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Preliminary investigations on Arctic microalgae by joint application of fluorescent instruments

ZHANG Fang*, HE Jianfeng & GUANG Yingzhi

Key Laboratory for Polar Science of State Oceanic Administration, Polar Research Institute of China, Shanghai 200136, China

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Abstract *In vivo* fluorescence has a wide application in analyzing microalgae, including assessing phytoplankton biomass, rates of primary production and physiological status. This study describes a preliminary investigation on the joint application of the three kinds of fluorescence analysis in the physiological study of microalgae. Flow cytometry and fluorescence spectrometry were used to obtain the *in vivo* static fluorescence information of pigments, and a Pulsed-Amplitude-Modulation chlorophyll fluorometer was used to detect the dynamic fluorescence of chlorophyll. The validity of the joint application was proved by analyzing two laboratory cultured Arctic microalgae, *Pseudo-nitzschia delicatissima* (*Bacillariophyceae*) and *Thalassiosira* sp. The higher value of minimum fluorescence yield in dark-adapted state (*Fo*), actual photochemical efficiency of PSII (ΦPSII), and electron transport rate (ETR) exhibited positive results in a higher cell abundance and chlorophyll *a* content of *P. delicatissima*; whereas higher *β*-carotene content of *Thalassiosira* sp. played an important role in the protection of photosynthesis.

Keywords **Arctic microalgae, flow cytometer, fluorescence spectrum, PAM chlorophyll fluorometer, joint application**

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0 Introduction

While microalgae are important primary producers in the Arctic Ocean[1], they are very sensitive to environmental variations. Their growth rate, abundance, and cell pigment content vary with even slight changes in environment[2]. Accordingly, monitoring of microalgae serves as an extremely useful measure of ocean environmental health[2]. Scientists have shown a correlation between rapidly changing fluorescence emitted from microalgae and environmental variation[3-5]. Cell pigments and metabolic processes within living microalgae communities cause the fluorescence effect. Fluorescence can therefore be affected by the composition of a microalgae population, individual cell size, photosynthesis status, and physiochemical properties of the surrounding water, such as temperature, light, pH, and nutrient levels[6-7]. In addition, because analysis of *in vivo* fluorescence is fast, sensitive, and easy to perform[8-9], it is widely used for phytoplankton pigment analysis in both laboratories and site studies[11-14].

Flow cytometry (FCM) assesses the number of phytoplankton cells in a given population. In addition, FCM provides comprehensive fluorescence information on all pigments present in the single cell that emits fluorescence at any of three constant wavelengths[15]. This technique is primarily applied to analyze picophytoplankton community composition, especially in abundance detection[16-17]. However, few studies have been performed on phytoplankton in the Arctic Ocean[18]. Olson et al.[19] classified microalgae from the Arctic Ocean into several categories by analyzing their FCM fluorescence signal characteristics. Full-range fluorescence spectrometry is another analytical technique that can be used to identify and count living phytoplankton by acquiring fine fluorescence information on different photosynthetic pigments in a phytoplankton community[20-23]. Both methods use static fluorescence to provide qualitative and quantitative information on pigments. In

* Corresponding author (email: zhangfang@pric.gov.cn)

contrast, pulsed amplitude modulation (PAM), assesses phytoplankton chlorophyll dynamic fluorescence information, which shows instantaneous variations in pigments that occurs with environmental changes. Certain studies have compared differences in the photosynthesis process between Arctic cryophilic phytoplankton and temperate-zone algae by means of PAM, successfully elucidating the ways in which the xanthophyll cycle differs from species to species[24-25]. This mechanism demonstrates various adaptive strategies to sunlight availability exhibited by different species of phytoplankton. In 2009, Seppälä[14] initially applied *in vivo* fluorescence techniques to Baltic Sea phytoplankton biomass detection. He used both rapid qualitative and semi-quantitative methods to monitor the phytoplankton population, and additionally, he measured the chlorophyll dynamic fluorescence. After making a comprehensive physiological and ecological evaluation, he concluded that *in vivo* fluorescence techniques were appropriate to the monitoring of Baltic Sea phytoplankton.

