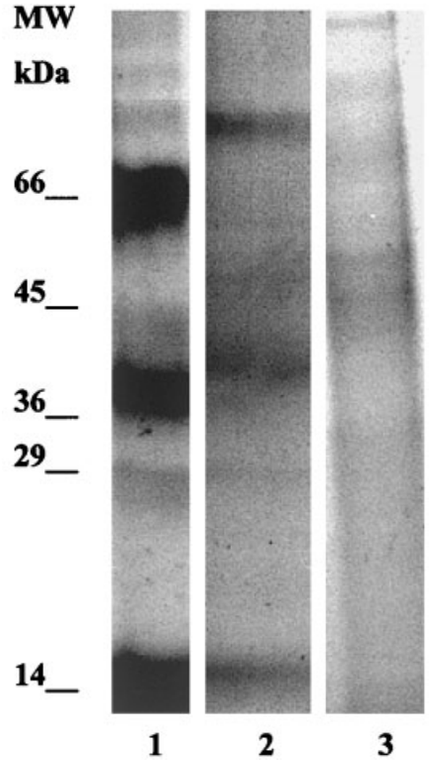

Figure 2. SDS-PAGE separation of phytoplankton proteins in samples collected in the summer (lane 2) and samples collected in the spring (lane 3) at Varna Bay. Lane 1 shows molecular weight markers (see Experimental section for details).

It is known that, at an excitation wavelength of 295 nm, tryptophan appears to be the only aromatic amino acid residue capable of absorbing light. Also, that the wavelengths of maximum emission for tryptophan tyrosine and phenylalanine are situated at 346, 348, and 303 and 282 nm, respectively (Khan *et al.*, 1980). Hence, the maximum observed at 346 nm may derive from the contribution of both tryptophan and tyrosine residues, or from tryptophan alone. In the literature there are no data on the fluorescence of phytoplankton proteins but our finding correlate well with data for tryptophan emission of vicilin and other plant globulins, which have been reported to occur around 347 nm (Arntfield *et al*., 1987).

The fluorescence spectra of the summertime sample collected at Varna Bay was different to the springtime sample (Fig. 1; traces 1 and 2). Such seasonal variation in fluorescence has been previously reported for algal and bacterial plankton (Determann *et al*., 1998) and may derive from differences in amino acid composition in the extractable protein resulting from adaptations to environmental changes, in agreement with Laney *et al.* (2001) and Churilova (2001), who reported that natural fluorescence varies in response to changes in the physical and chemical environment.

Determination of the UV-excited fluorescence is useful for revealing the occurrence of certain aromatic amino acids (Gorinstein *et al*., 1996). By applying this technique to protein material extracted from phytoplankton, we have been able to produce spectral evidence of the seasonal changes occurring in phytoplankton protein composition at Varna Bay.

Electrophoretic separation of phytoplankton protein

Average intensity bands for the extracted phytoplankton protein collected in the middle of July are presented in Fig. 2 (lane 2) and show several bands with apparent molecular weights of ca. 14, 29, 37, 48 and 70 kDa. Diffused and very weak bands were observed in the sample obtained in the middle of April (Fig. 2; lane 3), mostly with average molecular weights of around 38–48 kDa. These results are similar to those of Tanoue (1995, 1996) and Tanoue *et al.* (1996) reporting two different groups of proteins with molecular weights of 45 and 66 kDa. Palenik and Koke (1995) also reported that a 82 kDa sub-unit could be associated with a nitrogen-regulated protein, but the highest molecular weight protein detected in our samples was 70 kDa (Fig. 2; lane 2). Thus, it appears that the processes by which specific proteins from marine organisms are transferred to and accumulated in the pools of dissolved and particulate organic matter are identical throughout the world's oceans.

Quantitative structure of phytoplankton communities, particulate proteins and carbohydrates

Whilst phytoplankton is present all the year round, its contribution to the total cell biomass varies according to the season and its nature. In summertime, protein and carbohydrate composition of phytoplankton reveal increased amounts in comparison with the springtime. Thus particulate carbohydrates increased from a spring

