The *Journal of Marine Research* is an online peer-reviewed journal that publishes original research on a broad array of topics in physical, biological, and chemical oceanography. In publication since 1937, it is one of the oldest journals in American marine science and occupies a unique niche within the ocean sciences, with a rich tradition and distinguished history as part of the Sears Foundation for Marine Research at Yale University.

Past and current issues are available at [journalofmarineresearch.org](http://journalofmarineresearch.org).

Yale University provides access to these materials for educational and research purposes only. Copyright or other proprietary rights to content contained in this document may be held by individuals or entities other than, or in addition to, Yale University. You are solely responsible for determining the ownership of the copyright, and for obtaining permission for your intended use. Yale University makes no warranty that your distribution, reproduction, or other use of these materials will not infringe the rights of third parties.

This work is licensed under the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. To view a copy of this license, visit [http://creativecommons.org/licenses/by-nc-sa/4.0/](http://creativecommons.org/licenses/by-nc-sa/4.0/) or send a letter to Creative Commons, PO Box 1866, Mountain View, CA 94042, USA.
Fluorescence spectral signatures: The characterization of phytoplankton populations by the use of excitation and emission spectra

by Charles S. Yentsch¹ and Clarice M. Yentsch¹

ABSTRACT

The early trichromatic method of chlorophyll analysis featured the possibility of distinguishing abundance of chloroplastic pigments specific to phyla of algae in the population. The method was not successful however, because in natural populations there are major interferences due to pigments or their degradation products.

Much of this can be circumvented using *in vivo* fluorescence techniques. The advantages are that fluorescence from chlorophyll *a* can be excited by wavelengths specific to accessory pigments such as fucoxanthin in diatoms, chromoproteins of the bluegreens and peridinin in the dinoflagellates with only a negligible amount of wavelength overlap. The technique appears to be promising for gross characterization of phytoplankton populations and would be a valuable addition to *in situ* studies where continuous monitoring is employed.

1. Introduction

This paper demonstrates that a characterization of phytoplankton populations using specific excitation and emission wavelengths of fluorescent light is possible. The concept: “characterization of populations by means other than a microscope” is not new. It was the basis for the development of the trichromatic method for measuring chloroplastic pigments. Knowledge of the various photosynthetic pigments for different algal groups is essential (Fig. 1). The trichromatic method, as well as the method proposed here, bears only a superficial resemblance to classical taxonomy.

Why use the technique of fluoresced light for the characterization? Are there any advantages over older techniques? First and maybe foremost, its use allows a possibility of continuous monitoring. This has been demonstrated by remotely sensing algal blooms by multiwavelength laser induced fluorescence (Mumola *et al*., 1975). Also, fluorescence has been useful in monitoring the growth of algal cultures (see Mook, 1970). Secondly, the major groups of algae have quite different excitation spectra for chlorophyll *a* fluorescence emission. Further, wavelength bands specific

¹. Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, 04575, U.S.A.
to each group are widely separated in the spectra—which, for the most part, mini-
mized the effect of overlap in either excitation or emission wavelengths. Thirdly,
the presence of degraded chlorophylls, namely phaeophorbide and/or chlorophyll-
ide, is not a problem since they are very weakly fluorescent in vivo (Yentsch, 1974).
This is important since in extracts of algal material, the degraded pigments are
highly fluorescent and create errors in light absorption.

There are problems associated with the use of fluorescence for characterization:
1) there is a difference in the total amount of light fluoresced from different algae
(see Heaney, 1978) which is possibly an optical effect associated with the composi-
tion of the cell wall (Lorenzen, 1966; Loftus and Seliger, 1975); and 2) variability
in the amount of fluoresced light is associated with physiological state which is
largely in response to nutrient and light stress (Flemor, 1969; Berman, 1972; Kiefer,
1973 a, b, c; Blasco, 1973; Schimura and Fujita, 1975; Heaney, 1978). These re-
searchers point out some of the problems encountered in the attempt to relate in vivo
fluorescence and chlorophyll content. To date, the uncertainties of this approach
have not caused workers to discontinue its use.

The research reported here was designed to establish the specifics of spectra for
different algae in the oceans in an attempt to evaluate the potential for distin-
guishing among; a) diatoms, dinoflagellates and green algae, b) diatoms and dinoflagellates
and c) procaryote and eucaryote algae having phycobilin pigments.

2. Methods
The unit used for spectral measurements of fluorescence was built by Baird-
Atomic and consists of two double beam monochrometers, a xenon light source and a red sensitive photomultiplier. Output from the photomultiplier is fed to an $x$-$y$ recorder which traces the excitation or emission spectrum as selected by the operator.

The amount of algae is generally too low in raw seawater samples to obtain reliable spectra. To obtain the necessary sensitivity, 1 to 5 liters of water is filtered through a Gelman type A glass fiber filter 2-5 cm in diameter. The filter is held in a moist condition upright in the light beam by a square of rigid black plastic.

