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**SPECTROFLUOROMETRIC DIFFERENTIATION
OF THE RED TIDE ALGA, GONYAULAX TAMARENSIS
FROM OTHER ALGAE
COMMON TO NEW ENGLAND WATERS**

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Report No. MITSG 77-18

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ABSTRACT

Cultures of Gonyaulax tamarensis were subject to spectrofluorometric analyses for discrimination from common endemic algae of the New England area for possible use in an airborne spectrofluorometric system. Extracts of G. tamarensis and other dinoflagellates display a fluorescence emission different from other algae tested. Live cultures did not display the unique emission with a laser of excitation source limited frequencies. Detection limits of G. tamarensis by chlorophyll a determination is 60 cells per milliliter in live cultures.

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LIST OF SYMBOLS

cm	centimeter
ft.	feet
m	meter
m ³	cubic meter
mg	milligram
nm	nanometer
PSP	paralytic shellfish poisoning
mu	millimicrons
V	volts
W	watts

A. INTRODUCTION

Outbreaks of paralytic shellfish poisoning (PSP) have occurred in several localities for many years. These outbreaks pose serious public health problems which require the closing, by patrol, of productive shellfish areas, removal of shellfish from markets, and publicity efforts to reduce shellfish consumption. Preventative action creates economic disaster, not only for the shellfish harvester, but for the whole seaside community.

Present monitoring efforts for PSP are slow, expensive, and do not provide sufficient information for forecasting of outbreaks. They depend upon shellfish collection and subsequent bioassay to determine if PSP is present. By the time the test is positive, irreversible damage has usually been done and the beds must be closed. Prakash (1971) and others have demonstrated that PSP is due to a dinoflagellate toxin (saxitoxin) that accumulates in shellfish as they feed upon the dinoflagellate algae. In the New England areas the dinoflagellates are specifically Gonyaulax tamarensis. Since a correlation exists between G. tamarensis and PSP, Prakash (1971) suggests that a direct measurement of the algal population would give useful information concerning the toxicity of a shellfish bed.

Spectrofluorometry is a technique in use for identifying specific organic compounds by their ability to absorb light at one wavelength and to re-emit it at another. Techniques have been developed by Yentsch(1963) as others using spectrofluorometry as a tool to measure

chlorophyll production in ocean waters. Since the Red Tide dinoflagellates, G. tamarensis, are plants which have light sensitive pigments, it could be possible to define their presence rapidly over wide areas in the sea by an airborne spectrofluorometer. Several airborne spectrofluorometers using fixed wavelength lasers have been described by O'Neil et. al. (1975), Kim (1973) and Mumola (1973). These units were designed to detect oil and chlorophyll.

Several different approaches to algal discrimination are presented in the literature. Mumola (1973) states in his development of techniques for chlorophyll a detection that the excitation cross-sections, or the pattern of light absorbed by the algae which later is re-emitted as fluorescence emission, differs for algal color groups, although overlaps occur. Horning and Eastwood (1973) present similar views. O'Neil (1975) discusses the possibility of time discrimination by differences between the time of the entrance of the excitation light into a fluorescence molecule and the time of the exit of the emitted fluorescence. Udenfriend (1962) describes the differences of fluorescence emission with respect to differences in plant pigmentation.

The utilization of airborne spectrofluorometry to identify a specific alga such as G. tamarensis or the group to which it belongs, the dinoflagellates, would be a powerful tool for two reasons. The first and more desirable, from a public health viewpoint, would be the development of monitoring techniques for contaminated shellfish beds. This is estimated to require the sensitivity to detect about 10 contaminating algae cells per milliliter (ml) of water (Al Sylvia -

personal communication). Sensitivity is needed, because with time, the shellfish will concentrate the PSP to dangerous levels. A second advantage would be the ability to map the areal distribution of algal blooms, defining the likely movements of algal masses along shorelines and into inshore regions.

This paper presents an approach to the problem of the differentiation of Gonyaulax tamarensis from some other common endemic algae of the New England area. Fluorescence emissions are discussed, sensitivities of detection are stated, and limitations imposed by interfering substances in possible field use are mentioned.

B. METHODOLOGY

1. Instrumentation

A Perkin-Elmer 204 A Fluorescence Spectrophotometer with a 150 W xenon lamp was used with spectrally matched cuvettes. The photomultiplier used was an R-107, which has sensitivity to approximately 710 nm. The entrance and exit slits in the Model 204 are fixed at 10 nm (nanometers). Wavelengths reported are uncorrected for spectral response. For studies into regions where the desired emission wavelength was greater than 700 nm, or different slitwidths were desired, a Perkin-Elmer MPF-2A Fluorescence Spectrophotometer was used with a R-136 photomultiplier tube, which gives better sensitivity in the near infrared region.

To increase light intensity a Spectrophysics Model 164 argon laser was used. Frequencies were fixed to 460 nm, 476 nm, and 488 nm. Optical filters, Wratten 12 and 23A employed were tested for fluorescence, but none was noted.

In Appendix 1 the specifications of the Model 204 Fluorescence Spectrophotometer are presented.

2. Cultured Algae

Cultures of algae were obtained from Dr. Christopher Martin of the University of Massachusetts Marine Station at Gloucester, and Dr. Robert Guillard of Woods Hole Oceanographic Institution. When the cultures were analyzed directly with the spectrofluorometer blanks of water filtered from the culture were used for comparisons.

3. Acetone Extracts of Algal Pigments

Acetone extracts of algal pigments were obtained by filtering a species of microscopic algae on a 3 micron Nuclepore filter and placing the filter in 10 ml of acetone. Macroscopic algae were ground in a Waring blender, filtered and similarly placed in 10 ml of acetone. Blanks for background comparisons and possible fluorescence by the Nuclepore filter were prepared by placing a Nuclepore filter in 10 ml of acetone from the same source that the algal extracts were prepared.

4. Frozen Extracts

Gonyaulax tamarensis cells were exposed to freezing temperatures for at least 24 hours in order to break the cell walls and extract pigmentation. Blanks were prepared from the water in which the algae were growing.

C. EXPERIMENTAL DETERMINATIONS OF FLUORESCENCE EMISSION PEAKS

Live cultures of Gonyaulax tamarensis were analyzed or scanned for spectral activity. Initially, due to the light scattering properties of G. tamarensis cells acting similarly like colloidal particles, a great deal of spectral noise, or the reflections of non-monochromatic light from the xenon excitation lamp passing by the monochromator, occluded any meaningful signals even from the relatively strong fluorescence of chlorophyll (see Figure 1). This spectral noise was reduced two ways: 1) by the extraction of the plant's pigmentation by chemicals and freezing, 2) the use of an optical filter.

Acetone extracts of the G. tamarensis pigmentation, following the procedure of Yentsch(1963), revealed two reproducible and distinguishably separate peaks (Figure 2). One peak occurs at 670 nm and was identified as the chlorophyll a peak. The other peak at 640 nm is an accessory pigment, which in extracts, is unique to the dinoflagellates tested and can be separated by changing the excitation frequency to 450 nm (Figure 3). The 640 nm peak did not occur with other algae tested. Table 1 lists the algae tested. Figures 4 to 14 show results of scans for both extracts and live cultures. Although the list is not extensive, it is representative of the algae of different color groupings which are very likely to be found in the New England waters.

Scans of extracts of other spectral regions were made possible by the reduction of scattering noise and the subsequent establishment of

TABLE 1. List of Algae Tested

<u>Algae</u>	<u>Phylum</u>
Skeltonema costatum	Bacillariophceae
Cylindrotheca (Nitzschia) closterium	"
Nannochloris atomus	Chlorophyceae
Unnamed Genus	Eustigmatophyceae
Chroomonas	Cryptophyceae
Platymonas (Tetraselinis) succica	Prasinophyceae
Fucus	Phaeophyta
Ascophyllum	"
Enteromorpha	Chlorophyta

FIGURE 1.

EXCITATION 410 nm
EMISSION SCAN nm

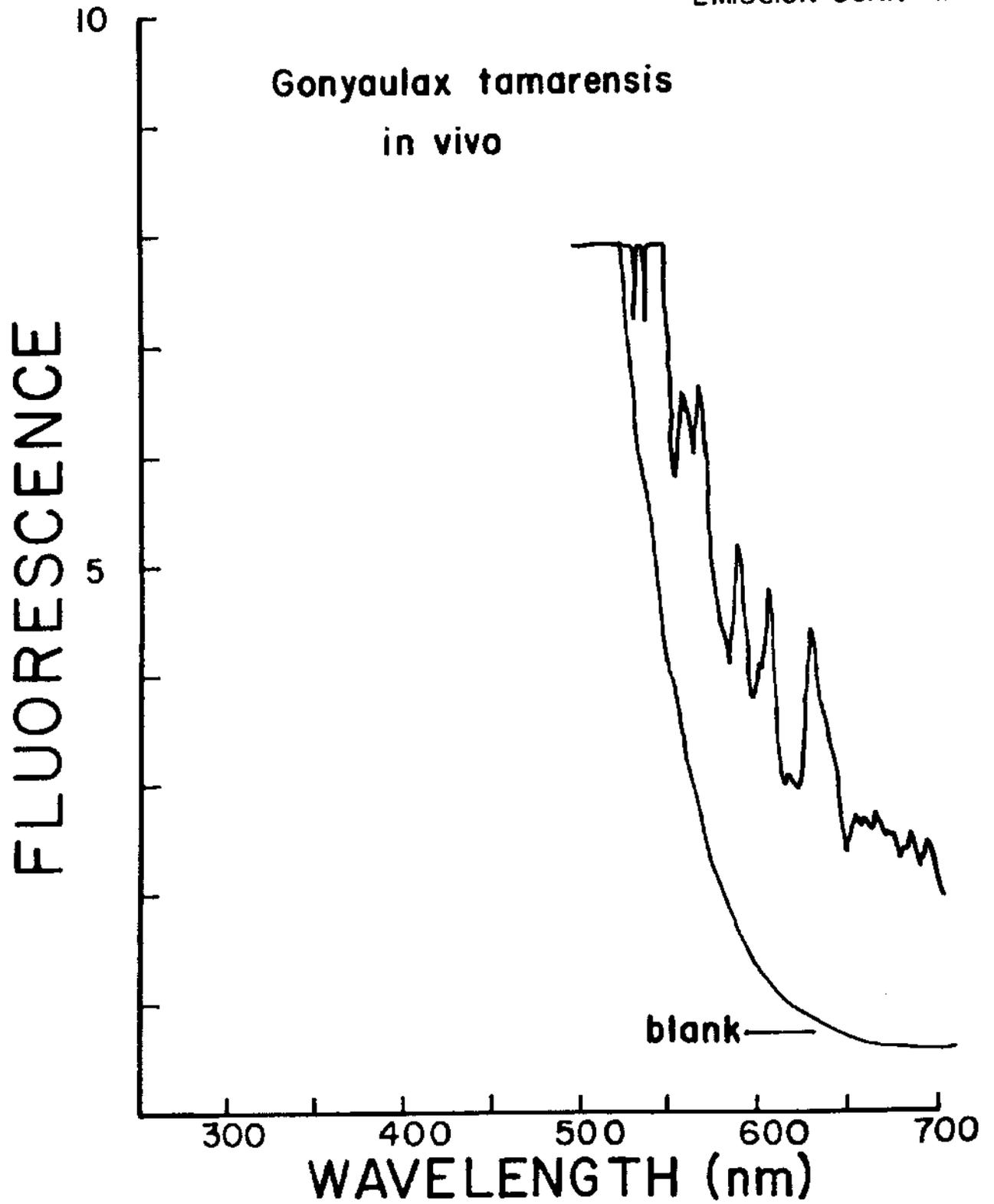


FIGURE 2.

EXCITATION 410 nm
EMISSION SCAN nm

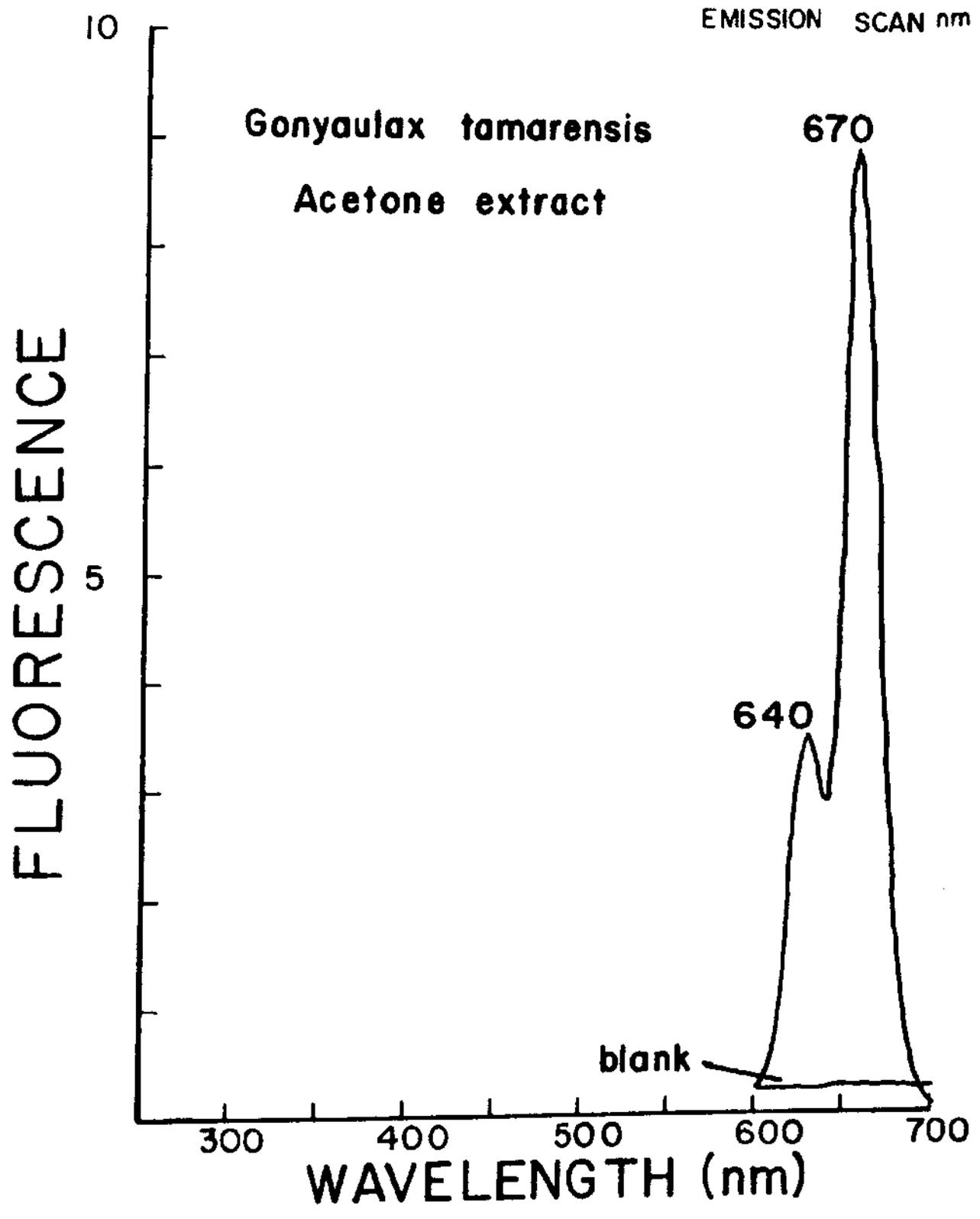


FIGURE 3.

