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PART I

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A STUDY OF MARINE LUMINESCENCE SIGNATURES

By Arthur W. Hornig and DeLyle Eastwood

March 1973

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NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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1. INTRODUCTION AND SUMMARY

1.1 Introduction

This is the Final Report under Contract Number NAS2-6048, A STUDY OF MARINE LUMINESCENCE SIGNATURES, for the National Aeronautics and Space Administration, Ames Research Center, Moffett Field, California. The work was performed by the Applied Research Department in the Government Systems Division of Baird-Atomic, Inc., under the direction of Dr. Arthur W. Hornig, Director of Research.

The objective of this program was to develop data on the luminescence of natural waters as a prelude to the development of airborne and satellite techniques for the measurement of ocean productivity and pollution. Success of the technique depends on the fact that chlorophyll and several other algal pigments belong to the general class of aromatic organics which exhibit efficient luminescence when stimulated in their absorption bands. Certain organic decay products (Gelbstoffe) and water pollutants such as oil also contain materials which can be stimulated to luminesce.

Remote sensing allows wide-area, repetitive measurements of photosynthesis-induced productivity which will yield a data base for evaluating changes in ocean and estuarine quality. Total biomass may also be determined.

Measurement of chlorophyll concentration may be used as an index of phytoplankton concentration and marine food production. Regions with high phytoplankton abundance can support large concentrations of herbivores and successive links in the animal food chain. Such measurements may be useful in delineating areas of upwelling, waterbody boundaries and currents. This information may, in turn, lead to more effective global fish harvesting.

Monitoring of pollutants is important not only during episodes, but to check water quality following abatement action.

Luminescence techniques are valuable because of their high sensitivity, and because of the dual specificity afforded by the parameters of excitation and emission wavelength.

1.2 Summary

As a result of consultations with marine biologists, literature search, and laboratory measurements on time-dependent changes in natural water samples, it was determined that measurements on old water samples would not be representative of natural waters. Therefore the planned program of laboratory measurements on samples sent from remote sites was modified to field measurements at representative sites. This required modification of a laboratory instrument for field use.

In the work here reported, we have demonstrated that a laboratory fluorescence spectrophotometer, modified for field use, has sufficient sensitivity to monitor the lowest concentrations of chlorophyll encountered in coastal waters (less than 0.2mg/l). At the same time, there is enough specificity to distinguish between water samples and establish some identification.

We have surveyed waters on-site at five locations along the Atlantic and Gulf Coasts. Waters from four Pacific Ocean locations were examined in our laboratory because of insufficient time and funding for field measurement. The on-site examinations included chlorophyll excitation/emission and Gelbstoff excitation/emission. The samples mailed to our laboratory were monitored only for Gelbstoff.

A broad literature search has been conducted, directed principally at finding recent work in on-site luminescence of natural waters. A representative Bibliography has been assembled and appears as Appendix B of this report. As expected, there is very little data available on measurements in-situ, except for data from filter fluorimeters. The recent data are discussed separately and spectra reproduced for comparison with the present work.

The excitation/emission data from all sites for both chlorophyll and Gelbstoff have been compared (both inter- and intra-site). Special attention is given to an analysis of chlorophyll excitation data because of possible use

in identifying algal types. As a result of this analysis, we have proposed a model of particulates excitation/emission which assumes total absorption of ultraviolet and most visible radiation, resulting in quantum counter response. This concept leads to a new approach to the identification problem which is discussed in detail.

The data have been assembled into a Compendium of Marine Luminescence Signatures which is published separately. The Compendium forms Appendix C of the Final Report.

2. ORIGINS OF MARINE LUMINESCENCE

2.1 Luminescence Principles

A discussion of the General Principles of Fluorescence Analysis is found in Appendix A. In the following discussion we focus on the application of luminescence methods to seawater.

Principal luminescing moieties in seawater will be aromatic unsaturated organic compounds, either dissolved in the water or as constituents of phytoplankton. In general, these materials will not phosphoresce at ambient temperatures; therefore the luminescence is usually fluorescence. An exception is bioluminescence, which is a special case of chemiluminescence.

The excitation spectrum of a given fluorescent material is usually very similar to the absorption spectrum. Hence, comparison of excitation spectra with tabulated absorption spectra may afford useful identification. The excitation spectrum of a mixture of non-interacting fluorescent materials will be the weighted superposition of the individual spectra. However, for interacting species, the excitation spectrum of a single fluorescing moiety may show excitation characteristics of other components. The interaction we speak of is known as energy transfer.

Energy transfer is of particular importance in algae where the interacting species are the chlorophylls and accessory pigments. In this case, excitation throughout the spectrum results in typical chlorophyll emission. The excitation spectrum for chlorophyll emission may contain indications of the absorbing pigments and hence serve to identify (however roughly) the algal species. The variations in chlorophyll excitation spectra tabulated in the compendium of spectra and elsewhere in this report are one of the most significant aspects of this work.

2.2 Luminescent Materials in Seawater

Seawater forms the matrix of a complex ecological system. The inorganic portion consists primarily of water and eleven organic ions

(Culkin, F1965), accounting for the predominating characteristic of salinity. In fact, trace amounts of almost all of the elements can be found, and many are involved in some of the more important inorganic biochemical reactions in the marine environment. However, fluorescence related to trace materials would usually be too weak to be of interest for remote detection and identification.

The principal fluorescent inorganic materials are dissolved rare earth and uranyl salts, or suspended undissolved phosphors such as calcium tungstate or zinc sulfide. These materials are rarely found in sizeable concentrations in marine waters. Examination of a typical "artificial" seawater in our laboratory has shown no significant fluorescence due to the inorganic ions, and we have never seen seawater luminescence clearly attributable to inorganic materials.

The living organic constituents of seawater range from large fish and plants to the minute phytoplankton and zooplankton. Fluorescence of these "particulates" is related to aromatic pigments present in the surface layer of the organism. In this laboratory we have observed the fluorescence of freely-swimming fish, macroscopic plants, phytoplankton, and dissolved materials. The last two are of greatest interest in this study.

Non-living organic materials may be dissolved or particulate. They may result from excretion or shedding by living organisms, or they may be decay products of dead organisms. Suspended matter of organic origin, permanently incapable of reproduction, is termed detritus (Strickland, E1965). The dissolved decayed organic materials are often referred to as "Gelbstoffe" (Kalle, B1949) because of the yellow color when concentrated. These have a very complex composition which varies with location.

It is convenient to classify fluorescence as that derived from particulate organic matter or from dissolved organic matter; however, it is also interesting to note that the emissions from particulate matter are predominantly at wave-

lengths in the red region of t' spectrum, whereas emissions from dissolved substances are predominantly at wavelengths in the blue-green region of the spectrum.

In addition to the above "natural" components of seawater, there are now significant components introduced by man. The most common pollutants include petroleum oil, sewage, and industrial wastes such as lignin sulfonates. These materials may also contribute to fluorescence background, which must be understood if it is to be subtracted out.

In the preceding we have discussed the general sources of stimulable fluorescence. To these may be added a special category of luminescence which could also be detected remotely (and misinterpreted)--namely bioluminescence. This is a type of auto-luminescence which is properly a form of chemiluminescence, displayed by certain dinoflagellates.

In the following subsections, the specific sources of luminescence in seawater will be discussed by categories important to this study.

2.3 Phytoplankton

If one isolates particulate matter from natural w[illegible]y filtration, and then examines the fluorescence from the particulate mat[illegible]etained on the filter, the most striking feature is the strong emission band occurring at wavelengths around 680 nanometers. This emission is strongest when the excitation beam is centered at 400-500 nanometers. This emission band is due to the chloroplastic pigment chlorophyll, which occurs universally in the marine phytoplankton. As far as can be ascertained from the measurements, the emission characteristics associated with this pigment are identical for a wide range of organisms. There is a wide variety of species composing marine phytoplankton; however, most of the organisms are diatoms and dinoflagellates. There are some exceptions, and these differences should be noted. First of all, red and blue-green algae, which occasionally occur in ocean waters, have a different fluorescence emission spectrum, principally due to the presence of a group of pigments known as phycobilins. Depending upon the

type of organism, and its pigment content, excitation at short wavelengths will produce emissions generally shorter than that for chlorophyll a.

Although there has been considerable interest in the study of fluorescence emission, principally in the connection with the emission from chlorophyll a, no general or clarifying statement can be made as to the factors regulating the intensity of the emission. Yet it can be said that the intensity of the emission is a crude measure of the concentration of the phytoplankton organisms. There also exists the possibility of distinguishing the type of algae by examination of the excitation characteristics responsible for emission.

In vivo fluorescence from phytoplankton is directly or indirectly related to the presence of plant pigments. All pigments will absorb light in specific spectral regions. Some fluoresce directly. Others will transfer energy to another emitting moiety. The main pigments to be found in marine phytoplankton are: chlorophylls, biliproteins, and carotenoids. Xanthophylls, which are oxygenated products of carotenes, are sometimes considered a separate pigment group (Strickland, E1965). Table I, taken from Bogorad (A1962), shows the distribution of pigments among algae.

2.3.1 Chlorophylls

The literature on chlorophylls is voluminous because of the importance and complexity of the subject. Almost all topics are considered in the book edited by Vernon and Seely (A1966). The five principal chlorophyll types (Bogorad, A1962) are labeled a through e. Their concentrations vary widely among algal types; however, all algae contain chlorophyll a. Further, Yentsch (G1971) has determined that only chlorophyll a is observed when algae are observed in vivo. Since we are interested in marine luminescence in natural waters, our attention will be restricted to chlorophyll a.

Chlorophyll a is built from a dihydroporphyrin ring which contains a central non-ionizable magnesium atom. In addition to the four pyrrole

Table I. Distribution of Chlorophylls, Biliproteins, and Carotenoids Among Algae

Pigments	Charophyta	Chlorophyta: "Siphonales" b	Chlorophyta: Others	Euglenophyta	Xanthophyta	Chrysophyta	Bacillariophyta	Phaeophyta	Pyrrophyta	Cryptophyta	Cyanophyta	Rhodophyta
Chlorophylls												
a	+	+	+	+	+	+	+	+	+	+	+	+
b	+	+	+	+	-	-	-	-	-	-	-	-
c	-	-	-	-	-	?	+	+	+	+	-	-
d	-	-	-	-	-	?	-	-	-	-	-	±
e	-	-	-	-	a	?	-	-	-	-	-	-
Biliproteins												
Phycocyanin				-	-	-	-	-	-	b	+	+
Phycoerythrin			c	-	-	-	-	-	-		+	+
Carotenes												
α-Carotene			±				±	±		+		±
β-Carotene	+	+	+	+	+	+	+	+	+		+	+
γ-Carotene	±											
Lycopene	+											
ε-Carotene							+			+		
Unknown				+								
Xanthophylls		L N, S Sx V, Z	As L N V Z	As L, N, Un		F L Un	Dd Dt F	Flx, F, L, V	Dd Dn P	? Z	Apn, Apl Fle, ±l Mn. Ml, O, Z	T, ±Z l

(After Bogorad, 1962)

Key

+ = Present
- = Absent
? = Insufficient Information
Apn = Aphanicin
Apl = Asphanizophyll
As = Astaxanthin (euglenarhodone)
Dd = Diadinoxanthin
Dt = Diatoxanthin
Dn = Dinoxanthin
Fle = Flavacin
Flx = Flavoxanthin
F = Fucoxanthin
L = Lutein
Mn = Muxoxanthin (aphanin, echinenone)
Ml = Myxoxanthophyll
N = Neoxanthin
N = Neoxanthin
O = Oscilloxanthin
P = Peridinin (sulcatoxanthin)
S = Siphonein
Sx = Siphonoxanthin
T = Taraxanthin
V = Violaxanthin
Z = Zeaxanthin
Un = Unknown

a Only observed in Tribonema bombycinum
b Reported also in Cyanidium caldarium
c Reported in Palmellococcus miniatus

nuclei surrounding the magnesium atom, there is a cyclopentanone ring with a methyl ester group attached. A propionic acid side-chain, esterified with phytol, is attached to one of the pyrrole nuclein. A structural diagram is given in Figure 1.

In an algal cell, the chlorophyll is bonded to a protein and to a lipid. The details of the bonding affect the exact position and shape of chlorophyll emission and excitation (or absorption). Thus spectra of extracts may be quite different from those of intact living algae. Rabinowitch (A1951) has noted that the absorption maxima of chlorophylls in vivo were at wavelengths about 10 to 20 nm longer than those in organic solvents. Emission maxima may be similarly shifted, accounting for some of the apparent discrepancies in the literature.

The absorption of chlorophyll a is characterized by two principal absorption bands, one in the blue and one in the red region of the spectrum. The emission is peaked in the red, just beyond the red absorption, with a minor peak at longer wavelengths. The absorption and emission of chlorophyll a extract in ether is given in Figure 2, taken from Goedheer (A1966). The peak absorption is at 430 nm, with a second major peak at 662 nm. The emission is characterized by a peak at 669 nm, with a substantial shoulder at about 370 nm. The emission of chlorophyll a in a green leaf is given in Figure 3, taken from French and Young (A1952). The larger peak is for a partially greened leaf thought tocontain little chlorophyll and hence representing a dilute sample. The peak is at approximately 676 nm, with a minor shoulder at 730 nm. The major peak is considerably shifted when compared with Figure 2. The emission of the darker green leaf of Figure 3 shows the 676 nm peak shifted to 685 nm and reduced, whereas the 730 nm shoulder has developed into a main peak, unshifted. French and Young attribute the difference in shape of the two peaks of Figure 3 to reabsorption of emitted light due to high chlorophyll concentrations. (We have observed similar results in other fluorescing systems as concentration has increased.) Thus observed fluorescence may depend on all parameters affecting the

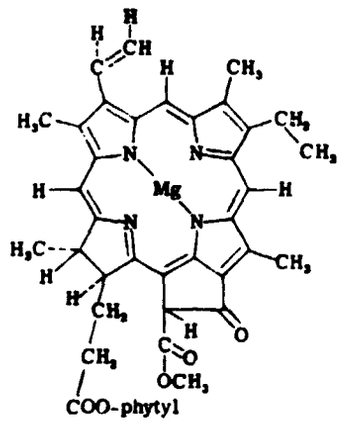


FIGURE 1. Chlorophyll a.

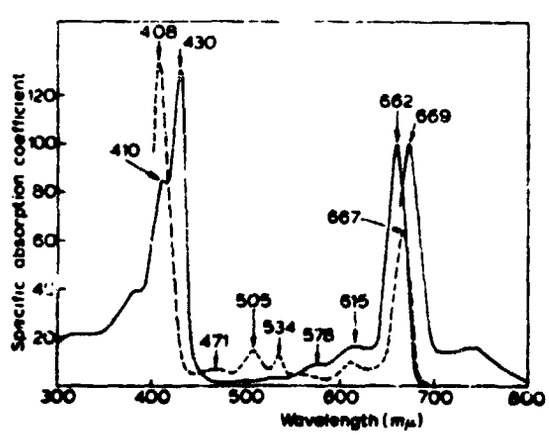


FIGURE 2. Absorption and fluorescence spectra of Chlorophyll a (———) and absorption spectrum of pheophytin a (-----) dissolved in ether (Goedheer, A1966).

concentration of chlorophyll, e.g, age, temperature, light exposure, etc. Corresponding data on the in vivo excitation spectra for chlorophyll a do not exist because chlorophyll is always accompanied by other accessory pigments in living algae, and the excitation spectrum for chlorophyll emission contains absorption bands associated with the pigments.

The previous discussion of this section has referred exclusively to live algae. In a real marine sample, there will also be dead cells and degradation products of chlorophyll a. There are three important decomposition products, chlorophyllide, pheophytin, and pheophorbide. Chlorophyllide is produced from chlorophyll by the loss of the phytol group (see Figure 1). Chlorophyllide a has an absorption spectrum very similar to chlorophyll a: no data are available on its fluorescence. On general principles, the distant phytol group should have little effect on the fluorescence which is related to the aromatic ring structure. Pheophytin a is formed when chlorophyll becomes acidic and the magnesium is replaced by hydrogen. The absorption spectrum differs markedly from chlorophyll in that the intensity of the red peak decreases and the blue peak shifts to slightly longer wavelengths. The fluorescence of pheophytin a is also shifted to longer wavelengths. Pheophorbide is formed by removal of the phytol group from pheophytin. The corresponding absorption is similar to pheophytin and presumably the fluorescence too.

2.3.2 Biliproteins

The biliproteins are composed of phycobilins and a protein fragment. The phycobilins are tetrapyrroles, the principal classes being phycoerythrins and phycocyanins. The phycobilins are not readily released from their associated proteins (OhEocha, A1962), unlike chlorophylls, and hence most measurements are made on the biliproteins, rather than on the phycobilins. The complexity of the protein bonding, etc., has made it impossible to precisely define the structure of the phycobilins. Figure 4 depicts the structure of mesobiliviolin which Lemberg and Bader (A1933) felt was the

chromophore of C-phycocyanin. While it is now felt that this is probably an artifact formed from phycocyanobilin in concentrated hydrochloric acid (OhEocha, A1960), the diagram serves to indicate the general structure of phycobilins.

The absorption spectra of biliproteins in aqueous solution correspond very closely to spectra of intact algae. The absorption spectra of three phycobilins from Prophyra naiadum are given in Figure 5, taken from French et al. (A1956). The peaks at 545 nm (phycoerythrin) 616 nm (phycocyanin) and 654 nm (allophycocyanin) all lie below the longwave chlorophyll absorption, but well above the blue chlorophyll absorption. Figures 6 and 7, taken from OhEocha (A1962), show more detailed absorption spectra of varieties of phycoerythrins and phycocyanins.

In aqueous solution, the phycobilins are strongly fluorescent. Phycoerythrins emit in the orange (578 nm), and allophycocyanin deep red (663 nm). Phycocanin has been reported to emit from 637 nm to 655 nm (French and Young, A1952; Duysens, A1952). The fluorescence of intact algae is much weaker, probably because of energy transfer to chlorophyll. The solution fluorescence of three phycobilins is given in Figure 8, taken from French et al. (A1956).

Biliproteins are generally found in Rhodophyta, Cyanophyta, and Cryptophyta.

2.3.3 Carotenoids

Carotenoids are yellow, orange, or red pigments of aliphatic or alicyclic structure composed of isoprene units, usually eight, linked so that the methyl groups nearest the center of the molecule are in the 1,6-position, while all other lateral methyl groups are in the 1,5-position. The series of conjugated double bonds constitutes the chromophoric system of the carotenoid (Karrer and Jucker, A1950). The structure of α-carotene is given in Figure 9.

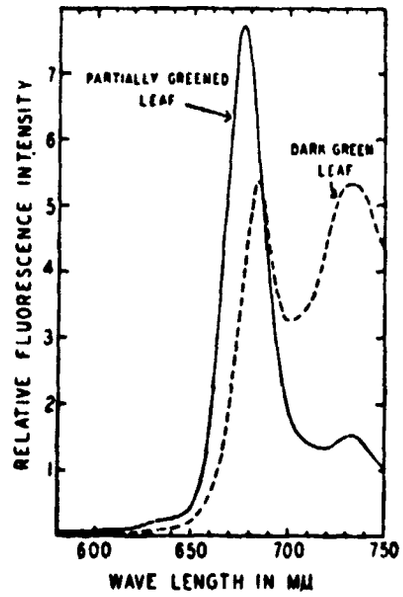


FIGURE 3. The fluorescence spectrum of a leaf containing very little chlorophyll compared with that of a leaf containing a large amount of chlorophyll. Excitation, 436 nm. (French and Young, A1952.)

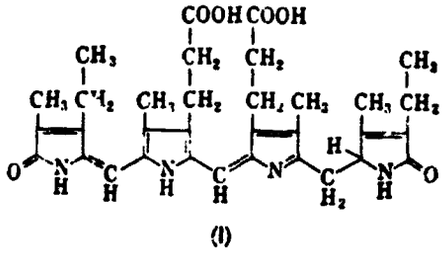


FIGURE 4. Mesobiliviolin (phycocyanobilin)

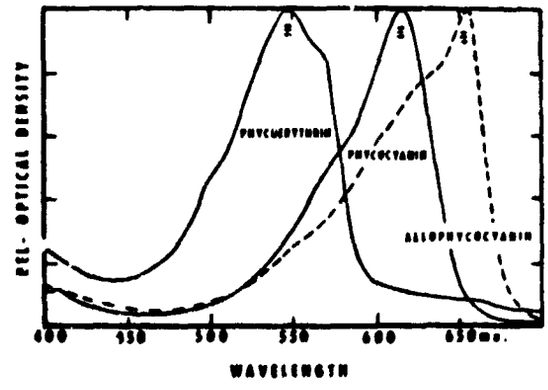


FIGURE 5. Absorption spectra of the phycobilin pigments (French et al., 1956).