The fluorescence data have not been corrected for spectral change resulting from lamps, photomultiplier, and/or the monochromators for the following reasons: 1) we anticipate that for continuous measurement, different lamps and broad-band pass filters will be used, hence calibration of fluorescence emission will be similar to that presently used in the *in vivo* chlorophyll fluorescence method (Lorenzen, 1966); 2) the excitation region of concern is between 400-530 nm, where spectral corrections for the xenon lamp emission through the excitation monochromater are slight. In some cases, the attenuation spectra were obtained by the method described by Yentsch and Truper (1967). Essentially the filters (a moist blank and sample) are held upright in the light beam of a Beckman DK-1A spectrophotometer.

When reported, chlorophyll measurements were made by the method of Yentsch and Menzel (1963). The chlorophyll $a$ standard (source: spinach) was obtained from Sigma. A few measurements reported have resulted from use of the Turner fluorometer with interference filters (band width $\pm 10$nm) with lamps and secondary filters the same as described.

With regard to the pigment terminology used in this text, "carotenoid-protein-complex" refers to carotenoids such as fucoxanthin and peridinin which have markedly altered spectra when denatured. We use the term "chromoprotein" or "bile-protein" when referring to the water-soluble pigments phycoerythrin or phycocyanin.

3. Results

*Fluorescence excitation and emission spectra from algae cultures and marine isolates.* Among eucaryotic algae there is considerable difference in the wavelengths of excitation for chlorophyll $a$ fluorescence between organisms with a carotenoid-protein-complex and those without. This distinction can clearly be seen by comparing excitation spectra for diatoms, dinoflagellates, and green algae (Fig. 2). The major difference is that the presence of fucoxanthin in diatoms or peridinin in dinoflagellates allows chlorophyll $a$ to be excited efficiently, at wavelengths of 525-530 nm in addition to wavelengths of 450 nm. The absence of the carotenoid-protein-complex in green algae confines the spectrum of excitation to the region of 450 nm.

2. The carotenoid siphonaxanthin has been found in some benthic green algae: its excitation spectrum is similar to that for fucoxanthin (Kageyama, 1977).
Figure 2. The excitation spectra for chlorophyll $a$ fluorescence for three groups of algae. Diatoms: 1) *Skeletonema costatum*, 2) *Biddulphia mobiliensis*; Dinoflagellates: 3) *Gonyaulax tamarensis*, 4) *Peridinium* sp.; and Chlorophytes: 5) *Dunaliella tertiolecta* and 6) *Ulva lactuca*.

In all of the so-called phaeophytes tested, the ratio 530:450 nm averages around 0.85.

The marked difference between diatoms and green algae is shown in the spectral overlay in Figure 3. Note the difference in the amount of chlorophyll $a$ fluorescence obtained from using excitation light of wavelengths at 450 and 530 nm in green algae and diatoms. In green algae, wavelengths of 530 nm are very ineffective, whereas for the diatoms they are highly effective. Included in this overlay is a spectrum for a natural population. The amount of chlorophyll $a$ fluorescence obtained by excitation at 530 nm appears to be intermediate between the range set by diatoms and green algae. In suspensions of the two types of algae, the proportions can be assayed using the 530:450 nm ratio. This is demonstrated in Figure 4, where the ratio has been measured in varying suspensions of a diatom and green alga in the Turner fluorometer fitted with interference filters to isolate wavelengths of excitation light at 450 nm and 530 nm for chlorophyll $a$ fluorescence.
Figure 3. Overlay of excitation spectrum for chlorophyll a fluorescence for a diatom (Skeletonema costatum) and a green flagellate (Dunaliella tertiolecta), and a natural population collected in the coastal waters of the Gulf of Maine.

We have been unable to demonstrate any distinctive difference in the excitation spectra of diatoms as opposed to dinoflagellates (see Fig. 5a), which is a disappointment, as we had hoped to find a simple means of distinguishing between these. Too, this is surprising, since cultures of dinoflagellates (e.g. Gonyaulax tamarensis) appear more red-brown than do cultures of diatoms (e.g. Skeletonema costatum). This difference in color is borne out by their in vivo absorption spectra (Fig. 5). When

Figure 4. Relationship between chlorophyll a fluorescence excitation ratio 530:450 nm and the percentage of green algae (Dunaliella) or diatoms (Skeletonema) in the suspension. Zero percent on the lower axis refers to 100% diatoms whereas 100% on this scale refers to 100% green algae. Dots and triangles are two separate experiments. The lines are fitted by eye.
the two organisms are compared on an equal chlorophyll basis, the difference spectra (Fig. 5) show that the dinoflagellate is a better absorber of light at wavelengths longer than 500 nm and is particularly effective in the near u-v. This additional absorption does not appear to participate in the excitation of chlorophyll $a$ fluorescence.