EXCITATION 450 nm
EMISSION SCAN nm

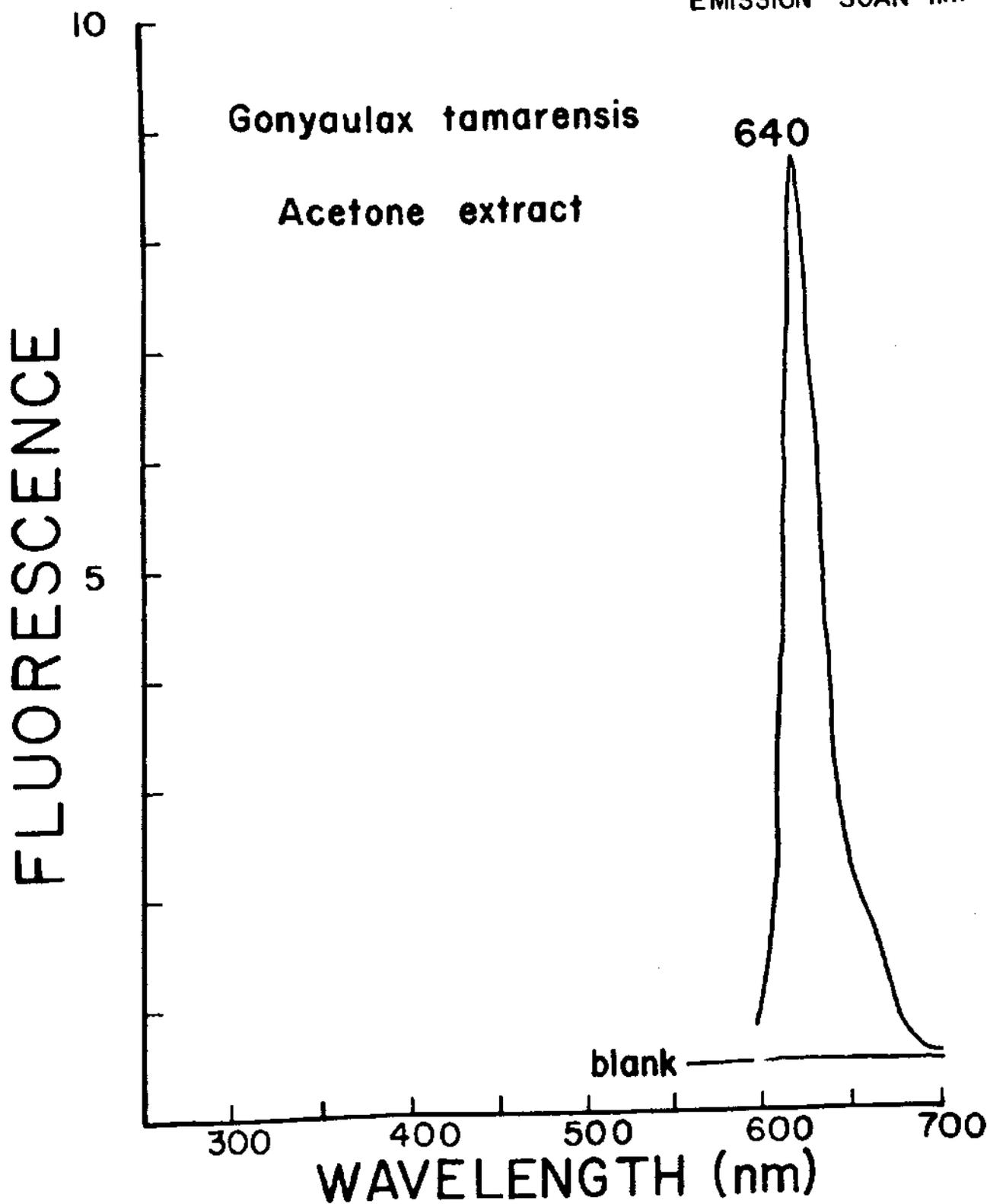


FIGURE 4.

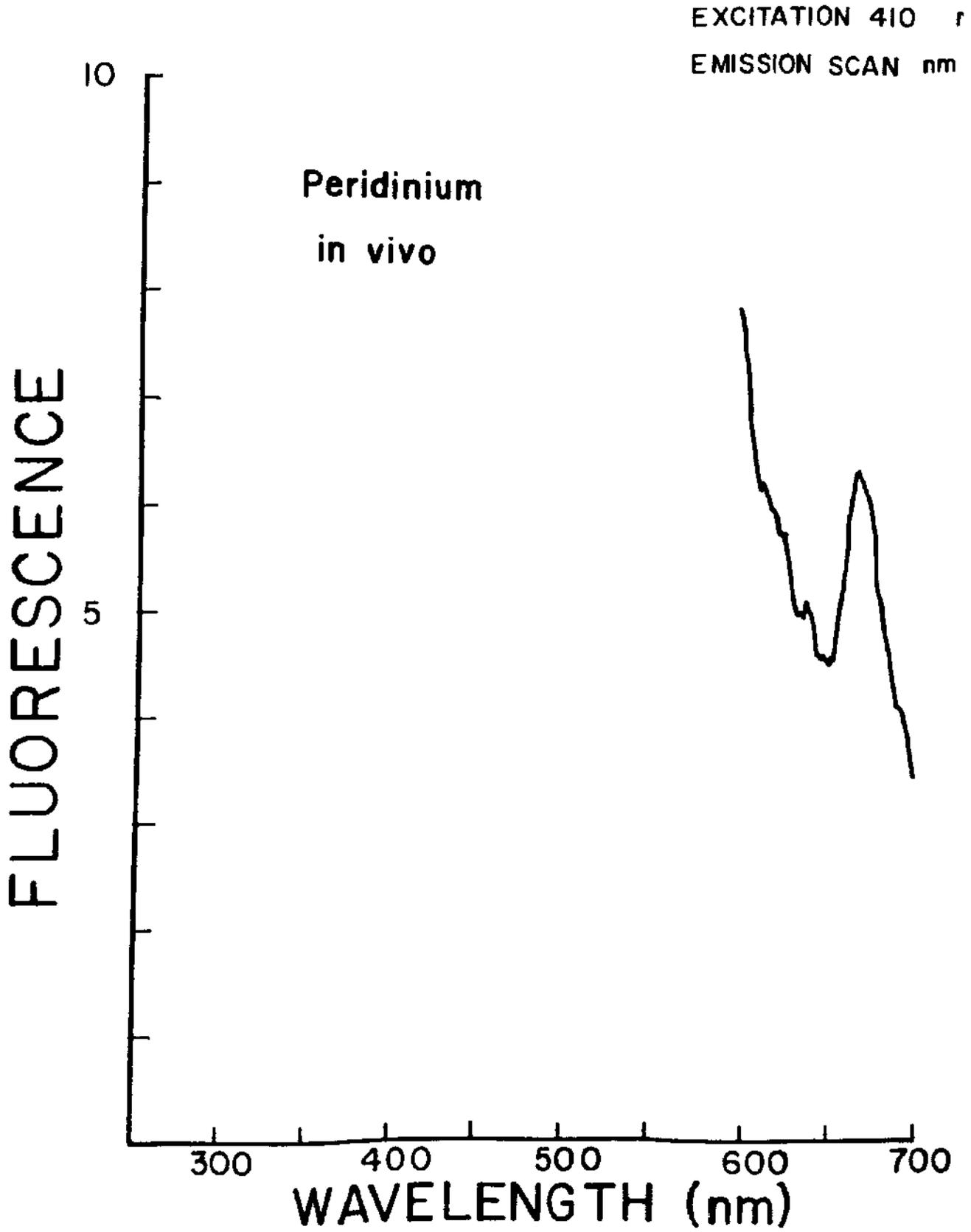


FIGURE 5.

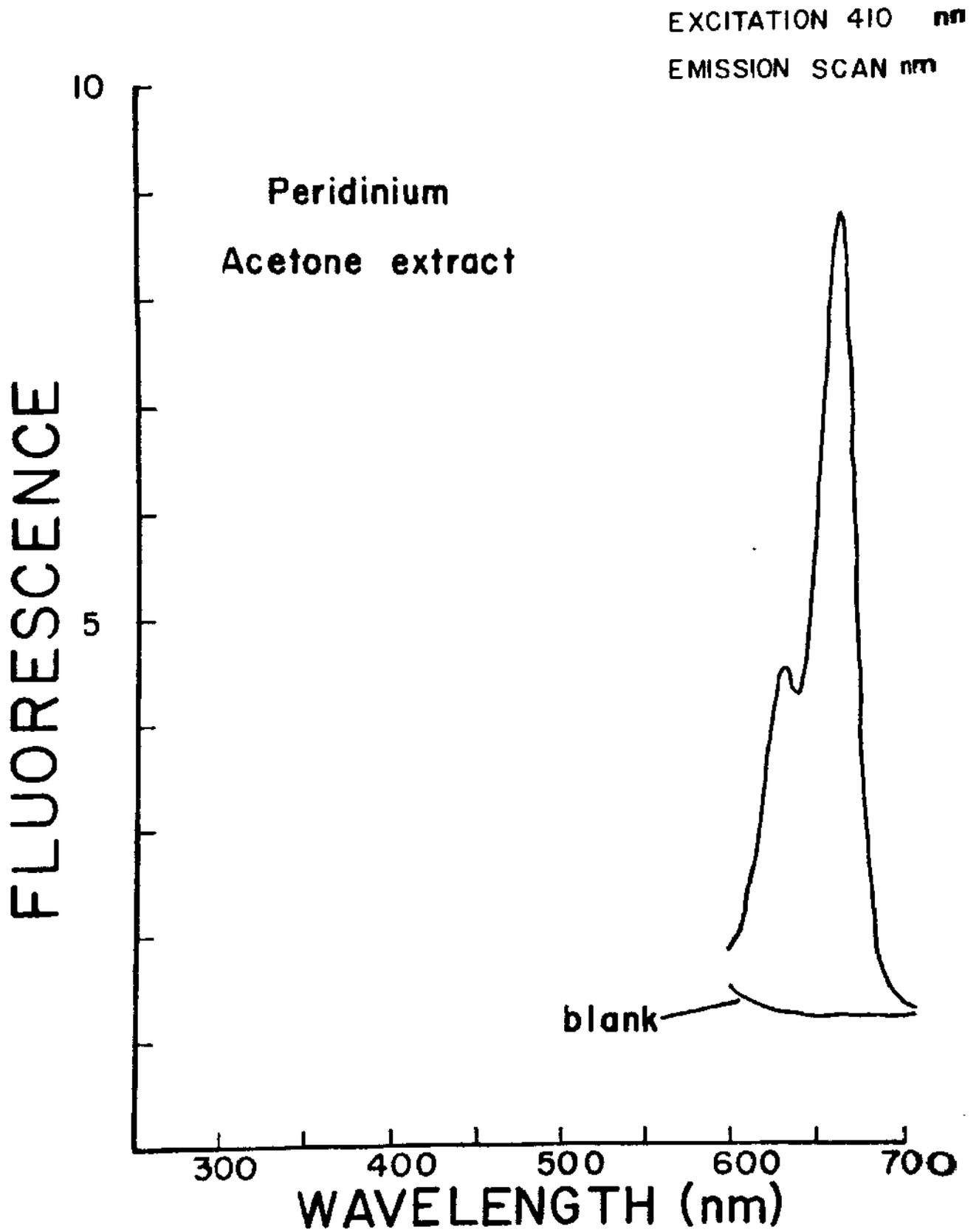


FIGURE 6.

