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Steady state and Lifetime Measurements of Primary Fluorescence from Phytoplanktons

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식물플랑크톤 색소의 형광 특성과 lifetime 측정

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The steady state and decay characteristics of primary fluorescenece of phytoplanktons including Cyanophyceae and Cryptophyceae were investigated *in vivo*. At 580~640 *nm* region, fluorescence emission spectra were obtained from all algae examined. The observed fluorescence emission maxima were similiar(± 3 *nm*) except *Synechococcus sp.* (SYN). Considered λ_{max} of emission spectra of phycobiliproteins and the excitation spectra with λ_{max}=540~560 *nm*, it seems to be originated from biliproteins. Fluorescence lifetimes (τ) and decay curves were compared with standard solution of candidate organic compounds, b-phycoerythrin. The τ values obtained for phytoplankton with λ_{max}=580 *nm* were different depending upon the species of algae. The observed τ values were ranged from 1.39 *ns* to 1.95 *ns*. These are considerably shorter than τ(3.23 *ns*) for standard solution of b-phycoerythrin. The reduction of τ for phycoerythrin *in vivo* seems to be originated from effective energy transfer system between Chl. a and phycobiliprotein in intact cell. There are subtantial differences in fluorsecence spectra and lifetimes at the class level. At the species level, differences seems to be much smaller. The result of experiment suggests that measurement of fluorescence lifetimes may be helpful in the rapid characterization of algae. Direct application will likely be found in combination with the measurement of other luminescence parameters.

Introduction

At present methods for identification of algae are based on morphology. However, there have been several examples of confusions in classification and identification of algae based upon visual characterization(Drouet, 1968). Especially, in case of blue-green algae difficulties were found not only because of small size but also their morphological adaptation to their different environment(Humm and Wicks, 1980). In order to investigate a practicality of fluorescence spectroscopic method to detection and identification of algae, fluorescence characteristics of algae were studied. Fluorescence spectroscopic analyses of microorganisms such as bacteria and algae show promise as means of rapid detection and identification(Dalterio et al., 1986; Baek et al., 1988). Advantages include excellent sensitivity and selectivity and non-destructive sampling.

In generl, algae shows strong fluorescence emisson between 320 and 680 *nm* when excited by UV or visible light. The known fluorophores present in algae are chlorophylls(Govindjee and Papageorgiou,

1971), phycobiliproteins(Gantt et al., 1966; Goodwin, 1976) and tryptophan(Baek et al., 1988). They are of special interest because the emission maxima for their fluorescence spectra tend to be widely separated from each other and the fluorophores have a tendency to fluoresce independently. Most previous studies have used chlorophyll a fluorescence to determine only total number of algae present in a sample(Strickland and Parsons, 1968). Only recently, attempts have been made to differentiate phytoplankton on the basis of their fluorescence(Oldham et al., 1985; Yentsch et al., 1979). Oldham et al.(1985) reported the unique and specific combination of pigments of algae are very useful for rapid fluorescence characterization of algae. In fact, many studies have been conducted to relate the pigment composition of algae to their taxonomy(Glazer et al., 1977; Yentsch et al., 1979).

The phycobiliproteins are found in three genera of algae: Rhodophyceae, Cyanophyceae, and Cryptophyceae(Stewart et al., 1974; O'Carra et al., 1976; Robert and Deborah et al., 1987). Most species of Rhodophyceae, Cyanophyceae, and Cryptophyceae contain both a PE(phycoerythrin) and one or more PC(phycocyanin), although a single biliprotein usually predominates(Stewart et al., 1974). An extensive review of algal biliproteins and phycobilins including molecular weights, structure, distribution, fluorescence and absorption properties has been made(O'Carra et al., 1976; Parsons et al., 1984; Robert and Deborah et al., 1987). Specifically, Yentsch et al.(1979) pointed out that the previous lack of emphasis on the identification of organisms by using biliproteins stemmed from difficulties in extracting these pigments intact from algae, and in particular, from blue-green algae. In the native state these spectral properties are considerably altered by the microenvironments of the polypeptide chains compared to those of extracted PE solution. Generally, the diversity of these protein environments makes it possible for a given phycobilin to possess different spectral properties depending on the environment in which it is located. It means that fluorescence spectra of algae *in vivo* can give specific information about PE and its microenvironment.