In this study, FCM, fluorescence spectrometry and PAM were employed to detect *in vivo* fluorescence of two kinds of microalgae from the Arctic Ocean. The aims of this paper are to investigate the feasibility of comprehensive *in vivo* fluorescence analysis for identifying pigment and classifying phytoplankton, and to explore the mechanisms behind photosynthesis in phytoplankton from the perspective of pigment theory. This is the first study of polar phytoplankton to utilize the joint fluorescent methods.

1 Material and methods

1.1 Microalgae cultures

Pseudo-nitzschia delicatissima (*P. delicatissima*, CCMP 1309) and *Thalassiosira* sp. (CCMP 1056) with diameters <20 μm were purchased from U.S. CCMP (Bigelow Laboratory for Ocean Sciences) and cultured. Growth media f/2 and L1 was used for *P. delicatissima* and *Thalassiosira* sp., respectively. The initial seeding density was approximately 1×10^5 cells·mL^{-1}. The culture temperature was maintained at 4±2℃and the culture irradiance at 30 μmol·m^{-2}·s^{-1}. The light:dark cycle was maintained at 12:12 h. Three identical experiments were conducted. Three samples were collected from each of the three parallel experiments during the dark stage and measured when the microalgae reached the stationary period (22 d).

1.2 Instrumentation

FCM measurement was performed using a FACSAria (Becton Dickinson, NJ, U.S.). The machine parameters were checked and adjusted to confirm that the signal intensity was within the detection region. Samples of 2.5 mL in volume were filtered through a mesh of 20 μm and then placed in the machine. A laser of 488 nm was used to excite the cells. Assessment of the fluorescence of the samples at 530±15 nm, 585±21 nm, and 695±20 nm was performed.

A fluorescence spectrophotometer (Hitachi F-7000) was used to measure spectra. Samples of 2.5 mL in volume were filtered through a mesh of 20 μm and transferred to a 1 cm quartz cuvette before being placed in the machine. The operation conditions were as follows: The range of excitation spectra wavelength was 200—600 nm when emitted at 680 nm; the range of emission spectra wavelength was 200—800 nm when excited at 488 nm; the step widths and slit widths of excitation and emission were 1 nm and 5 nm, respectively; the scanning velocity was 200 nm·s^{-1}.

Additionally, 2.5 mL samples filtered through a mesh of 20 μm were measured by WATER-PAM (Walz, Germany). Some parameters, such as maximum quantum yield (*Fv*/*Fm*) of PSII, effective quantum yield (ΦPSII), relative rate of electron transport (ETR), and non-photochemical quenching (NPQ), could be read directly from the meter. The fundamental and maximum fluorescence, *Fo* and *Fm* respectively, were obtained under measuring light of 0.01 μmol·m^{-2}·s^{-1} and saturating light pulse excitation (4 000 μmol·m^{-2}·s^{-1} lasting 0.4 s). The potential activity (*Fv*/*Fo*) of PSII was calculated using the equation (*Fm*−*Fo*)/*Fo* = *Fv*/*Fo*$_{PS}$.

2 Results and discussion

2.1 FCM analysis

Table 1 shows the *in vivo* fluorescence intensities at three wavelengths for the two kinds of Arctic microalgae. Fluorescence acquired at 695±20 nm, 585±21 nm, and 530±15 nm is red, orange, and green, respectively. Generally, red fluorescence is generated by chlorophyll, and a generally weak orange by phycobilin or carotenoids. However, green fluorescence is rarely emitted from living microalgae[26]. Based on the intensely red and relatively weak orange fluorescence (Table 1), we conclude that the two kinds of microalgae possessed the characteristics of eukaryotes[27]. The red fluorescence intensity from *P. delicatissima* was much higher than that from *Thalassiosira* sp., whereas the orange fluorescence intensity was lower. These indicate that *P. delicatissima* cells had large amount of chlorophyll *a* but small amounts of pigments emitting orange fluorescence. The green fluorescence intensity was extremely low (<300) and could not be analyzed here.

2.2 Spectrometry

Figure 1 shows the excitation spectra emitted at 680 nm and emission spectra excited at 488 nm for both species: The spectral shapes for the two species were very similar. However, higher fluorescence peaks were observed for *P. delicatissima* than for those of *Thalassiosira* sp. Pigment concentration was in direct proportion to the peak height[21]. The cell abundance for *P. delicatissima* (2.2×10^6 cell·mL^{-1}) was higher than that for *Thalassiosira* sp. (8×10^5 cell·mL^{-1}). This implies a higher biomass (in both cell abundance and chlorophyll *a*) for the *P. delicatissima* cultures than for the *Thalassiosira* sp. cultures.