EXCITATION 410 nm

EMISSION SCAN nm

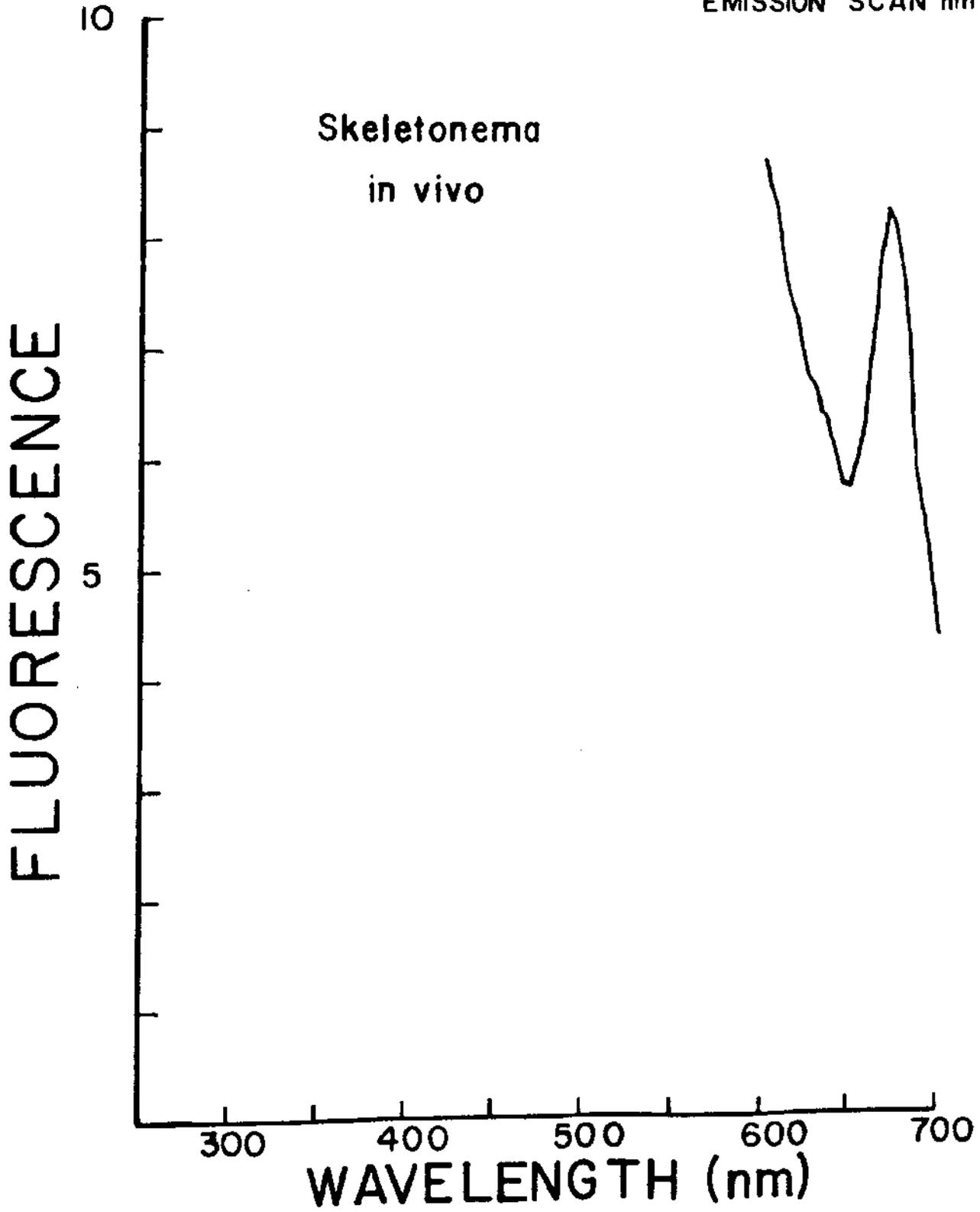


FIGURE 7.

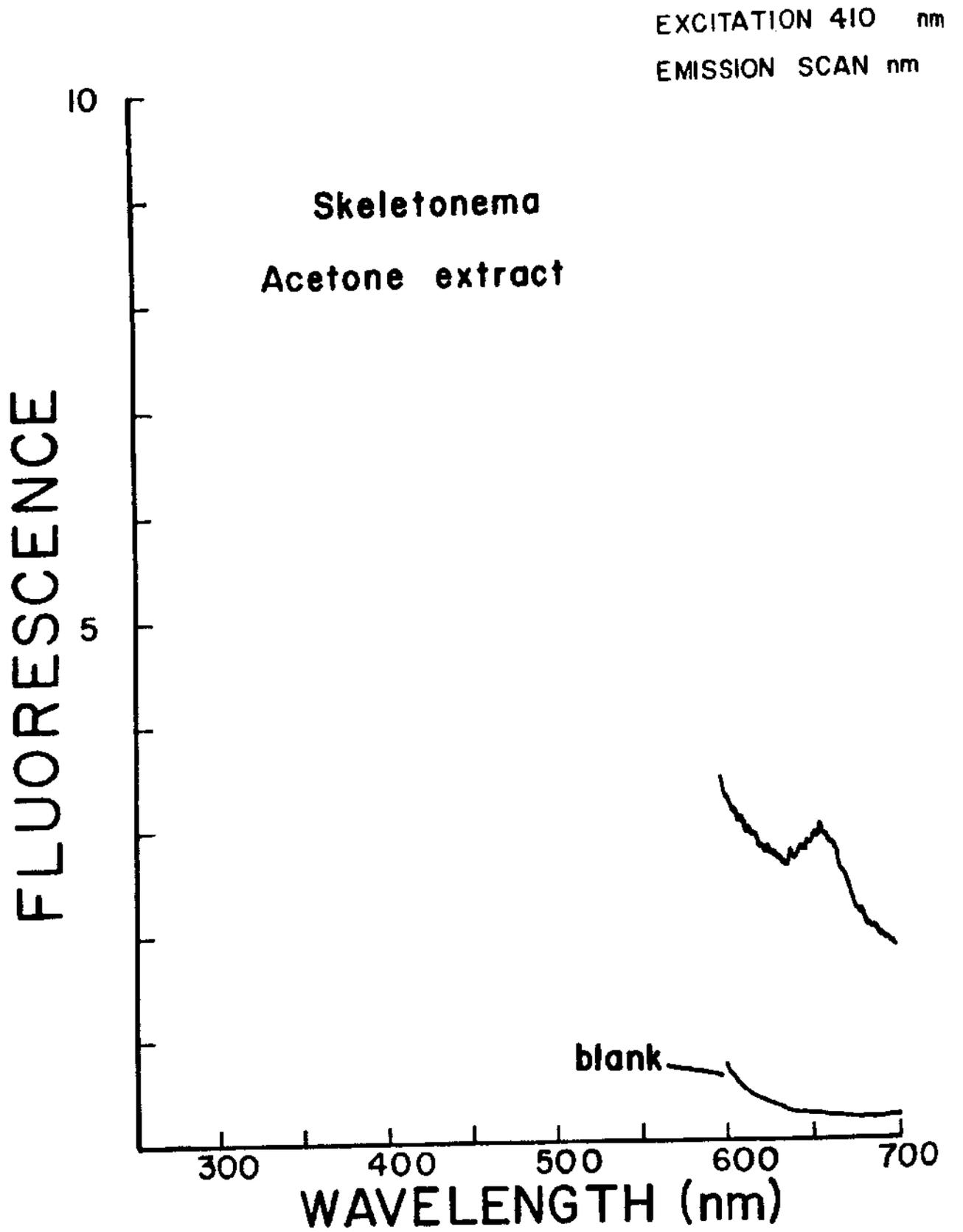


FIGURE 8.

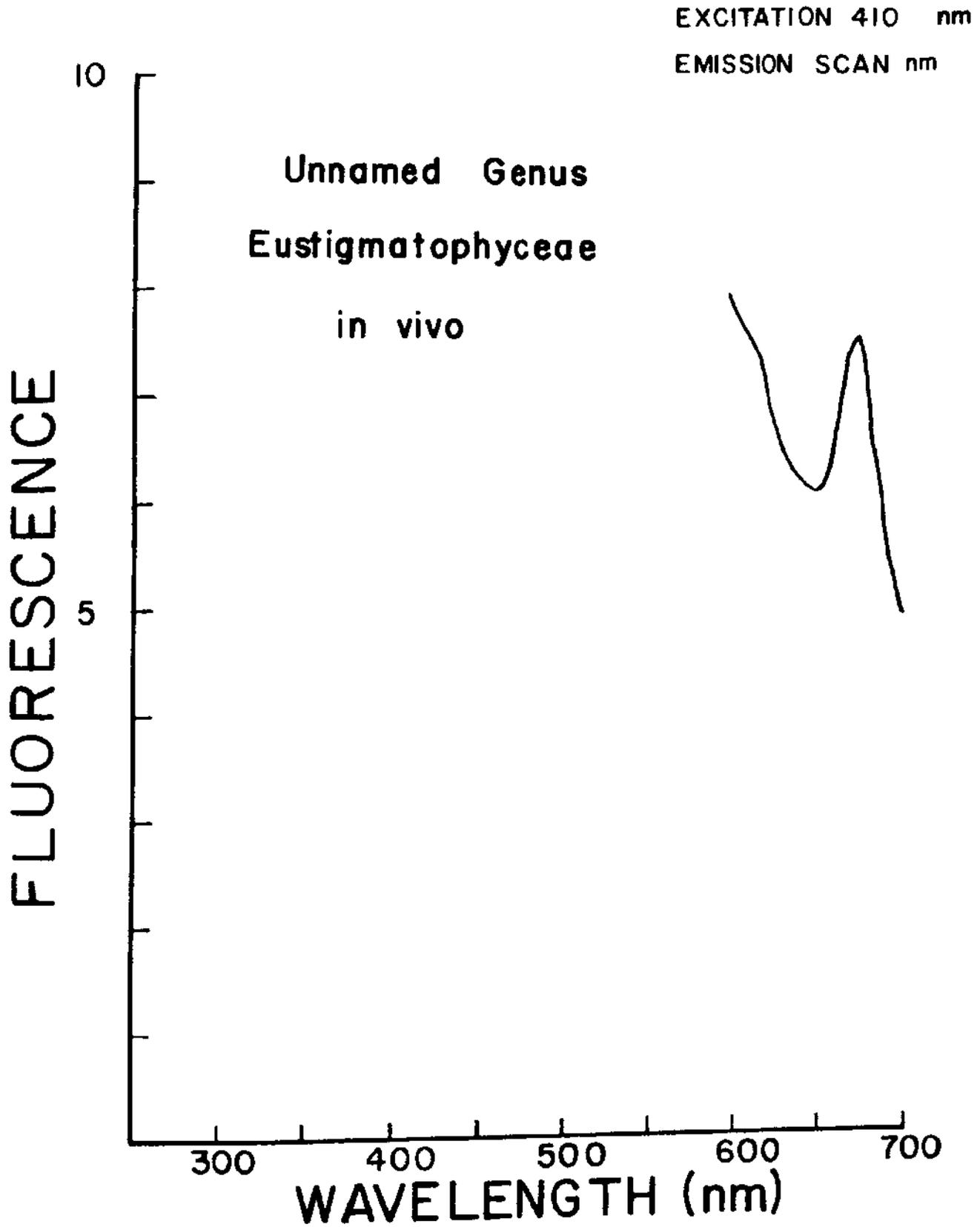


FIGURE 9.

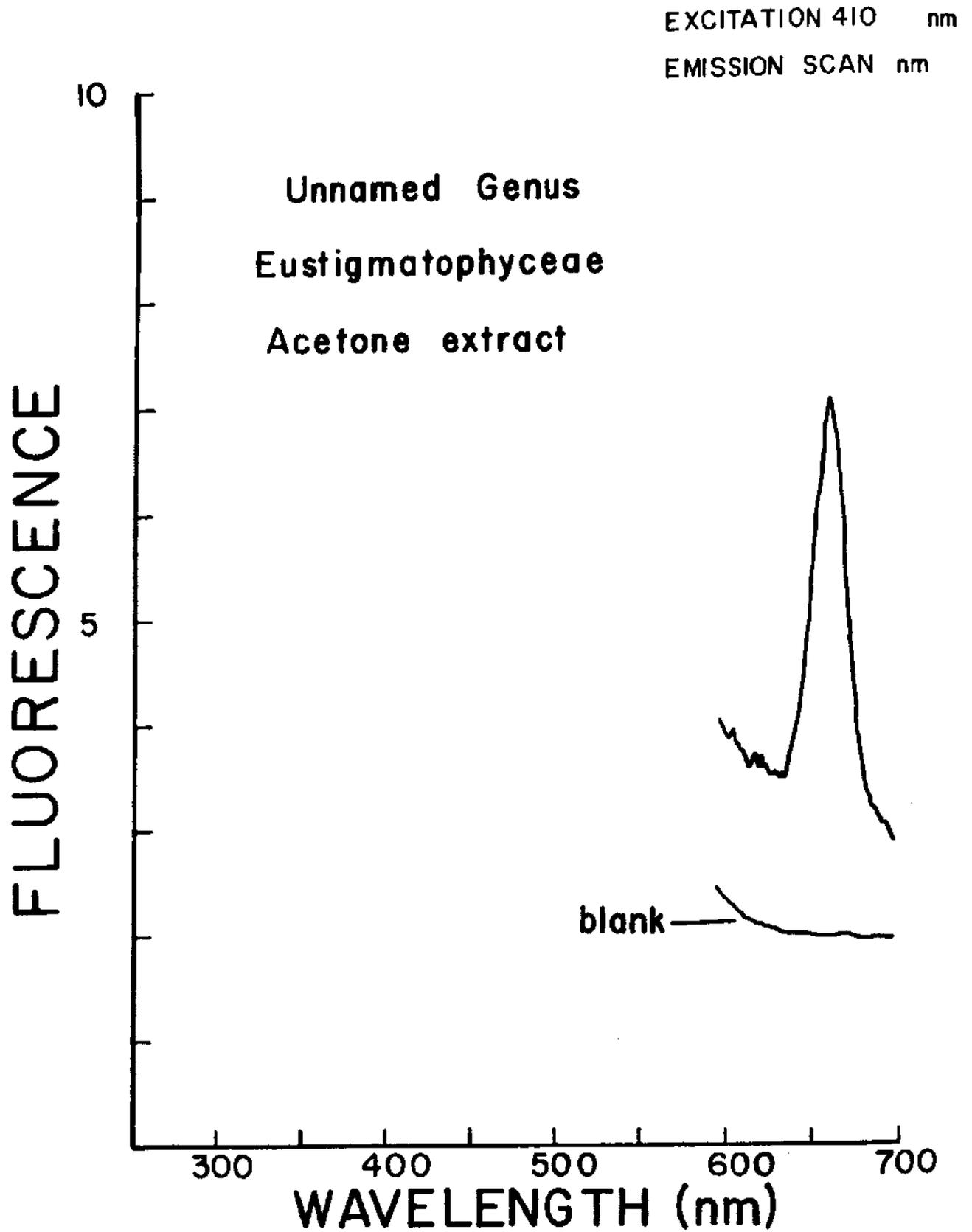


FIGURE 10.