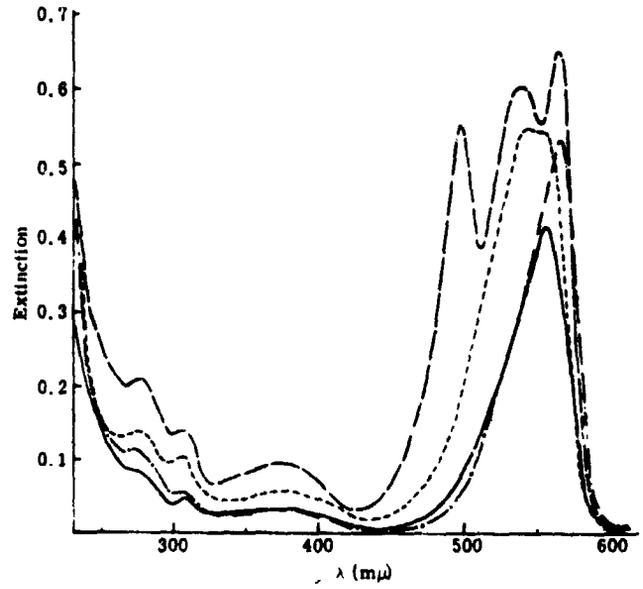


FIGURE 6. Absorption spectra of aqueous solutions of phycoerythrins (pH 6-7):

- - - - - - R-phycoerythrin
- - - - - - - - B-phycoerythrin
—·—·—·— C-phycoerythrin
— — — — cryptomonad phycoerythrin (OhEocha, A1962)

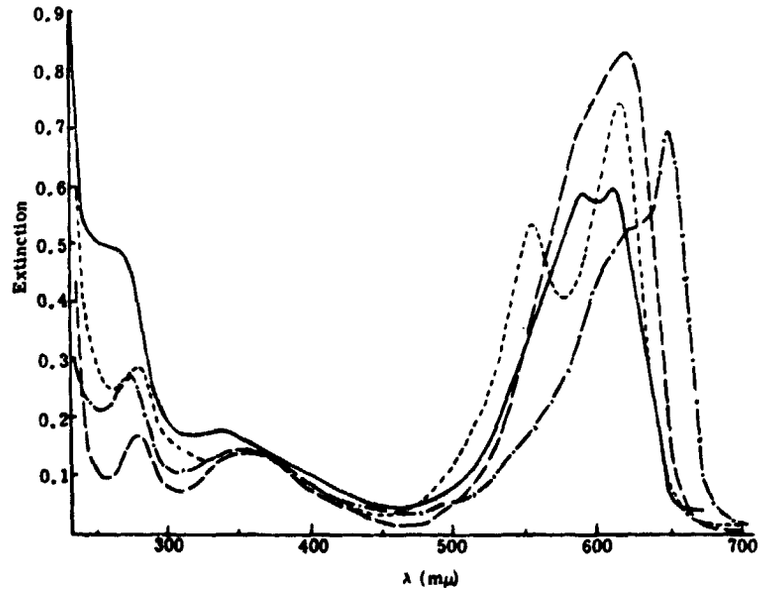


FIGURE 7. Absorption spectra of aqueous solutions of phycocyanins (pH 6-7):

- - - - - - - - R-phycocyanin
— — — — C-phycocyanin
—·—·—·— Allophycocyanin
— — — — Cryptomonad phycocyanin, Plymouth strain No. 157)

(OhEocha, A1962)

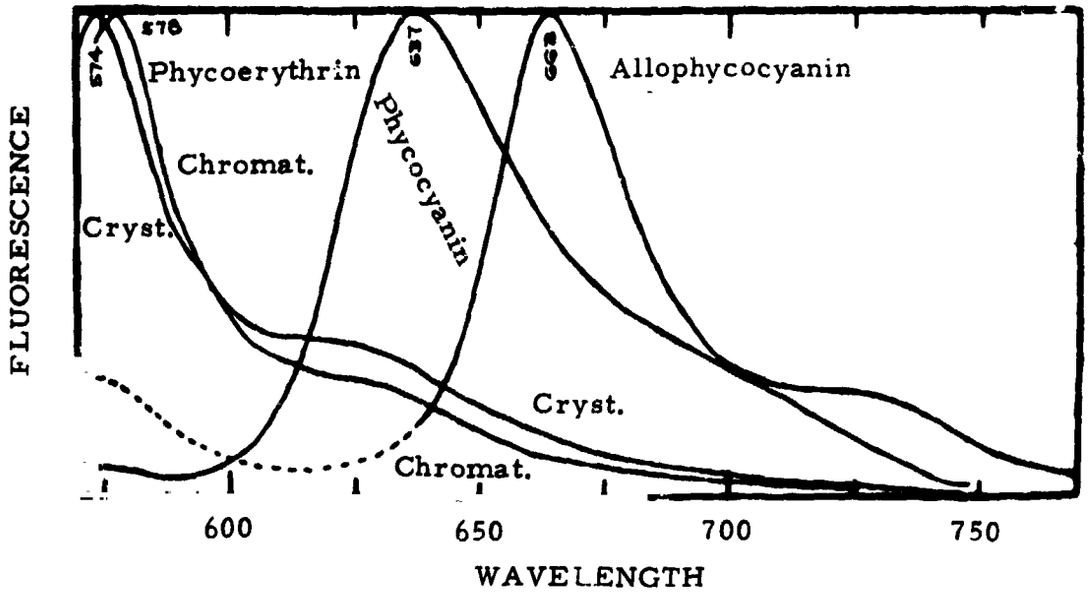


FIGURE 8. The fluorescence spectra of the phycobilins from Porphyra naiadum (French, et al., 1956).

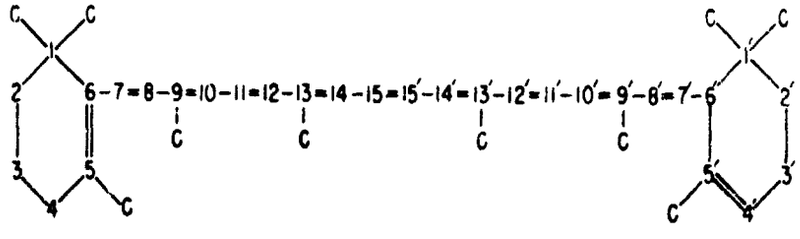


FIGURE 9. The numbering system of carotenoids illustrated for α-carotene according to the American Chemical Society Committee on Nomenclature.

The carotenes do not contain oxygen, while the xanthophylls are oxygenated. In vivo carotenes are associated with proteins and lipids, which gives rise to a "red-shift" of 1-20 nm in absorption. β-carotene predominates in all phytoplankton except Cryptophyta (Strickland, E1965). The absorption spectrum of β-carotene in acetone peaks at about 455 nm.

The xanthophylls have the most complex distribution in algae and hence are of most use for characterization of classes. Carotenoids do not usually fluoresce, but are known to protect chlorophylls from damaging radiation (Clayton, A1966). Fucoxanthin, an important xanthophyll found in brown algae, has an absorption band at 530 nm (Yentsch, D1971).

2.3.4 Energy Transfer

Intermolecular energy transfer is of great importance because of its involvement in photosynthesis. It has now been well established (Brody, A1962) that the role of accessory pigments in photosynthesis is to absorb energy and transfer it to chlorophyll a, whose role remains that of the only photocatalyst. Energy transfer has been established indirectly by oxygen evolution and directly by fluorescence experiments. Dutton et al. (A1943) demonstrated that some of the energy absorbed by the carotenoid fucoxanthin in "Nitzschia closterium" was emitted as fluorescence of chlorophyll to about the same extent as energy directly absorbed by the chlorophyll. Brody and Rabinowitch (A1957) measured the time for energy to be transferred from phycoerythrin to chlorophyll a (probably via phycocyanin) as 0.5×10^{-9} sec. Haxe (A1960) has measured the action and absorption spectra of the red alga Porphyridium aerugineum and compared these with the absorption spectrum of an aqueous extract which contained principally C-phycocyanin. Figure 10 demonstrates that the action spectrum is very similar to the absorption by phycocyanin, while chlorophylls and carotenoids are relatively inactive. Thus, the photosynthesis occurring for excitation between 420 and 445 nm and at 680 nm, where chlorophyll has its maxima, is extremely low. From this it would seem that the energy is absorbed by phycocyanin and passed on to chlorophyll.

Because of the energy transfer from the accessory pigments to chlorophyll, the excitation spectrum for chlorophyll emission may be the most useful single measurement which will yield information about the type of algae present in a given seawater specimen. Since energy transfer is not total, some excitation remains in the accessory pigments which may appear as characteristic fluorescence. It is known that this fluorescence efficiency is not very high. Nevertheless, it may also be useful to monitor emission at wavelengths corresponding to phycobilins, for example (see Figure 8), when exciting in phycobilin or possibly carotenoid absorption bands. This last is illustrated in Figure 11, taken from French and Young (A1952). Here the observed fluorescence from Porphyridium excited at 530 nm is decomposed into the sum of emissions assigned to phycoerythrin, phycocyanin, and chlorophyll.

2.4 Dissolved Organic Materials (Gelbstoffe)

Blue-green fluorescing material is introduced into ocean waters when organic matter is decomposed, and also by the production of brown "exudates" by aquatic plants and marine seaweeds. Spectral examination of those yellow-brown substances, which are almost identical spectrally, shows that their color is due to a high u.v. absorption, which tails over into the visible. Studies of C. Yentsch and collaborators (D1971) have shown that the blue-green fluorescence is mostly associated with these yellow compounds; that is, when the yellow compounds are removed from the seawater (by extraction), there is no fluorescence. It should be noted that there is, apparently, no band in the absorption spectrum corresponding to the excitation spectrum.

The identity and chemical composition of these substances have been of great interest to oceanographers. In 1963, the German chemist Kalle (B1963) showed that fluorescent substances similar to that in seawater could be formed from carbohydrates and amino acids. It is not clear, however, how these materials are introduced, or whether or not they are differentially altered by bacteria in water. Yentsch has observed that the

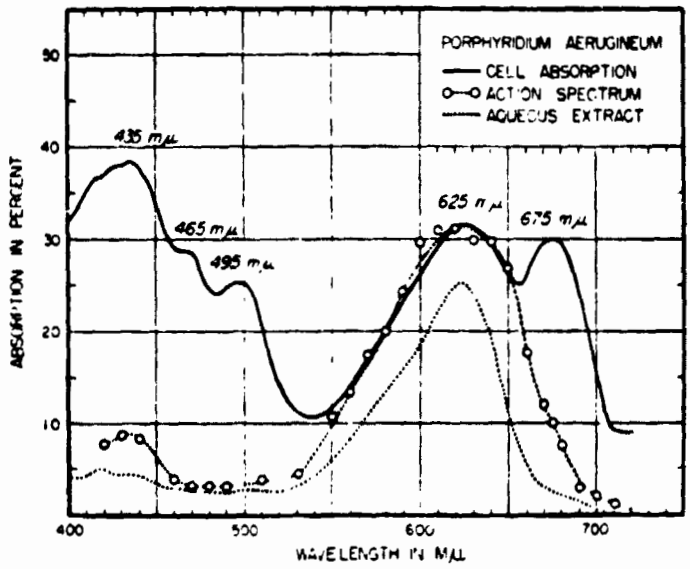


FIGURE 10. Cell absorption and photosynthetic action spectra of the red alga Porphyridium aerugineum, which contains C-phycocyanin as its principal accessory pigment (Haxo, A1960).

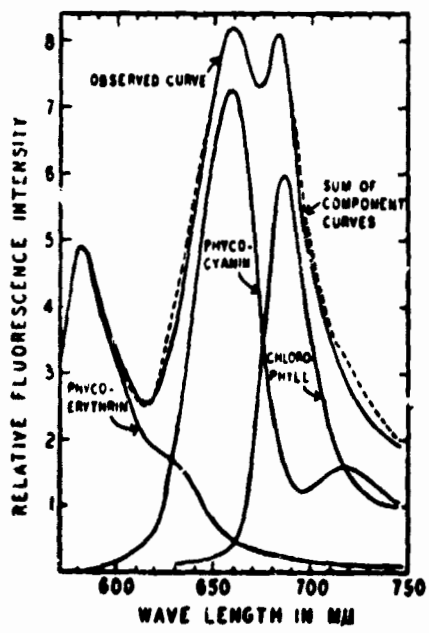


FIGURE 11. The derived curves for the fluorescence spectra in Porphyridium of phycoerythrin, phycocyanin, and chlorophyll a. Their summation is compared with the fluorescence spectrum of Porphyridium illuminated by a wave length of 530 nm. The sum of the individual curves is indicated by dotted lines (French and Young, A1952).

amino acid tryptophane photo-oxidizes rapidly, resulting in a yellow, highly fluorescent photoproduct. The stability or chemical nature of this photoproduct is not known. There are a number of other compounds that can be extracted from marine organisms that are highly fluorescent. Most of these are aromatic, polycyclic hydrocarbons such as anthracene, phenanthrene, chrysene and fluoranthene. It should also be noted that some of these are components of crude oils. There are also many intermediates of the metabolic mechanisms in the organisms that are highly fluorescent. Pigments such as pterins, flavones, and coumarins are highly fluorescent. It is not clear that these extracts or pigments are observed directly in situ.

In the remainder of this report, we shall often use Kalle's term "Gelbstoff" to signify the entire complex mixture of blue-fluorescing natural materials in seawater.

2.5 Bioluminescence

Bioluminescence is not fluorescence but a type of chemiluminescence. Most of the bioluminescence seen in the oceans is termed "stimulable" bioluminescence--meaning light emission after mechanical agitation of the organism. The most common source of bioluminescence is the dinoflagellates. Typical emission spectra are shown in Figure 12, taken from the thesis of M. Kelly (C1968). In general, peak emission is in the 470-480 nm region, rising sharply from between 435 and 450 nm and dropping off by 550 nm. While typical chlorophyll luminescence can be light-stimulated from bioluminescent dinoflagellates, it is unclear whether a true fluorescence, identical to the bioluminescence, can be light-stimulated.

2.6 Fish Oils

It is known that schooling fish emit characteristic oils which sometimes form slicks. In a study performed in this laboratory (Hornig, G1970), the excitation/fluorescence signatures of acetone extracts of a number of fish oils were examined. Typical multicomponent spectra are given in Figures 13 and 14. It has also been observed (Hornig, D1971) that the skins of live

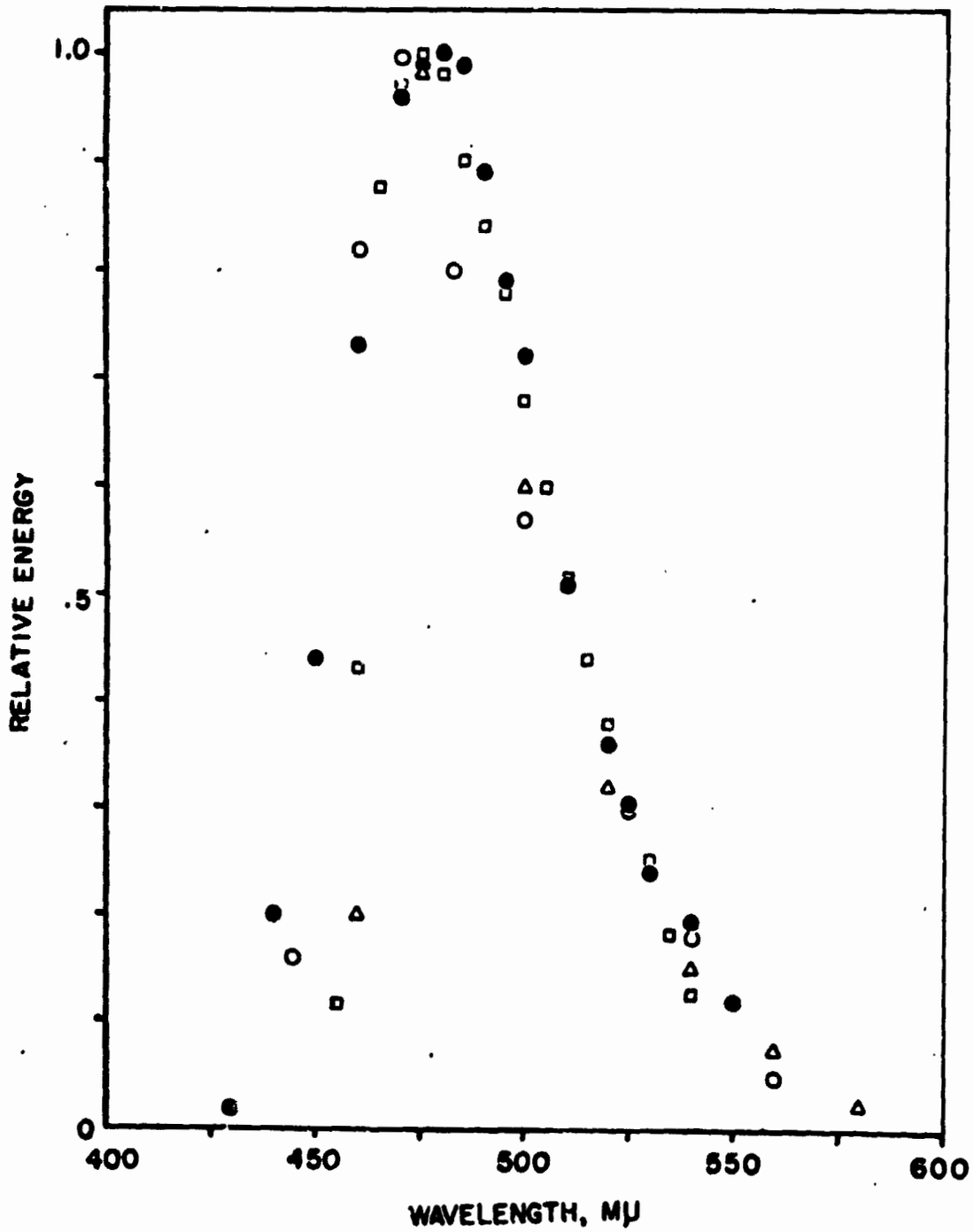


FIGURE 12. Bioluminescence of several dinoflagellates (Kelly, C1968).

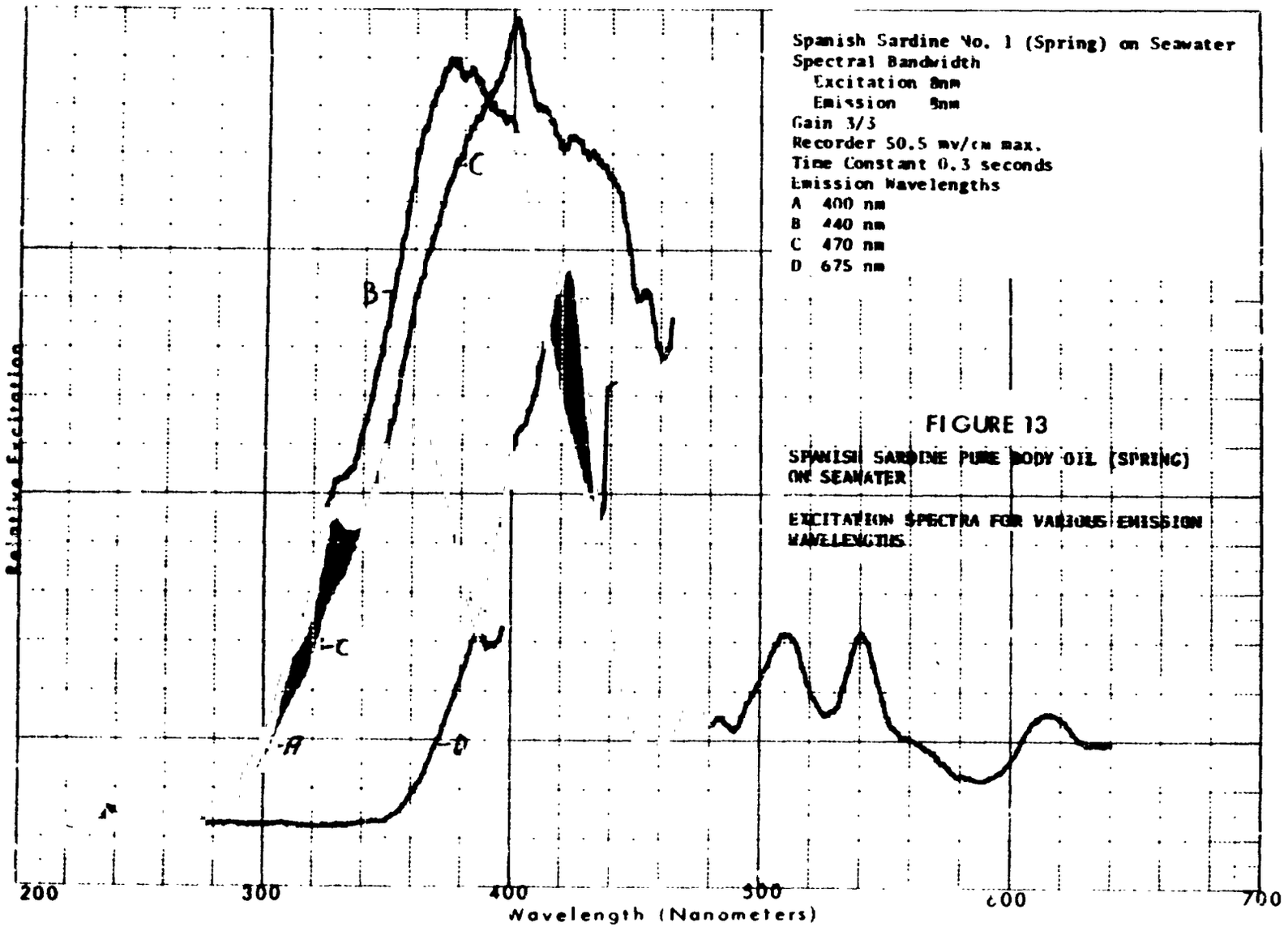


FIGURE 13

SPANISH SARDINE PURE BODY OIL (SPRING) ON SEAWATER

EXCITATION SPECTRA FOR VARIOUS EMISSION WAVELENGTHS

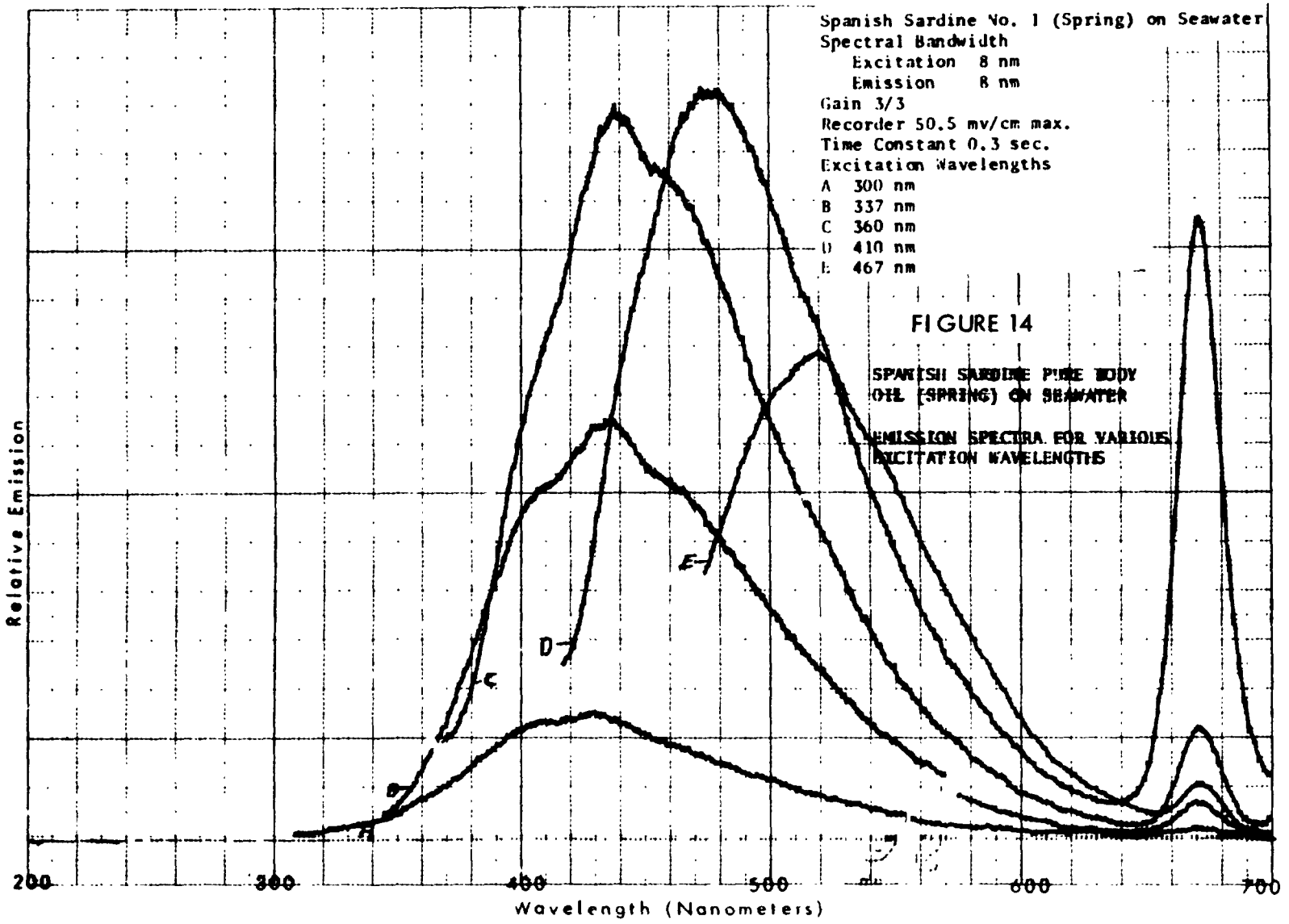


FIGURE 14

SPANISH SARDINE PURE BODY OIL (SPRING) ON SEAWATER

EMISSION SPECTRA FOR VARIOUS EXCITATION WAVELENGTHS

fish fluoresce. In Figure 15, three different emission spectra are presented, corresponding to three excitation wavelengths, for fresh smelt.