In this study, in order to maximize differences in primary fluorescence and lifetimes of different species of algae for detection and identification, the fluorescence characteristics of algae were measured *in vivo*, without extraction of pigment which is characteristic for its microenvironments in live organism. Emission spectra of algae *in vivo* have been obtained at about 580 *nm* when selectively excited at about 540 *nm*. At 580 *nm*, the fluorescence lifetimes have been measured. The primary fluorescence observed from algae has been compared to the known fluorescence properties including lifetimes of molecular components which are known for their wide-spread occurrence in algae. Attempts have been made to attribute the algal fluorescence to molecular components known to exist in algae. In addition, the extent to which fluorescence lifetimes can be used in combination with fluorescence excitaton and emission data to identify algae is reported.

Materials and Methods

All phytoplankton were grown at 15℃, in 12:12 light-dark cycle, 80 $\mu mol/m^2/sec$ in a Sherer incubator. These cultures were obtained from the Graduate School of Oceanography, University of Rhode Island, Kingston, RI. The sources for these algae were listed in Table 1. The culture medium was of 30 ppt salinity with 1/2 strength nutrients of Guilliard's 'f' medium(Guillard et al., 1962). Bacterized cultures were made axenic using a mixture of penicillin and streptomycin.

In order to get more biomass of algae per volume, algal samples were either sedimented or centrifuged. After the concentration of the samples, the culture medium was decanted. The algal suspensions used for experimental measurements were prepared by diluting the concentrated algal samples with 0.05 M phosphate buffer(pH 6.6). The phosphate buffer solution was made by adjusting a solution containing 0.025 M Na_2HPO_4 and 0.025 M NaH_2PO_4 to pH 6.6 with additional NaH_2PO_4, while monitoring the pH with a meter. Algal suspensions were always stored at 4℃ and were equilibrated to room temperature(between 20℃ and 23℃) just before the fluoresence measurements were

made.

Distilled water was used to prepare all solutions in this study. Anhydrous Na_2HPO_4 was obtained from J. T. Baker Chemical Company. $NaH_2PO_4 \cdot H_2O$ was obtained from Mallinckrodt, Inc. Anthracene and all other chemicals including b-phycoerythrin were all obtained from Sigma Chemical Co., St. Luis, MO.

In order to prepare the b-phycoerythrin solution, 1 *mg* of b-phycoerythrin was diluted in 6 *ml* of pH 6.8 phosphate buffer solution at 25℃. Since b-phycoerythrin is a light-sensitive chemical, the solution was stored at 5℃ and equilibrated with room temperature(between 20℃ and 23℃) before the steady-state fluorescence and the decay measurements were made.

Steady-state fluorescence spectra were obtained with a Perkin-Elmer MPF-2A spectrofluorometer. Spectra were uncorrected. Quartz cells(1 *cm*) were used for all spectroscopic measurements. Anthracene as external standard was used to monitor instrumental changes for wavelength by its known three emission maxima at 377, 398, and 420 *nm*. The anthracene solution used as standard was 10^{-5} M in MeOH. A typical excitation band width was 10 *nm*.

Fluorescence decay measurements were obtained with a Photochemical Research Associates System 3000. Excitation was provided by a hydrogen flash lamp with a pulse time of 2.5 ns, full width at half maximum. Interference filters with 8 *nm* bandpass were used for wavelength discrimination in the excitation and emission beams. A single-photon-counting detection system was used. To optimize the signal-to-noise ratio, 5000 counts at the peak of the sample decay profile were collected. The excitation-time-intensity profile and the sample fluorescence decay profile were collected in 256 channels in a multichannel analyzer(Tracor-Northern Model TN-7200).