EXCITATION 410 nm

EMISSION SCAN nm

Nanochloris atomus
in vivo

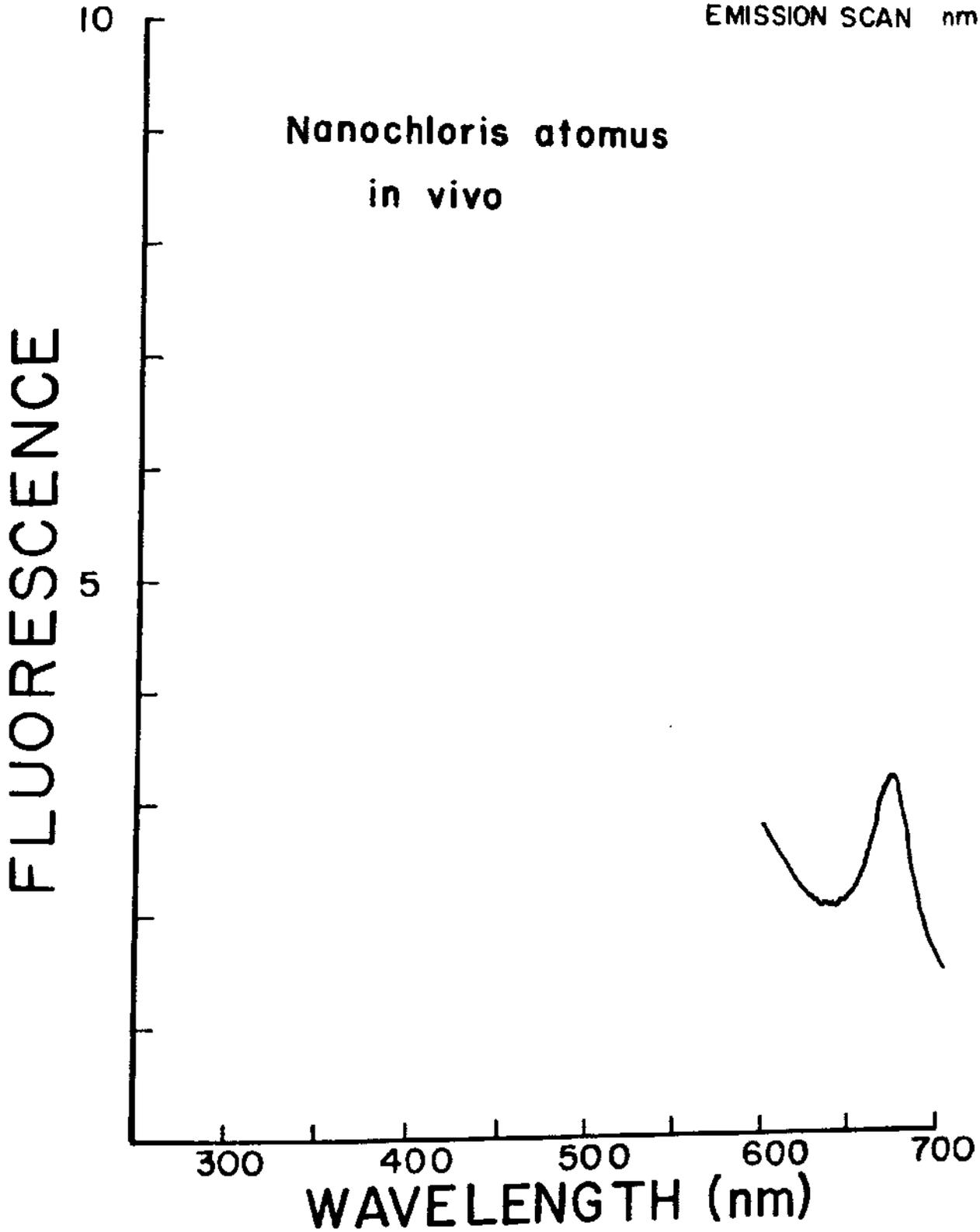


FIGURE 11.

EXCITATION 410 nm

EMISSION SCAN nm

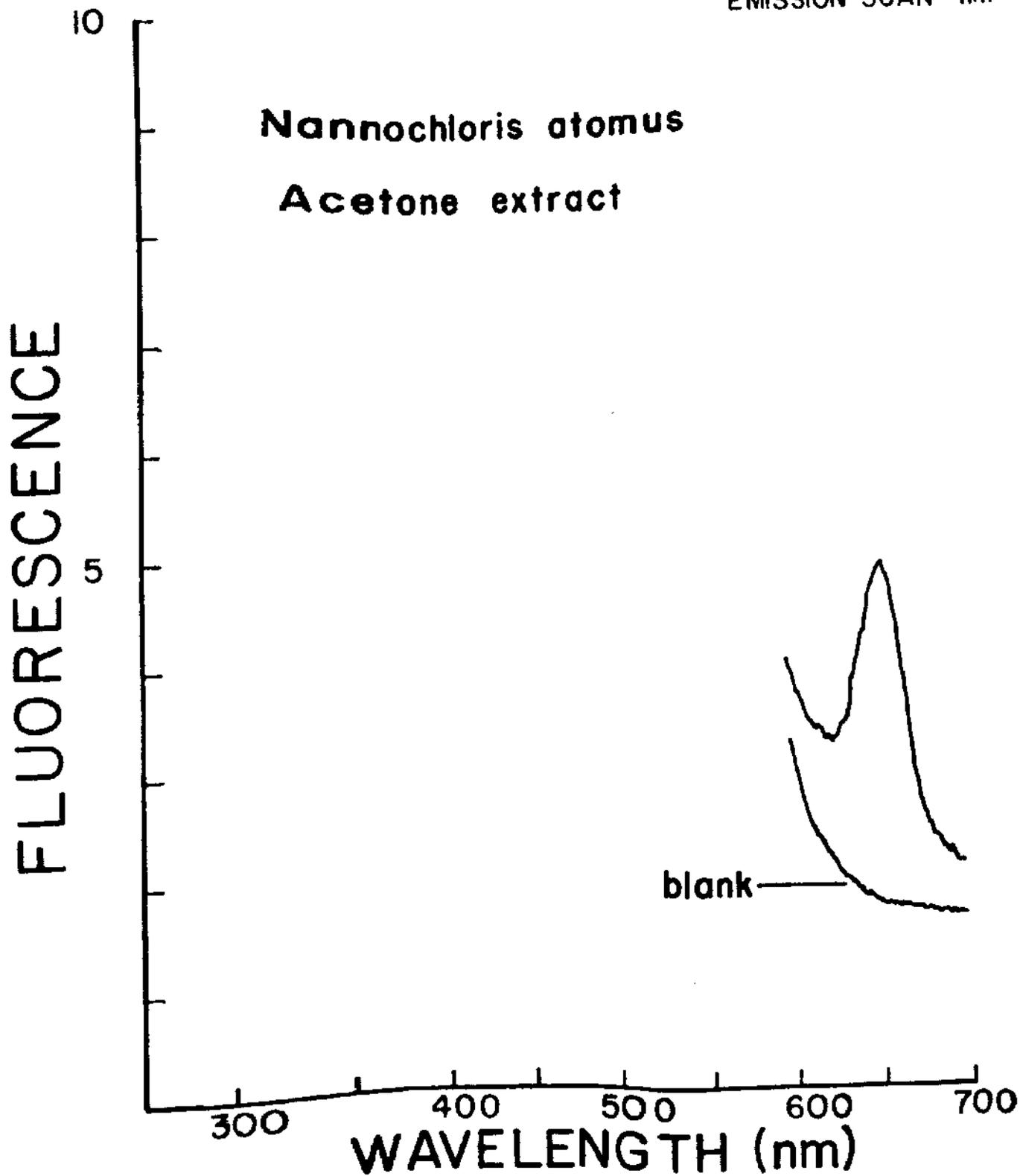


FIGURE 12.

EXCITATION 410 nm

EMISSION SCAN nm

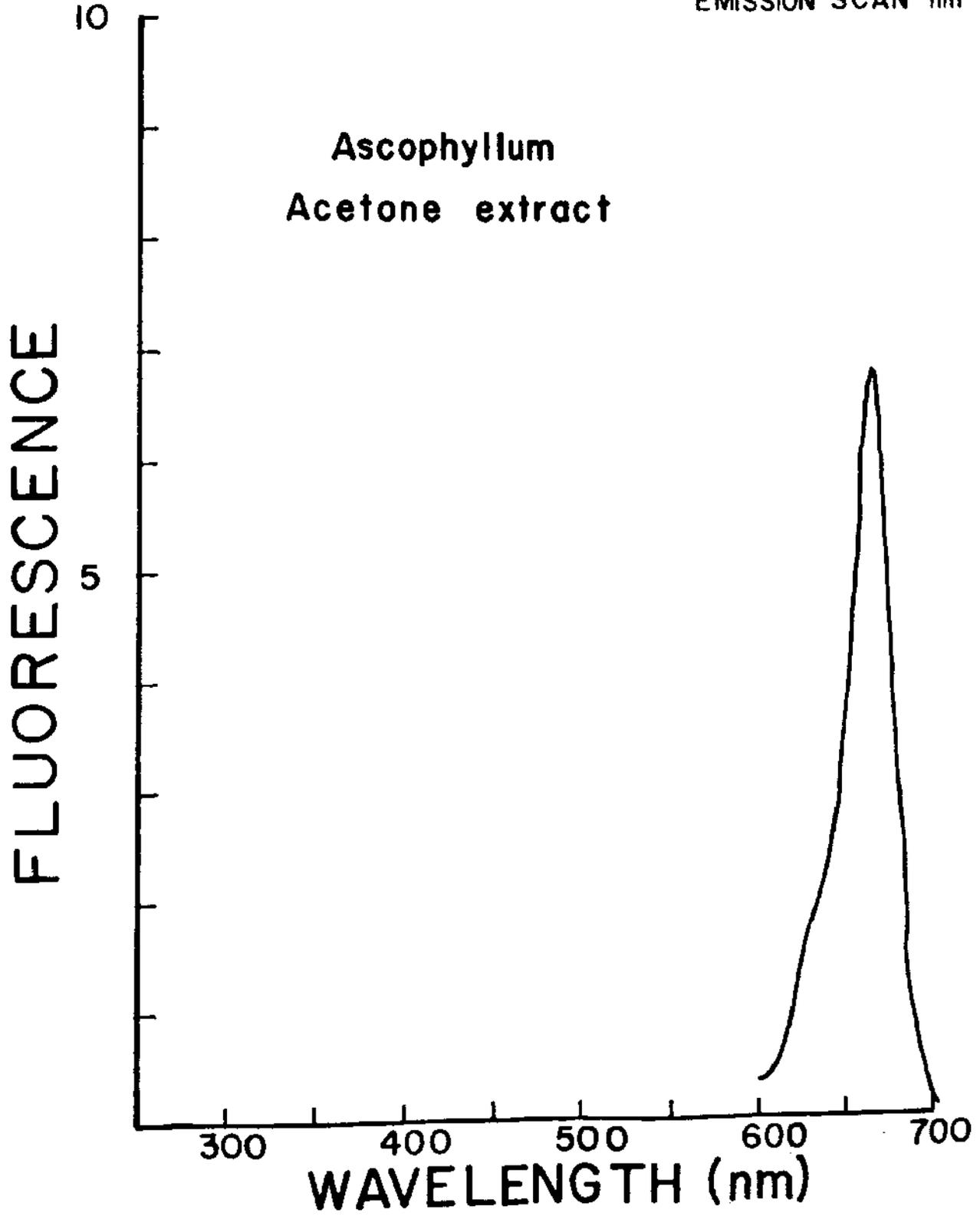


FIGURE 13:

