

ANALYSIS OF PHYTOPLANKTON PIGMENTS BY EXCITATION SPECTRA OF FLUORESCENCE

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ABSTRACT

The advantages and problems of the application of actively excited fluorescence in natural phytoplankton analysis are discussed. The focus is made to a correct prediction of pigment concentrations by fluorescence data. The results of high resolution mapping of chlorophylls and phycobilins in the Gotland Basin (Baltic Sea) during Cyanobacterial blooms in 1997 and 1998 are presented. Dynamical spatial maps of phytoplankton pigments were registered *on line* with the flow-through spectrofluorimeter FLUO-IMAGER and the shipboard laser remote sensing spectrometer FLS-S. Characteristic patterns of the phytoplankton distribution in the study area and their evolution in time are discussed.

Field studies confirmed that *on line* spectrofluorimetry can be effectively used to diagnose phytoplankton pigments in application to the tasks of phytoplankton bloom detection, analysis of phytoplankton development and spatial mapping of photosynthetic pigments. Considering the tasks of monitoring large water areas or temporary processes in a spot area, the most productive way is a balanced combination of *on line* continuous fluorescence measurements and sampling procedures, which allows to decrease the time-consuming manual analysis of water samples in the laboratory.

INTRODUCTION

Fluorescence methods are widely applied for characterization of the phytoplankton community status *in vivo* in the marine and freshwater environment. Various techniques are used to estimate the concentration of chlorophyll *a*, biomass and primary production. Based on the method to induce the fluorescence of photosynthetic pigments of phytoplankton *in vivo* in a discrete sample or directly in a water habitat different scientific systems and commercial instruments exist. The broadest class of devices that began to be applied in phytoplankton analysis was connected with measurements of fluorescence based on fixed wavelength fluorimetry with a filter system for excitation and detection of fluorescence (1), and scanning spectrofluorimetry based on recording the excitation or emission spectra of fluorescence (2). In the last decade a number of new fluorescence techniques has been intensively developed, including fast repetition rate fluorometry (3) and the pump and probe technique (4).

The common feature of all these techniques is that the concentration of pigments is estimated through the correlation of *in vivo* fluorescence with the isolated photosynthetic pigment values. Attempts to quantify the phytoplankton pigments *in situ* using fluorescence characteristics run against the fact that photosynthetic pigments in a living cell are bound to protein. Due to that, the *in vivo* fluorescence of phytoplankton should be considered in connection with the specific pigment-protein structure of the cells. Various pigment-protein units in a cell have a different structure and play a different role in photosynthesis (5). Some of the pigment-protein units are located in photosynthetic reaction centres and participate in the light reactions of photosystem I and photosystem II. The bulk of the chlorophyll/carotenoid-protein consists of various light-harvesting pigment-proteins whose role is to collect light and transfer the absorbed energy to the reaction centres. Therefore, the characteristics of phytoplankton *in-vivo* fluorescence, including its fluorescence efficiency, depend on the taxonomy.

There have been numerous attempts to achieve a taxonomic identification based on *in vivo* bio-optical characteristics of phytoplankton, including fluorescence excitation and absorption spectra. Earlier investigations, introducing the chlorophyll *a*/accessory pigment ratio technique (1,6) in excitation spectra, have shown that the identification of the alga class or species in natural mixed phytoplankton population is quite problematic. Fluorescence becomes a powerful tool in cell identification when the cells can be separated and analysed individually, e.g. with flow cytometry (7,8).

Phytoplankton *in vivo* fluorescence excitation spectra depend not only on the taxonomic position of algae, but also on the photoadaptation state. The cellular pigment content, the ratio of total chlorophyll *a* to accessory pigments, and the efficiency of energy transfer to chlorophyll *a* are sensitive to the light condition of culture growth (9,10,11). The effect of photoadaptation on fluorescence excitation spectra of algae can be of the same level of significance as the influence of taxonomy (12). In particular, photoadaptation effects must be taken into account in interpreting vertical profiles of phytoplankton, when the gradient of ambient light may be quite high. The fluorescence yield depends also on the stage of growth of phytoplankton (13). The analysis of fluorescence of different alga species at different stages of growth in controlled light and nutrient conditions has shown that the predicted chlorophyll by fluorescence can be lower than the real concentration at the growth stage of phytoplankton. This is mainly due to a change of the cellular pigment content. As the intensive growth of phytoplankton is typically registered in bloom processes, it is important to quantify such development when considering the task of diagnostics of phytoplankton bloom (14).