2.7 Pollutants

Marine waters have become increasingly polluted by petroleum oil spills, industrial wastes, and sewage, to name a few common substances. Since these all may contain aromatic materials, fluorescence may be the rule. Typical excitation/emission spectra for thin films of a variety of petroleum oils (Hornig, H1971) are given in Figures 16 and 17. Similar spectra for a lignin sulfonate, a waste product from the paper industry, are given in Figure 18.

2.8 Summary

The principal natural fluorescence to be encountered in marine water is from algae and Gelbstoff. The absorption, excitation, and fluorescence of principal components are nicely summarized in Figures 19 and 20, taken from Yentsch (D1971).

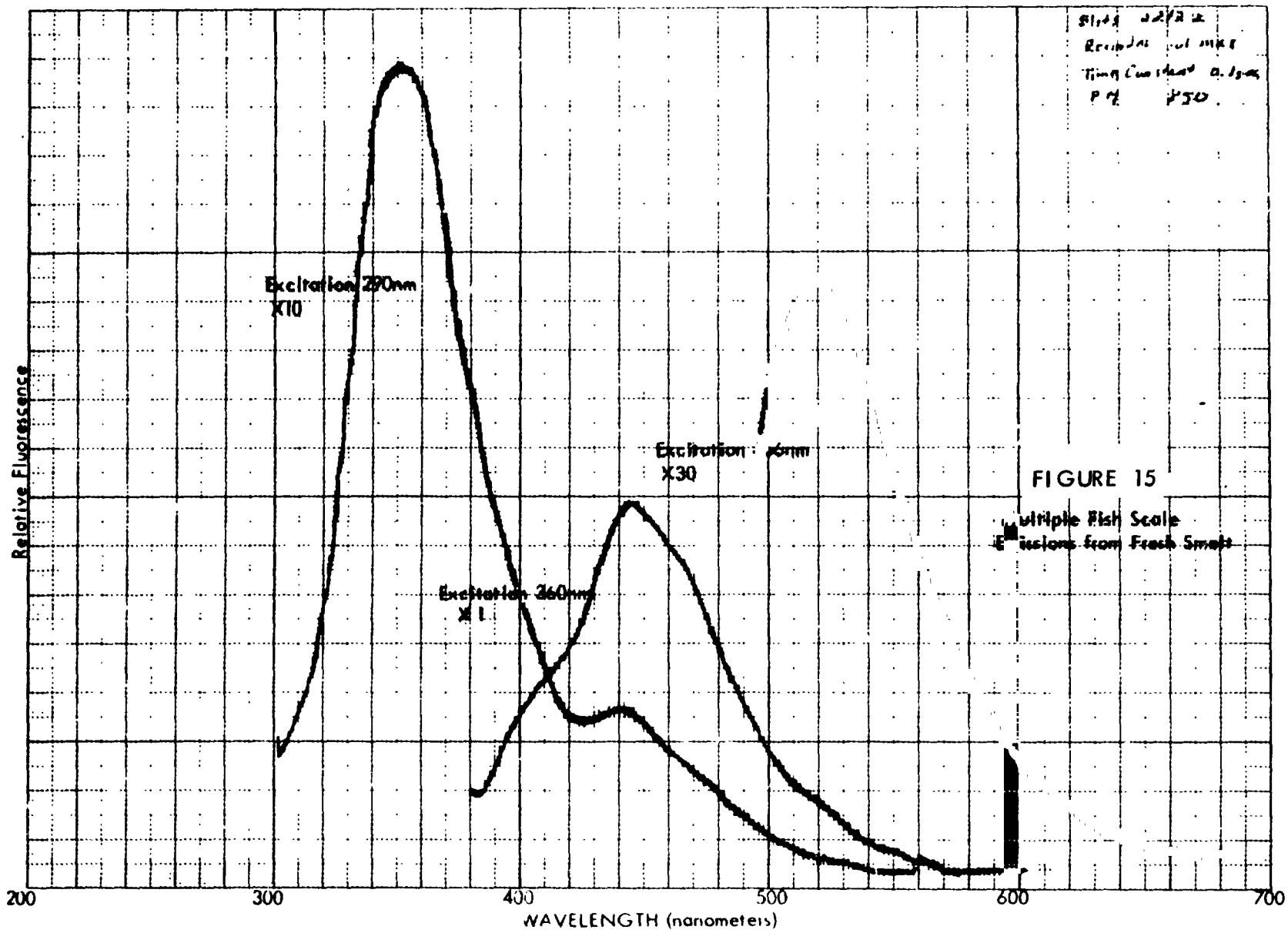
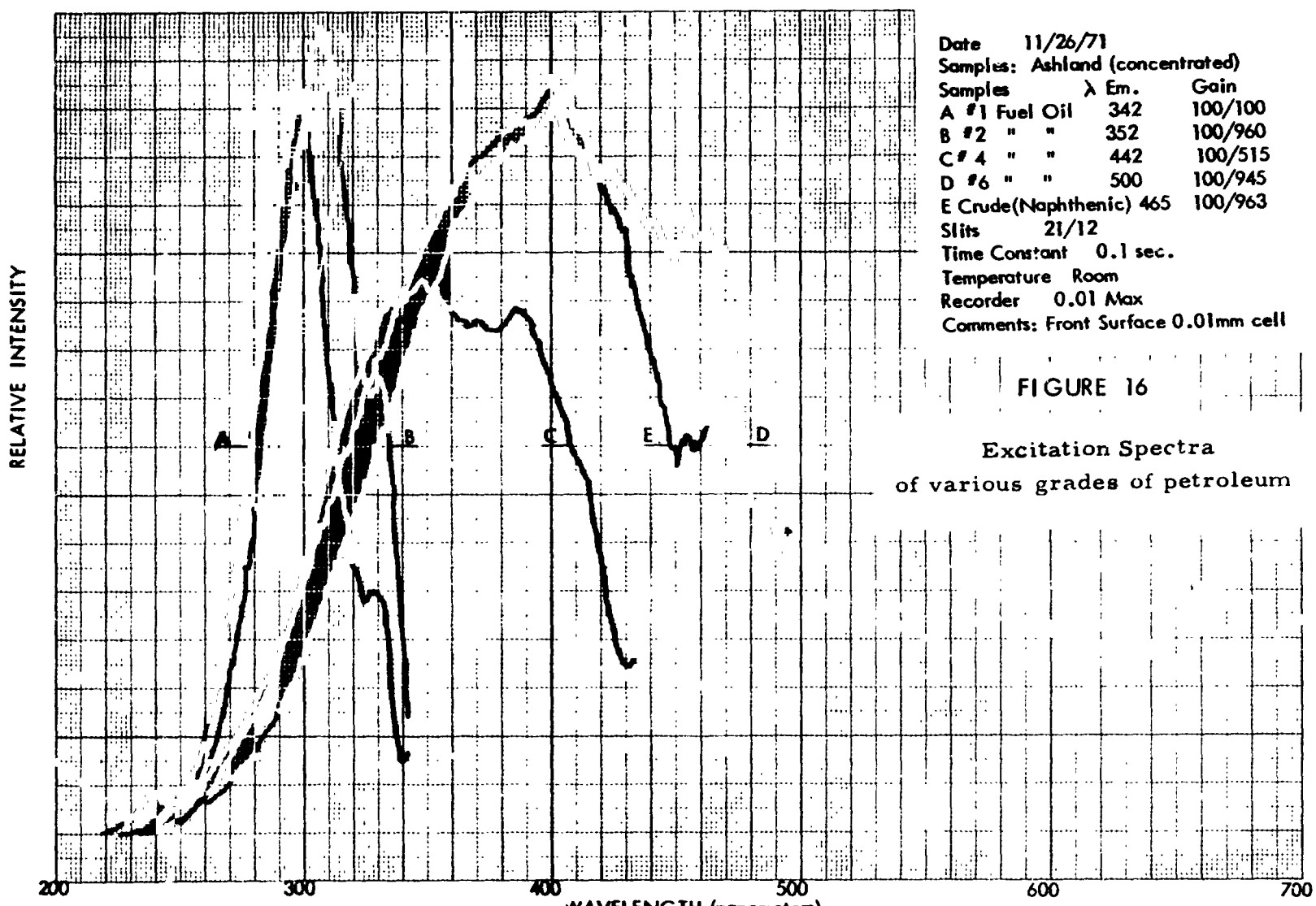


FIGURE 15

Multiple Fish Scale Emissions from Fresh Smelt



Date 11/26/71
Samples: Ashland (concentrated)

Samples	λ Em.	Gain
A #1 Fuel Oil	342	100/100
B #2 " "	352	100/960
C # 4 " "	442	100/515
D #6 " "	500	100/945
E Crude(Naphthenic)	465	100/963

Slits 21/12
Time Constant 0.1 sec.
Temperature Room
Recorder 0.01 Max
Comments: Front Surface 0.01mm cell

FIGURE 16

Excitation Spectra
of various grades of petroleum

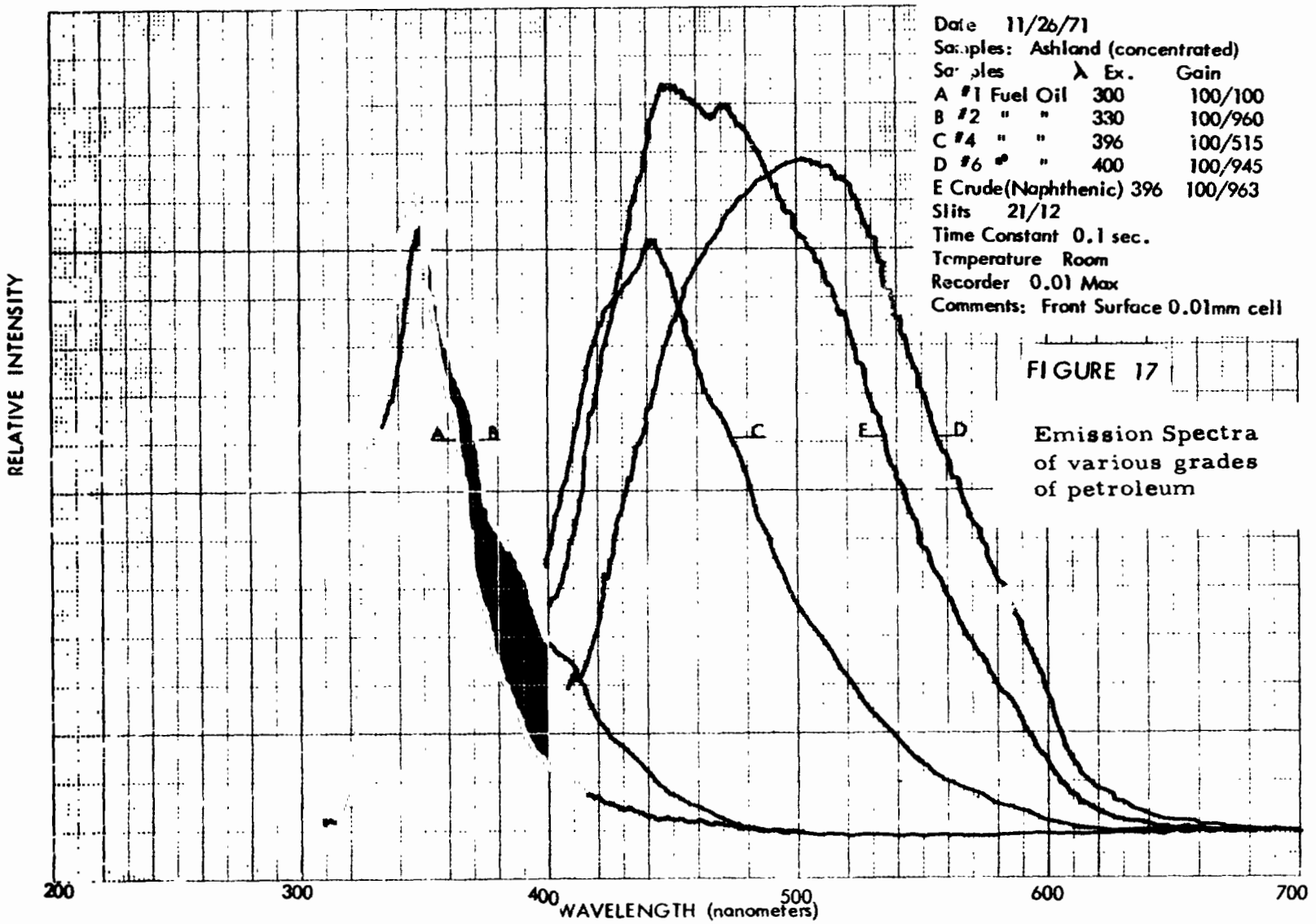


FIGURE 17

Emission Spectra of various grades of petroleum

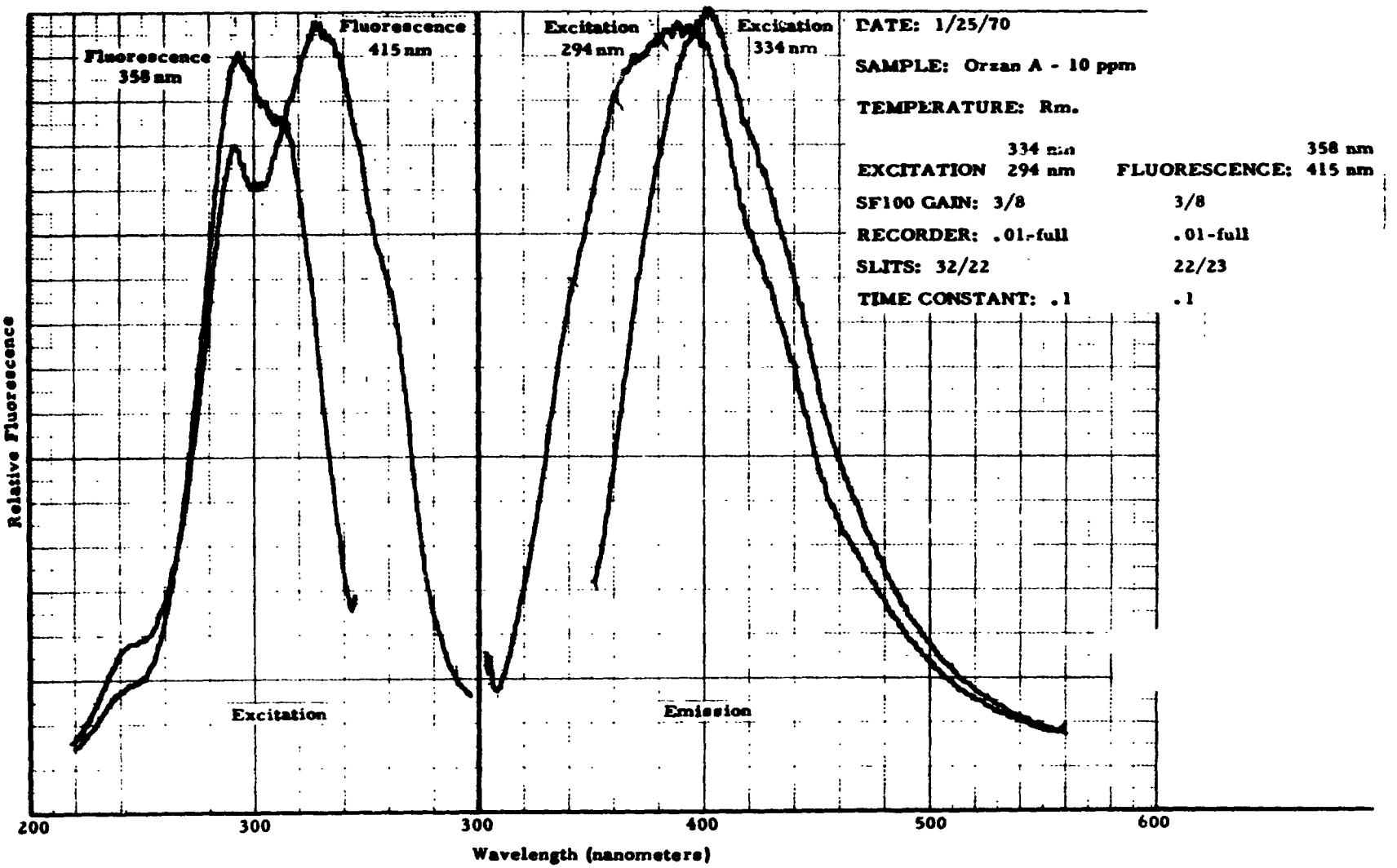
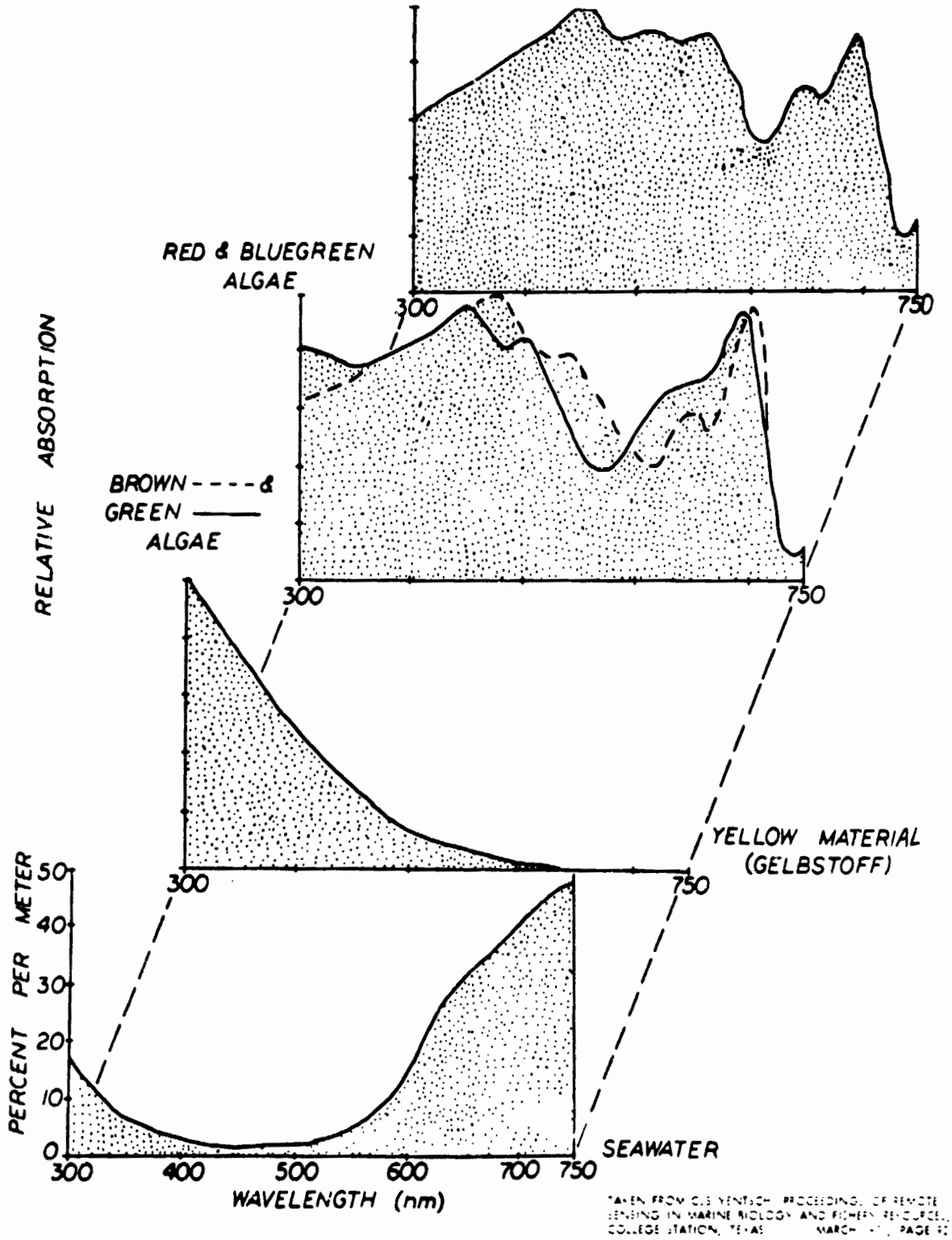


FIGURE 18 : Multicomponent Excitation / Emission Spectra of 10 ppm Orzan A in Distilled Water



TAKEN FROM C.S. YENTSCH, PROCEEDINGS OF REMOTE SENSING IN MARINE BIOLOGY AND FISHERY RESOURCES, COLLEGE STATION, TEXAS MARCH [illegible], PAGE [illegible]

FIGURE 19 : Absorption Spectra for Substances in Natural Waters

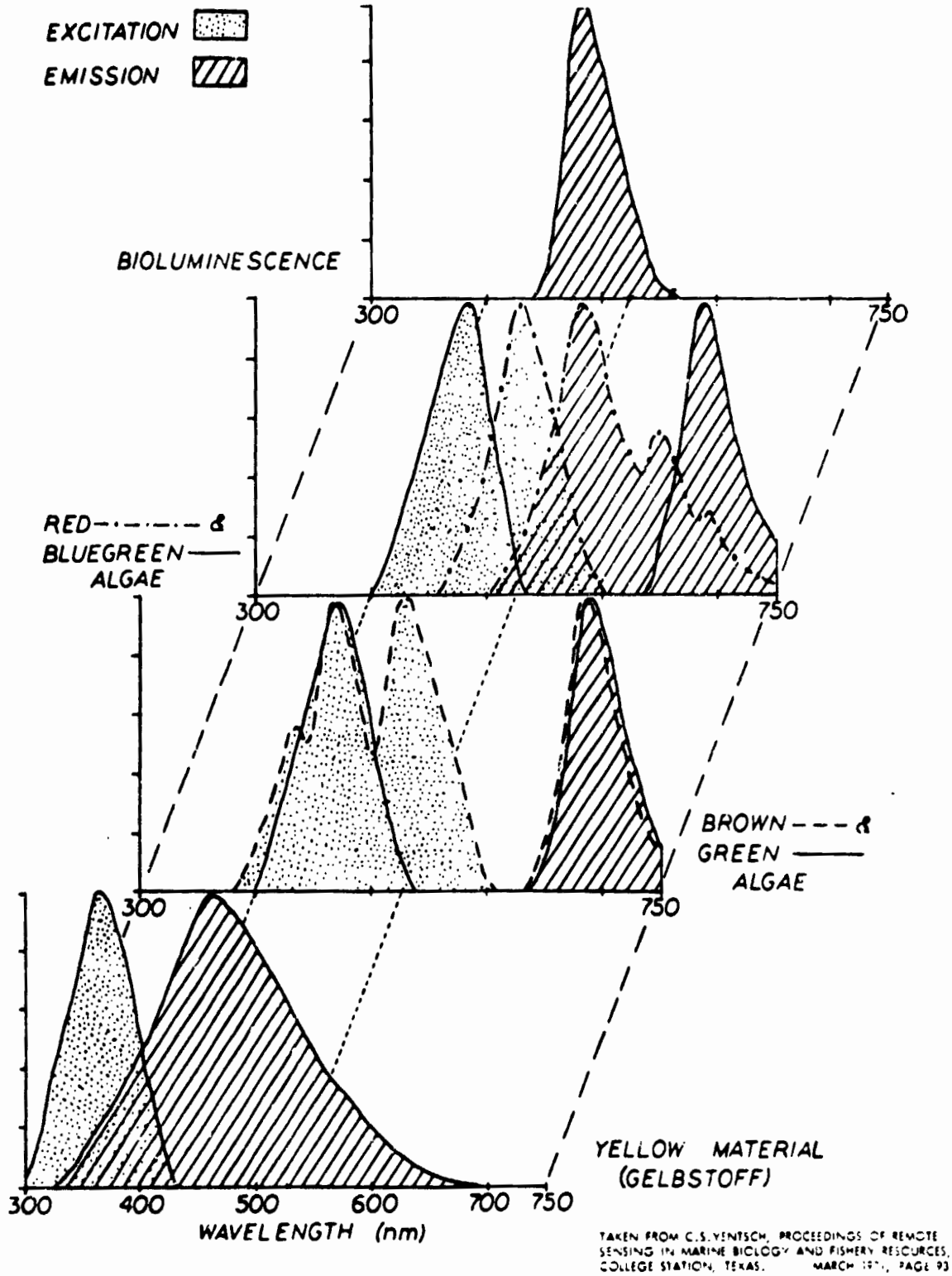


FIGURE 20 . Excitation/Emission Spectra for Principal Sources of Light Emission in Natural Waters

3. EXPERIMENTAL METHOD

At the inception of this project it was planned to perform all spectral measurements at our laboratories in Bedford, Massachusetts. Samples from coastal areas of the U. S. were to be mailed to Bedford by cooperating institutions. These plans were predicated on the existence of a simple, reliable sample treatment and packaging scheme which would preserve sample integrity. Consultation with marine biologists, literature search, and measurements on real samples demonstrated that marine samples are too fragile to permit shipment without drastic changes in luminescing components--especially those involving chlorophyll. Therefore it was decided that the entire modus operandi would have to be changed in order to ensure valid data. Specifically, it would be necessary to revamp laboratory instrumentation for measurement on fresh samples at field sites.