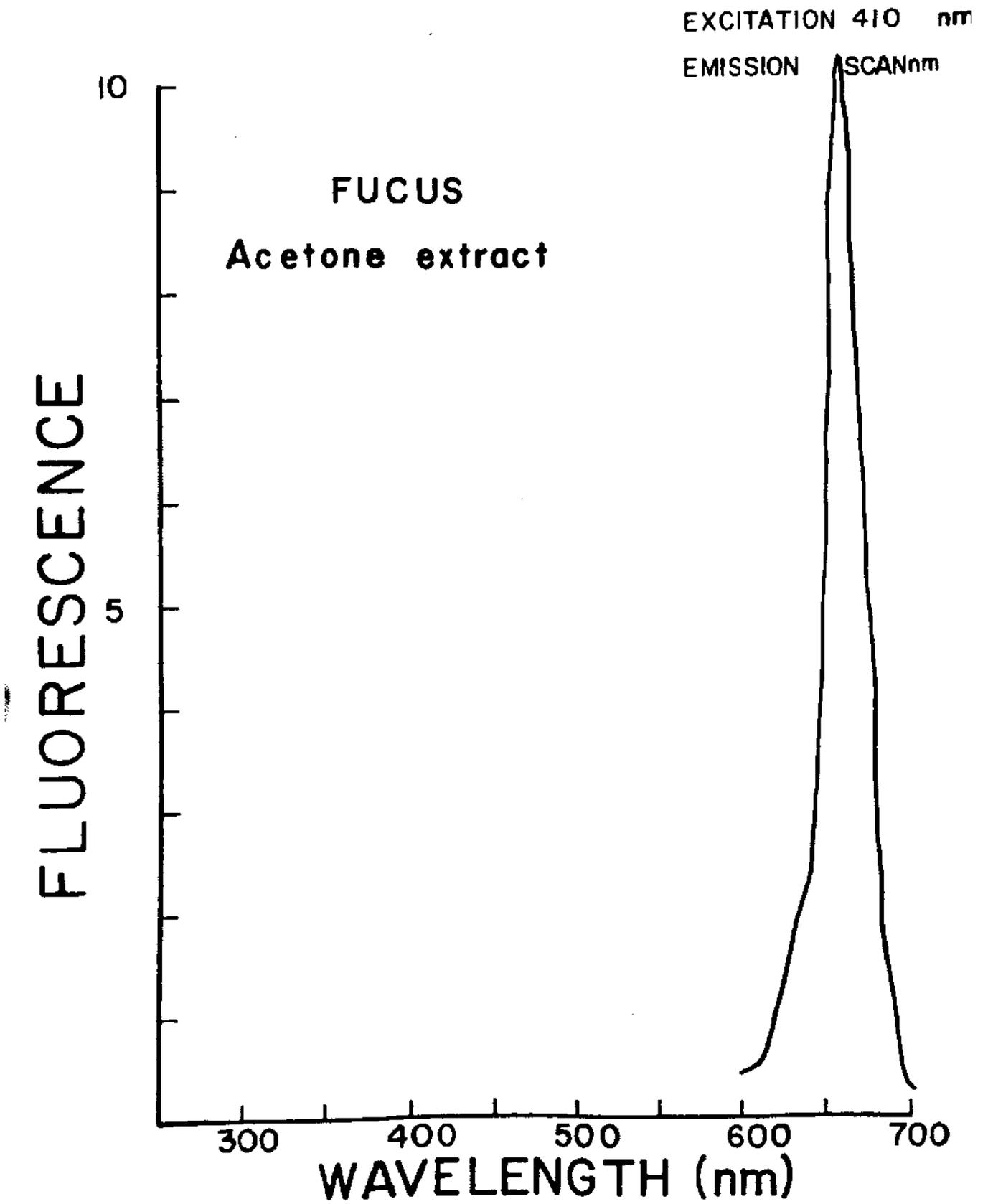
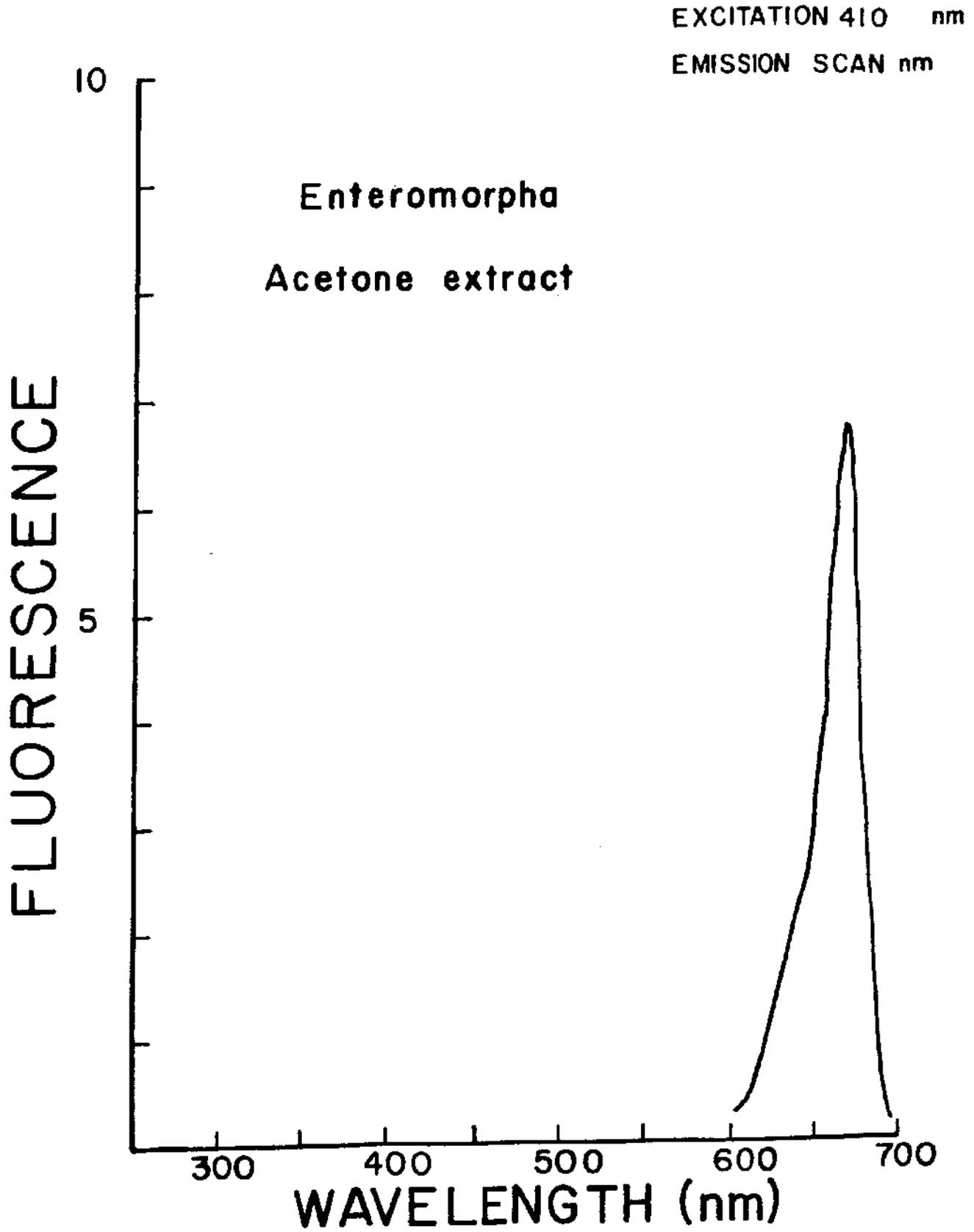


FIGURE 14.



the chlorophyll a response. A series of emission scans from 220 nm to 700 nm were performed by changing the exciting wavelength 10 nm with each emission scan (Figure 15). Strong spectral activity exists in two major areas, 590 nm to 700 nm and 420 nm to 480 nm. The excitation wavelength of 390 nm produced a Raman scatter peak at 445nm. The Raman peak position with respect to wavelength will vary as a function of the excitation frequency. The difference between the acetone blank and the G. tamarensis extract may be caused by fine particle scattering or true fluorescence as in the 600 nm to 700 nm region. The position of the peaks at the 600-700 nm region, being fluorescence peaks, remained constant. However, the peak height varied as a function of the excitation wavelength.

Other solvents, such as chloroform, produced similar peaks at 640 nm and 670nm. To ascertain that no chemical reaction was producing the peak at 640 nm, extracts were prepared by freezing, and thus rupturing the G. tamarensis cells and releasing the pigment responsible for the 640 nm peak. Figure 16 clearly shows the peak at 640 nm in a frozen extract. The ratio of the heights of the 640/670 nm peaks is reduced and there was no way to separate the two peaks by changing the excitation light as in Figures 2 & 3.

Optical correction for the scattered non-monochromatic light in live cultures was accomplished by the use of filters, Wratten 12 and 23A placed in front of the analyzer monochromator (Figure 17). An easily distinguishable and reproducible chlorophyll a peak at

FIGURE 15.

EXCITATION 390 nm
EMISSION SCAN nm

Gonyaulax tamarensis
Acetone extract

FLUORESCENCE

10

5

blank

300

400

500

600

700

WAVELENGTH (nm)

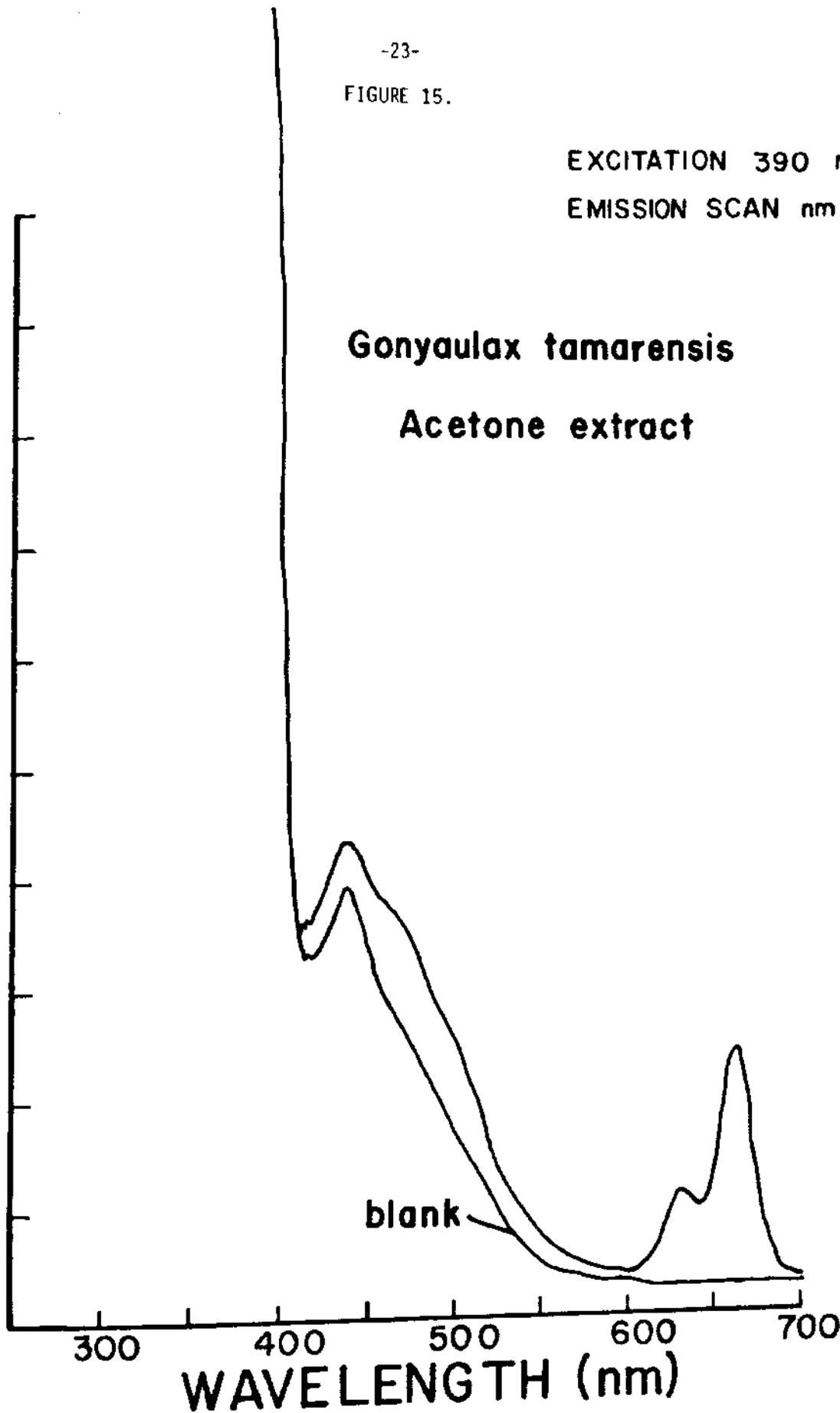


FIGURE 16.

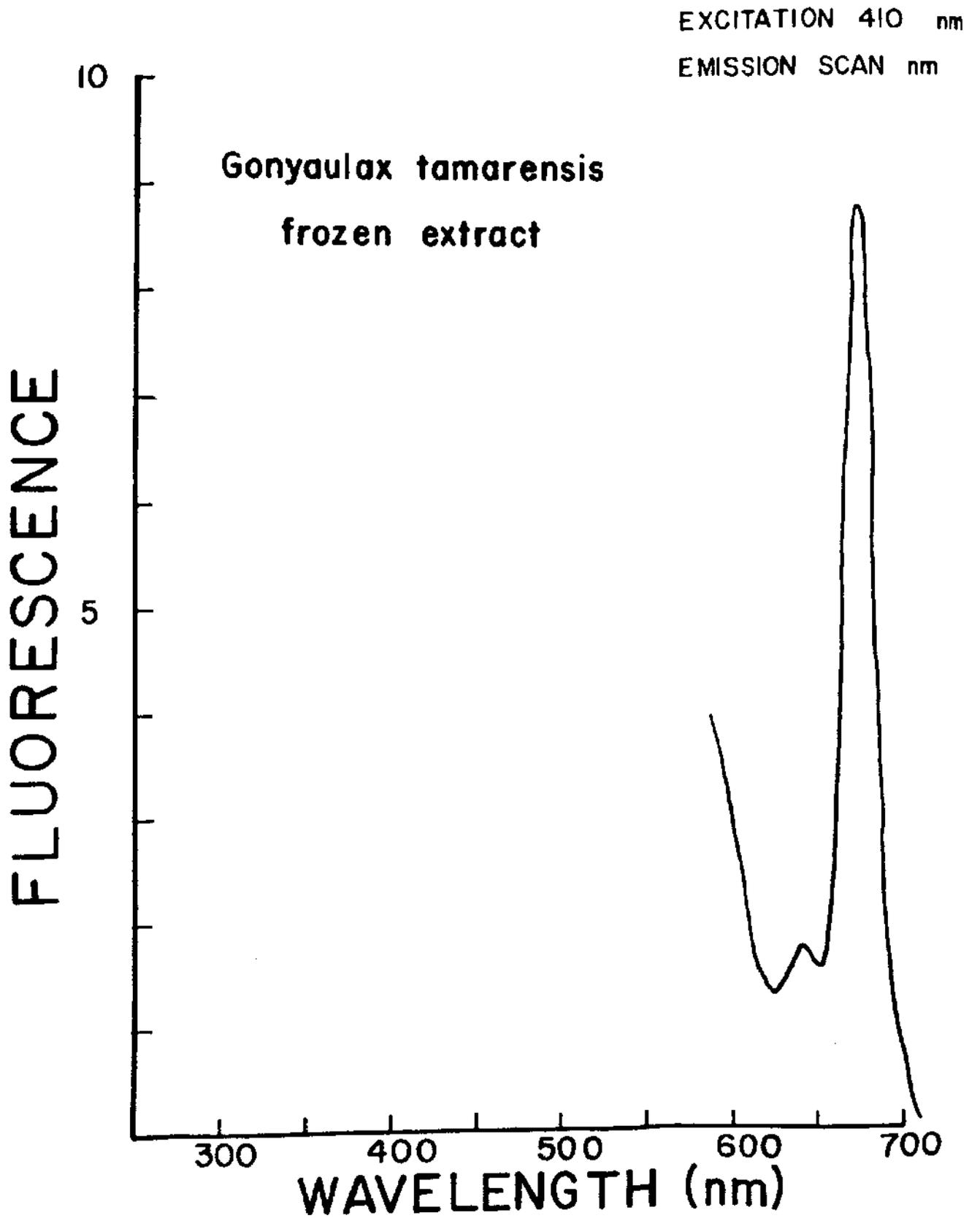
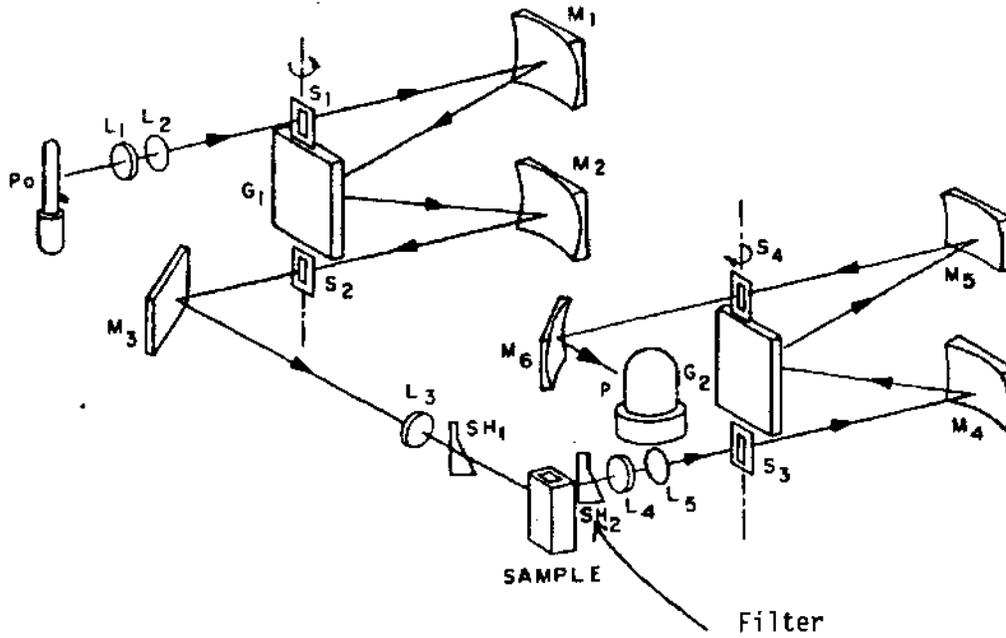
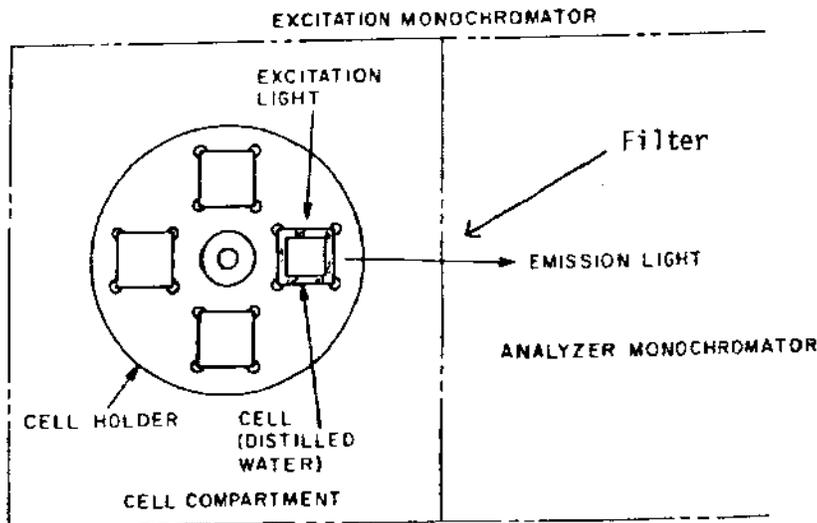