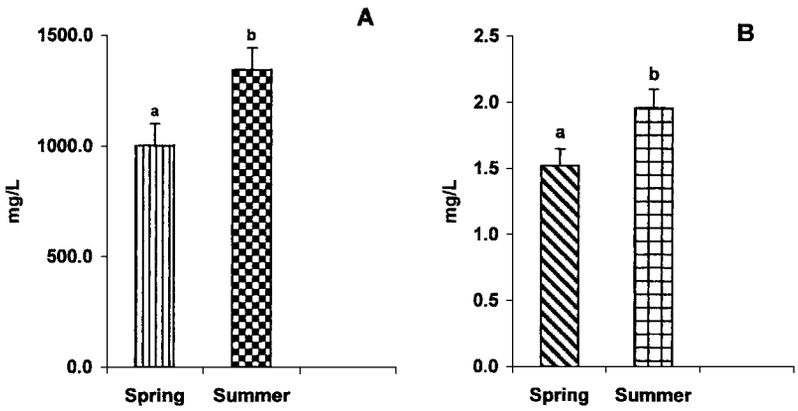

Figure 3. Seasonal changes in the content (mean values; $n = 5$) of the suspended proteins (A) and carbohydrates (B) in water samples collected in spring and summer at Varna Bay. Vertical lines represent standard deviations; bars with different letters are significantly different ($p < 0.05$).

value of 1.52 ± 0.13 mg/L to a summer level of 1.96 ± 0.14 mg/L [Fig. 3(B)]. Our results on particulate carbohydrates are similar to those previously reported (Tepic *et al.*, 2000), showing that their concentration was well correlated ($p < 0.05$) with the distribution of phytoplankton biomass during different bloom seasons. A similar relationship was shown [Fig. 3(A)] in the increase of particulate proteins in the summer. The results of fluorometric measurements (Fig. 1) are also in correspondence with the changes in particulate proteins.

The applied analytical methods such as fluorometry (showing a decrease in the fluorescence intensity of phytoplankton proteins in spring in comparison with summer, and a decrease in protein stability to 29%), electrophoresis (demonstrating more distinct electrophoretic patterns in summer than in spring) and elemental analysis (indicating an increase of carbohydrates and proteins in the summer) can be used to detect seasonal changes. It was also necessary to investigate whether the results obtained using these analytical methods would correlate with the composition and the quantity of various classes of phytoplankton present in spring and summer. For this purpose data (not shown) was collected over four years concerning the distribution of the phytoplankton groups Bacillariophyceae, Dinophyceae, Euglenophyceae and Chrysophyceae at Varna Bay. Bacillariophyceae and Dinophyceae were the most abundant groups in terms of cell numbers and biomass, and were also the most diverse. Between November 1995 and early March 1996, however, there was a depletion in both biomass (Fig. 4A; dates 16, 19, 22 and 28) and the number of cells (Fig. 4B) for these two groups. This could have been caused by a sudden decrease in water temperature (with an obvious correlation with ambient temperature) and probably a depletion in nutrient quantity as well. As may be observed in Fig. 4, samples were not taken exhaustively during this period for the other years of the study. During the spring and summer periods studied, there was a clear pattern of alternation with respect to both biomass values and cell numbers of Bacillariophyceae and Dinophyceae i.e. when one group showed increased values the other showed lowered values. Specifically, the parameters for Bacillariophyceae were elevated (and those for Dinophyceae decreased) from March through April in 1996, 1997 and 1998 (Fig. 4; dates 28 and 37), and vice versa from June through July in 1996, 1997 and 1998 (Fig. 4; dates 31, 34, 40 and 43). The alternation of algal groups could be explained by seasonal differences in the maximum and minimum ambient temperatures at Varna Bay, and these are also depicted in Fig. 4. As the ambient temperature increases, Bacillariophyceae dominate over Dinophyceae; the opposite situation is observed when ambient temperatures decrease. The increase in cell number and biomass is an obvious consequence of the continuous input of nutrient to the water which generally occurs when ambient temperatures are high. It is well known that diatoms and dinoflagellates acquire different distributions according to water column stratification, upwelling and consequent mixing. The seasonal variation in the distribution of phytoplankton outlined here support previous conclusions (Morrison *et al.*, 2001) that, while there is a significant decrease (or uptake) of inorganic nitrogen, phosphate and silicate associated with the seasonal phytoplankton bloom, none of the nutrients, except perhaps silicate, are actually depleted within the euphotic zone. At the end of the growing season, nutrient concentrations rapidly approached their pre-bloom levels.