The channel width was 0.352 ns per channel. Deconvolutions were performed on the decay data from the channel containing the maximum number of counts to a channel where the counts were less than 1% of the maximum with a PDP 11/03 computer utilizing PRA software with Decay V 3.0 deconvolution routines(Dalterio et al., 1986).

Results

Emission and Excitation Spectra

The fluorescence characteristics of six different marine plankton algae have been examined. The fluorescence emission and excitation maxima for six algae and b-phycoerythrin in the visible region are listed in Table 2. Depending upon the species of algae, the maxima for emission and excitation bands are different. Figure 1 shows representative algal fluorescence excitation and emission profiles. A characteristic fluorescence emission-excitation spectrum of b-phycoerythrin in pH 6.8 phosphate buffer solution is also shown in Figure 2. The excitation maximum is located at about 520 *nm*. The

Table 1. Sources for algae

	Name	Clone	Source
1.	*Chroomonas sp.*	8C	Hargraves-N. Y.
2.	*Chroomonas sp.*	J6F	Hargraves-(Narragansett Bay)
3.	*Synechococcus sp.*	DC-2	CCMP
4.	*Synechococcus sp.*	SYN	CCMP
5.	*Gloeotrichia sp.*	86W-2010	Ward's*
6.	*cf. Synechococcus sp.*	TPBG	Steele, EPA
7.	*cf. Synechococcus sp.*	CYAN	Steele, EPA
8.	*Spirulina sp.*	PJ/SPIR	Johnson, URI

(*): Purchased from Ward's Natural Science Establishment, Inc.
Class of algae: *Cryptophyceae* : 1, 2
Cyanophyceae : 3, 4, 5, 6, 7, 8

excitation maxima of spectra obtained from six algae are located from 540 to 560 *nm* depending on the species. The emission maxima range from 576 to 640 *nm*. All the emission and excitation spectra are quite broad and featureless.

Table 2. Fluorescence excitation and emission maxima for b-phycoerythrin (b-PE) and algae[a]

Sample	Emission(*nm*)	Excitation(*nm*)
8C	582*[b]	545*
SYN	640*	560*
DC2	580*	542*
86W-2010	579*	560*
PJ/SPIR	576*	541*, 484(*sm*)[c]
J6F	582*	546*, 556(*m*)[d]
b-PE	577*	523*, 566(*sh*)[e]

a: Excitation and emission slits were 10 *nm* for scans. Average two scans, ± 1 *nm*.
b: (*) indicates the most intense band.
c: (*sm*) indicates a small peak.
d: (*m*) indicates a moderate peak.
e: (*sh*) indicates a shoulder peak.

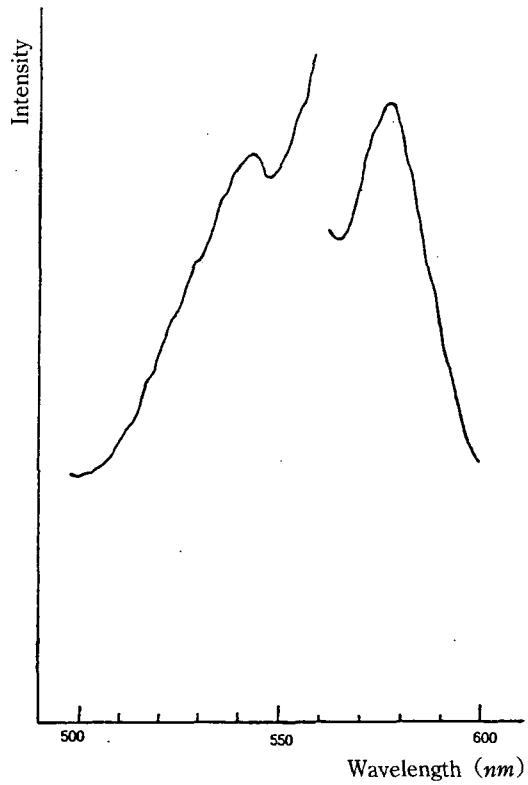

Fig. 1. Fluorescence excitation and emission profiles of PJ/SPIR

Lifetime Data

Table 3. lists the fluorescence decay times of the algae and b-phycoertythrin obtained with the use of 540 *nm* excitation and emission at 580 *nm*. The fluorescence decay times of the algae were characteristic depending upon species of algae. The fluorescence decay of b-phycoerythrin was fit with a monoexponential function($\chi^2=1.08$) with lifetime of 3.23 ns.(Figure 3) In the case of DC2, the fluorescence decay time was measured with the use of 520 *nm* excitation and emission at 600 *nm*. The

