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Discrimination of marine algal taxonomic groups using delayed fluorescence spectroscopy

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ABSTRACT

We present a method for in situ monitoring of phytoplankton composition changes in a marine environment. The method is based on delayed fluorescence excitation spectra analyzed with CHEMTAX software, which is generally used for determination of phytoplankton communities with HPLC pigment data. Delayed fluorescence (DF) is a photosynthetic parameter that can only be measured in living cells. Algal DF excitation spectra are group-specific, based on their composition of photosynthetic pigments.

DF excitation spectra of 14 marine algal species from different families were measured with a delayed fluorescence spectrometer. Mixtures were prepared from northern Adriatic algal species representing six taxonomic groups: dinoflagellates (*Prorocentrum minimum*), diatoms (*Skeletonema costatum*), cyanobacteria (*Synechococcus* sp.), prasinophytes (*Micromonas* sp.), cryptophytes (*Teleaulax* sp.), and prymnesiophytes (*Isochrysis galbana*). The DF excitation spectra (DFS) and HPLC pigment compositions of the mixtures were analyzed with CHEMTAX software. The prediction power of DFS–CHEMTAX method was comparable to HPLC–CHEMTAX.

1. Introduction

Good evaluation protocols are critical for understanding phytoplankton population dynamics. More than one method is usually needed to gain sufficient information for good quality analysis. Although many laboratory and in situ methods are available, they have method-specific disadvantages. Thus, research goals must be defined before the method is selected.

When studying changes in phytoplankton composition, fast methods with reasonable resolution of algal taxonomic groups can be used for in situ monitoring. A method that has been used for more than 10 years in routine freshwater phytoplankton dynamics studies is delayed fluorescence spectroscopy (Krause and Gerhardt, 1984; Yacobi et al., 1998; Istvanovics et al., 2005; Greisberger and Teubner, 2007). Delayed fluorescence (DF) is the long-term emission of light from cells triggered by illumination (Strehler and Arnold, 1951; Berden-Zrimec et al., 2010). It has the same emission spectrum as chlorophyll *a* fluorescence, but occurs with a time delay (from milliseconds to minutes) (Arnold and Davidson, 1954). DF originates from repopulation of excited states of chlorophyll from stored energy after charge separation (Joliot et al., 1971). It has hyperbolic decay kinetics during the first seconds, which is sometimes followed by a more or less pronounced peak (Bertsch, 1962; Zrimec et al., 2005; Berden-Zrimec et al., 2010). The main source of DF are back reactions in the photosystem II (PSII) (Rutherford and Inoue, 1984), whereas the photosystem I contributes much less to the DF emission (Jursinic, 1986). In PSII, charge pairs are generated during the illumination with positive charges located on the oxygen evolving complex (OEC) and negative charges on quinone acceptors (Q_A and Q_B). The slow components of DF originate in back reactions between the S_2 and S_3 states of the OEC and quinones Q_A and Q_B (Joliot et al., 1971). The half-times of these reactions in isolated chloroplasts are 1.5 s for $Q_A + S_{2/3}$ and 25 s for $Q_B + S_{2/3}$ (Rutherford and Inoue, 1984).

The major advantage of DF is that it is emitted only from cells that are photosynthetically active; that is, alive. Thus additional signals from dead cell debris do not interfere with the measurements. Long-term DF emission (measured for seconds or minutes) also prevents interference problems with fluorescent backgrounds in natural samples (Istvanovics et al., 2005).

Delayed fluorescence excitation spectra (DFS) of algae are based on pigment composition. Chlorophyll *a* is present in all algal classes; chlorophyll *b* in chlorophytes and euglenophytes; chlorophyll *c* in diatoms, chrysophytes, dinoflagellates, and cryptophytes; and phycobiliproteins such as phycoerythrin and phycocyanin in cryptophytes and cyanobacteria, or allophycocyanin in cyanobacteria

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(Barlow et al., 1993; Larkum, 2003). Chlorophylls affect the action spectra for photosynthesis with absorption in blue (430–470 nm) and red (630–680 nm) part of the spectrum (Strain et al., 1963; Jeffrey, 1963). Carotenoids absorb in 450–550 nm and phycobilins in 500–600 nm spectral regions (Haxo and Blinks, 1950). The resulting action spectra of certain algae are a combination of several absorption bands forming a specific spectral fingerprint. The method and spectra analysis used to date distinguish only four "color groups" of phytoplankton: (i) cyanophytes, (ii) chlorophytes (e.g., Chlorophyceae, Euglenophyceae, and Conjugatophyceae), (iii) chromophytes (e.g., Bacillariophyceae, Chrysophyceae, Dinophyceae, Xanthophyceae, and Haptophyceae) and (iv) cryptophytes (Gerhardt and Bodemer, 2005). This method has been used only in freshwater phytoplankton. In a marine environment, these four classes are not sufficient to monitor phytoplankton changes. Several algal taxonomic groups included in the chromophytes color group often prevail in marine phytoplankton populations and must be detected separately for successful monitoring of phytoplankton composition. Therefore we set out to develop a new protocol for monitoring phytoplankton changes using CHEMTAX analysis in addition to a new DF phytoplankton meter developed at our institute.

Analysis of phytoplankton pigments by HPLC and data processing using CHEMTAX has been proposed as an alternative to time-consuming microscopic cell counting to determine taxonomic algal composition (Mackey et al., 1996). The resolution of taxonomic groups by this method is generally coarse (e.g., diatoms, dinoflagellates, cryptophytes, cyanobacteria, chlorophytes, prasinophytes, and haptophytes) compared to direct microscopic counting that can distinguish species within these broader groups.