In conclusion, we have shown that during the summer there are significantly ($p < 0.0005$) more particulate carbohydrates and greater biomass of the phytoplankton groups Bacillariophyceae and Dinophyceae than in the spring. These results are similar to previous reports (Determann *et al.*, 1998; Fabiano and Pusceddu, 1998; Rios *et al.*, 1998; Morrison *et al.*, 2001). Differences in the emission peak response and fluorescence intensity in phytoplankton proteins were also found. It is tempting to speculate that the changes in the protein composition of phytoplankton species observed by fluorescence measurements could result from the partial seasonal changes. The electrophoretic analysis of phytoplankton samples collected in spring and summer has also shown some differences which are clearly related to the described seasonal changes in the community structure of the phytoplankton carbohydrates and proteins. Electrophoretic separation of phytoplankton proteins does not give information about their classification but shows the seasonal changes in the quantity and quality of the protein bands. These data are in correspondence with other investigations

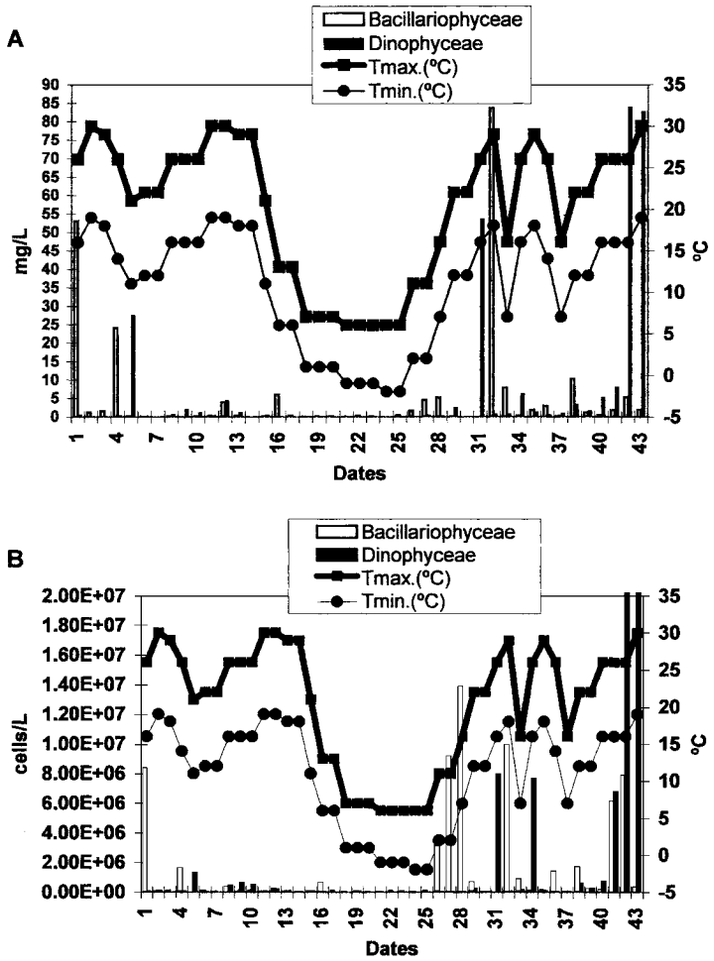

Figure 4. The algal biomass (mg/L) (A) and abundance of algal cells (cells/L) (B) of Bacillariophyceae and Dinophyceae, together with the minimum and maximum ambient average temperatures (°C), at Varna Bay at various collection times during a four year period. Dates 1, 10, 31, 34, 40 and 43 represent summer collections, dates 7, 28 and 37 represent spring collections, and dates 4, 5, 13, 16, 19, 22 and 25 represent autumn collections.

(Sreepada *et al*., 1996). Initially favourable conditions for phytoplankton growth, such as sufficient supply of sunlight and nutrients, are not permanent values since they undergo seasonal changes (Lardinois *et al*., 1995; Stilinovic and Plenkovic-Moraj, 1995). The established quantitative and qualitative characterisation of phytoplankton proteins could to a great extent be related to the seasonal changes in the chemical and physical parameters of the environment, the recurrent summer blooms of the species and anthropogenic eutrophication in the coastal Black Sea zone (Moncheva *et al*., 1995). The applied analytical methods could be used for partial characterisation of phytoplankton proteins.

REFERENCES

Arntfield SD, Ismond MAH, Murray ED. 1987. Use of intrinsic fluorescence to follow the denaturation of vicilin, a storage protein from *Vicia faba*. *Int J Pept Prot Res* **29**: 9–20.

Churilova TY. 2001. Light absorption by phytoplankton and detritus in the Black Sea in spring. *Oceanology* **41**: 687–695.

Determann S, Lobbes JM, Reuter R, Rullkoetter J. 1998. Ultraviolet fluorescence excitation and emission spectroscopy of marine algae and bacteria. *Marine Chem* **62**: 137–156.

Fabiano M, Pusceddu A. 1998. Total and hydrolysable particulate organic matter (carbohydrates, proteins and lipids) at a coastal station in Terra Nova bay (Poss Sea, Antartica). *Polar Biol* **19**: 125–132.