FIGURE 17.



- | | |
|--|---|
| P ₀ - Lamp Source | G ₁ , G ₂ - Grating |
| M ₁ , L ₂ , L ₃ , L ₄ , L ₅ - Lens | SH ₁ , SH ₂ - Shutter |
| M ₁ , M ₂ , M ₃ , M ₄ , M ₅ , M ₆ - Mirror | P - Photomultiplier |
| S ₁ , S ₂ , S ₃ , S ₄ - Slit | |

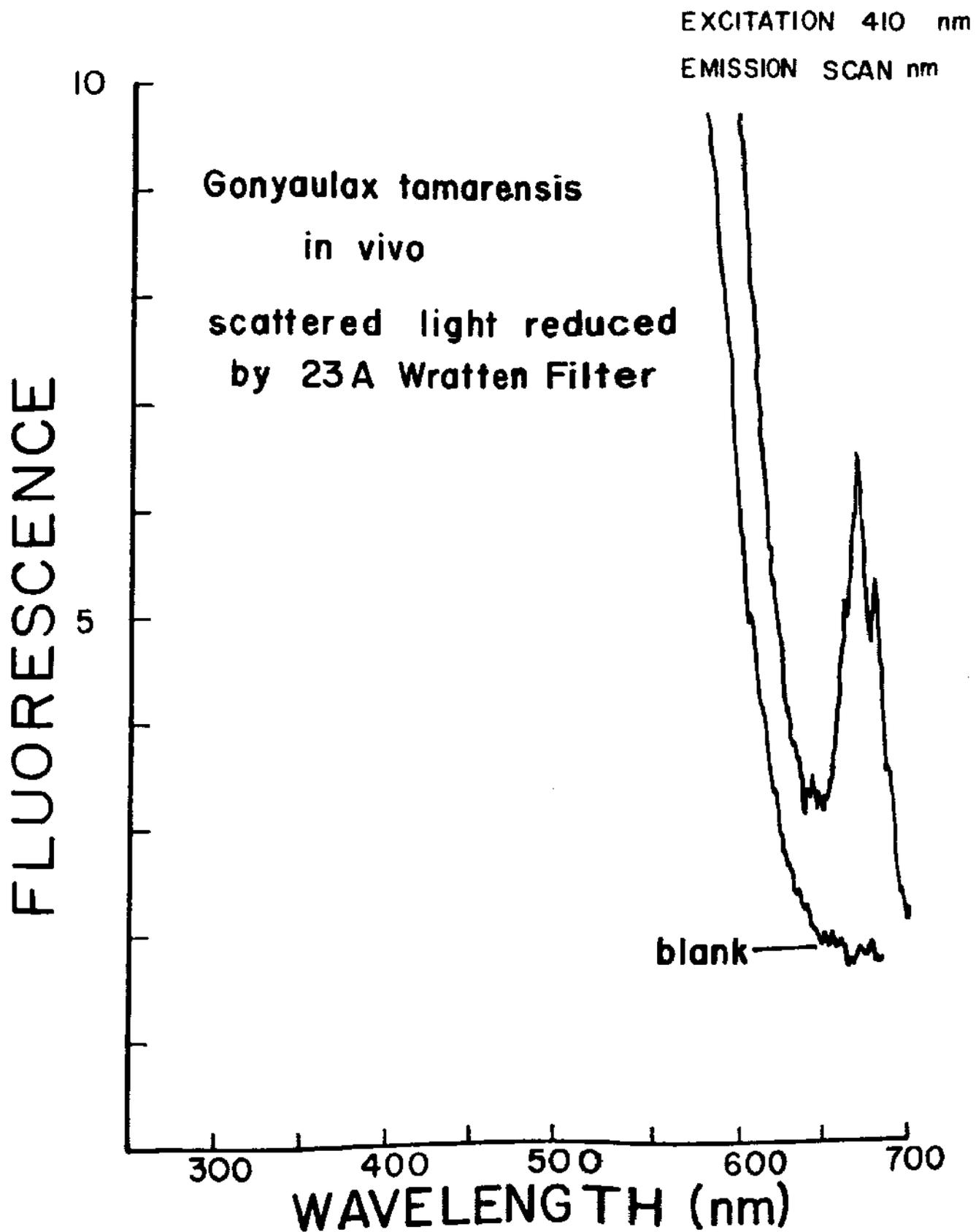
Optical Schematic



Installation of the Sample Cell

670 nm resulting from the filter use can be seen in Figure 18. However, attempts to distinguish the secondary peak at 640 nm were not successful in scans of the live G. tamarensis. Figure 18 presents the maximum sensitivity the spectrofluorometer could provide using the xenon lamp excitation configuration. Detection limits with the xenon lamp were about 250 cells per ml.

FIGURE 18.



D. USE OF LASER TO INCREASE SENSITIVITY

Instrumental sensitivity for live cultures of G. tamarensis was increased by the use of a 2 watt argon laser, Spectrophysics Model 164. The useful excitation wavelengths for G. tamarensis were limited to 460 nm, 476 nm, and 488nm. Emission scans of the 600 to 700 nm region using the 460 nm excitation wavelength at 6 milliwatts, projected onto an approximately 1 sq. cm area, revealed a four-fold increase in sensitivity over the maximum sensitivity displayed by the xenon lamp configuration. Figure 19 shows the increase in sensitivity for chlorophyll a 670 peak. The intensity of the laser excitation wavelength 460 nm was increased incrementally as depicted in Figure 20 from 6 milliwatts to 80 milliwatts. The response was linear. A detection limit of 60 algal cells per ml was established at the 460 nm excitation frequency using 80 milliwatts and monitoring the emission at 670 nm, the chlorophyll a peak (Figure 21). The 640 nm peak unique to dinoflagellates in the extracts did not become evident when using the laser at the 460 nm, 476 nm, or 488 nm excitation frequencies throughout the ranges of power each frequency was capable of producing.

Characteristics of natural blooms studied by Yentsch in 1972 and 1974 present the following data:

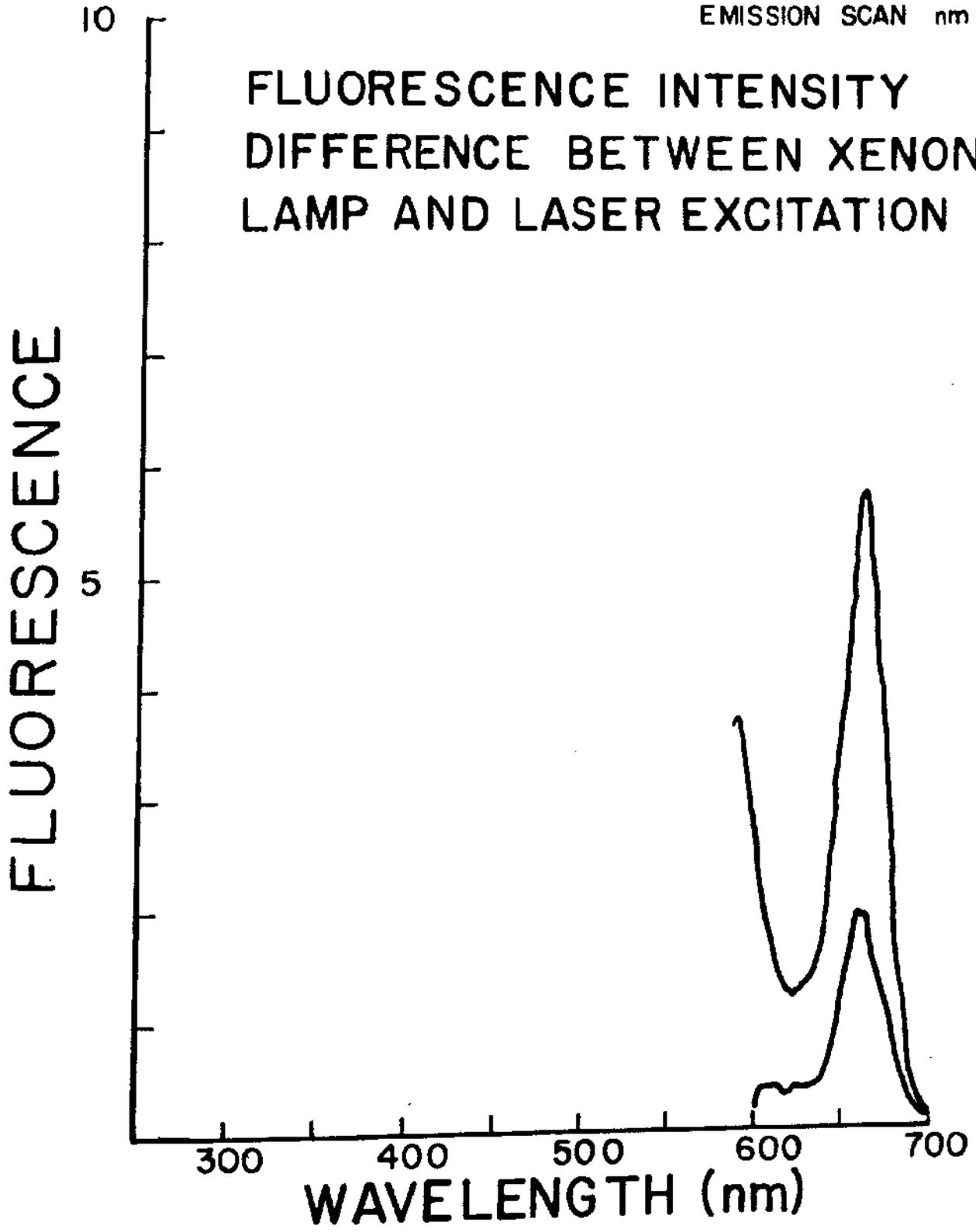
<u>Algal population (cells/l)</u>	<u>1972</u>	<u>1974</u>
G. tamarensis (50u)	1.78×10^6	5.2×10^6
S. costatum (5u)	1.87×10^6	8.2×10^4
<u>Chlorophyll (mg/m³)</u>		
in patch of bloom	60-100	45
outside patch of bloom	31	<2

FIGURE 19.