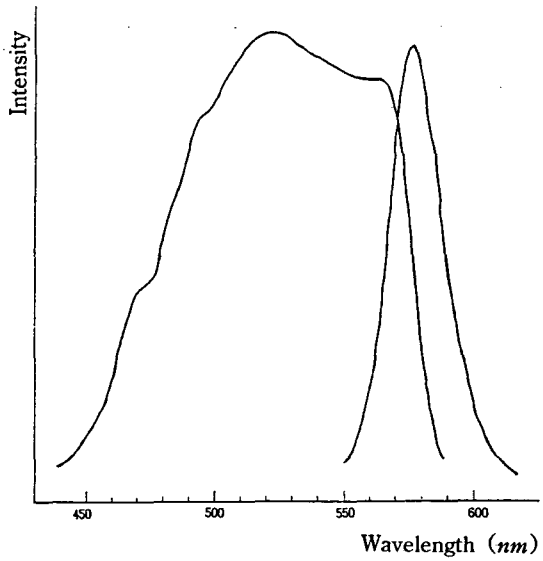

Fig. 2. Fluorescence excitation and emission profiles of b-phycoerythrin (b-PE)

Table 3. Fluorescence decay times(ns) of biliproteins of algae and b-phycoerythrin(b-PE)[a]

Sample	τ_1	τ_2	χ^2
CYAN	1.39	*	1.80
TPBG	0.19	1.77	1.02
DC2[b]	1.95	*	2.93
b-PE[c]	3.23	*	1.08

a: λex=540 *nm*, λem=580 *nm*.
b: λex=520 *nm*, λem=600 *nm*.
c: 1 *mg* of b-PE was diluted in 6 *mg* of pH 6.8 phosphate buffer solution at 25℃.

fluorescence decay times of b-phycoerythrin obtained in this study are also listed in Table 3. For CYAN and DC2, the fluorescence decays were best described by monoexponential decay functions(Figure 4).

The two-exponential decay of TPBG at 580 *nm* emission seems to be an artifact of the instrument since the short lifetime(0.19 ns) is suspect. The limit of the instrument's resolution is about 0.5 ns. Other organisms such as 8C, DC2 and SYN were also examined in the visible region. Even though they have shown measurable fluorescence emission bands at about 580 *nm* when excited at about 540~560 *nm*, the fluorescence intensities were too low to measure meaningful lifetimes. Measurements were difficult because of intrinsically low intensity of the fluorescence and the low intensity of the hydrogen excitation lamp in this particular visible region.

For CYAN and DC2, one-exponential decays were observed with the lifetimes of 1.39 and 1.95 ns, respectively. Although χ^2 values for these measurements are much greater than 1, the fluorescence decays of the algae, CYAN and DC2 were fit by neither two- nor three-exponential decay functions. Difficulties in deconvolution of their decays arise from the fact that the decay curves of algal fluorescence were just sitting on the top of the excitation lamp profile, i.e., similar to lamp decay times. The algae TPBG has shown a two-exponential decay function with a very short lifetime of 0.19 ns and a longer lifetime of 1.77 ns and its χ^2 values were 1.02.