Most measurements documented in the Compendium were taken at field sites on fresh samples. Since the necessary instrument improvement was evolutionary, performance varied at different sites depending on the local circumstances. Often the conditions were less than ideal, instrument operation was not always up to normal, and press of time sometimes prevented use of filters or taking of as much multi-component data as we would have wished. Thus there is a certain unevenness in the results.

It was also expected that a computer-correction scheme would be available for presentation of corrected curves in the Compendium. Difficulties with the computer program and dependence on borrowed instrumentation delayed the availability of this facility. As a result the data of the Compendium are not corrected.

The instrumental evolution and typical results will be discussed in the remainder of this section.

3.1 Initial Approach

Measurements were to be performed on an extended range version of a Baird-Atomic Model SF-100 Fluorescence Spectrophotometer. This

instrument employs double monochromators for both excitation and emission, uses a 150-watt xenon lamp for excitation, and is calibrated from 220-1400 nm on both excitation and emission. An RCA 1P28 is normally used in the visible, while a cooled RCA 7102 is used for spectra in the red and near infrared.

3.1.1 Sample Degradation

The question of how to collect, preserve, and ship a water sample from a distant source to our Bedford laboratory--and preserve sample integrity insofar as luminescence signatures are concerned--is very difficult. Further, such a method must be simple and inexpensive enough to permit volunteer personnel to provide such samples.

As soon as a sample has been collected, it begins to change due to several factors, including:

nature and intensity of illumination during shipment
dissolved oxygen content
pressure and temperature
lack of equilibrium with a marine environment
interaction with the sample container

If living cells are present (as is the case for samples of interest to this project), they may continue to grow, and the composition of the sample may change if oxygen concentration and lighting conditions are favorable. If cells containing chlorophyll are killed, or the photosynthetic process is inhibited by lack of light or for other reasons, the chlorophyll fluorescence may increase by a factor of 2000 (Oppenheimer, E1966). If the sample is kept in darkness, the organism may bleach. On the other hand, if the sample is exposed to light of wavelength shorter than 400 nm, it may degrade in a matter of minutes, depending on the organism.

Samples may be stabilized for a few hours by cooling to near 0°C, but certain algae may grow at or just below 0°C. Further cooling may rupture cell walls, allowing soluble pigments to dissolve in the water, leaving an altered system.

Chlorophylls are exceptionally labile and readily form green and gray-brown alteration products when cells are injured or killed, subjected to various reagents, or exposed to various unfavorable conditions (Strain and Svec, in Vernon and Seely, A1966). Chlorophylls often isomerize spontaneously in solution, especially at elevated temperatures. They are oxidized (allomerized) rapidly when dissolved in relatively inert solvents such as alcohols in the presence of air.

For these reasons we conclude that it may be practically impossible to preserve sample integrity for plankton measurements or for any living organisms, unless measurements are made immediately after sample collection. This is particularly true where the measurements must duplicate those on raw water. Shipping and handling are expected to have less effect on Gelbstoff and certain pollutants such as petroleum or lignins.

3.1.2 Shipping Methods and Experiments

A useful shipping method must not only preserve sample integrity, but be simple and inexpensive enough to permit practical shipment from many remote sources. We first investigated a commercial biomailer consisting of a cubical styrofoam box fitted into a cardboard mailing carton. Such a box, filled with CO_2 powder and tightly sealed, lasted less than one day. Increased wall thickness and use of solid carbon dioxide would have increased cold time, but hardly more than a day or so.

Mr. J. E. Warinner of the Virginia Institute of Marine Science at Gloucester Point, Virginia, reported that he had had some success in mailing samples cooled in dry ice, with the samples frozen after several days. He kindly agreed to provide us with a variety of samples of waters from the Chesapeake Bay, all shipped in different containers.

Frozen samples were sent in polypropylene bags and polystyrene (Nalgene) petri dishes. Uncooled samples were sent in the above plus a glass vial. Samples were all gathered from the same place and at the same time; however, three different samples were sent in each type container to

check reproducibility. Distilled water samples were also sent in each type of container (frozen and unfrozen) as a control. The sample containers were packed in a styrofoam box with four-inch walls top and bottom and two-inch walls on the side. The sample/dry ice space was 4" x 6-1/2" x 4", allowing ample space for a good load of dry ice. The uncooled samples were also imbedded in thick styrofoam to maintain ambient temperature during shipment.

Despite the care in setting up this experiment, it was largely disappointing due to the extraordinarily slow air parcel post service. The cooled samples arrived five days after they were shipped: the uncooled eight! Needless to say, the dry ice was gone, and all samples were warm upon arrival, thus vitiating the primary purpose of the experiment. It is clear that shipping times of up to a week must be expected, casting further doubt on the expected sample validity.

3.1.3 Initial Luminescence Measurements

In order to check changes in sample luminescence, with time it was decided to work in cooperation with Dr. Charles Yentsch of the Marine Institute of the University of Massachusetts at Gloucester, Massachusetts. In an effort to use a single photomultiplier for all measurements, and so simplify experimental procedures when using the instrument at Yentsch's laboratory, an EMI 95580 tube with an S-20 response was employed. Unfortunately this broke during a cooling experiment. We next returned to an S-1 tube, but used an experimental tube, an RCA C70007A, rather than our 7102. This tube requires cooling and hence could not easily be used in measurements at Yentsch's laboratory.

First measurements were on the samples from VIMS. Examining samples nine or ten days old we could not detect emission in the 680 nm (chlorophyll) region using the cooled C70007A and a standard cuvette. Using the 1P28 we did detect emission corresponding to the Gelbstoff of Figure 20. As expected, we found several variant emissions, depending on the excitation

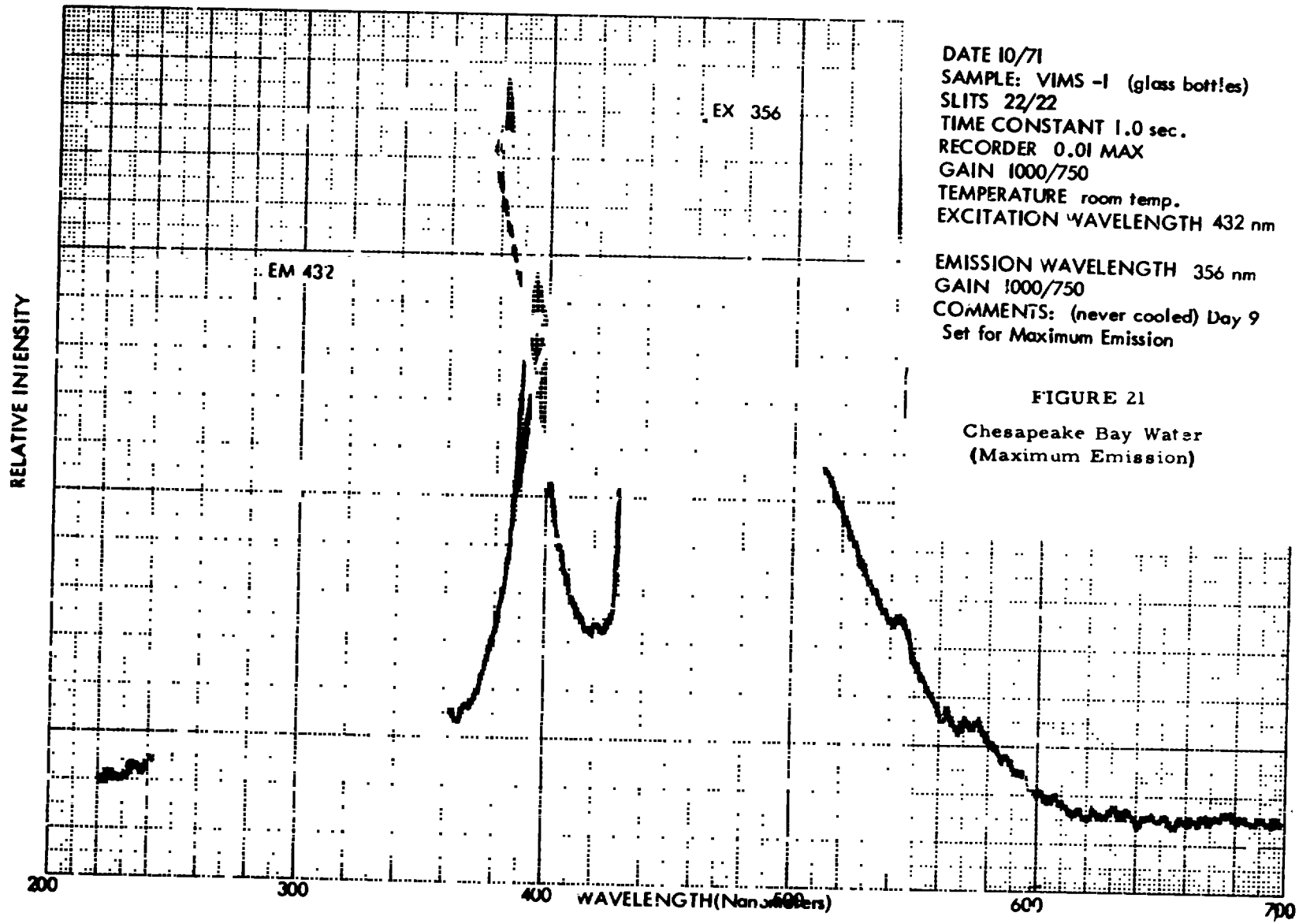
chosen. Correspondingly, the excitation spectrum varied, depending on the monitoring wavelength. Our initial results are given in Figure 21. Multicomponent excitation and emission spectra for the same sample are given in Figures 22 and 23. (Raman peaks are identified with the symbol "R" in the figures.)

Considering the emission spectra of Figure 23, it is apparent the chief emission is rather structureless (instrumental bandwidth, 8 nm) peaked near 450 nm. The rather sharp peak at 340 nm, corresponding to excitation at 295 nm, is very possibly due to a light oil pollutant (cf. traces A and B in Figure 17).

The excitation spectra of Figure 22 are likewise structureless, although the excitation peak moves to shorter wavelengths as the monitoring wavelength decreases. Again, the excitation trace when monitoring at 340 nm has a peak resembling that of the lightest oil of Figure 16, suggesting very strongly that the Chesapeake Bay sample was polluted. This is not unlikely since Gloucester Point is located close to busy docking facilities.

The emission data of Figure 24 were taken on the same sample as Figure 23 but on the tenth day--one day later. Comparing relative intensities and shapes of curves, it would appear that the light oil has decreased in intensity, but the general shape of the remaining curves is similar. The oil peak may have decreased from evaporation, but more probably it was present as a fine emulsion when we first examined it, but had coalesced on standing to larger droplets which do not give as intense a spectrum. We conclude that the blue emission (which we attribute to "Gelbstoff" for convenience) is rather stable in time, as opposed to the red "chlorophyll" emission which we did not detect.

A further conclusion of this experiment concerned shipping containers. Glass vials, polypropylene bags and polystyrene petri dishes were used. Examination of the ten-day-old distilled water samples revealed that the water stored in polypropylene bags had a much larger background fluorescence

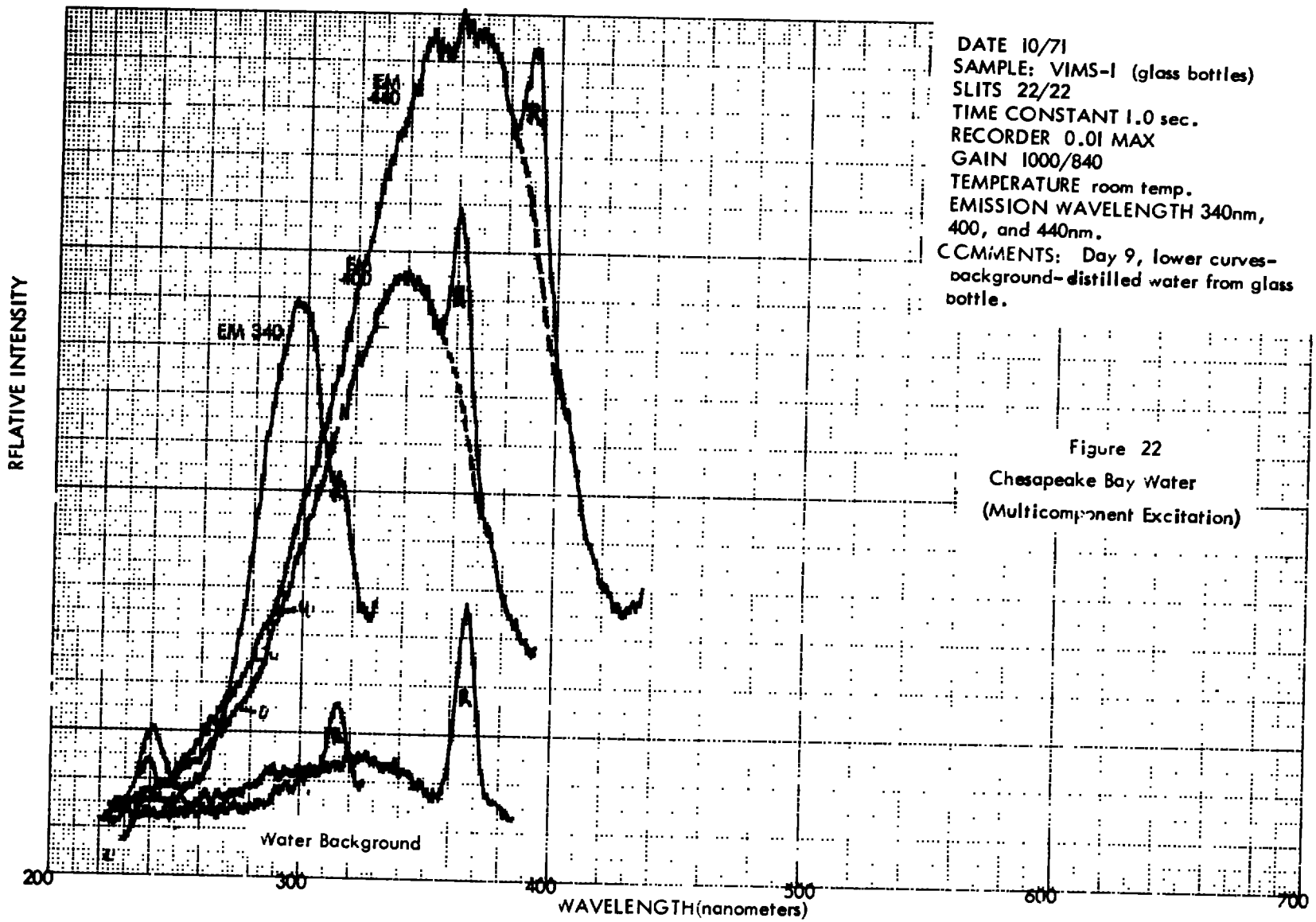


DATE 10/71
SAMPLE: VIMS -I (glass bottles)
SLITS 22/22
TIME CONSTANT 1.0 sec.
RECORDER 0.01 MAX
GAIN 1000/750
TEMPERATURE room temp.
EXCITATION WAVELENGTH 432 nm

EMISSION WAVELENGTH 356 nm
GAIN 1000/750
COMMENTS: (never cooled) Day 9
Set for Maximum Emission

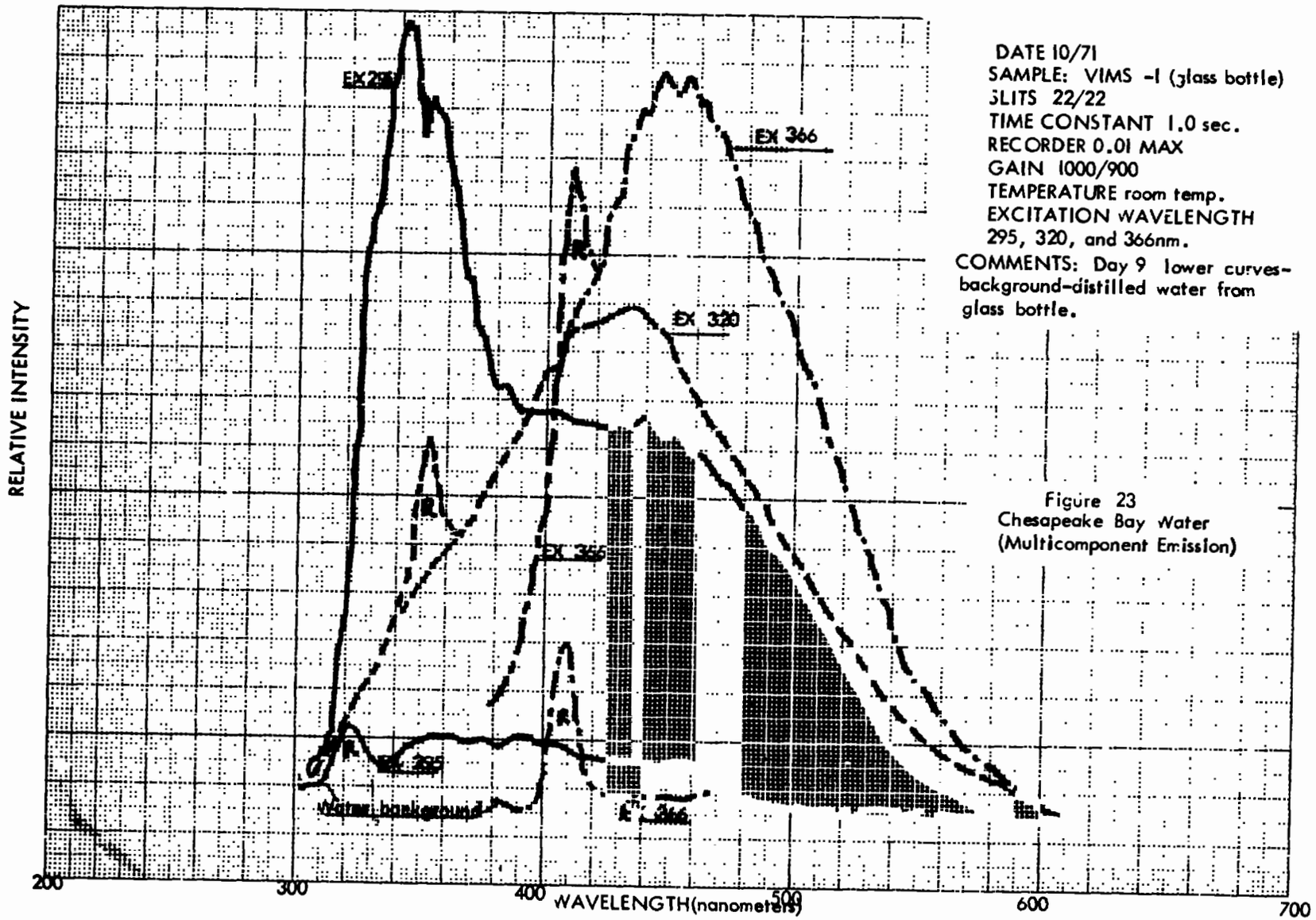
FIGURE 21

Chesapeake Bay Water
(Maximum Emission)



DATE 10/71
SAMPLE: VIMS-I (glass bottles)
SLITS 22/22
TIME CONSTANT 1.0 sec.
RECORDER 0.01 MAX
GAIN 1000/840
TEMPERATURE room temp.
EMISSION WAVELENGTH 340nm, 400, and 440nm.
COMMENTS: Day 9, lower curves-background-distilled water from glass bottle.

Figure 22
Chesapeake Bay Water
(Multicomponent Excitation)



DATE 10/71
SAMPLE: VIMS -I (glass bottle)
SLITS 22/22
TIME CONSTANT 1.0 sec.
RECORDER 0.01 MAX
GAIN 1000/900
TEMPERATURE room temp.
EXCITATION WAVELENGTH
295, 320, and 366nm.
COMMENTS: Day 9 lower curves-background-distilled water from glass bottle.