EXCITATION 460 nm

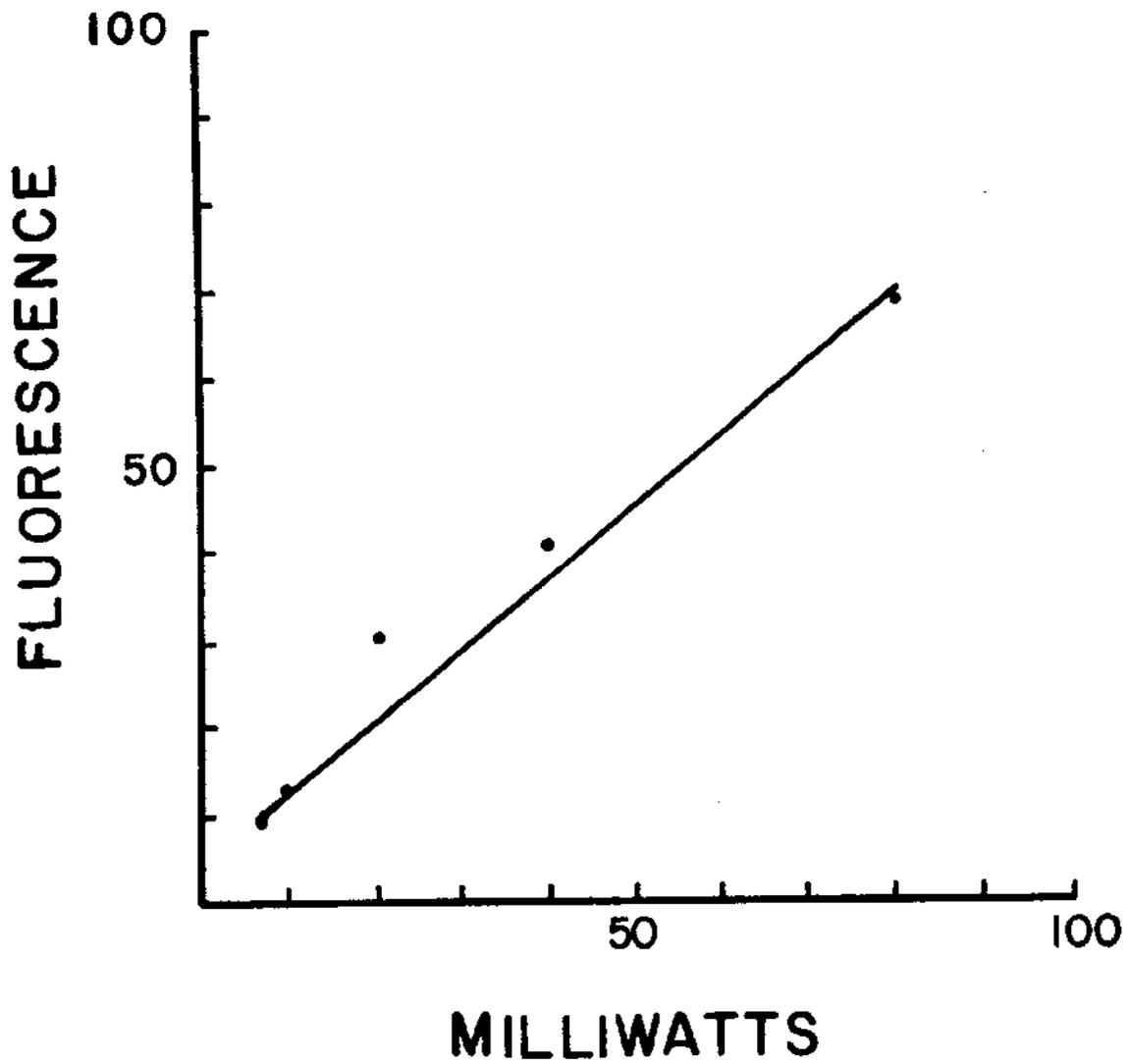
EMISSION SCAN nm

FLUORESCENCE INTENSITY
DIFFERENCE BETWEEN XENON
LAMP AND LASER EXCITATION



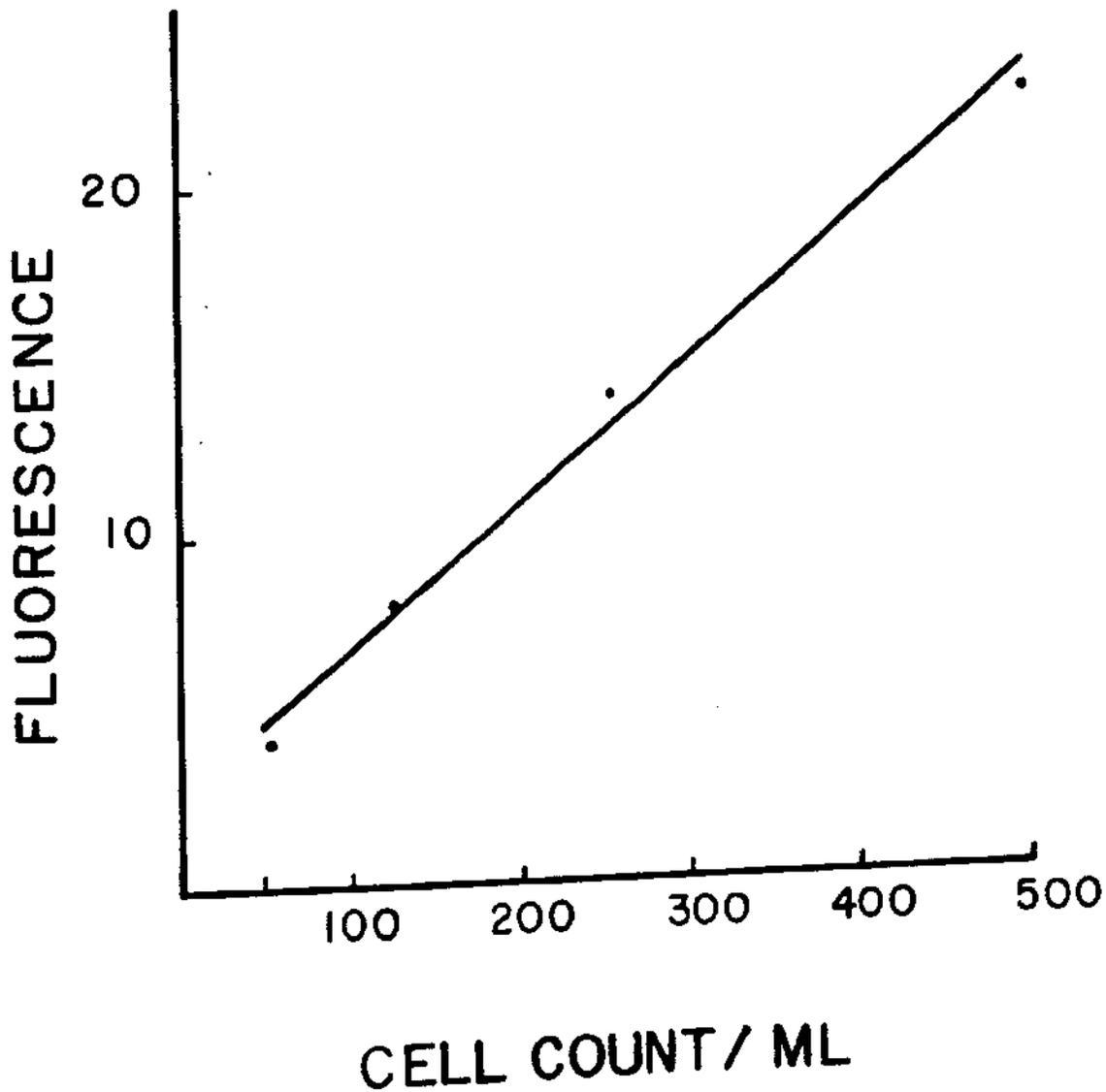
FLUORESCENCE RESPONSE TO INCREASED LASER INTENSITY

FIGURE 20.



DETECTION LIMIT OF *G. TAMARENSIS* BY THE USE OF LASER EXCITATION OF CHLOROPHYLL

FIGURE 21.



Bloom conditions in 1974 show 5.2×10^6 cells/liter or 5.2×10^3 cells/ml of G. tamarensis. Detection limits for G. tamarensis using spectrofluorometry can be set at 6.0×10^1 cells/ml. In chlorophyll counts 6.0×10^1 cells/ml is approximately 1 mg/m^3 . Normal background conditions for New England waters is 1 to 3 mg/m^3 . Sensitivity based on chlorophyll detection is adequate to detect blooms. The critical factor in identifying G. tamarensis is the ability to discriminate it from other algae.

E. BRIEF THEORETICAL DISCUSSION OF PLANT PIGMENTATION AND FLUORESCENCE

Udenfriend (1964) suggests that a chlorophyll molecule when absorbing light energy will be changed to an excited state CHL^* and be further converted to a triplet state CHL^{***} . Energy from the active triplet state is passed on to an acceptor which initiates the chains of metabolic activities necessary for the life of the plant. Accessory pigments transfer energy not absorbed by chlorophyll to chlorophyll, and not directly into the metabolism of the plant. Some energy is lost as heat or fluorescence. In live plant cells therefore, the fluorescence efficiency is not as high as in extracts because of the tendency of the chlorophyll molecule to transfer its energy to metabolic activities.

The decrease in the relative fluorescence of the emission peaks was expected and observed. The increase in sensitivity was needed in live cultures of G. tamarensis and accomplished by the use of a strong excitation light provided by the laser. However, the disappearance of the 640 nm emission peak when subjected to the intense radiation of a laser was unexpected. The laser was limited in its ability to produce only narrow bandwidth light with frequencies no lower than 460 nm.

F. ATTENUATION OF LIGHT AND G. TAMARENSIS DISTRIBUTION FOR MASSACHUSETTS WATERS

The advantage of the intensive research into the 600 to 700 nm fluorescence region for G. tamarensis is the importance of this region to algal photosynthesis, the differences in fluorescence frequencies in the region, and the relative strength of the fluorescence as stated by Udenfriend. One possible drawback for eventual use of the 600 to 700 nm emission region in an ocean application is that the diffuse red fluorescence is absorbed by water. Table 2 gives the attenuation in percent of the various wavelengths of light discussed for waters near the Boston area (G.L. Clarke, 1976). The excitation frequencies in the 400 nm region will penetrate seawater with little attenuation to a 5 meter (16.4 ft.) depth. Diffuse radiation of 625 nm and nearby frequencies has little attenuation likewise to 5 meters. A depth penetration to 5 meters would be adequate for detection of G. tamarensis blooms as these are found close to the surface in the daytime. It has been noted by Martin (1972) that concentrations of algal cells on the immediate sea surface are much higher than at depths directly below. It has been speculated that this phenomenon might be due to entrapment of the cells by surface tension. There are differences in the concentrations of G. tamarensis cells and depth with respect to daytime-nighttime light regimes according to Martin. The motile G. tamarensis cells tend to migrate down the water column at night and migrate to surface during the day.

TABLE 2. Attenuation Properties of Downwelling Light for Seawater Near the Boston Light Ship (9-8-67)

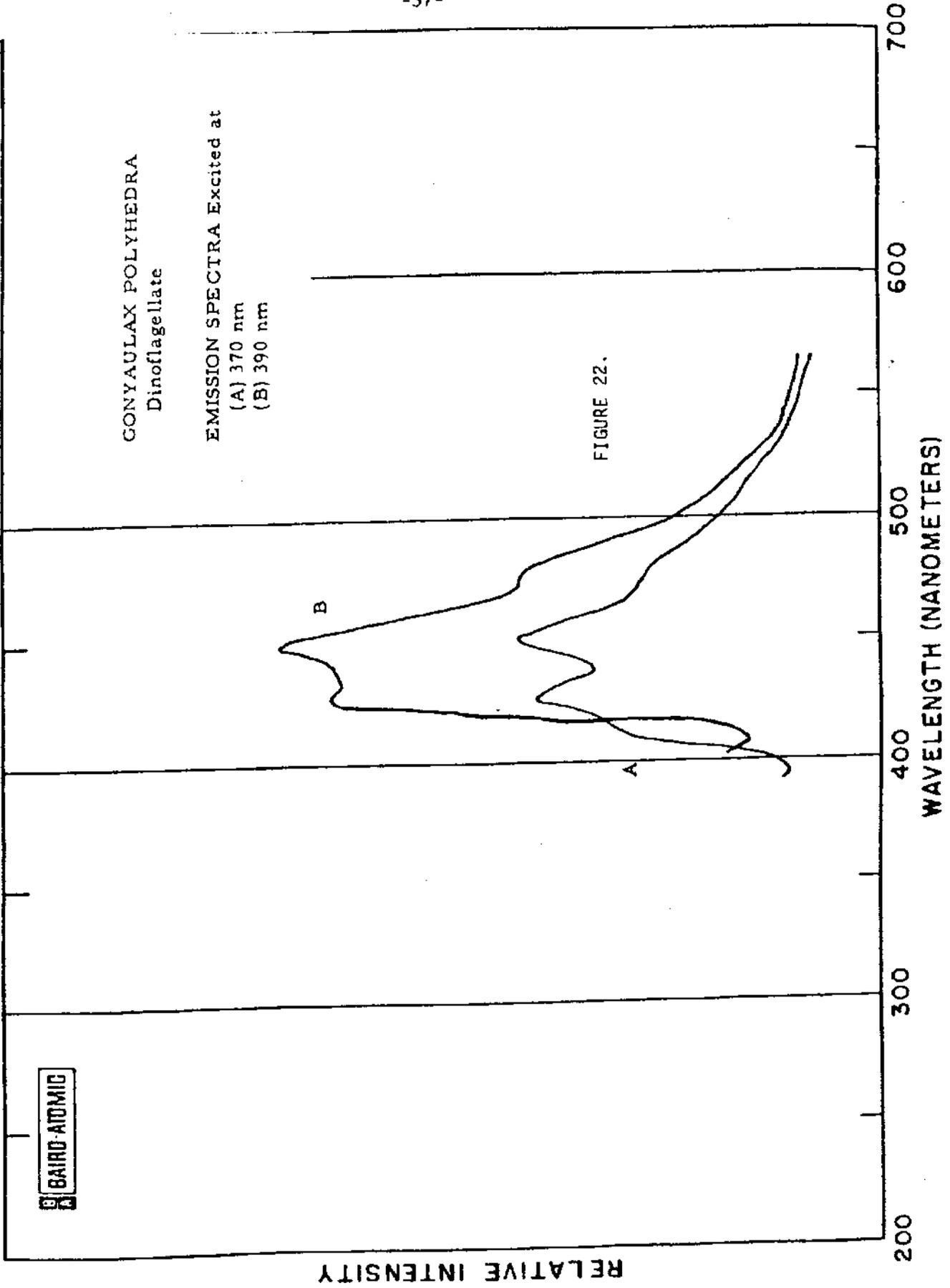
<u>Wavelength</u>	<u>Depth in meters (feet)</u>	<u>Radits*</u>	<u>Percent Transmission</u>
410 nm	5m (16.4 ft.)	5.7	100
	10m (32.8 ft.)	1.0	75.5
450 nm	5m (16.4 ft.)	10.0	100
	10m (32.8 ft.)	5.6	56
	20m (65.6 ft.)	.6	6
625 nm	5m (16.4 ft.)	3.5	100
	10m (32.8 ft.)	.7	20