In spite of the complicated correlation of *in-vivo* fluorescence and phytoplankton pigments further development of the technique continued to distinguish the fluorescence of different pigments in the phytoplankton community (15). Species/class-specific bio-optical and photoacclimation characteristics were overviewed and related to the pigment composition of phytoplankton for major marine phytoplankton groups (16). These numerous studies were connected with the definition of class-specific marker pigments for the indication of bloom-forming and toxic species. Due to the fact that phycobiliproteins of cyanobacteria are strongly auto-fluorescent and do not induce a dominant chlorophyll *a* signal, the fluorescence technique was successfully used for vertical and horizontal *in-situ* profiling of phycobiliproteins in the marine environment (17).

Up to now the method of *in vivo* fluorescence excitation spectra remains the most specific to the variability of pigment composition among other bio-optical techniques to characterise living phytoplankton and could be used for on line monitoring (18). The excitation spectra are little affected by light scattering (19) and do not depend on absorption of light by photoprotective pigments (20). Recent laboratory investigations have shown that the ratio of spectral peaks in excitation spectra of fluorescence are less sensitive to growth stage and light conditions than the fluorescence intensity (21).

The aim of the present work was to study the capabilities of the fluorescence excitation spectra technique for a rapid characterisation of living phytoplankton: 1) to reveal the common features of class-specific excitation spectra, 2) to investigate the possibilities of *in vivo* phytoplankton pigments quantification, 3) to consider the approach of phytoplankton excitation spectra application for on-line monitoring.

MATERIALS AND METHODS

Algae collection

The culture collection of the Institute of Biology of Southern Seas (Sevastopol, Ukraine) was used. Algae cultures of 32 phytoplankton species covering major marine taxa were studied (Table I). Batch cultures were maintained in a modified Goldberg medium (22) and an enriched seawater medium (23) at room temperature (18-22^o C) under daylight. *Synecococcus sp.* strains were obtained from Black Sea picoplankton. All trials were conducted in the late log-phase or in the stationary phase of the cultures.

Instruments

Fluorescence excitation spectra of culture samples were measured with a Fluo-Imager M32B spectrofluorimeter at three emission wavelengths. The first spectral range (excitation 400 to 660 nm, emission 680 nm) was aimed to reveal fluorescence of chlorophyll *a* induced via accessory pigments. The second spectral range (excitation 400 to 620 nm, emission 630 nm) was used to record separately the phycocyanin (PC) fluorescence. The third spectral range (excitation 400 to 550 nm, emission 580 nm) was used to record the phycoerythrin (PE) fluorescence. A xenon lamp (150 W) was used as a light source. The fluorescence of the sample in a flow-through quartz cuvette is induced via the excitation monochromator and recorded with a photomultiplier tube (PMT) with further digital processing. An interference filter system that contains three filters (678, 630, 580 nm) was used to select the spectral bands for registration. Specially designed software provided spectral data analysis and control of instruments. The excitation spectra were not corrected for the spectral distribution of the lamp source.

In the field studies the fluorescence excitation spectra were measured continuously in a flow-through mode. Water from 3 to 5 m depth was pumped and analysed every 2 minutes providing an averaged spatial resolution of approx. 250 m at a cruise speed of about 7 knots. The software analysed the fluorescence excitation spectra in real-time to reveal the fluorescence intensities corresponding to different phytoplankton pigments. The intensity of pigment fluorescence was stored according to the time and coordinates of the measurements.