Figure 23
Chesapeake Bay Water
(Multicomponent Emission)

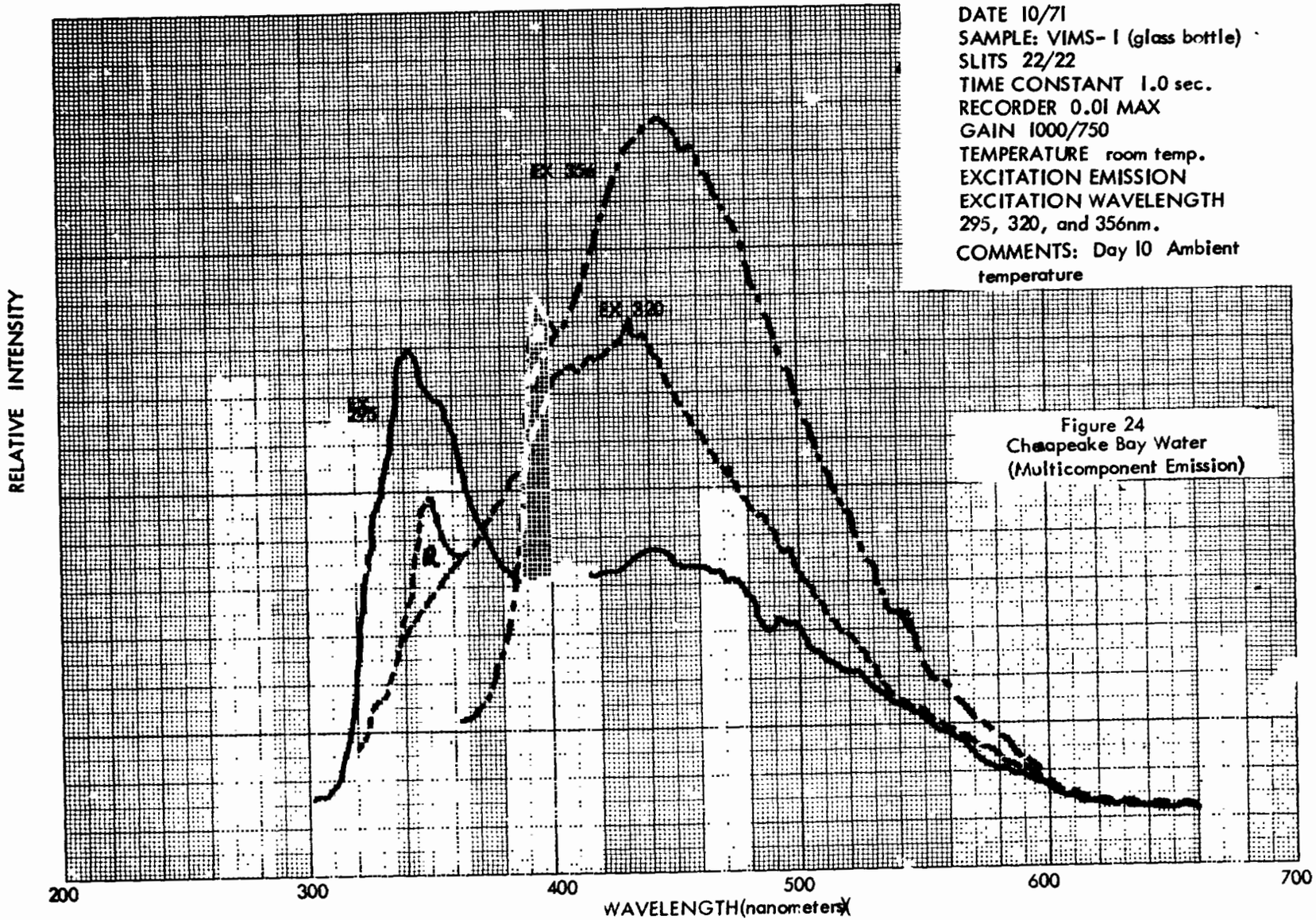


Figure 24
Chesapeake Bay Water
(Multicomponent Emission)

than water stored in either glass or polystyrene. The peak background fluorescence occurred at approximately 415 nm for an excitation of 310 nm. The polystyrene dish seemed equally as good as glass.

3.2 Development of a Field Instrument

The standard laboratory instrument described in the previous section cannot be used for field measurements of chlorophyll. The 1P28 detector, which does not have to be cooled, is too insensitive in the 680 nm region. The C70007A (or 7102) needs cooling, which is utterly impractical in field situations. Our solution to the problem involves use of a new photomultiplier with a GaAs photocathode. This new tube, an RCA C31025C, does not need cooling and has a flat response in the chlorophyll region. Figure 25 contains comparative data on the quantum efficiencies of the RCA C31025C and the EMI 95580. It will be noted that the former has a higher quantum efficiency at 685 nm (5.9%) than the latter (3.3%), and the wavelength dependence is less severe. Thus, the C31025C should display better signal/noise and less wavelength distortion.

The C31025C has the same geometry as the 1P28 and hence can be substituted in the instrument directly. Hence, it was first felt that this substitution would result in good performance from below 300 nm to beyond 800 nm. Unfortunately, the gain of the C31025C is less than that of the 1P28 by a factor of about 100, which puts a strain on the first stage of amplification. Despite use of special amplifiers, it was found by much testing that the performance of the C31025C in the visible was always much inferior to the 1P28. Field tests on waters off Cape Ann, Massachusetts (University of Massachusetts Marine Station) showed the sensitivity in the visible to be unacceptably low.

The decision was then made to mount both the C31025C and the 1P28 on the instrument with dual electronic controls for photomultiplier high voltage and dark current adjust. The two side-viewing tubes were mounted end to end in a vertical tube attached to the side of the SF-100. A quartz lens

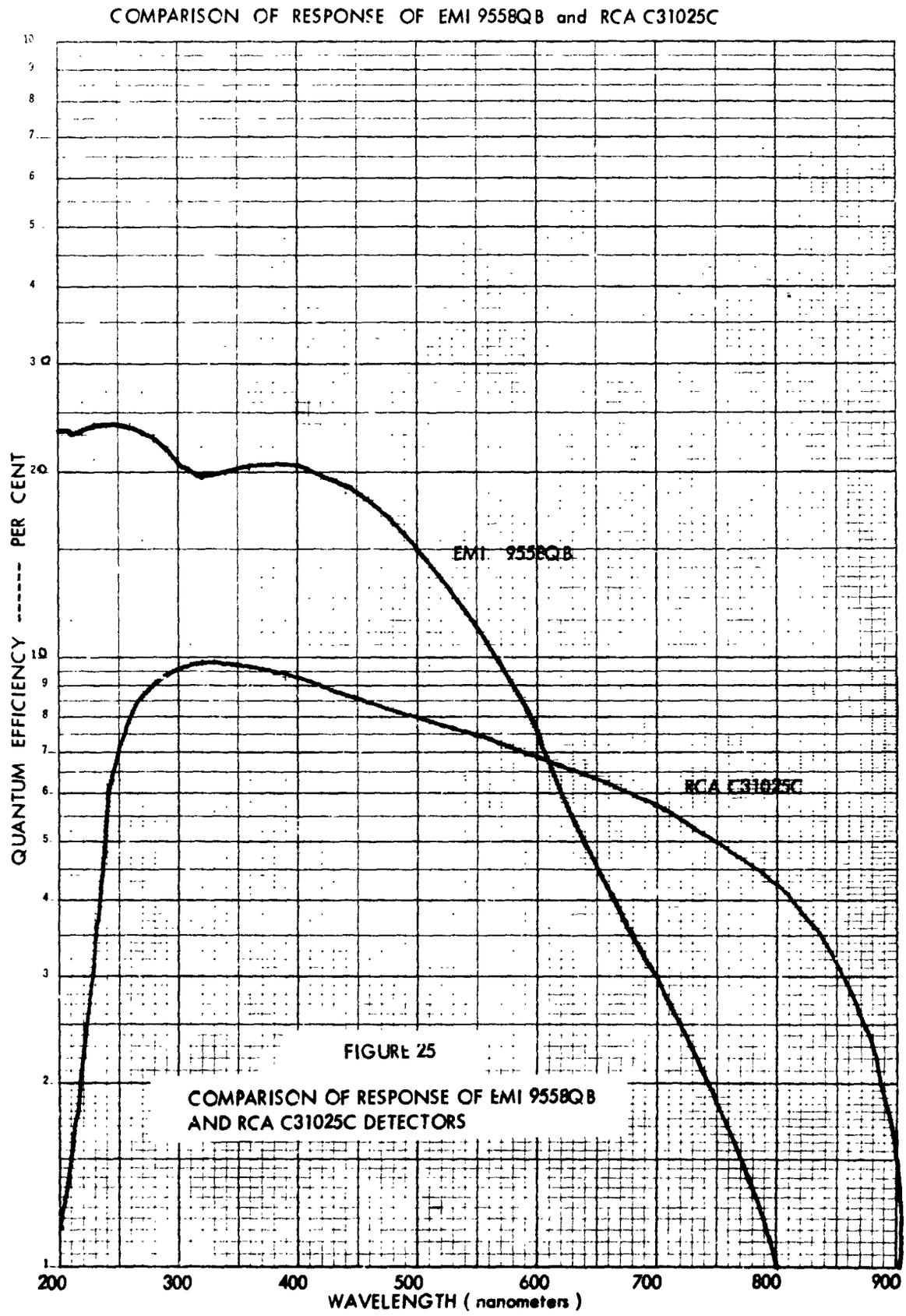


FIGURE 25

COMPARISON OF RESPONSE OF EMI 9558QB AND RCA C31025C DETECTORS

focuses the light from the exit slit onto the active photocathode area of whichever tube is positioned for use. The tubes are selected by manually sliding the desired detector into place. The housing is light-tight, allowing change of detectors in ambient light. A switch allows use of normal panel controls for the 1P28 and auxiliary controls for the C31025C. Once voltages are adjusted, it is possible to switch back and forth between tubes without further resetting of dark current and high voltage.

Figure 26 is a photograph of the modified instrument showing the external mounting of the two detectors together with electronic controls.

The basic instrument available for field use was an SF-100, usable only to 700 nm. While the chlorophyll a peak is near 685 nm, the long wavelength tail extends well beyond 700 nm. Therefore a camspacer was provided to change the standard range to 420-900 nm when desired. This spacer is moved into position by means of a small lever located within the sample compartment. This wavelength shift is applied only to the emission monochromator, although it could be applied to the excitation side as well.

The resulting modified instrument thus allows the use of the 1P28 for Gelbstoff and other uv-visible emission measurements in the 220-700 nm range, and use of the C31025C for red and near-infrared measurements in the range of 420-900 nm.

A single difficulty with the C31025C is the time required to achieve low dark current after the tube has been exposed to light, or stored without high-voltage applied.

While the revamped instrument had sensitivity enough to monitor less than one gram of chlorophyll per liter, there was some indication during measurements at Gloucester that some amplifier instability was evident at high gain. This became more evident at Nova University and finally became a real problem at Carrabelle. As a result, the feedback on the high-voltage supply for the photomultiplier was revised, resulting in good stability for all remaining measurements.

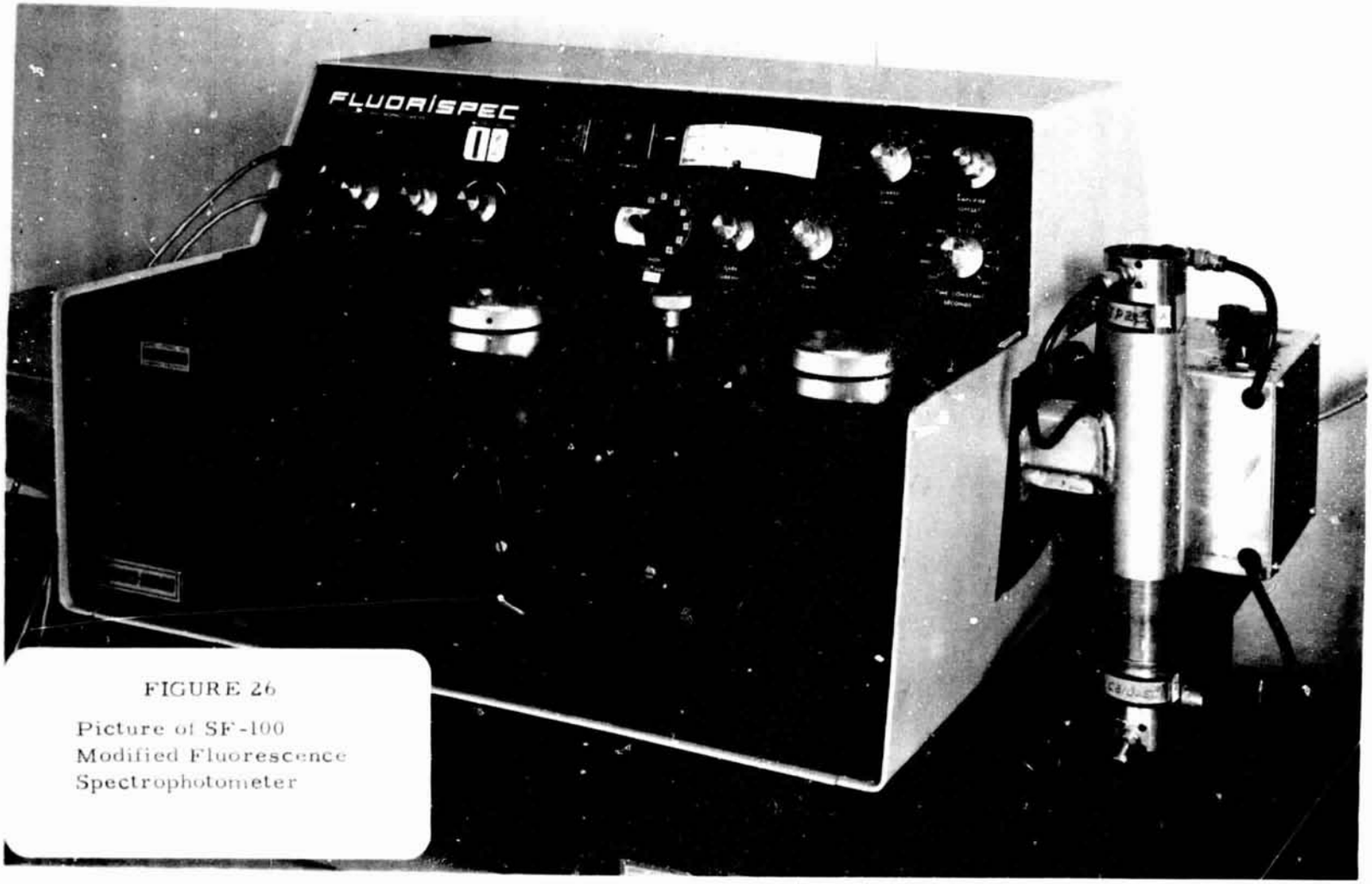


FIGURE 26

Picture of SF-100
Modified Fluorescence
Spectrophotometer

3.3 Field Testing at VIMS

The completed instrument was tested at two field sites, the Virginia Institute of Marine Science laboratory at Gloucester Point, Virginia, and the University of Massachusetts Marine Station at Gloucester, Massachusetts. The VIMS experiment was arranged through Dr. Paul L. Zubkoff, Chairman of the Department of Physiology, and Mr. J. Ernest Warinner III, Assistant Marine Scientist. VIMS provided laboratory facilities for luminescence studies and provided space on their 47-foot boat for a day-long study at four stations in Chesapeake Bay.

While the results of these measurements are included in the Compendium, we shall discuss them in some detail here as an example of the type of measurements carried out elsewhere.

The laboratory is located five nautical miles from the mouth of the York River and thirty miles from the mouth of the Chesapeake Bay, as indicated in Figure 27. Also indicated are four stations, labeled A, B, D, E, at which samples were taken.

3.3.1 Laboratory Seawater Studies

Laboratory studies were carried out on seawater samples taken from off the VIMS pier. These studies were made to check the instrument and develop diagnostic excitation/emission wavelength settings.

In Figures 28a and 28b, we document the Gelbstoff emission for various excitation wavelengths. Both excitation and emission bandwidths were set at approximately 16 nm. Water samples were examined in a standard 10 x 10 mm^2 cuvette immediately after collection. The broad emission is peaked in the 450 nm region, showing some variation with excitation. At lowest excitation (280 nm), the peak is located at about 445 nm; at an excitation of 320 nm, the peak has shifted to 438 nm; at higher wavelengths the peak shifts slightly to longer wavelengths again with suggestions of a new broad peak at 470 nm. As excitation wavelength is increased, the water Raman emission is seen

REPRODUCIBILITY OF THE ORIGINAL PAGE IS POOR.

FIGURE 27 TAKEN FROM CHART 15

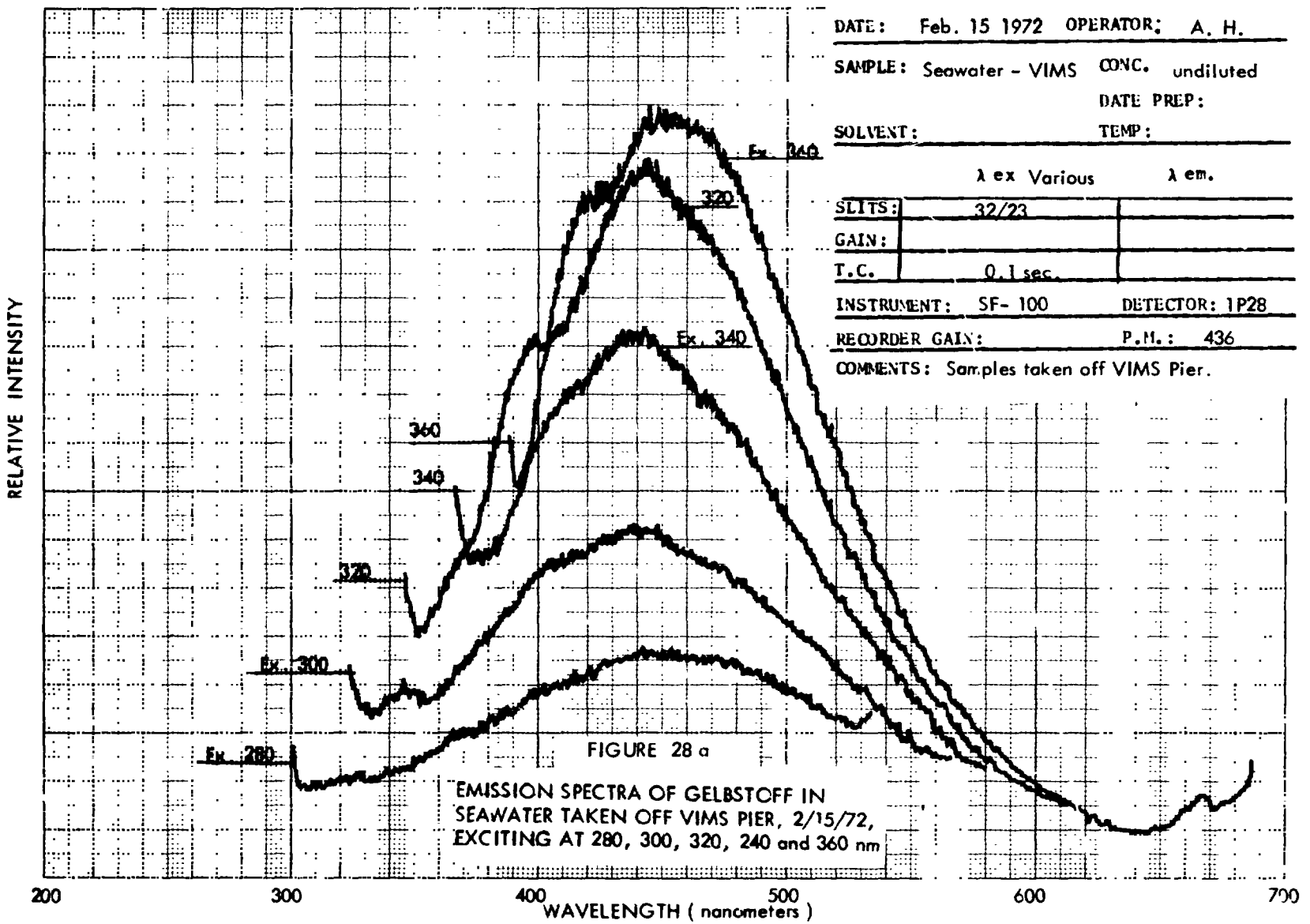


FIGURE 28 a

EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN OFF VIMS PIER, 2/15/72, EXCITING AT 280, 300, 320, 240 and 360 nm

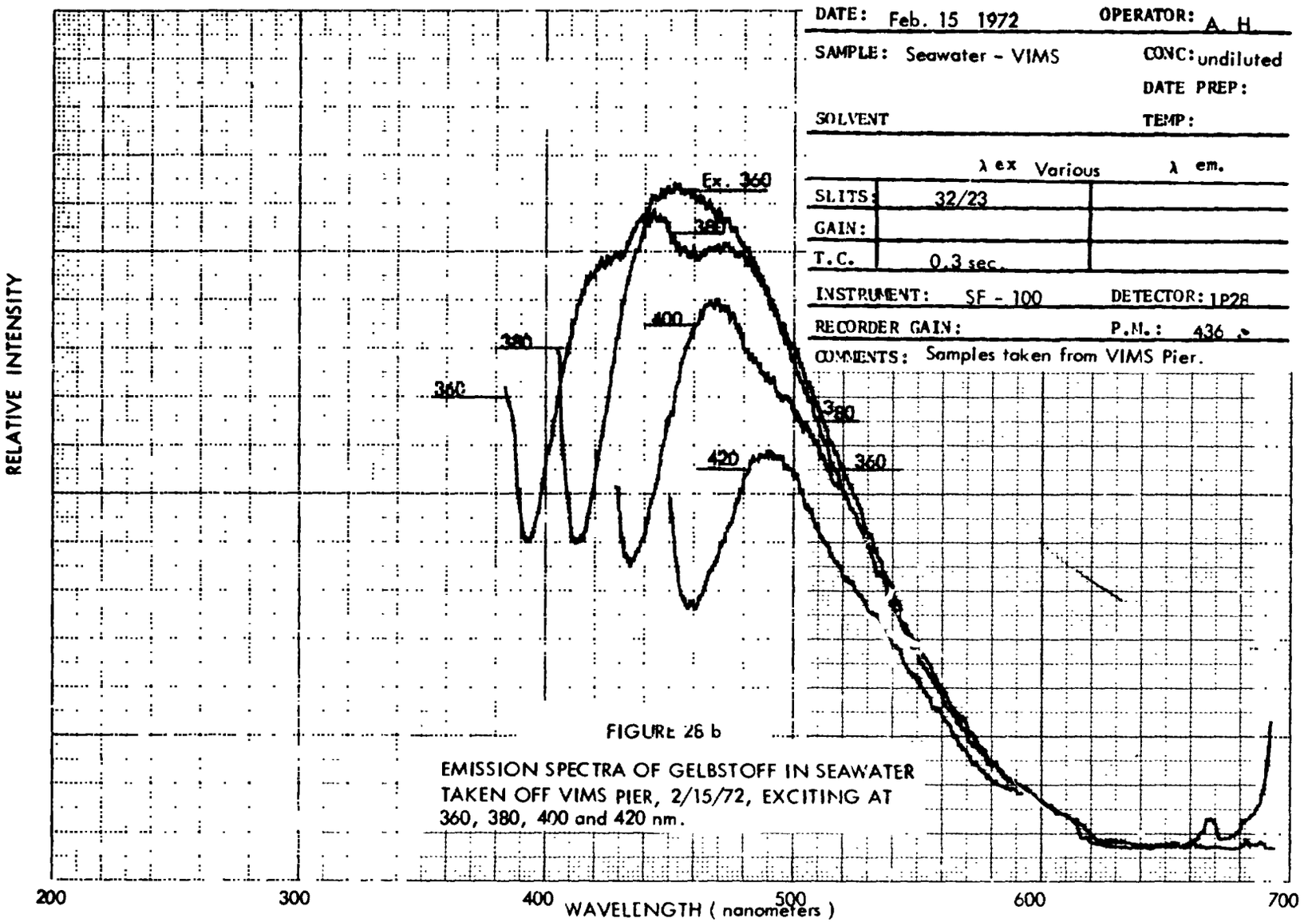


FIGURE 26 b

EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN OFF VIMS PIER, 2/15/72, EXCITING AT 360, 380, 400 and 420 nm.