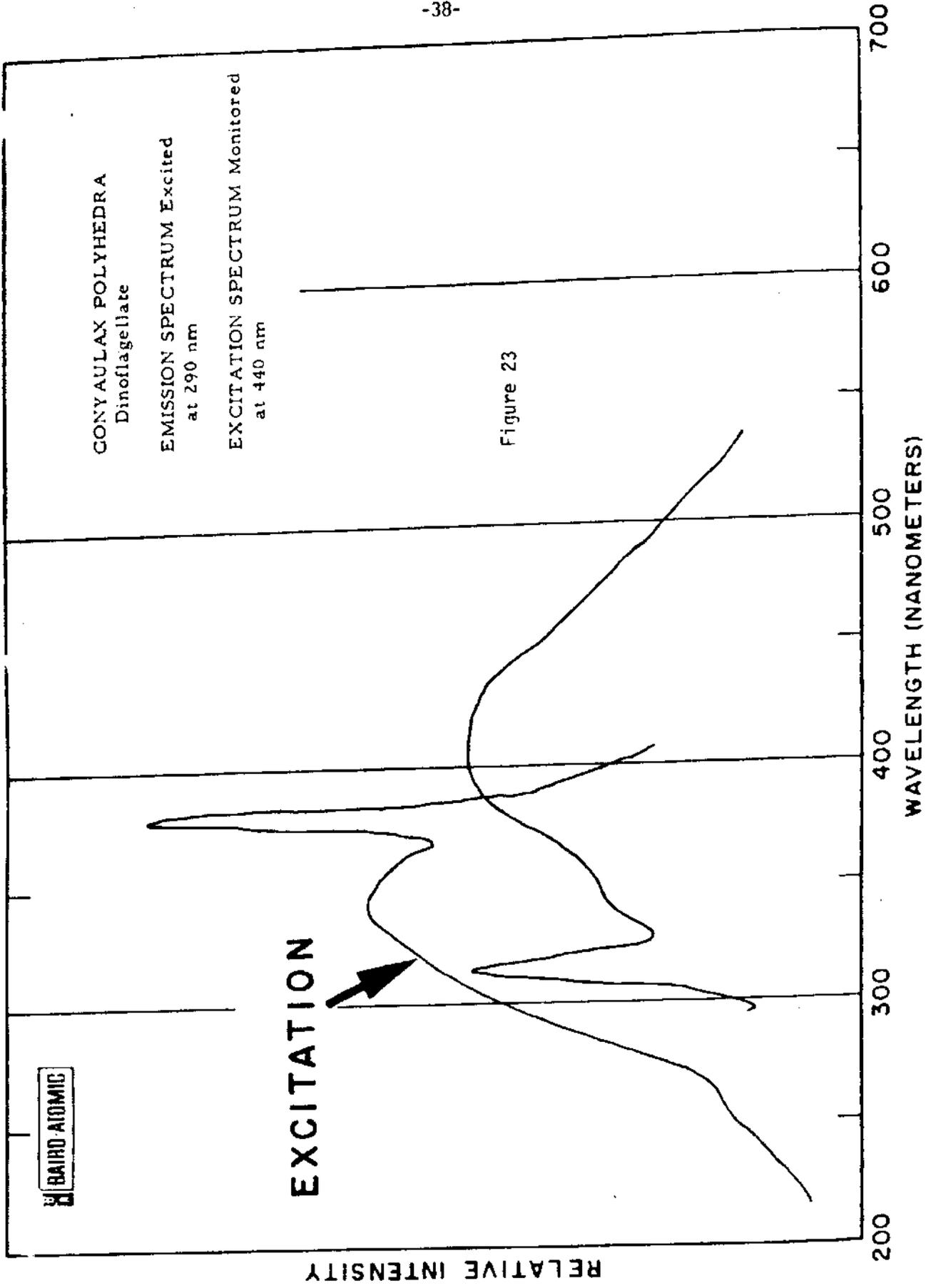
*Radits = $(uw/mm^2/sterad/mu) \cdot X 10^2$

G. POSSIBLE ALTERNATE METHODS OF DETERMINATION

Hornig and Eastwood (1973), investigating other methods and spectral regions, have concluded that recording the pattern of the changing excitation frequencies, or absorbed light, during a scan while monitoring a fixed emission frequency, will yield differentiation among algal types by comparing the excitation patterns. Figures 22 and 23 show the differences in excitation and emission scans by Hornig and Eastwood. Figure 22 presents an emission scan for Gonyaulax polyhedra, an alga in the same genera as G. tamarensis. The peak at 420 nm for line A and the peak at 440 nm for line B represent the Raman scattering of seawater with excitation of 370 nm and 390 nm respectively. The Raman peak is highly susceptible to particulate scatter interferences which can make analyses difficult. The excitation scan of G. polyhedra is presented in Figure 23. Mumola (1973) describes the utilization for the differences in the excitation cross-sections of patterns to maximize chlorophyll a detection. As he points out there is a similarity between the golden-brown algae, into which he classifies dinoflagellates, and green algae. The approach of monitoring excitation scans could be a viable method and certainly merits further investigation for possible use in algae discrimination instruments.

Other methods of differentiation of fluorescence materials mentioned by O'Neil (1975) have been the detection of a time delay between the entrance of the excitation light into a molecule and the exit of the fluorescence emission. Brody and Rabinowitch (1957) have presented some data on the excitation lifetimes of chlorophylls





and related pigments. This is another promising avenue to research, however, it would require more sophisticated instrumentation to be transported in an airborne platform.

H. INTERFERENCES

Possible interferences which might occur in the field with the application of an airborne spectrofluorometer would be turbidity and floating oil. Turbidity from colloidal particles will affect the fluorescence signal from algae in several ways:

1. It will make the solution containing the algae opaque so that no excitation light can pass.
2. It will interfere with the return fluorescence signal which is not as intense as the excitation light.
3. It will scatter any non-monochromatic light from the excitation light and register false peaks.

Figure 24 shows how various concentrations of bentonite clay, a source of very fine (less than 2 microns, .00008 in.) white particles, can raise signals in the 600 to 700 nm region by scattering. A red Wratten 23A filter greatly reduces the scattering as shown even though the excitation and emission light was passed through monochromators. Light from a good monochromatic source such as a laser also reduced scattering, but did not replace the filter with respect to signal to noise ratios.

The fluorescence spectral techniques for the detection of oil on water use excitation wavelengths of 340 nm and monitor emission wavelengths of 400 to 500 nm. These wavelengths are far enough away from the 600 to 700 nm wavelength region to plant pigment emission to be of little consequence.

FIGURE 24.

EXCITATION 410 nm

EMISSION SCAN nm

Light Scatter Caused
by Colloidal Bentonite
Removed by Red Filter
23A

FLUORESCENCE

10

5

.1 gram / liter

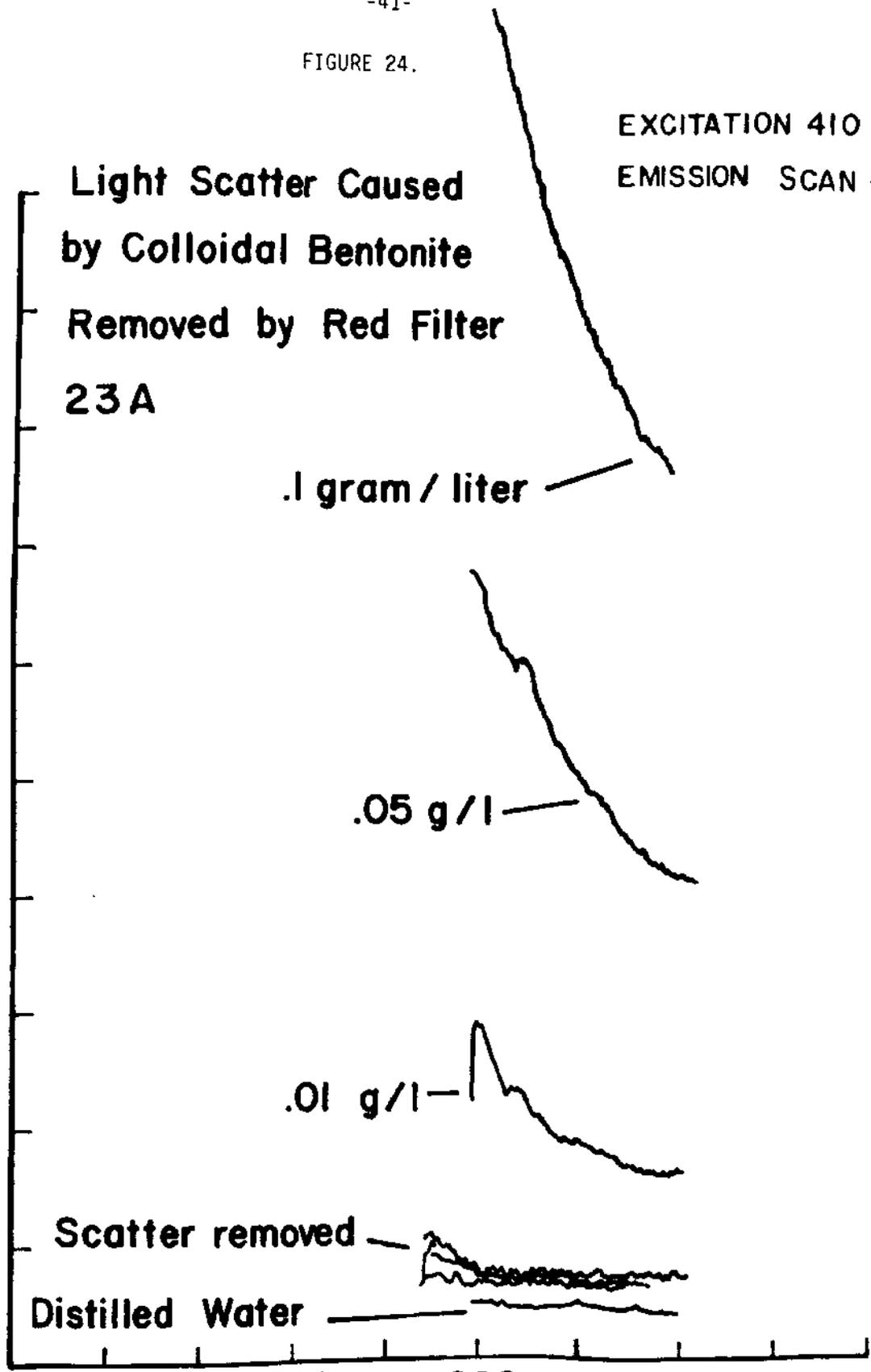
.05 g / l

.01 g / l

Scatter removed

Distilled Water

500 600 700
WAVELENGTH (nm)



I. AIRBORNE LASER SPECTROFLUOROMETRY

There are several airborne laser spectrofluorometers described in the literature. Instrumentation developed by O'Neil et. al. (1973) utilized a Helium-Cadmium laser whose blue line at 442 nm produced 25 milliwatts of power for excitation during field trials. Limitations of power are necessary for safe eye limits should a ground observer look directly at the laser beam as it passes. This factor is related to the determination of altitude. The receiver for the fluorescence return signals was a Schmitt Cassegrain telescope which gathered light into a series of filters. The instrumentation of Mumola (1973) and Kim (1973) had similar configuration, except Mumola utilized a four color light beam. This was constructed by placing dyes in separate cuvettes and using a rotating intercavity shutter to transmit a single laser pulse at the rate of .5 pulse per second. Mumola (1973) maintains that for a true measure of total chlorophyll in algal groups of different colors several frequencies of excitation light must be employed. During field trials laser frequencies were limited to 454.4 nm, 539.0 nm, 598.7 nm, and 617.8 nm. Reported sensitivities for chlorophyll in situ for the laser spectrofluorometers mentioned are less than 2 milligrams per cubic meter, well within the 1-3 mg/m³ range common to productive coastal waters. The design of these instruments was to measure chlorophyll rather than to distinguish a single algal type.

A second generation laser spectrofluorometer is under development by NASA's Wallops Island Flight Center and AVCO Everett Research

Laboratory Inc. The instrument has a tuneable dye laser excitation source, a system to monitor time-fluorescence discriminations, and a multichannel fluorescence analyzer. This may provide a powerful tool for algal discrimination.

J. CONCLUSION

1. Gonyaulax tamarensis, like other dinoflagellates tested, can be distinguished in extracts from other tested algae by a primary fluorescence emission peak at 670nm and a secondary fluorescence emission peak at 640 nm.
2. The secondary fluorescence emission peak at 640 nm was not evident in live cultures of G. tamarensis, or other tested dinoflagellates.
3. Fluorescence sensitivity may be enhanced by the use of a laser excitation light.
4. The detection limit for G. tamarensis by monitoring the chlorophyll peak (670 nm) is 60 cells per ml.
5. Turbidity, caused by colloidal particles, is a major interference factor, but may be corrected for by use of selective filters.
6. Other areas of investigation, such as time-fluorescence studies or excitation pattern differentiation, may provide further differentiation of G. tamarensis from other common endemic algae.
7. Second generation airborne laser spectrofluorometers possess flexible capabilities, which include the ability to change laser excitation frequencies continuously, nanosecond time discrimination, multi-channel emission monitors. This could prove to be a very powerful tool for further discrimination of specific algae.

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L. APPENDIX 1

Specifications of Perkin-Elmer Model 204 Fluorescence Spectrophotometer

Optical System:	Vertical type - Ebert
Wavelength Range:	Scale: 220-780 m μ Effective: Exciter - 220-780 m μ Analyzer - 220-650 m μ (standard detector); 220-780 m μ (with optional R-136 detector)
Relative Aperture:	F3
Dispersion Element:	Grating - 600 lines/mm, blazed at 300 m μ . Same grating type on each monochromator.
Grating Scan Speed:	60 m μ /min.
Fixed Slit:	Spectral bandwidth = 10 m μ
Dispersion:	12.5 m μ /mm
Light Source:	150W Xenon arc
Sample Compartment:	Holds four 10-mm square cells in rotatable turret selected by external lever.
Readout:	Direct reading microammeter, scale 0-110 units and Model 056 strip chart recorder.
Detector:	Photomultiplier tube R-107
Power Consumption:	250 W at 110-115 V.