Gorinstein S, Paccola-Meirelles LD, Belo VA, Azevedo JL. 1996. Characterisation of *Beauveria bassiana, Metarhizium anisopliae* and *Aspergillus nidulans* through electrophoretic patterns of their protein fractions. *J Ferm Bioeng* **2**: 89–92.

Khan SM, Darnall DW, Birnbaum ER. 1980. Tyrosine fluorescence as a measure of denaturation in thermolysin. *Biochim Biophys Acta* **624**: 1–12.

Kieleck C, Bousquet B, Le Brun G, Cariou J, Lotrian J. 2001. Laser induced fluorescence imaging: application to groups of macroalgae identification. *J Phys D Appl Phys* **34**: 2561–2571.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Laney SR, Letelier RM, Desiderio RA, Abbott MR, Kiefer DA, Booth CR. 2001. Measuring the natural fluorescence of phytoplankton cultures. *J Atmos Oceanic Technol* **18**: 1924–1934.

Lardinois D, Eisma D, Chen S. 1995. Seasonal differences in concentrations of particulate lipids, proteins and chitin in the North Sea. *Netherlands J Sea Res* **33**: 147–161.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–270.

Moncheva S, Petrova-Karadjova V, Palasov A. 1995. Harmful algal blooms along the Bulgarian Black sea coast and possible patterns of fish and zoobentic mortalities. In *Harmful Marine Algal Blooms*, Lassus P, Arzul G, Denn E, Gentien P (eds). Lavoisier Publ. Inc: Paris. 193–198.

Morrison JM, Gaurin S, Codispoti LA, Takahashi T, Millero FJ, Gardner WD, Richardson MJ. 2001. Seasonal evolution of hydrographic properties in the Antarctic circumpolar current at 170 degrees W during 1997–1998. *Deep Sea Res II* **48**: 3943–3972.

Nguyen RN, Harvey HR. 1997. Protein and amino acid cycling during phytoplankton decomposition in oxic and anoxic waters. *Org Geochem* **27**:115–128.

Nguyen RN, Harvey HR. 2001. Preservation of protein in marine systems: hydrophobic and other non-covalent associations as major stabilising forces. *Geochim Cosmochim Acta* **65**: 1467–1480.

Palenik B, Koke JA. 1995. Characterisation of a nitrogen-regulated protein identified by cell-surface biotinylation of a marine-phytoplankton. *Appl Environ Microbiol* **61**: 3311–3315.

Rios AF, Fraga F, Perez FF, Figueiras FG. 1998. Chemical composition of phytoplankton and particulate organic matter in the Rio de Vigo (NW Spain). *Sci Marina* **62**: 257–271.

Rochelle-Newall EJ, Fisher TR. 2002. Production of chromophoric dissolved organic matter fluorescence in marine and estuarine environments: an investigation into the role of phytoplankton. *Marine Chem* **77**: 7–21.

Romankevich E. (Ed.) 1990. Methods for the Investigation of Organic Matter in the Ocean. Nauka; Moscow: 99–101.

Schumann R, Rentsch D. 1998. Staining particulate organic matter with DTAF—a fluorescence dye for carbohydrates and protein: a new approach and application of a 2D image analysis system. *Marine Ecol Progr Ser* **163**: 77–88.

Shtereva G, Moncheva S, Doncheva V, Christova O, Shterev I. 1998. Changes in chemical parameters in the close coastal Black Sea area (Bulgarian part) as an indication of the ecological characteristic of the environment. *Water Sci Technol* **39**: 37–45.

Sreepada RA, Rivonkar CU, Parulekar AH. 1996. Particulate carbohydrate and proteins in the Bay of Bengal. *Estur Coast Shelf Sci* **43**: 295–310.

Stilinovic B, Plenkovic-Moraj A. 1995. Bacterial and phytoplanktonic research of Ponikve artificial lake on the island of Krk. *Periodicum Biologorum* **97**: 351–357.

Tanoue E. 1995. Detection of dissolved protein molecules in oceanic waters. *Marine Chem* **51**: 239–252.

Tanoue E. 1996. Characterisation of the particulate protein in Pacific surface waters. *J Marine Res* **54**: 967–990.

Tanoue E, Ishii M, Midorikawa T. 1996. Discrete dissolved and particulate proteins in oceanic waters. *Limnol Oceanogr* **41**: 1334–1343.

Tepic N, Terzic S, Ahel M. 2000. Distribution of dissolved and particulate carbohydrates in the northern Adriatic during the 1997 mucilage event. *Periodicum Biologorum* **102**: 179–187.

Wu FC, Tanoue E. 2001. Isolation and partial characterization of dissolved copper-complexing ligands in stream waters. *Environ Sci Technol* **35**: 3646–3652.