Algae with phycobilin pigments, mainly phycoerythrin, are frequently reported in the oceans. In the open sea, the bluegreen *Oscillatoria erythraea* (*Trichodesmium*) is at times very abundant. In coastal waters, blooms of the ciliate with a cryptomonad symbiont, *Mesodinium rubrum*, are abundant. At this time, the excitation and emission characteristics for an isolated marine cryptomonad are not available. However, the spectral characteristics of phycoerythrin from bluegreens and cryptomonads appear to be identical. Figure 6 contains the absorption characteristics and fluorescence excitation and emission of *Oscillatoria erythraea* for whole cells (filaments) and phosphate buffer extracts. This alga was collected in the central region of the Gulf of Mexico by towing a 35 $\mu$m plankton net through a surface patch. From this net sample, individual filaments were pipetted onto a filter and the spectra measured as described previously. The fluorescence from this organism is dominated by the presence of phycoerythrin (Moreth and Yentsch, 1970).

*Spectral signals in natural populations.* From observations using the light micro-
scope, one would predict that the carotenoid-protein-complex spectra should dominate spectral signatures for natural populations. Our observations (about 100 measurements of fluorescence spectra, mainly on populations in the New England coastal waters) support this. Examples are presented in Figure 7. In these cases, the excitation spectra are characterized by two bands; one at 450 nm and the other at 525-530 nm. The emission spectra are at times characterized by two peaks; one from chlorophyll $a$ (680 nm) and the other due to phycoerythrin at 560-570 nm.

In addition to these measurements, observations in the Gulf of Mexico provide a
comparison between populations in eutrophic coastal regions to populations in oligotrophic "blue water" offshore. The spectral examples of these extremes are shown in Figures 8 and 9. Again, the excitation spectra of the coastal populations are distinguished by the broad region of excitation with peaks of 450 and 530 nm. By contrast, the 530 nm excitation band is not pronounced in the offshore populations. Figure 10 is a plot of the surface chlorophyll content (ug/1) against the 530:450 ratio for all stations sampled in the Gulf. The changes in chlorophyll content (0-7.0 ug/1) indicates the range between oligotrophic to eutrophic conditions present in the Mississippi Delta area. This plot suggests that offshore populations absorb light at 530 nm less effectively than do the eutrophic populations of coastal waters.
Figure 10. Excitation ratio (530:450 nm) plotted against chlorophyll biomass (µg/1) for stations in the Gulf of Mexico. Observations are for surface waters only. Stations having low chlorophyll concentrations are from the oligotrophic waters of the central region, while the high chlorophyll values are from around the Mississippi Delta.

The suggestion is that eutrophic populations are dominated by organisms rich in fucoxanthin and/or peridinin, while the organisms in the oligotrophic open Gulf are not. This hypothesis is supported by microscopic examination of cell concentrates. The eutrophic populations were dominated by diatoms and dinoflagellates (El-Sayed, personal communication). Note that the transition between eutrophic and oligotrophic conditions in Figure 10 is not linear, but hyperbolic, which would suggest that as conditions progress from oligotrophic to eutrophic, the populations “saturate” in terms of the dominance of species which absorb light more efficiently at 530 nm. Note too that phycoerythrin (see Fig. 8) was present in most of the samples. The level varied considerably, and showed no pattern consistent with the total biomass of chlorophyll.

Spectral signatures as related to algal classification. The surprise here concerned fluorescence emission, specifically the occurrence and persistence of the phycoerythrin emission at 575-580 nm. This emission was observed more often than not. It was present in local Boothbay waters during the winter and spring months (Fig.
Table 1. The fluorescence excitation and emission wavelengths specific to chromatic groups in marine phytoplankton.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Wavelength (nm)</th>
<th>Accessory pigments of chromatic group</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
<td>Mainly accessory chlorophylls</td>
<td>all algae</td>
</tr>
<tr>
<td>2</td>
<td>525-530</td>
<td>Carotenoid proteins, i.e. fucoxanthin</td>
<td>diatoms and dinoflagellates</td>
</tr>
<tr>
<td>3</td>
<td>490*</td>
<td>Biliproteins, i.e. phycoerythrin</td>
<td>blue-greens and cryptomonads</td>
</tr>
</tbody>
</table>

* This is not the maximum excitation wavelength for phycoerythrin. It has been selected by Moreth and Yentsch (1970) for use in simple fluorometers using broad band colored filters.

7) and was observed in populations off the Mississippi Delta. One suspects that the previous lack of emphasis on identification of organisms with biliproteins stems from the difficulties in identifying small organisms and their destruction during preservation. These observations of fluorescence are useful in demonstrating that certain species need study by taxonomists. It is also clear that the phytoplankton ecologist should be measuring phycoerythrin as intensely as one does chlorophyll a.