Table 1. List of phytoplankton cultures.

| Class | No | Species | Label | Class | No | Species | Label |
|--------------------------|----|----------------------------------|-------|-----------------------|-----------------------------|----------------------------------|-------|
| <i>Bacillariophyceae</i> | 1 | <i>Thalassiosira pseudonana</i> | pl59 | <i>Chlorophyceae</i> | | <i>Stichococcus bacillaris</i> | pl63 |
| | 2 | <i>Phaeodactylum tricornutum</i> | pl64 | | 3 | <i>Chlorella minutissima</i> | pl215 |
| | | | | 4 | <i>Chlorella elipsoidea</i> | pl216 | |
| <i>Dynophyceae</i> | 3 | <i>Exuviaella pusilla</i> | pl61 | 6 | <i>Chlarococcum infus.</i> | pl218 | |
| | 4 | <i>Prorocentrum cordata</i> | pl56 | 8 | <i>Dunaliella salina</i> | pl221 | |
| | 5 | <i>Gymnodinium kowalewskii</i> | pl57 | 9 | <i>Dunaliella viridis</i> | pl224 | |
| | 6 | <i>Amphidinium klebsii</i> | pl55 | 11 | <i>Dunaliella maritima</i> | pl226 | |
| | 7 | <i>Gymnodinium lanskaja</i> | pl202 | 12 | <i>Dunaliella tertiol.</i> | pl227 | |
| | 9 | <i>Peridinium trochoidea</i> | pl212 | | | | |
| | 10 | <i>Peridinium triquetrum</i> | pl213 | <i>Prasinophyceae</i> | 10 | <i>Tetraselmis sp.</i> | pl225 |
| | | | 13 | | <i>Platimonas viridis</i> | pl223 | |
| <i>Chrysophyceae</i> | 12 | <i>Olistodiscus luteus</i> | pl60 | | | | |
| | | | | <i>Cyanophyceae</i> | | <i>Coccochloris sp. (LL)</i> | pl203 |
| <i>Prymnesiophyceae</i> | 13 | <i>Pavlova luteri</i> | pl65 | | | <i>Coccochloris sp. (HL)</i> | pl204 |
| | 14 | <i>Isochrysis galbana</i> | pl220 | | | <i>Synechococcus aeruginosus</i> | pl205 |
| | 15 | <i>Monochrysis luteri</i> | pl228 | | | <i>Oscillatoria sp.</i> | pl206 |
| | 16 | <i>Gephyrocapsa huxleyi</i> | pl52 | | | <i>Synechococcus sp.1</i> | pl207 |
| | 17 | <i>Emiliania huxleyi</i> | pl209 | | | <i>Synechococcus sp.2</i> | pl208 |

Pigment concentration

Seawater samples were filtered through Whatman GF/F glass fiber filters. After breaking the cells with ultrasonication, the pigments were extracted in 96% ethanol. Chlorophyll *a* was measured using a Shimadzu UV-2101PC spectrophotometer and calculated for concentration according to (24).

RESULTS AND DISCUSSION

In vivo fluorescence excitation spectra of phytoplankton cultures were measured at the emission wavelength 680 nm. The spectra are shown in Figure 1.