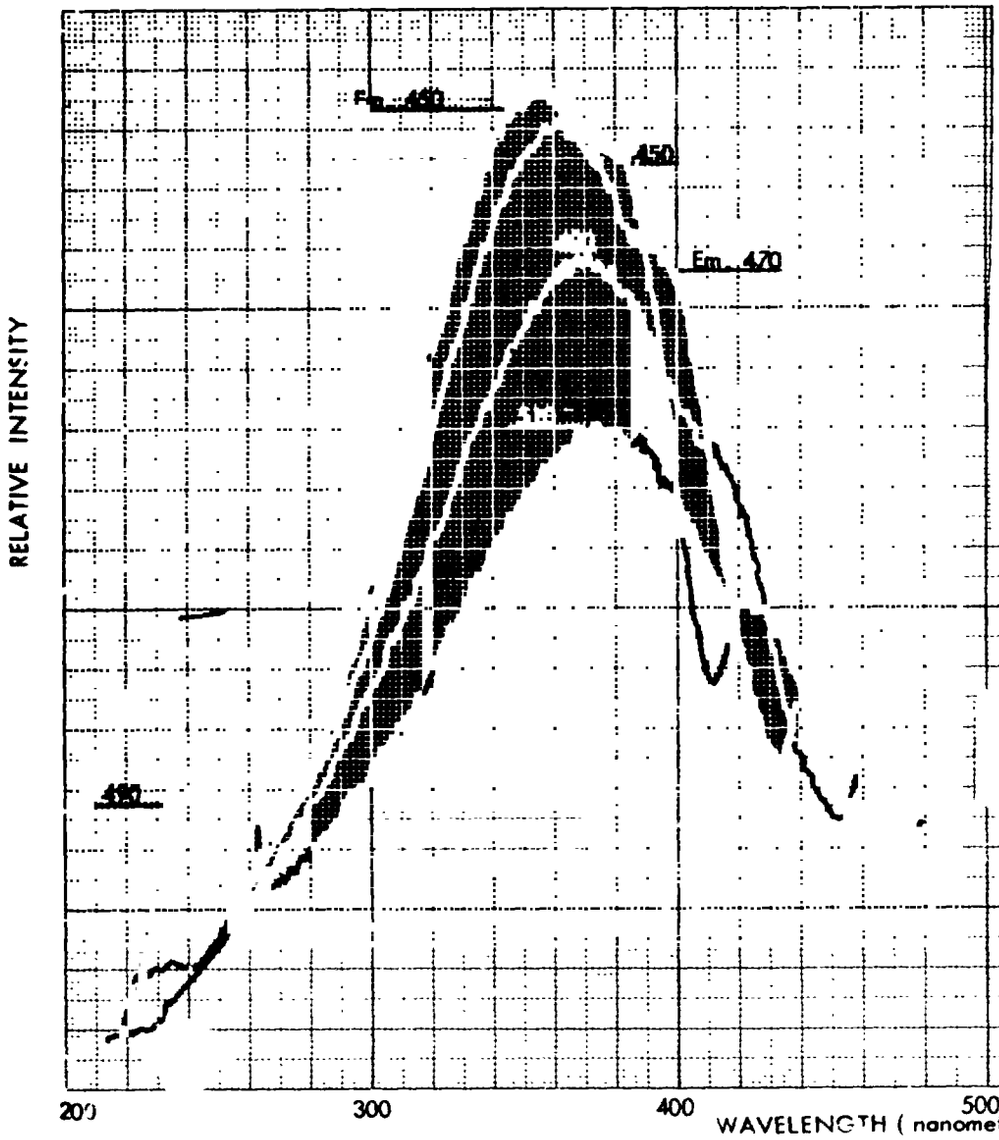
creeping up the fluorescence traces. At an excitation of 300 nm, it is barely visible at 345 nm. At an excitation of 360 nm (the highest trace in the series), the Raman line is at about 420 nm, approaching the fluorescence peak. At greater excitation wavelengths, the Raman interferes with the determination of the peak position. Thus, for an excitation of 400 nm, the Raman at 468 nm dominates the fluorescence emission which is still discernible by the inflection of 480 nm. At an excitation of 420 nm, the emission is principally Raman. Note that there is no indication of light oil emission as seen in Figure 23.

Figure 29 contains several corresponding excitation spectra for different monitoring wavelengths. The Raman again climbs the excitation spectra as the monitored wavelength becomes shorter. The excitation peak appears to shift from 375 nm (when monitored at 510 nm) to 355 nm (monitored at 450 nm).

We conclude that this Gelfstoff emission,* which is probably fairly typical, is centered in the 440-450 nm region with a bandwidth of approximately 150 nm. The slight shift in emission peak with excitation suggests it is composed of many emitting moieties which probably exchange energy to some degree, presenting the structureless spectrum. The excitation maximum (for our uncorrected instrument) is in the 345-355 nm region, with an apparent bandwidth of about 110 nm. The more severe instrumental corrections applicable in this region drastically reduce excitation spectra at short wavelengths, shifting peaks to longer wavelengths and producing narrower bandwidths.

In Figures 30a and 30b, we show excitation and emission spectra of the "chlorophyll" emission in the same sample. In this case the C31025C is the detector and bandwidths on both emission and excitation are approximately

*Here and throughout this report we refer to "Gelbstoff emission" as a convenient designation of the broad emission peaking in the 440-450 nm region which occurs in most natural waters. We also use the term "chlorophyll emission" to conveniently designate emission occurring in the 685 nm region. The emission may not be due to chlorophyll, but to pheophytin, etc.



DATE: Feb. 15, 1972		OPERATOR: A. H.	
SAMPLE: Seawater - VIMS		CONC: undiluted	
		DATE PREP:	
SOLVENT:		TEMP:	
	λ ex	λ em Various	
SLITS:		32/23	
GAIN:			
T.C.		0.3 sec	
INSTRUMENT: SF - 100		DETECTOR: C31025C	
RECORDER GAIN:		P.M.: 1000	
COMMENTS: Samples taken off VIMS Pier.			

FIGURE 29
EXCITATION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN OFF VIMS PIER 2/15/72, DETECTING AT 450, 470, 490 and 510 nm.

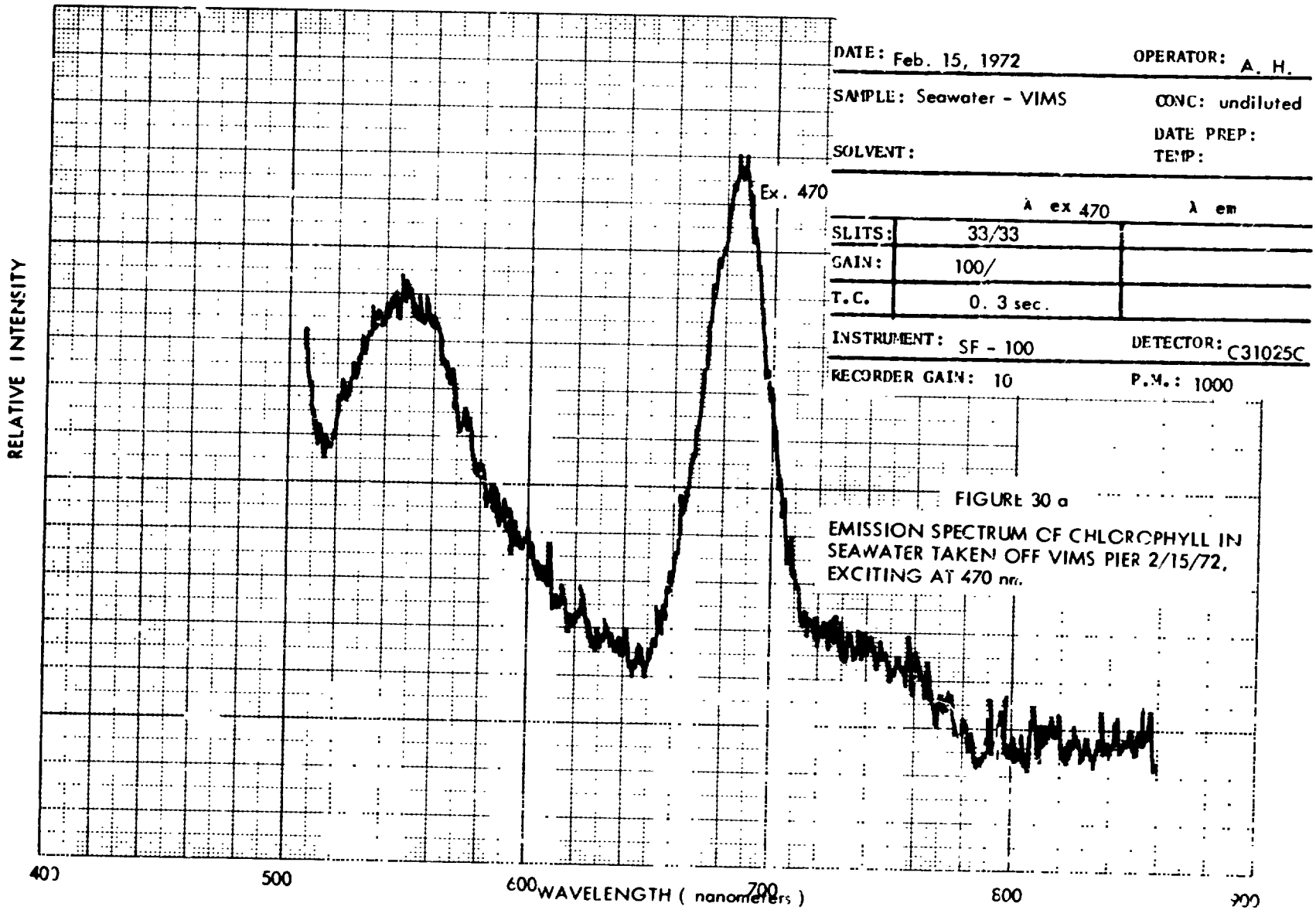


FIGURE 30 a

EMISSION SPECTRUM OF CHLOROPHYLL IN SEAWATER TAKEN OFF VIMS PIER 2/15/72, EXCITING AT 470 nm.

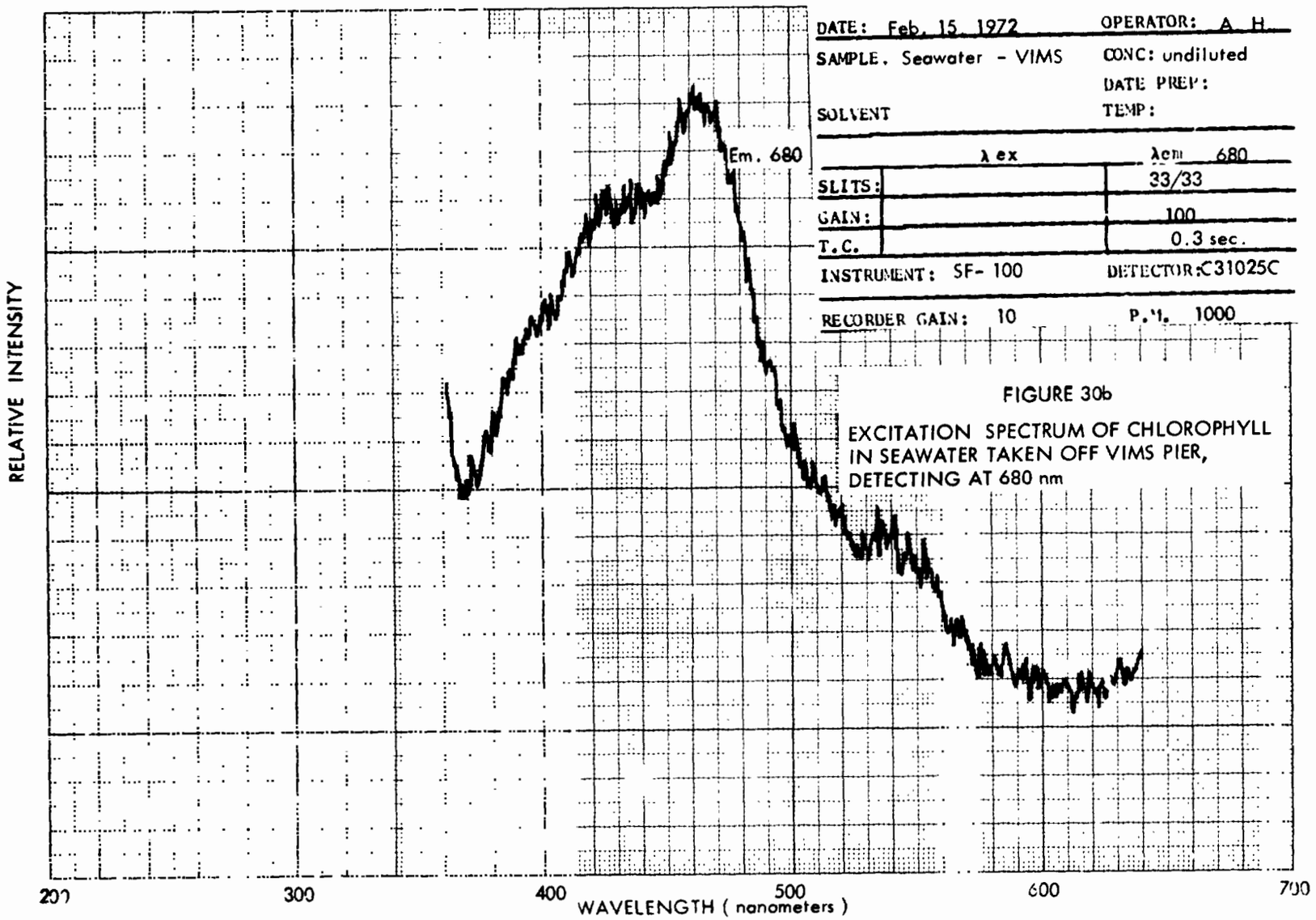


FIGURE 30b

EXCITATION SPECTRUM OF CHLOROPHYLL IN SEAWATER TAKEN OFF VIMS PIER, DETECTING AT 680 nm

24 nm. (These traces were made using a 1500-watt Sears motor-generator to power the SF-100, preparatory to experimentation aboard ship. The noise is acceptable, though use of a one-second time constant could have reduced it further. Frequency and voltage variations were found to affect the recorder span, resulting in some uncertainty in wavelength.)

The prominent emission in Figure 30a, with peak at 685 nm, is assumed to be due to chlorophyll a. The peak at 550 nm is the Raman associated with excitation at 470 nm. The Raman is located on a rising background which is the tail of the Rayleigh/Tyndall scattering peak. Since we are looking at particulates suspended in the water, we expect to see scattering. The long wavelength shoulder in the 730 nm region is real and is observed in other samples to varying degrees.

The corresponding excitation spectrum in Figure 30b monitoring the chlorophyll emission peak, is peaked at 460 nm with shoulders at 400 and 430 nm. There is also a long wavelength shoulder at 540 nm. The wide slits preclude examining the excitation close to the emission, thus missing the chlorophyll absorption band at 670 nm. The short wavelength termination of the excitation curve at 365 nm is due to the presence of second order scatter. (The emission monochromator set to detect 680 nm in first order will detect exciting light at 340 nm in second order.) This scatter peak can be removed with a filter; however, through an oversight, we did not have filters with us.

Uncorrected excitation spectra must be viewed very critically, especially if a xenon source is used, because of the fine structure in the xenon spectrum between 400 nm and 500 nm. The details of the correction necessary depend strongly on the slits used. In Figure 31 we show the relative quanta per unit wavelength bandwidth arriving at the sample with the slits used for Figure 30. This was obtained by inserting a solution of 5 g/l of Rhodamine B in ethylene glycol into the sample chamber in the transmission mode (using a front surface mirror). Note that all excitation spectra will reflect the structure at 400 nm, 540 nm, and particularly at 470 nm.

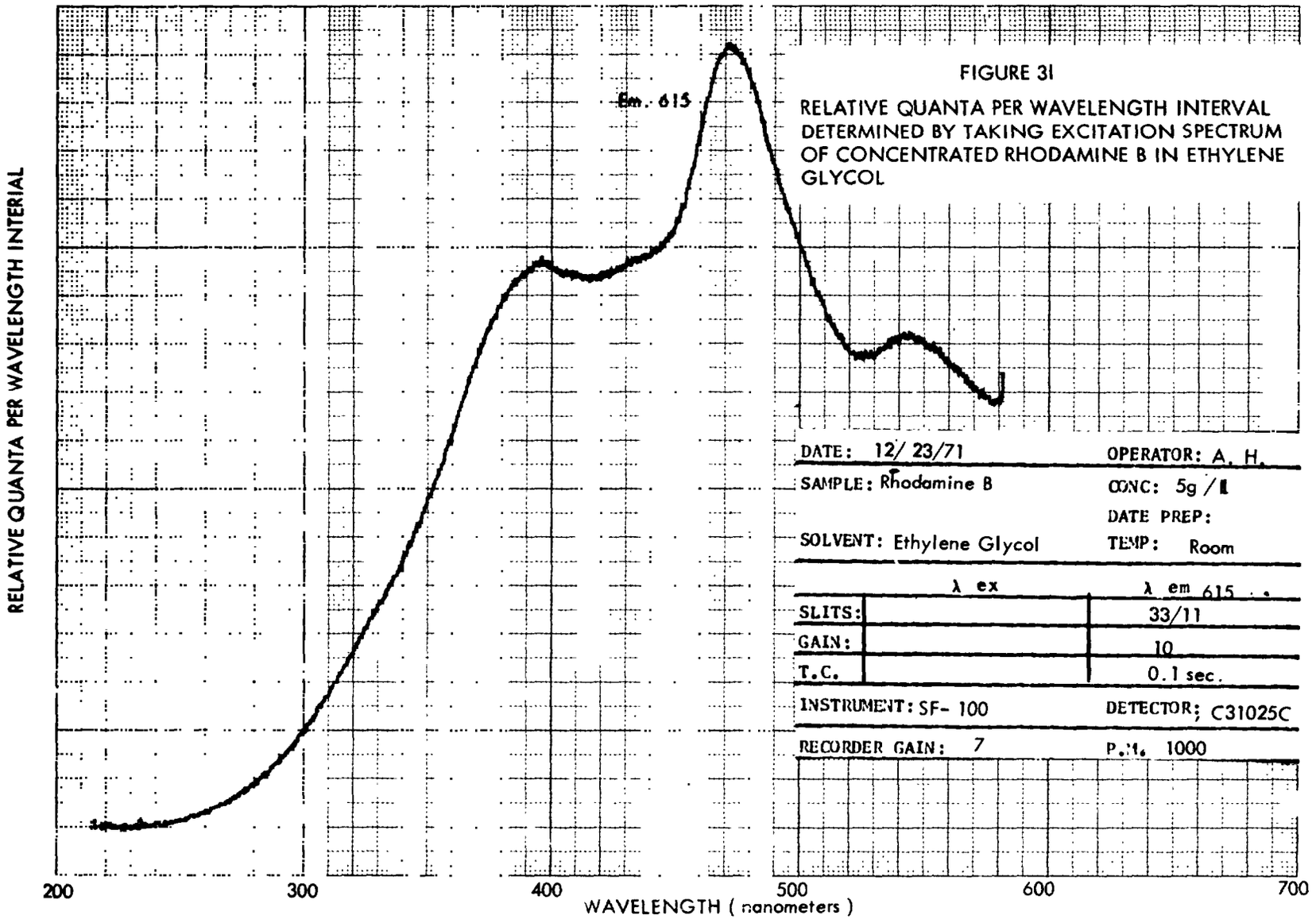


FIGURE 31

RELATIVE QUANTA PER WAVELENGTH INTERVAL DETERMINED BY TAKING EXCITATION SPECTRUM OF CONCENTRATED RHODAMINE B IN ETHYLENE GLYCOL

DATE: 12/ 23/71 OPERATOR: A. H.

SAMPLE: Rhodamine B CONC: 5g / l

DATE PREP:

SOLVENT: Ethylene Glycol TEMP: Room

	λ ex	λ em 615
SLITS:		33/11
GAIN:		10
T.C.		0.1 sec.

INSTRUMENT: SF- 100 DETECTOR; C31025C

RECORDER GAIN: 7 P.M. 1000

With the xenon source peaks in mind, it is easily deduced that the apparent peak at 460 nm in Figure 30b will be removed, leaving the general peak in the 440 nm region. This is in agreement with the action (excitation) spectra for chlorophyll a fluorescence of French and Young (A1952) and Yentsch. The shoulders at 400 nm and 540 nm are possibly due to the unequal distribution of source light. Despite the distortion of uncorrected excitation spectra, they can be valuable for the difference between them, as will become apparent.

3.3.2 Chesapeake Bay Studies

A primary purpose of the work at VIMS was to take the SF-100 (with x-y recorder and lamp power supply) to sea to measure freshly collected surface water samples. The VIMS boat was equipped with several gasoline generators to provide 110 v, 60 cycle power for instrumentation. This boat is taken out at least once a month in winter and weekly in summer to make measurements in Chesapeake Bay. These measurements include termperature salinity, dissolved oxygen, chlorophyll (a, b, c, d, and carotenoids), orthophosphate, nitrate, nitrite, plankton (dinoflagellates and diatoms) and light penetration.

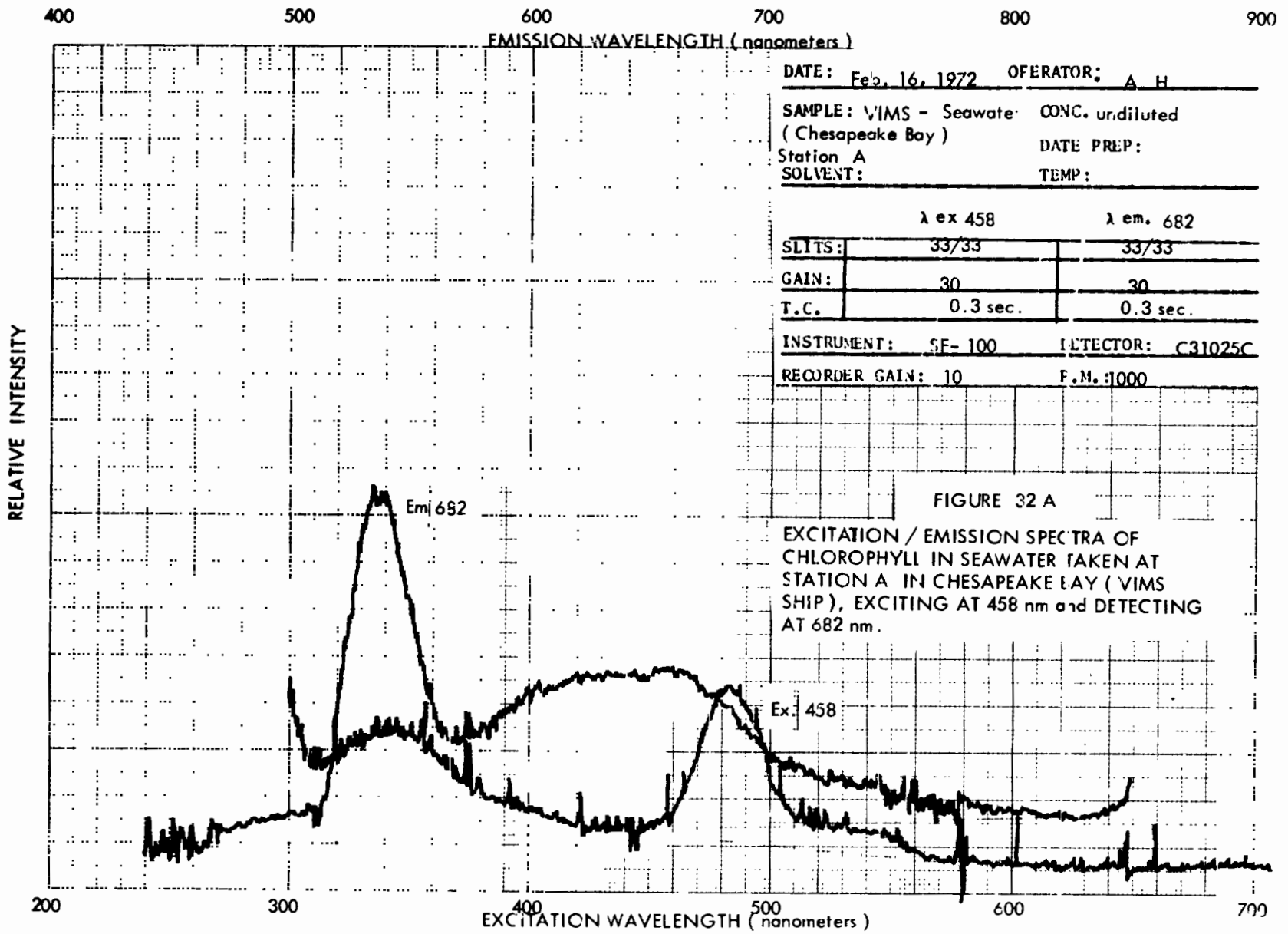
Water samples are filtered on the boat, and both filtrate and residue are immediately stored on dry ice for later analysis at the laboratory. Some chemical manipulations are performed on the boat before freezing samples.