Discussion

Emission Spectra and Excitation Spectra

A primary fluorescence with moderate or weak intensity was observed in the visible region from

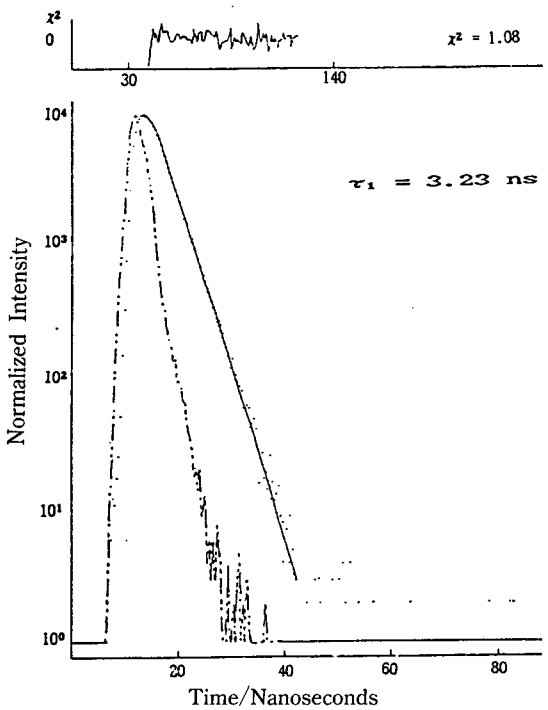

Fig. 3 Flashlamp excitation-time profile(---) and Fluorescence decay from b-PE. Upper plot is weighted residuals for the calculated emission decay profile after deconvolution of the emission decay from channels 41~120.

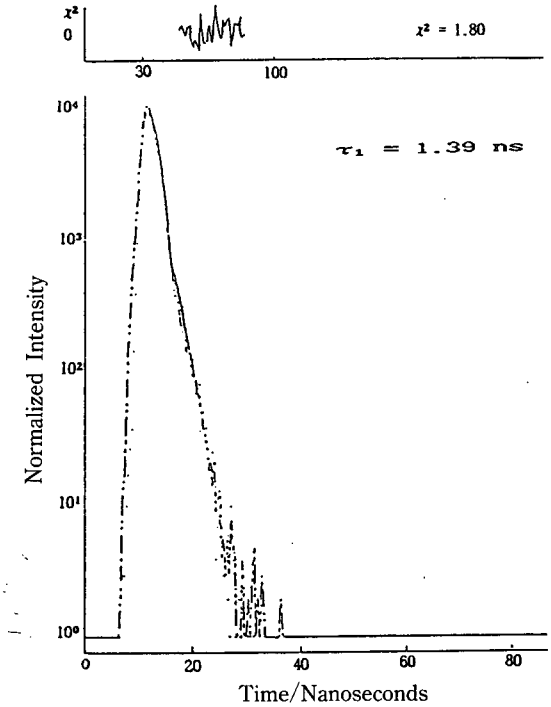

Fig. 4 Flashlamp excitation-time profile(---) and Fluorescence decay from CYAN. Upper plot is weighted residuals for the calculated emission decay profile after deconvolution of the emission decay from channels 34~75.

Cyanophyceae and Cryptophyceae. The emission maxima for these when excited at about 540 *nm* were about 580 *nm* except in one case. Only SYN has shown its emission maximum at 640 *nm* when excited at 560 *nm*. Each species showed measurably different emission and excitation maxima. The excitation maxima were located from 540 to 565 *nm*. The intensities of excitation and emission bands are much less than either those of algal protein tryptophan(Baek et al., 1988). Specifically, the fluorescence at 580 *nm* for most algae is about 30 times less intense than that of algal tryptophan fluorescence(Baek et al., 1988). All the emission and excitation spectra were also quite broad and featureless. Generally, the characteristics of these spectra are very similar to those of the phycobiliproteins, i.e., phycoerythrin and phycocyanin(Rowan, 1981).

Lifetime Data

The shorter lifetime of TPBG may be an artifact of the instrument, since the limit of resolution for the instrument is 0.5 ns. Alternatively, the rapid decay may be due to the fast energy transfer between biliproteins and chlorophyll a. It is well known(Gantt et al., 1973; Gantt et al., 1976) that there are very efficient energy transfer mechanisms between biliproteins and chlorophyll a. This energy transfer mechanism was first suggested by Gantt et al.(1973). By the development of picosecond time-resolved fluorescence spectroscopy, Porter et al.(1978) measured lifetimes of phycobiliproteins *in vivo*, and proposed an erergy transfer schems as PE→PC→APC→chlorophyll a by Föster mechanism involving dipole-dipole interaction. Additional evidence has been collected by observation of their consecutive rise time and sensitization by PE with excitation at 530 *nm* which transferred to chlorophyll a. Thus, the shorter lifetimes of TPBG may originate from the fast energy transfer of PE to either PC or chlorophyll a *in vivo*.