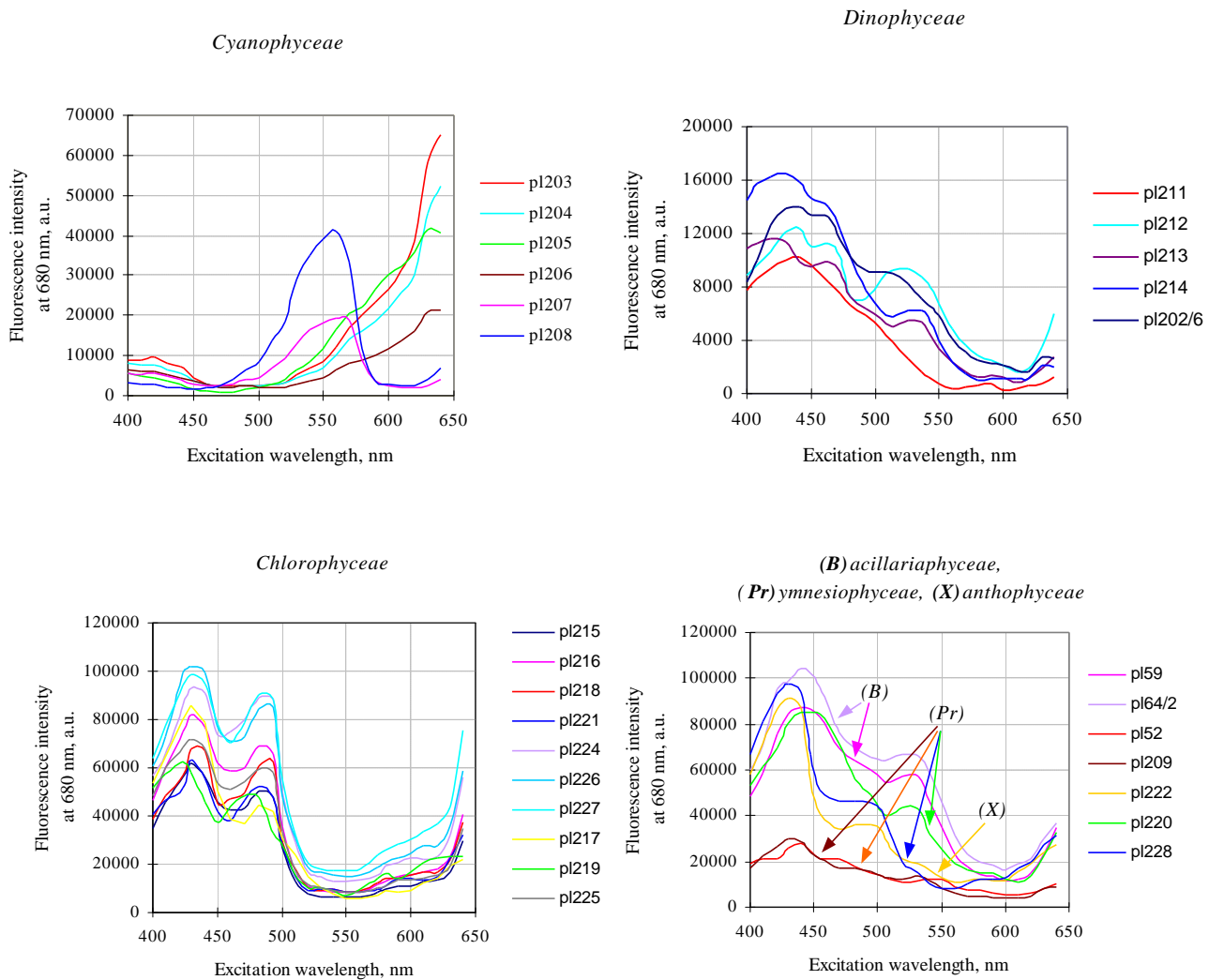


Figure 1: Fluorescence excitation spectra of phytoplankton cultures at the emission 680 nm.

The fluorescence excitation spectra of the cultures can be divided into three types of characteristic spectra according to pigment representation. The blue–green algae belong to the first type that is characterised by the absence of chlorophyll and carotenoid peaks in excitation spectra. The biliproteins play the role of accessory pigments to collect and transfer the light energy to the reaction centre. Biliproteins in blue–green algae are divided into three classes based on the position of their absorption bands: PE, PC, and allophycocyanins (in increasing order of wavelength). PE is displayed in the excitation spectra (Figure 1) as a broad band with a maximum near 560 nm (25), PC – as a shoulder of a band with a maximum near approx. 635 nm (26). In fact, the fluorescence recorded at the wavelength of chlorophyll *a* emission is a part of biliprotein autofluorescence. The second type of excitation spectra is specific for chlorophyll *b*–containing algae that are represented by species of *Chlorophyceae*. The single broad band in excitation spectra in the range 420–440 nm is connected with chlorophyll *a*. The spectral band in the range 460–490 nm is caused by a light-harvesting complex containing chlorophyll *b* and carotenoids. Chlorophyll *b* absorbs *in vivo* at 470 nm (27). A sharp decrease of intensity at 500 nm was observed in *in-vivo* excitation spectra for all *Chlorophyceae*. The last group comprises chlorophyll *c*–containing algae. Numerous peaks in different combinations fill the wide spectral range from 400 to 600 nm. Similar to chlorophyll *b*–containing algae, the spectral range 420–440 nm is connected mainly with chlorophyll *a*. The peaks in the range 460–470 nm are caused by diverse combinations of chlorophyll *c*₁, *c*₂ and *c*₃. The range 480–580 nm

contains the peaks of carotenoids. The frequently mentioned fucoxanthin (530 nm) is a predominant carotenoid for *Bacillariophyceae*, *Chrysophyceae* and *Dinophyceae*.