The boat normally leaves Sarah Creek, stopping successively at stations E, D, B, and A as indicated in Figure 27. At each station, water samples are collected at the surface, 1/2 m, 1 m, and larger increments to the bottom. The surface sampler consists of a frame with a stainless steel screen. The screen is placed flat on the water surface to pick up the top half-millimeter of water. It is then raised and drained into storage containers. Because of lack of time and experience, it was impossible to plot excitation/emission spectra of all water samples. Instead, the surface samples were all monitored as being of greatest inter to this program.

From laboratory work, it was decided to use certain diagnostic settings to contrast water luminescence. For the Gelbstoff measurements, excitation was set at 350 nm (16 nm bandwidth) and emission scanned with a 7 nm bandwidth. For excitation scans (7 nm bandwidth), the emission monochromator was set at 440 nm (16 nm bandwidth). These measurements employed a 1P28 detector. The other set of measurements made with the C31025C employed a 24 nm bandwidth for both excitation and emission monochromators. Excitation was at 458 nm and emission at 682 nm. These settings were felt to cover the principal emission and excitation regions (except for the long wavelength excitation of chlorophyll, etc.). The data were to be interpreted in terms of relative strength of emission and spectral distribution.

Figures 32_A, 32_B, 32_D, and 32_E record the excitation/emission spectra for surface waters at the corresponding stations in terms of chlorophyll type emission. Figures 33_A . . . 33_E record spectra for Gelbstoff type emission. Before considering specific results, we note that all spectra were taken with an instrumental time constant of 0.3 seconds. Further, instrumental gain was only medium in the worst cases. Signal-to-noise ratio was, in general, very good. In many cases the particular type of noise could be related to the starting or stopping of the ship's engines, or wave motion. The only difficulty involved wavelength inaccuracies of the x-y recorder due to slow changes in the output voltage and drequency of the gasoline generator. Low voltages also caused change in signal gain and sluggish response in the recorder. We now consider Figures 32 and 33 in some detail.

Figures 32 have two wavelength scales. That at the bottom of the page refers to excitation, while the scale at the top refers to emission. The emission trace is always characterized by a signal in the 685 nm region. The peak at 340 nm on excitation is second order excitation and can be used as an internal gain standard to some extent. (It will be affected if scatter is caused primarily by turbidity rather than Rayleigh scatter.)



DATE: Feb. 16, 1972 OPERATOR: A H

SAMPLE: VIMS - Seawater (Chesapeake Bay) Station A CONC. undiluted

DATE PREP:

SOLVENT: TEMP:

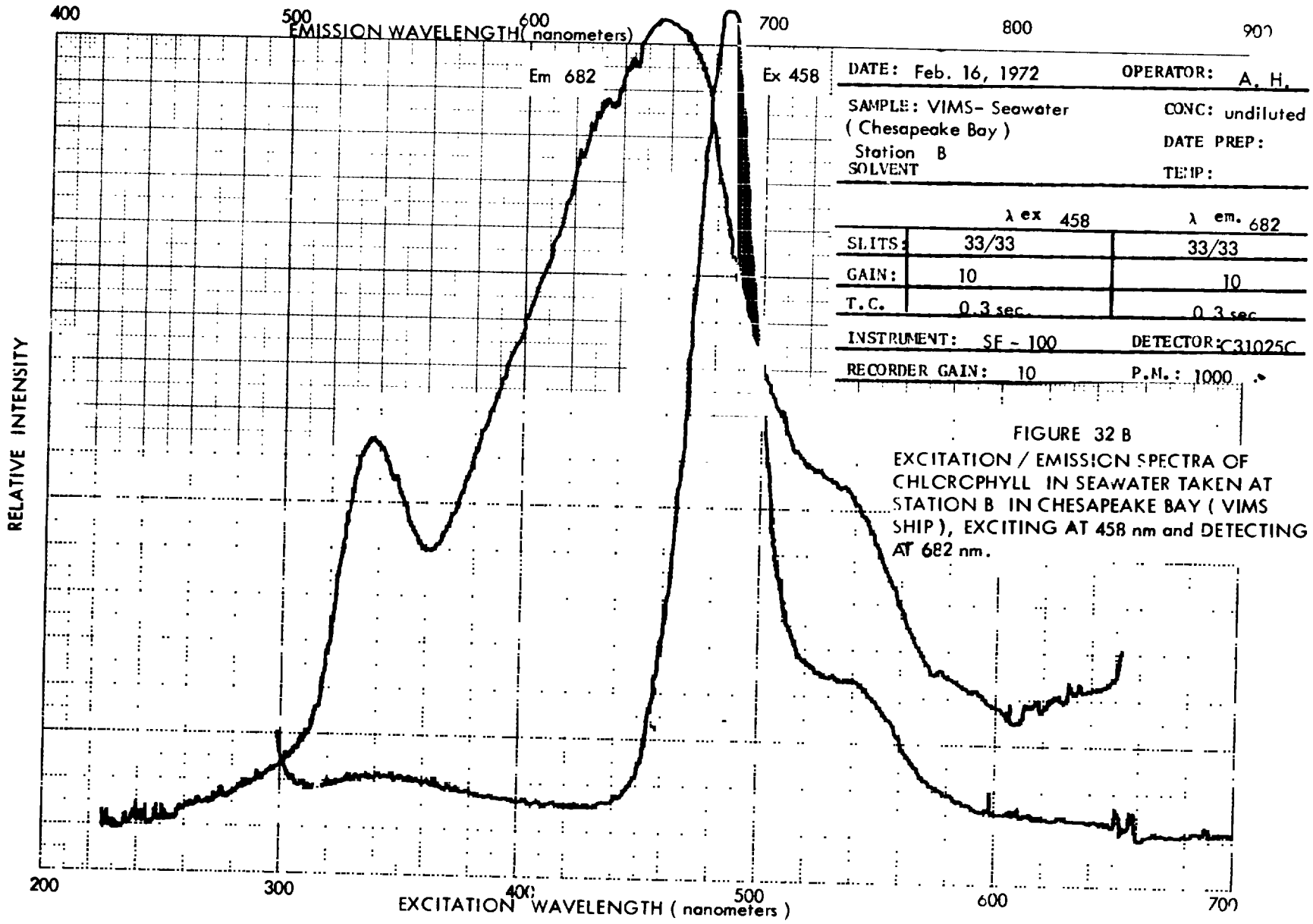
	λ ex 458	λ em. 682
SLITS:	33/33	33/33
GAIN:	30	30
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF- 100 DETECTOR: C31025C

RECORDER GAIN: 10 F.M.: 1000

FIGURE 32 A

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION A IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm.



DATE: Feb. 16, 1972 OPERATOR: A. H.

SAMPLE: VIMS- Seawater (Chesapeake Bay) Station B SOLVENT

CONC: undiluted

DATE PREP:

TEMP:

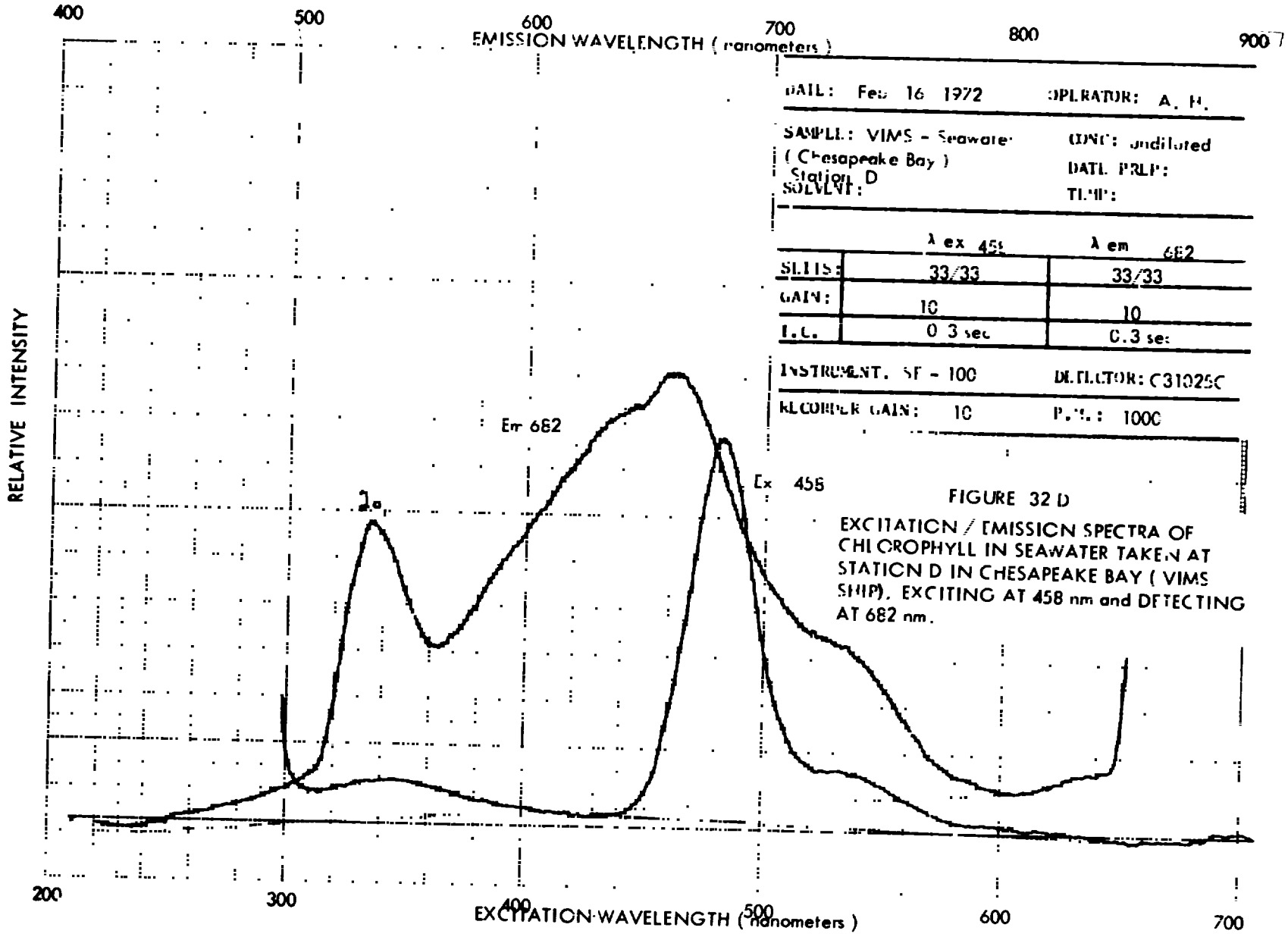
	λ ex 458	λ em. 682
SLITS:	33/33	33/33
GAIN:	10	10
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF - 100 DETECTOR: C31025C

RECORDER GAIN: 10 P.M.: 1000

FIGURE 32 B

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION B IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm.



DATE: Feb 16 1972 OPERATOR: A. H.

SAMPLE: VIMS - Seawater (Chesapeake Bay) Station D
SOLVENT:

CONC: undiluted
DATE PREP:
TEMP:

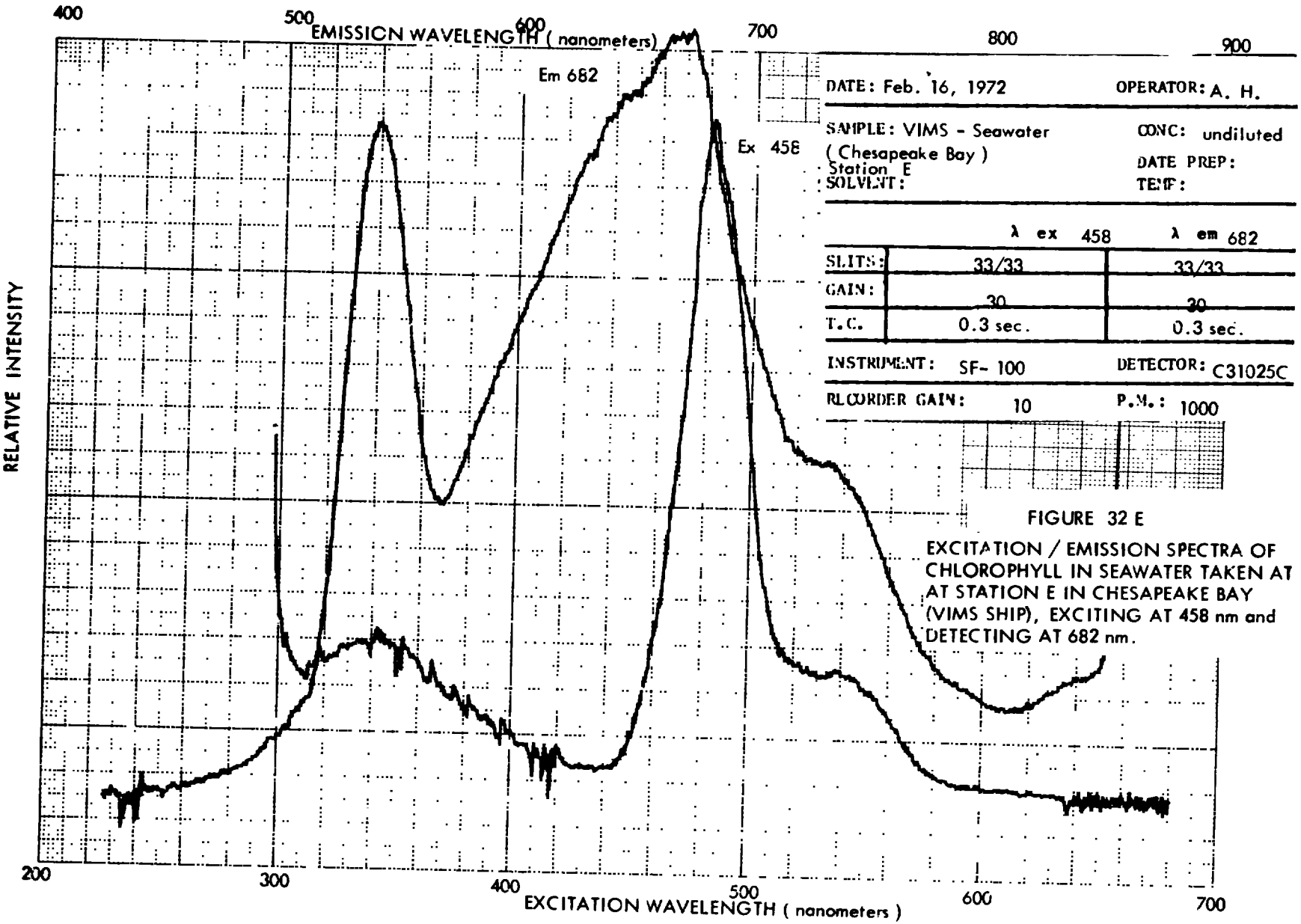
	λ ex 456	λ em 682
SLITS:	33/33	33/33
GAIN:	10	10
T.C.	0.3 sec	0.3 sec

INSTRUMENT: SF - 100 DETECTOR: C31025C

RECORDER GAIN: 10 P.M.: 1000

FIGURE 32 D

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION D IN CHESAPEAKE BAY (VIMS SHIP). EXCITING AT 458 nm and DETECTING AT 682 nm.



DATE: Feb. 16, 1972 OPERATOR: A. H.

SAMPLE: VIMS - Seawater (Chesapeake Bay) Station E CONC: undiluted

SOLVENT: DATE PREP:

TEMP:

	λ ex 458	λ em 682
SLITS:	33/33	33/33
GAIN:	30	30
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF- 100 DETECTOR: C31025C

RECORDER GAIN: 10 P.M.: 1000

FIGURE 32 E

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT AT STATION E IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm.

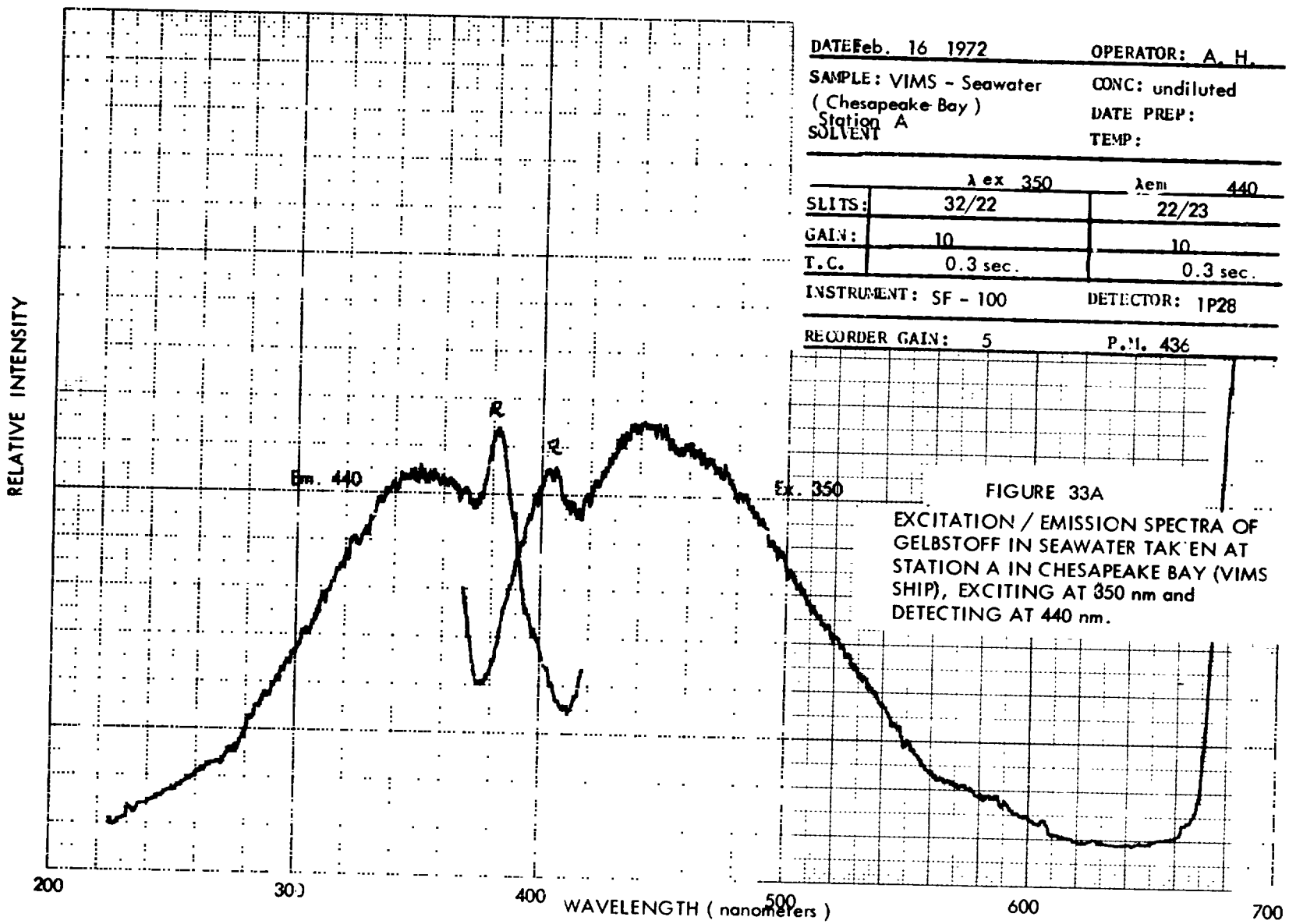


FIGURE 33A

EXCITATION / EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN AT STATION A IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 350 nm and DETECTING AT 440 nm.