As shown in case of chlorophyll a(Govindjee et al., 1986), not all PE in the organism may participate in energy transfer. Then two different types of PE and give two different lifetimes. In phosphate buffrer solution, b-PE has shown a fluorescence lifetimes of 3.23 ns.

The origin of the fluorescence observed at 580~640 *nm* is undoubtedly biliproteins, especially phycoerythrin and phycocyanin. The assignment can be made easily, because the unique fluorescence emission in the visible region of biliproteins does not overlap with that of other fluorophores and the existence of biliproteins in algae, especially in Cyanophyceae and Cryptophycea, is well-known(Govindjee et al., 1986). The lifetimes of biliproteins, especially that of PE, are shorter than the b-PE in pH 6.8 buffer solution. However, as mentioned above(Gantt et al., 1973; Robert and Deborah, 19 87), the efficient energy transfer which exists between biliproteins and chlorophyll a may be the reason for the shorter lifetimes of PE in live algae.

Conclusions

A standard solution of b-PE has shown a fluorescence lifetimes of 3.23 ns. Moderate fluorescence between 580~640 *nm* has been observed from all algae examined. This fluorescence is assigned to phycobiliproteins. The decay characteristics of the algal fluorescence at around 580 *nm* were described as either one- or two-exponential decay functions. The fluorescence lifetimes of algae were shorter than the corresponding fluorescence lifetimes of b-PE in pH 6.8 phosphate buffer solution. The shorter lifetimes are believed to originate from the energy transfer between biliproteins or between biliproteins and chlorophyll a in live organisms.

Clearly, the fluorescence characteristics at about 580 *nm* can be used to distinguish algae of different classes from Cyanophyceae and Cryptophyceae. Also, the observed fluorescence from phycobilin will give additional parameter for rapid detection and identification of microorganism. Possibilities of the use of this technique in remote sensing should be explored along with microfluorescence applications.

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식물플랑크톤 색소의 형광 특성과 lifetime 측정

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남조류와 crytomonads를 포함한 식물플랑크톤의 일차적 형광특성을 *in vivo* 상태에서 조사하였다. 형광과 exciatation 스펙트럼을 측정한 결과, 약 580~640 *nm* 영역에 걸친 형광 스펙트럼이, 조사된 모든 식물플랑크톤으로 부터 얻어졌다. 관찰된 형광 스펙트럼의 λ_{max} 값은, *Synechococcus sp.*(SYN)을 제외하고 모두 유사한 것으로 나타났다(±3nm). 관찰된 형광 스펙트럼의 형광소(fluorophore)를 밝히기 위하여, 식물플랑크톤의 형광 스펙트럼의 세기와 모양, λ_{max}를 식물플랑크톤에 존재하는 유기화합물의 표준용액의 것과 비교하였다. 식물플랑크톤의 fluorescence lifetime(τ)과 fluorescence decay curve를 식물플랑크톤에 존재하는 유기화합물의 표준용액의 τ값과 비교하였다. 580 *nm*의 형광 파장을 사용하여 얻은 식물플랑크톤의 fluorescence decay는 monoexponential과 biexponential decay를 보였으며, 식물플랑크톤의 τ(1.39~1.95ns)값은 b-phycoerythrin의 표준용액의 τ(3.23ns)값보다 현저히 작게 나타났다. 이는 이미 알려진 광합성 능력을 가진 intact cell 내부에서 엽록소 a와 biliprotein간의 효율적인 에너지 전달 체계에 의한 τ값의 단축으로 여겨지며, 580*nm*의 형광특성이 biliprotein에 의한 것임을 보여준다.