Table 1 Optical parameters from FCM

Phytoplankton species	FSC-A Mean	SSC-A Mean	PerCP-Cy5.5-A Mean/695±20	PE-A Mean/585±21	FITC-A Mean/530±15
Pseudo-nitzschia delicatissima_cf	72 021	2 265	29 671	9 371	180
Thalassiosira sp.	136 583	4 454	2 491	14 592	292

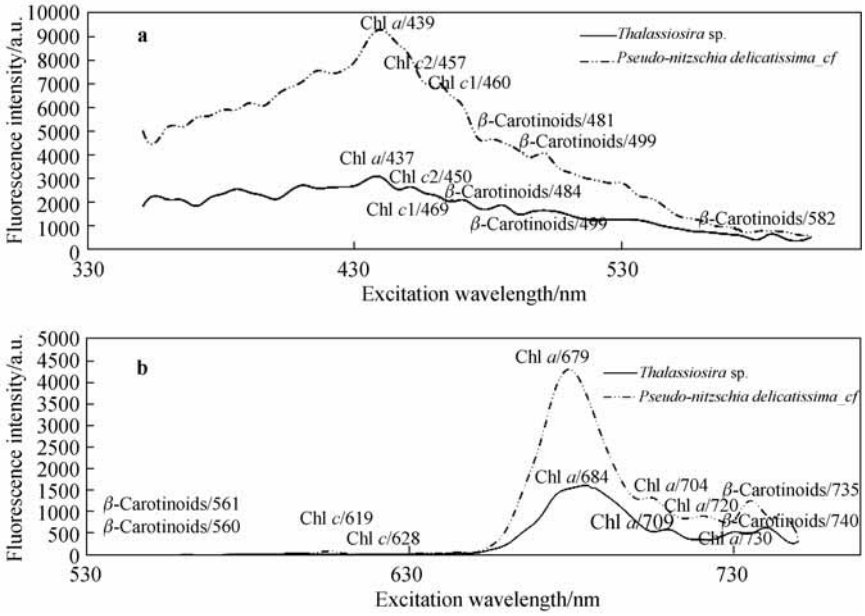

Figure 1 *In vivo* fluorescence spectra from different microalgal cultures. **a**, Excitation spectra. **b**, Emission spectra. Chl *a*: chlorophyll *a*, Chl *c*: chlorophyll *c*.

The maximum intensity on the excitation spectra for both microalgae appears to occur below 450 nm, which is in accordance with the expected values for diatoms[20]. Excitation fluorescence peaks around 460 nm and 500 nm represent chlorophyll *c* and *β*-carotenoid[28]. No fluorescence excitation peak for phycobilin was observed (575 nm). The maximum emission peak of chlorophyll *a* for *P. delicatissima* and *Thalassiosira* sp. appeared at 684 nm and 679 nm respectively. In addition, peaks for chlorophyll *c* and *β*-carotenoid also appeared, but no obvious phycoerithrin peak was detected (575 nm and a shoulder peak at 620 nm). Consequently, both the excitation and emission spectra indicated a lack of phycobilin in the two kinds of phytoplankton.

As can be seen in the above analysis, fluorescence data collected by FCM alone cannot precisely identify pigments in phytoplankton. Table 2 shows the pigment categories corresponding to FCM parameters provided by the fluorescence spectra: the peaks at 695±20 nm and 585±21 nm represent chlorophyll *a* and *β*-carotenoid, respectively. Taking together the data from Table 1, we find that the amount of chlorophyll *a* is higher and that of *β*-carotenoid is lower in *P. delicatissima* than those in *Thalassiosira* sp. The fluorescence intensities of chlorophyll *a* and *β*- carotenoid for each community is the intensity at 695 nm and 585 nm (fluorescence intensities measured by FCM from single cells) multiplied by the cell abundance (Figure 2). The total community fluorescence intensity of these two pigments is high in *P. delicatissima* but their ratio (0.17) is relatively low. This indicates that the *β*-carotenoid of the *P. delicatissima* community is low, in accordance with the results of the fluorescence spectrum (the corresponding ratio is 38.83/62.70). In addition, based on static fluorescence analysis, it can be concluded that the two kinds of phytoplankton are diatoms.