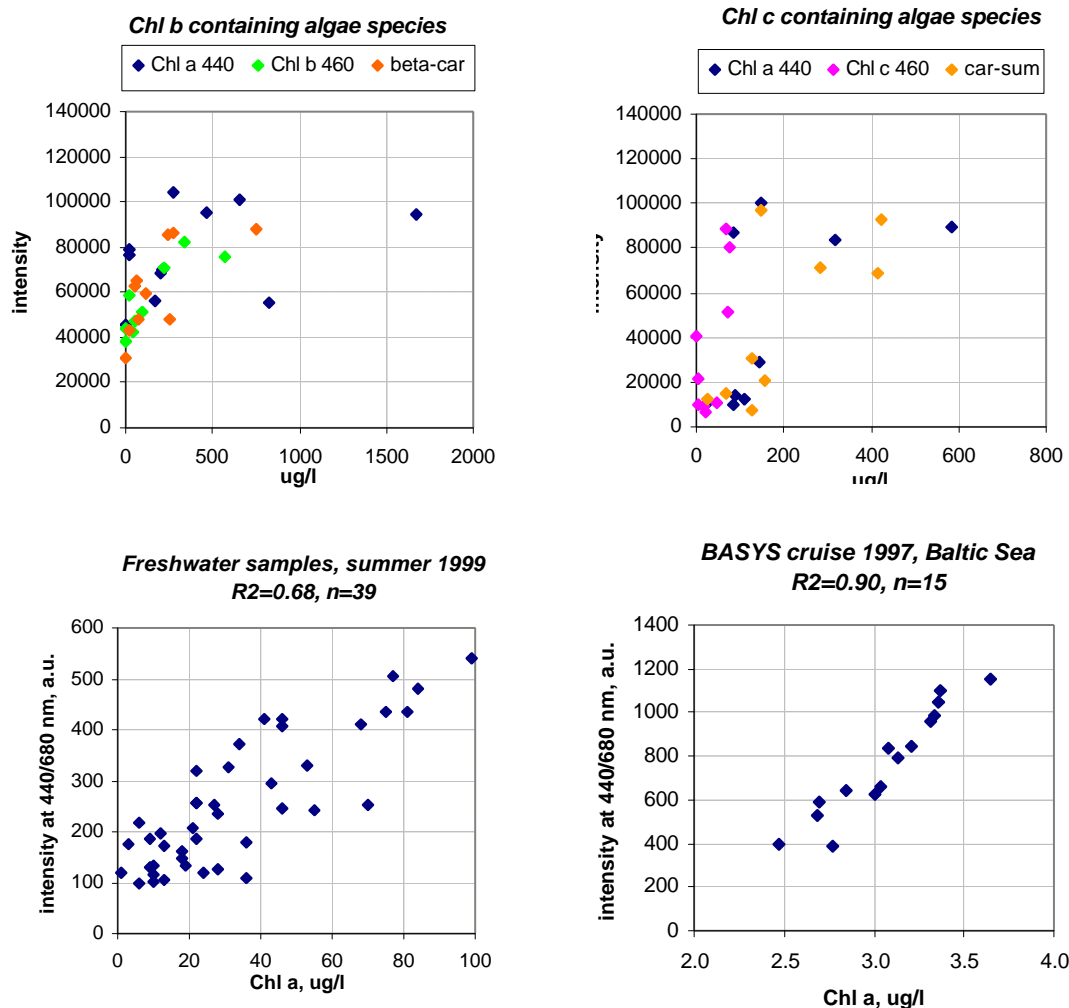


Figure 2: Calibration of fluorescence at 680 nm induced via a Soret band of chlorophyll a absorption and different accessory pigments for chlorophyll *b* and *c* containing cultures (upper diagrams); calibration of chlorophyll *a* fluorescence of samples collected from different freshwater reservoirs and in the Gotland Deep of the Baltic Sea (lower diagrams).