In a qualitative fashion, it is easy to distinguish the presence or absence of organisms with or without the carotenoid-protein-complex. Moreover, it is clear that the spectral signal for phycoerythrin is a distinctive feature which can be utilized to assess organisms that contain biliproteins. Table 1 summarizes the specific wavelengths for detection of two major groups of accessory pigmentation and relates these to different groups of algae. Scheme 1 is the conventional measurement of chlorophyll a fluorescence for all algae. In scheme 2 the excitation measurement at 530 nm allows the estimate of carotenoid proteins, whereas scheme 3 allows for the measurement of organisms using biliproteins. Modern systematics does not put that much emphasis on the presence or absence of accessory carotenoids and/or phycobilin pigmentation. What then can be said about the chlorophytes? The key to their detection centers around the measurement of chlorophyll excitation at 530 nm or more specifically the ratio of excitation at 530:450 nm. But, in natural populations, this ratio is complicated by the presence of cryptomonads and bluegreens which have biliproteins and not carotenoid proteins. Certainly, the absence of phycoerythrin emission can be construed to mean that no cryptomonads or cyanophytes are present, hence, chlorophytes and organisms with carotenoid proteins are the only organisms influencing excitation at 530 nm. Thus, by using the table, one has the initial basis for estimating the relative proportions of diatoms and dinoflagellates versus cryptomonads and bluegreens.

The case for quantitative estimates. The major problem of relating in vivo chlorophyll fluorescence to chlorophyll biomass is that the amount of light fluoresced per unit
of chlorophyll is highly variable. This was first documented in early studies of photosynthesis and was demonstrated to be related to changes in photosynthetic rate (see Rabinowitch, 1951). In a very general sense, it can be said that factors such as light or nutrient stress, which change the rate of photosynthesis, are reflected in the amount of fluorescence. Therefore, it is our opinion that utilization of ratios of excitation, whenever possible, is a better approach than strictly trying to compare emission after excitation with a measured quantity of chlorophyll. In our opinion, this ratio will be important to those interested in comparing areas of productivity in the oceans, and those interested in pollution problems.

The approach presented here features the measurement of fluorescence emission by phycoerythrin. When phycoerythrin fluorescence is measured at 570 nm, overlap is not a problem since the only pigment in this region, chlorophyll $a$, is weakly fluorescent at these wavelengths. One of the goals of the method must be to obtain a “pure” 530:450 nm ratio (no phycoerythrin absorption) in mixed populations. The notations for these corrections to the ratio are:

1) $E_{x450} = E_{x (cht')} + E_{x (phy)} \rightarrow E_{m450}$

2) $E_{x530} = E_{x (fuco)} + E_{x (phy)} \rightarrow E_{m530}$

where $E_{x (cht')}$ is the excitation due to light absorbed mostly by Soret bands of chlorophyll, $E_{x (phy)}$ and $E_{x (fuco)}$, the excitation from light absorbed by phycoerythrin and fucoxanthin respectively. The pure ratio is equal to:

3) \[
\frac{E_{x530} - E_{x (phy)}}{E_{x450} - E_{x (phy)}}
\]

Corrections for pigment overlap are simple in a system where the wavelength of excitation and emission can be measured by use of monochromators. One needs to select the fluorescence peaks of concern and measure the wavelengths of excitation to ascertain their contribution in light absorption. For continuous monitoring of phytoplankton in the open ocean, one anticipates the use of a series of fluorometers or a new fluorometric instrument for detecting emission and inducing excitation at several wavelengths.

In an attempt to characterize populations continuously by modifying conventional flow-through fluorometers, we have fitted these with interference filters to define the wavelengths of fluorescence emission and excitation. This system lacks sufficient sensitivity for open ocean studies. We are presently attempting to improve the sensitivity by the use of broader band sharp-cut dichroic filters. The system is calibrated in terms of the extracted chlorophyll $a$ and extracted phycoerythrin (Moreth and Yentsch, 1970).

For remote sensing, previous studies have shown that the attenuation—reflection spectral signal from particulate matter in natural waters is complicated by the presence of detritus (see Yentsch, 1962). Since most imaging satellite sensors are based
on the reflectance mode, it does not seem feasible at this time to sense accessory pigmentation from satellites. However, low flying aircraft or helicopters can use the multi-wavelength lasers (pioneered by Mumola et al., 1975) and thus can be a valuable addition to remote sensing characterization.

Acknowledgments. This research was supported by funds from the National Aeronautics and Space Administration, the National Science Foundation and the Food and Drug Administration. Technical assistance was given by Paul Sherman, Dave Hughes, Jim Rollins, and Vicki Jones. The paper is Bigelow Laboratory Contribution Number 78025.

REFERENCES


Received: 26 September, 1978; revised: 21 April, 1979.