Table 2 Comparison of pigmental fluorescence from FCM between different microalgal species

Phytoplankton species	Chlorophyll *a* (695±20 nm)/10^5	*β*-Carotinoids (585±21 nm)/10^5	Ratio (695/585)
Pseudo-nitzschia delicatissima_cf	432 762	206 162	2.10
Thalassiosira sp.	19 936	116 736	0.17

2.3 Photosynthetic activity

Table 3 shows the photosynthetic parameters obtained by PAM: The fundamental fluorescence intensity (*Fo*) is much

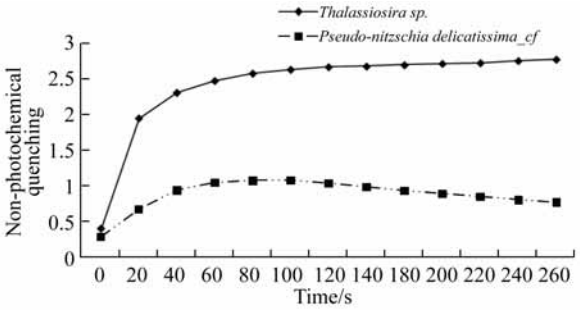

Figure 2 Non-photochemical quenching from different microalgal cultures.

higher in *P. delicatissima* than in *Thalassiosira* sp., which indicates that chlorophyll *a* is higher in *P. delicatissima* cells. These results are consistent with those of the static fluorescence analysis. Lower *Fv*/*Fm* and *Fv*/*Fo* ratios and higher ΦPSII and ETR values are obtained for *P. delicatissima* than for *Thalassiosira* sp. This indicated higher photosynthetic efficiency to the former than to the latter. As shown in Figure 2, the NPQ of the two algae increases significantly with actinic light (412 μmol·m^{-2}·s^{-1}). The NPQ values of *P. delicatissima* increased to the maximum value 1.1 and then gradually decreased. This demonstrated that high energy efficiency is achieved by a vigorous Calvin cycle in the algal cells. As for *Thalassiosira* sp., the NPQ rose to a higher value of 2.7 and subsequently changed little. This indicated that its heat dissipation of PSII is strong. Consequently, there are different light energy utilization processes for the two kinds of algae: *P. delicatissima* uses more energy in photosynthesis, relevant to high biomass (both cell abundance and chlorophyll *a*); and high chlorophyll *a* content keeps the community at a high level of photosynthetic activity. In contrast, *Thalassiosira* sp. spends more energy on heat dissipation than on the process of photosynthesis, exhibiting a positive correlation with low biomass. High levels of *β*-carotenoid are critical to high heat dissipation[29]. This is exactly in accordance with the results of the static fluorescence.

Table 3 Photosynthetical parameters from different microalgal cultures

Phytoplankton species	*Fo*	*Fv/Fo*	*Fv/Fm*	ΦPSII	ETR
Pseudo-nitzschia delicatissima_cf	844	1.187	0.547	0.247	42.8
Thalassiosira sp.	464	2.116	0.679	0.185	33.0

3 Conclusion

Three kinds of fluorescent methods inchuding FCM, fluorescence spectrometry, and dynamic fluorescence of chlorophyll were jointly applied to investigate the physiologisal status of two Arctic *Bacillariophyceae*, i.e., *Pseudo-nitzschia delicatissima* and *Thalassiosira* sp., and the conclusions are as follows:

(1) Cells of *P. delicatissima* contain more chlorophyll *a*, whereas cells of *Thalassiosira* sp. contain more *β*-carotenoid.

(2) *P. delicatissima* uses more energy in photosynthesis; and *Thalassiosira* sp. spends more energy on heat dissipation.

(3) Higher chlorophyll *a* content keeps the *P. delicatissima* community at a high level of photosynthetic activity; and higher *β*-carotenoid is critical to the high heat dissipation during the process of photosynthesis of *Thalassiosira* sp.

(4) The joint application of the three fluorescent methods is effective in analyzing pigmental identification and pigmental mechanisms behind photosynthesis of Arctic phytoplankton.

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