It is obvious that it is quite difficult to distinguish the single or even groups of marker-pigment for species/class identification. At the same time, it is possible to reveal some consecution of principal peaks in all chlorophyll *b* and *c*-containing algae: 440, 460, 490, 530 nm. In the studies connected with the reconstruction of *in vivo* absorption spectra (28) a good correlation of the spectral band at 440 nm with the chlorophyll *a* concentration was revealed. In these studies the peak at 460 nm was used to estimate chlorophyll *b* and *c* for chlorophyll *b* and *c* containing groups of algae. The peaks in the spectral region 480 to 560 nm were interpreted as a carotenoid absorption.

The results of fluorescence calibration for algae cultures using spectrophotometric measurements in extracted to ethanol pigments are shown in Figure 2. When considering the total set of studied algae cultures, a very low correlation between *in vivo* fluorescence and concentration of corresponding pigments has been revealed (Figures 2, upper diagrams). This was possibly due to the variability of fluorescence characteristics of different cultures caused by differences in their cellular pigment structure. A better correlation has been obtained for samples of natural phytoplankton collected simultaneously from different sampling spots in a limited area (Figure 2, freshwater samples). This

result was possibly due to a similar taxonomic composition for the majority of the collected samples. It has to be mentioned that even this correlation can become worse when including the samples collected in the further seasonal development of the phytoplankton. Apparently, this could be caused by the development of different dominant algae in different sampling points. The best result was obtained in trend measurements when the composition of the algae community did not change noticeably (Figure 2, Baltic Sea samples).

In order to analyse how the excitation spectra structure varies depending on the algae pigment composition, the portions of fluorescence intensities at excitation wavelength 440, 460, 490, 530 nm, and the amount of extracted pigments (chlorophyll a, b, c, and carotenoids) were investigated for chlorophyll b and c-containing algae. The corresponding diagrams are shown in Figures 3 and 4.

One can see that for chlorophyll b-containing algae the ratio of main peaks of excitation spectra is quite stable and does not depend much on the species (Figure 3A). The fluorescence of chlorophyll a is induced mainly at 440, 460, 490 nm and the relative efficiency of excitation is about the same. On the other hand the pigment composition, as defined by extraction (18) varies significantly from one species to another.

A similar situation is observed for chlorophyll c-containing species (Figure 4). The fluorescence composition is not so sensitive to the species as the pigment composition. However, the carotenoids that absorb the light in long-wavelength spectral range (i.e. fucoxanthin at 530 nm) start to play the considerable role in light collecting and energy transfer to chlorophyll a. The diagrams of fluorescence composition in Fig.3A and 4A show that it is possible to distinguish chlorophyll b and c-containing algae, taking into account the differences in carotenoids contribution to pigment composition and excitation spectra.