The advantage of CHEMTAX analysis is its speed and cost relative to other methods. On the other hand, a blind analysis can result in very significant errors because particular pigment compositions of certain species differ from the average (Irigoien et al., 2004). HPLC–CHEMTAX has been applied in combination with microscopy in the field in the Antarctic peninsula area (Rodriguez et al., 2002), southern Baltic Sea (Eker-Develi et al., 2008), southeast US estuaries (Lewitus et al., 2005), Belgian coastal zone of the North Sea (Muylaert et al., 2006), and others. It has been shown that the Mackey pigment matrix developed for the open ocean has to be optimized for local environments such as estuarine ecosystems (Lewitus et al., 2005).

Because CHEMTAX is a matrix factorization program it can be used with any type of data if the differences in vectors between the taxonomic groups are great enough. The DF action spectrum resembles the absorption spectra of the photosynthetic pigments. Compared to direct absorbance measurements, the DF excitation spectra measure only active pigments and not dissolved pigments, organic substances, and debris. The absorption spectra of various photosynthetic pigments are similar, and so the DFS–CHEMTAX method resolution was expected to be somewhat lower than that of the HPLC–CHEMTAX. On the other hand, the DFS method is an *in vivo* method less prone to errors at the extraction and pigment-analysis stages. Furthermore, DF measures nano- and pico-plankton, which can be lost during filtration or unaccounted for in the microscopic analysis.

The objective of this study was to indentify delayed fluorescence excitation spectra of the main marine algal taxonomic groups and use these spectra for their discrimination. The obtained spectra were analyzed using CHEMTAX deconvolution software to obtain the taxonomic composition of algae in prepared mixtures. The predicting power of DFS–CHEMTAX was compared with the standard HPLC–CHEMTAX approach.

2. Methods

2.1. Algal cultures

Algal cultures were maintained at the culture facility of the National Institute of Oceanography and Experimental Geophysics, Department of Biological Oceanography (OGS/BiO) in Trieste. All species were isolated from the Gulf of Trieste.

The algae were sustained as batch cultures in 100 ml borosilicate Erlenmeyer flasks with 50 ml of culture medium B (Agatha et al., 2004). The culture medium for the diatoms contained an addition of $Na_2SiO_3 \times 9H_2O$ at a final concentration of 1.07 mol l^{-1} (Guillard, 1975). All organisms were cultured at 15 °C for a 10:14 h dark:light cycle. The diatoms, *Teleaulax* sp., and *Synechococcus* sp. were grown at low irradiance of about 20 $\mu E\ m^{-2}\ s^{-1}$, and the remaining species were kept at about 50 $\mu E\ m^{-2}\ s^{-1}$.

The samples were taken from the batch cultures in their exponential phase of growth. Fourteen species of unicellular algae from nine different families were used for the DFS screening. Two different cultures of the cyanobacteria *Synechococcus* sp. were used for comparison. The samples were measured in three parallels.

2.2. Mixtures

The mixtures were prepared from representatives of the six different taxonomic groups that are most frequent in northern Adriatic Sea phytoplankton: dinoflagellates (*Prorocentrum minimum*), diatoms (*Skeletonema costatum*), cyanobacteria (*Synechococcus* sp.), prasinophytes (*Micromonas* sp.), cryptophytes (*Teleaulax* sp.), and prymnesiophytes (*Isochrysis galbana*). Aliquots of two or three taxonomic groups were mixed in all combinations to a final volume of 1 ml. Mixtures of all six species were prepared such that one of the species was always predominant (50% of 1 ml) and the other five each represented 10% of the total volume.

2.3. DF excitation spectrometry (DFS)

DF excitation spectra were measured with a custom-built delayed fluorescence spectrometer (Berden-Zrimec et al., 2010). DF excitation was performed with a halogen lamp and linear filter, providing monochromatic (25 nm half-width) illumination in the range between 400 and 700 nm with an intensity of 100 $\mu mol/m^2$ s PAR. A 1 ml sample of algal culture in a rectangular cuvette was illuminated for 0.6 s. The delayed fluorescence intensity (DFI) was measured in the interval of 0.4–1 s after the end of illumination. Light was detected with a Perkin Elmer C1393 channel photomultiplier in photon-counting mode. Two electromagnetic shutters were used to shield the photomultiplier from the excitation light. DFI was measured sequentially for 18 excitation wavelengths (430.0, 445.8, 493.2, 508.9, 524.7, 540.5, 556.3, 572.1, 587.9, 603.7, 619.5, 635.3, 651.1, 666.8, 682.6, 698.4, 714.2, and 730.0 nm).

2.4. Pigment composition

The qualitative and quantitative analysis of pigments in the samples was performed using the reverse-phase HPLC (High Performance Liquid Chromatography) method (Mantoura and Llewellyn, 1983; Barlow et al., 1993). The pure and mixed culture samples used for DF spectroscopy were filtered through Whatman GF/F filters and immediately frozen. For pure cultures (but only in these cases), all three parallel samples were filtered together through the same filter in order to have enough material for HPLC analysis. A control was made with 1 ml of culture media, filtered and prepared for analysis using the same procedure as for the samples.

Frozen samples were extracted in 4 ml of 90% acetone using sonication, and centrifuged for 10 min at 4000 rpm in order to