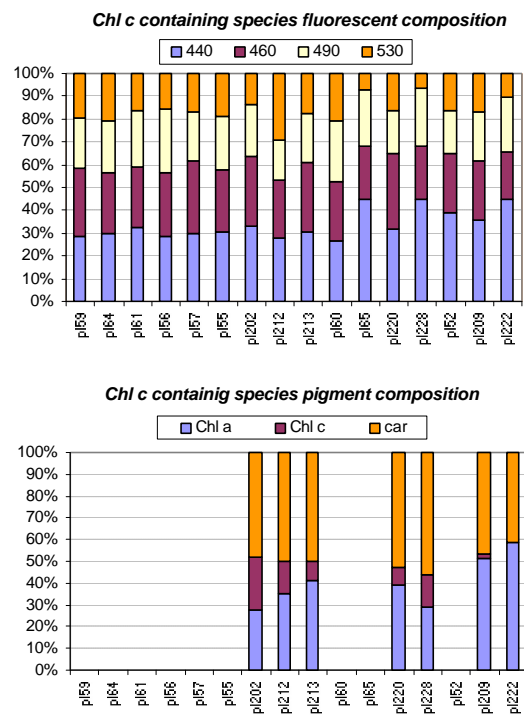
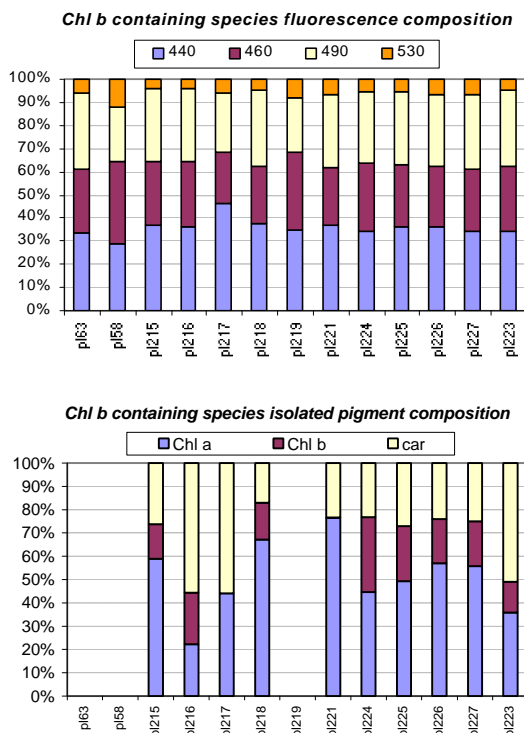


Figure 3: Composition of fluorescence at 680 nm induced at 440, 460, 490, 530 nm (above) and pigment composition of chlorophyll b-containing algae (below).

Figure 4: Composition of fluorescence at 680 nm induced at 440, 460, 490, 530 nm (above) and pigment composition of chlorophyll c-containing algae (below).

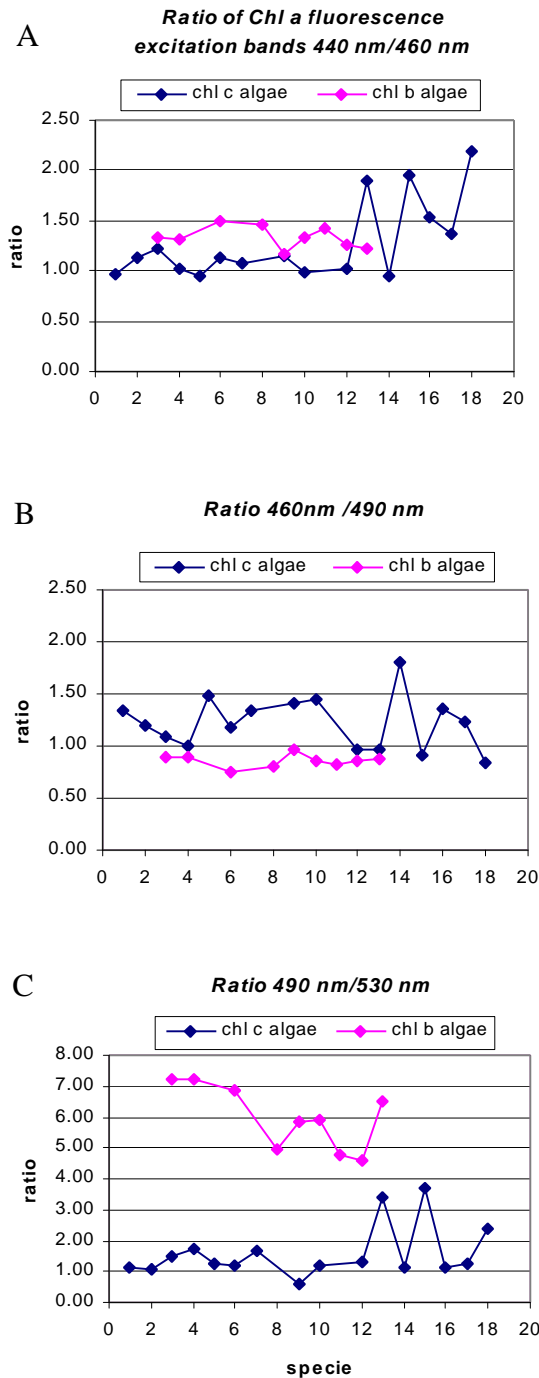


Fig.5. The ratio of fluorescence at 680 nm induced at different wavelengths.

$R_{440/460} = I_{440}/I_{460}$ (A); $R_{460/490} = I_{460}/I_{490}$ (B); $R_{490/530} = I_{490}/I_{530}$ (C).

Consideration of carotenoids is of principal importance for diagnostics of phytoplankton *in vivo*. Besides the property to specify the individual features of species, the *in vivo* absorption of carotenoids is shifted significantly to the longer wavelength from chlorophyll *a, b, c* in comparison with the same mixture of the isolated pigments in an organic solvent. This shift for carotenoids can be up to 90 nm, while for chlorophylls it is not more than 15 nm (29). This means that carotenoids can be recognised more successfully in the *in vivo* state.

Based on these diagrams the ratio of main intensity peaks for chlorophyll *b* and *c*-containing groups of algae were estimated and compared. The results are shown in Figure 5 and Table 2.

The ratio of the fluorescence induced at 440 and 460 nm ($R_{440/460}$) for *Bacillariophyceae* and *Dynophyceae* is quite stable and does not vary significantly (Figure 5A). Very close values were obtained for *Chlorophyceae*. The highest variability was observed for *Prymnesiophyceae*. This fact confirmed that it is difficult to distinguish chlorophyll *b* and *c*-containing algae *in vivo* in the mixed phytoplankton population using only the ratios 440/460 nm and 460/490 nm in excitation spectra. This is caused by very close values of absorption wavelengths of the chlorophyll *b* and chlorophyll *c*. Sometimes they practically coincide at 470 nm.

The ratio $R_{460/490}$ that characterises the accessory pigment complex is better to use, but some values for chlorophyll *b* and *c*-containing algae are very close (Figure 5B). However, this ratio does not include all carotenoids. The ratio $R_{490/530}$ demonstrates the best result (Figure 5C) possibly due to a better representation of light harvesting carotenoids. By this ratio the two groups of algae (chlorophyll *b* and *c*-containing) are distinguished properly.

CONCLUSIONS

The fluorescence excitation spectra of phytoplankton up to now remain one of the effective bio-optical techniques for rapid diagnostics of algae population *in vivo*. In spite of the diversity and complexity of the algae pigment composition the three main groups of phytoplankton can be defined in accordance with distinguishing fluorescent features: biliprotein-containing, chlorophyll *b*-containing and chlorophyll *c*-containing phytoplankton. These groups can be recognised using the fluorescence excitation spectra of the chlorophyll *a* and biliproteins.

The key problem for optical methods is still data quantification. All attempts to provide universal calibration of phytoplankton fluorescence have been doomed to failure. The only possibility has been to make the trend calibration, the so-called internal calibration, that works for permanent phytoplankton populations in specific temporal/spatial conditions. It is obvious that a method of phytoplankton quantification *in vivo* needs a different approach to those currently existing which has to include the step of algae identification.

Table 2. Ratios of fluorescence intensity at 680 nm in excitation spectra.

| Class of algae | 440/460 | 460/490 | 490/530 |
|----------------------------|----------------|----------------|----------------|
| <i>Bacillariophyceae</i> , | 0.95 - 1.33 | 1.20 - 1.35 | 1.05 - 1.13 |
| <i>Dynophyceae</i> | 0.97 - 1.13 | 1.0 - 1.49 | 0.6 - 1.75 |
| <i>Prymnesiophyceae</i> | 0.94 - 1.96 | 0.91 - 1.80 | 1.24 - 3.72 |
| <i>Chrysophyceae</i> | 1.0 | 1.1 | 1.24 |
| <i>Xanthophyceae</i> | 2.18 | 0.85 | 2.38 |
| | | | |
| <i>Chlorophyceae</i> , | 1.16 - 1.50 | 0.75 - 0.97 | 4.61 - 7.25 |
| <i>Prasinophyceae</i> | 1.22 | 0.87 | 6.5 |

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