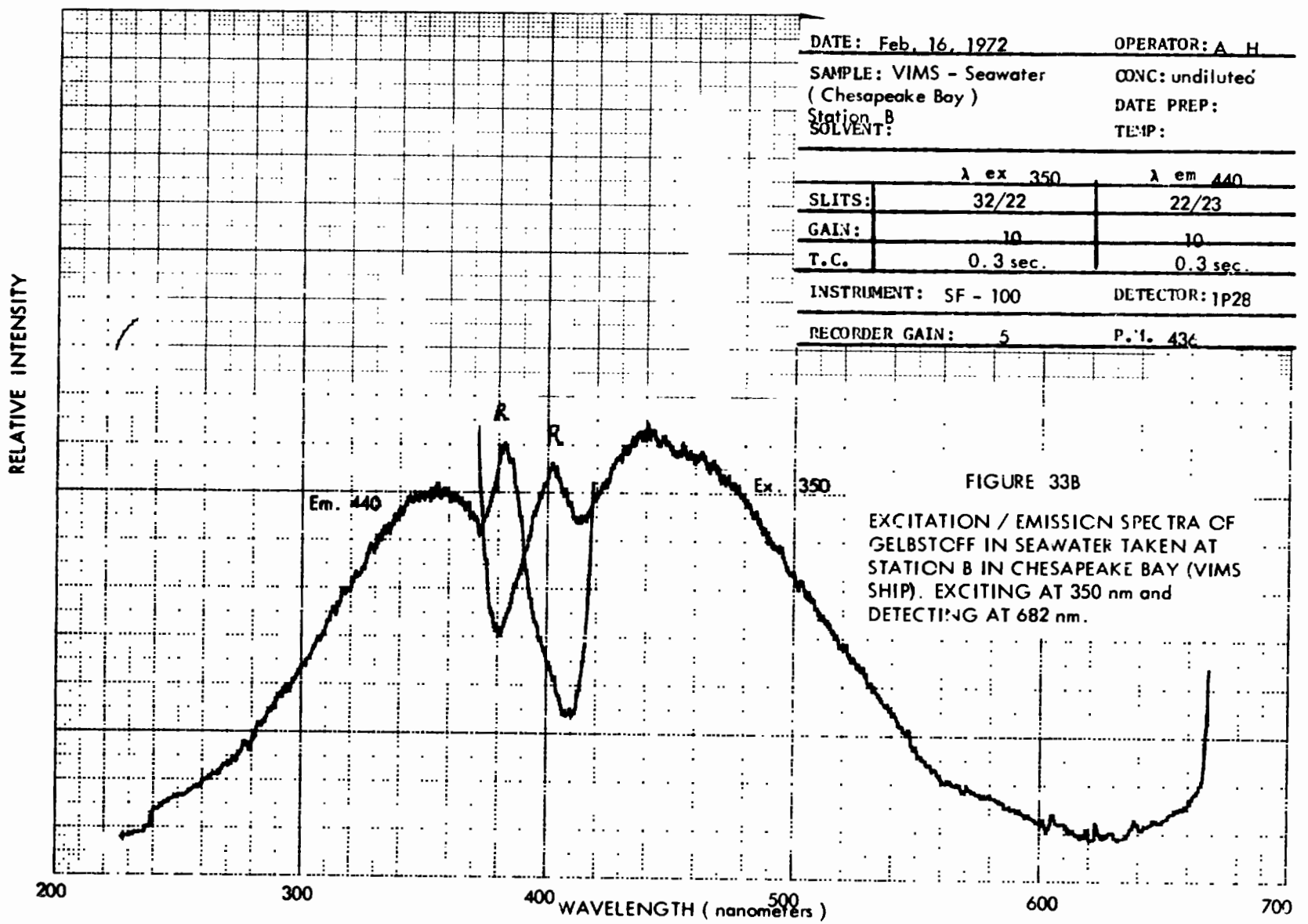
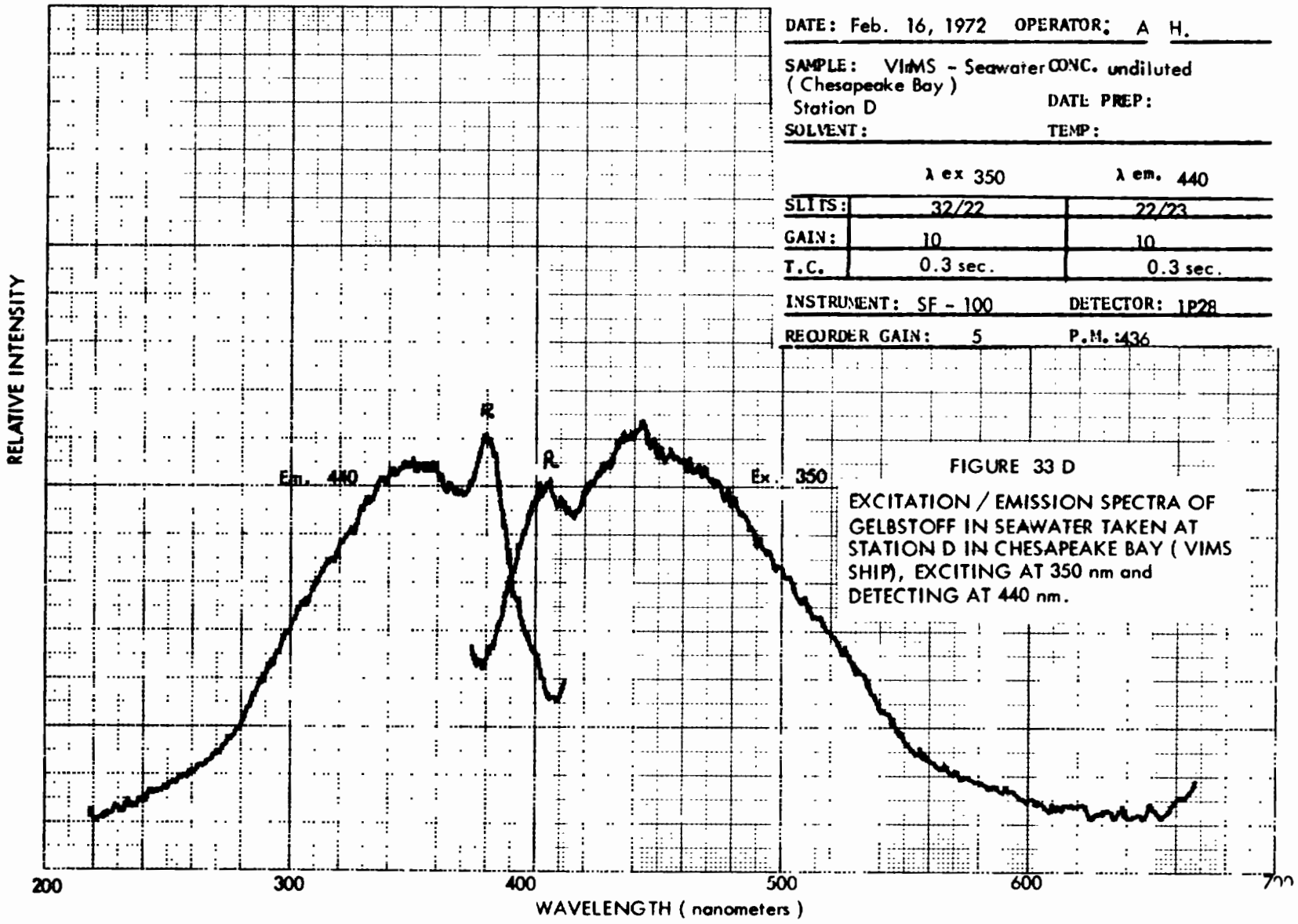


FIGURE 33B

EXCITATION / EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN AT STATION B IN CHESAPEAKE BAY (VIMS SHIP). EXCITING AT 350 nm and DETECTING AT 682 nm.



DATE: Feb. 16, 1972 OPERATOR: A H.

SAMPLE: VIMS - Seawater CONC. undiluted
(Chesapeake Bay)
Station D DATE PREP:
SOLVENT: TEMP:

	λ ex 350	λ em. 440
SLITS:	32/22	22/23
GAIN:	10	10
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF - 100 DETECTOR: 1P28

RECORDER GAIN: 5 P.M.: 436

FIGURE 33 D

EXCITATION / EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN AT STATION D IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 350 nm and DETECTING AT 440 nm.

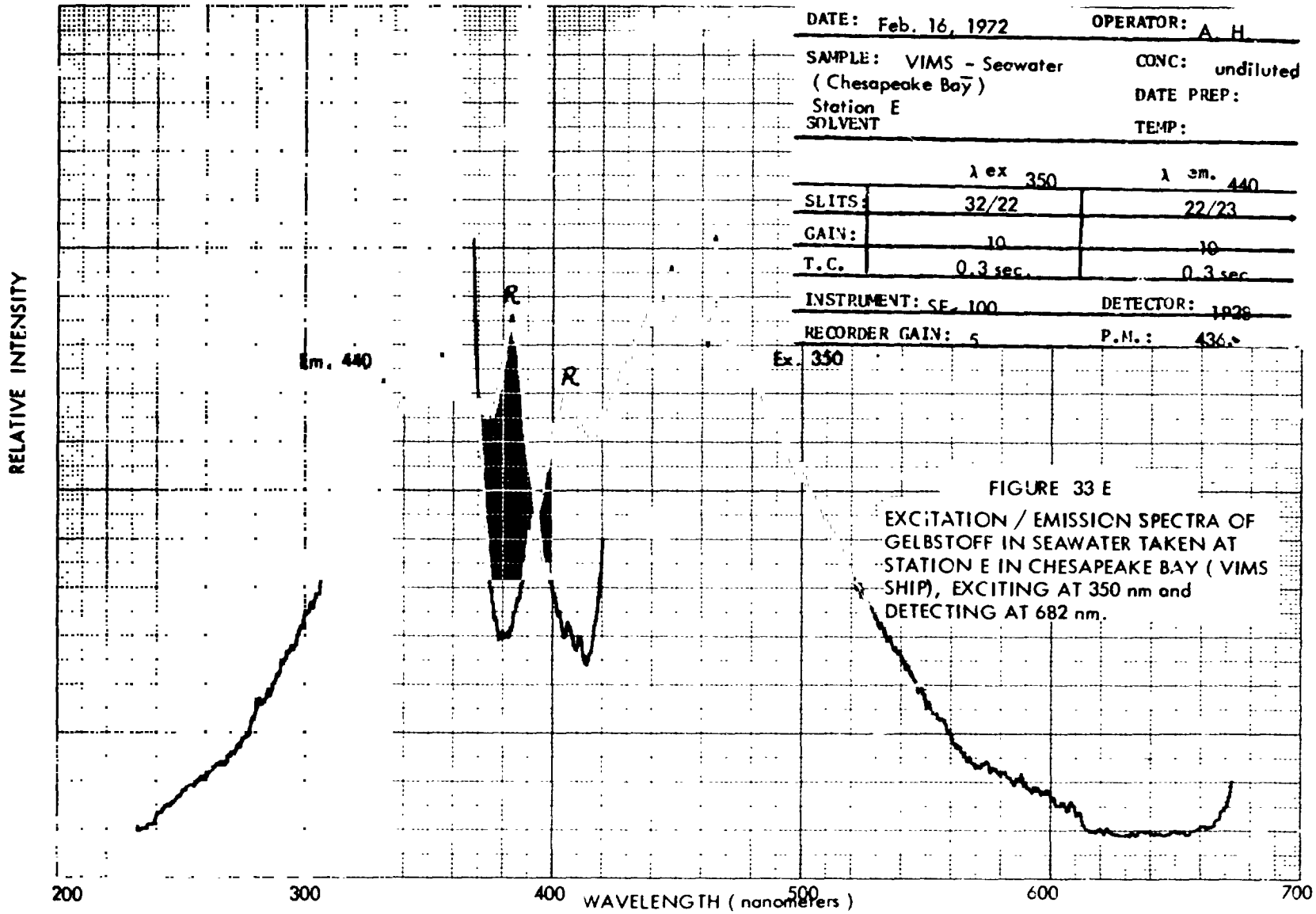


FIGURE 33 E

EXCITATION / EMISSION SPECTRA OF GELBSTOFF IN SEAWATER TAKEN AT STATION E IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 350 nm and DETECTING AT 682 nm.

The peculiar noise in 32_A and 32_E is associated with the ship's motor. Figure 32_D is noteworthy for the high quality of the trace taken at sea.

All traces show a primary emission at 685 nm with a secondary peak in the 730 nm region. The latter has been attributed to a chlorophyll aggregation effect, a different form of chlorophyll or vibrational band enhanced by reabsorption. The broad emission at 545 nm in all traces is Raman due to excitation at 458 nm. This Raman can be used as a monitor of instrument gain, fairly independent of small changes in turbidity. Thus, there is no other identifiable emission in the range 500-900 nm besides the chlorophyll emission at 685 nm with its shoulder at 730 nm.

The corresponding excitation traces of Figures 32 are similar to Figure 30b, exhibiting the (uncorrected) peak at 460 nm and the (uncorrected) shoulder at 540 nm. However, no anomaly appears at 400 nm (as in Figure 30b) which means the traces are different in that respect. We conclude that the excitation traces are all similar and show no indication of different accessory pigments, etc. This may be expected since all samples came from the same geographical area.

The spectra of Figures 33 were taken with the excitation and emission set at 350 nm and 440 nm respectively. These diagnostic settings were chosen from the data of Figures 28 and 29. The traces appear to be similar, all being broad with no evident structure. The excitation peak at 380 nm and the emission peak at 402 nm are Raman peaks. It was learned after the fact that there is a paper pulp mill 25 miles up the York River. Had we monitored with an excitation of 300 nm, we might have seen an emission in the 400 nm region which could be associated with lignin sulfonate. Such emission would have been detected at Station E, but not at Station A.

Thus, the discernible differences in excitation/emission spectra at all four stations involve intensities rather than spectral distribution. The intensity effects will be discussed in the next section in conjunction with other measurements made by VIMS.

3.3.3 Comparison of Fluorescence Intensity Data with Related VIMS Data

Before evaluating data, a few descriptive comments are in order concerning stations A-E. Referring to Figure 27, station A is located at the head of Mobjack Bay, the confluence of several small rivers. There is no known source of pollution; the bay is shallow (6m). Station B is at the mouth of Mobjack Bay on the north side of an underwater bar, York Spit. It is located in a fairway channel and has a depth of 8 m. Station D is located in the York River channel with a depth of 11 m. Station E is located in the channel further up the York River and has a depth of 20 m. The York River has a paper pulp mill twenty five miles up at its head, two naval installations, a power plant and an oil refinery. Thus, stations E and perhaps D are expected to have the greatest environmental strain.

In Table 2, we list the relative peak heights of the 460 nm and 685 nm emission taken from the data of Figures 32 and 33, together with data measured by VIMS for each of the surface and near surface samples. The luminescence data are normalized for the instrument gain. Some VIMS data are missing for station D because of loss of certain samples.

The 460 nm emission (gelbstoff) is essentially constant at all stations except E, where it is somewhat higher. This may be expected since E has the greatest pollutant load. The chlorophyll emission at 685 nm varies among the stations in the same way as chlorophyll a levels, though the relationship is not linear. Since, according to Yentsch (D1971), the 685 nm emission is due to particulates, the fluorescence will be dependent on the size of the particles, the distribution of chlorophyll a in the particles, and the concentration of particles. For the same total chlorophyll content, smaller particles are expected to yield higher fluorescence readings in vivo because of higher surface to volume ratio.

TABLE 2. SUMMARY OF BAIRD/VIMS DATA FOR CHESAPEAKE BAY SURFACE WATERS

Measurement	Sta. A	Sta. B	Sta. D	Sta. E
Luminescence at 685 nm (relative)	13	177	85	49
Luminescence at 460 nm (relative)	85	82	80	108
Chlorophyll a (μg/l)(Surface)	12	112.5	?	39.7
Chlorophyll a (μg/l)(1/2 meter)	6.5	16.6	?	13.3
Chlorophyll b (μg/l)	0.3	0	?	0
Chlorophyll c (μg/l)	4.9	63	?	20.2
Salinity (ppth)(at 1/2 meter)	18.9	19.06	19.51	19.64
Temperature (°C)(at 1/2 meter)	6.7	5.5	5.8	6.0
Light penetration (% at 1/2 meter)	65.7	49.2	45	54.2
Plankton		(Data unreduced)		
Weather (wind speed in knots)	NE 5 little chop	NE 13 choppy, few whitecaps	NNE 10 little chop	NNE 10-12 some chop

3.3.4 Sample Decay Measurements

While the primary purpose of the on-site measurements was to obtain data on fresh samples we were also interested in the change of spectral signature with time. In Figures 34_{A-D}, we show chlorophyll excitation/emission traces for one-day-old samples, stored at laboratory temperature. These were the same surface samples displayed fresh in Figures 32_{A-D}. In every case, there is a distinct drop in signal intensity. In Figure 34_A, the chlorophyll signal has decreased by a factor of 0.3, compared to 32_A. The traces are similar except for the appearance of a rise in the excitation spectrum at about 560 nm. In Figure 34_B, the signal has decreased by a factor of 0.074 with no apparent change in signatures. In Figure 34_D, the decrease is about 0.1 with no obvious change in signature. Unfortunately,

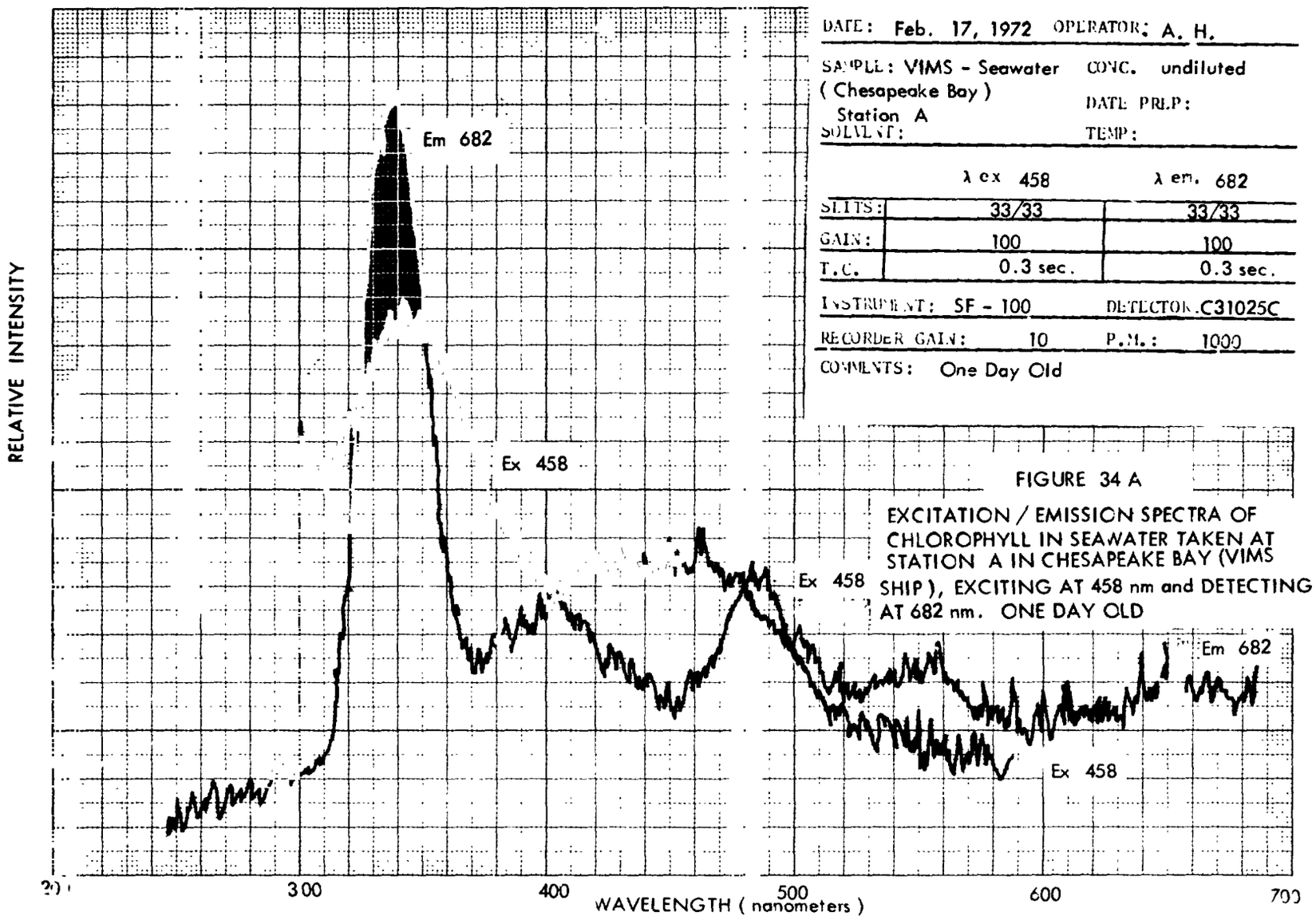
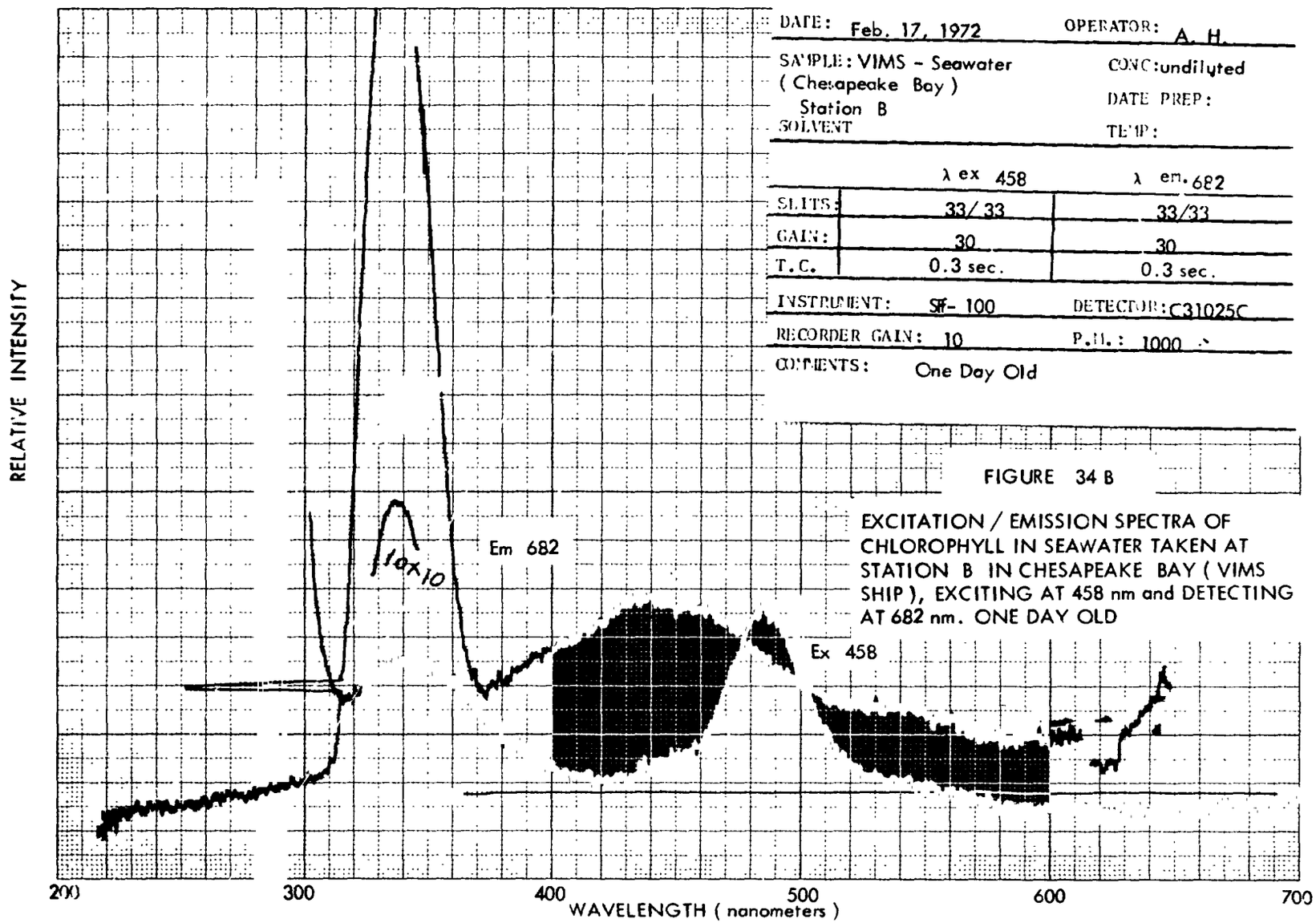


FIGURE 34 A

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION A IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm. ONE DAY OLD



DATE: Feb. 17, 1972 OPERATOR: A. H.

SAMPLE: VIMS - Seawater (Chesapeake Bay) Station B

CONC: undiluted

DATE PREP:

SOLVENT

TEMP:

	λ ex 458	λ em. 682
SLITS:	33/33	33/33
GAIN:	30	30
T.C.	0.3 sec.	0.3 sec.

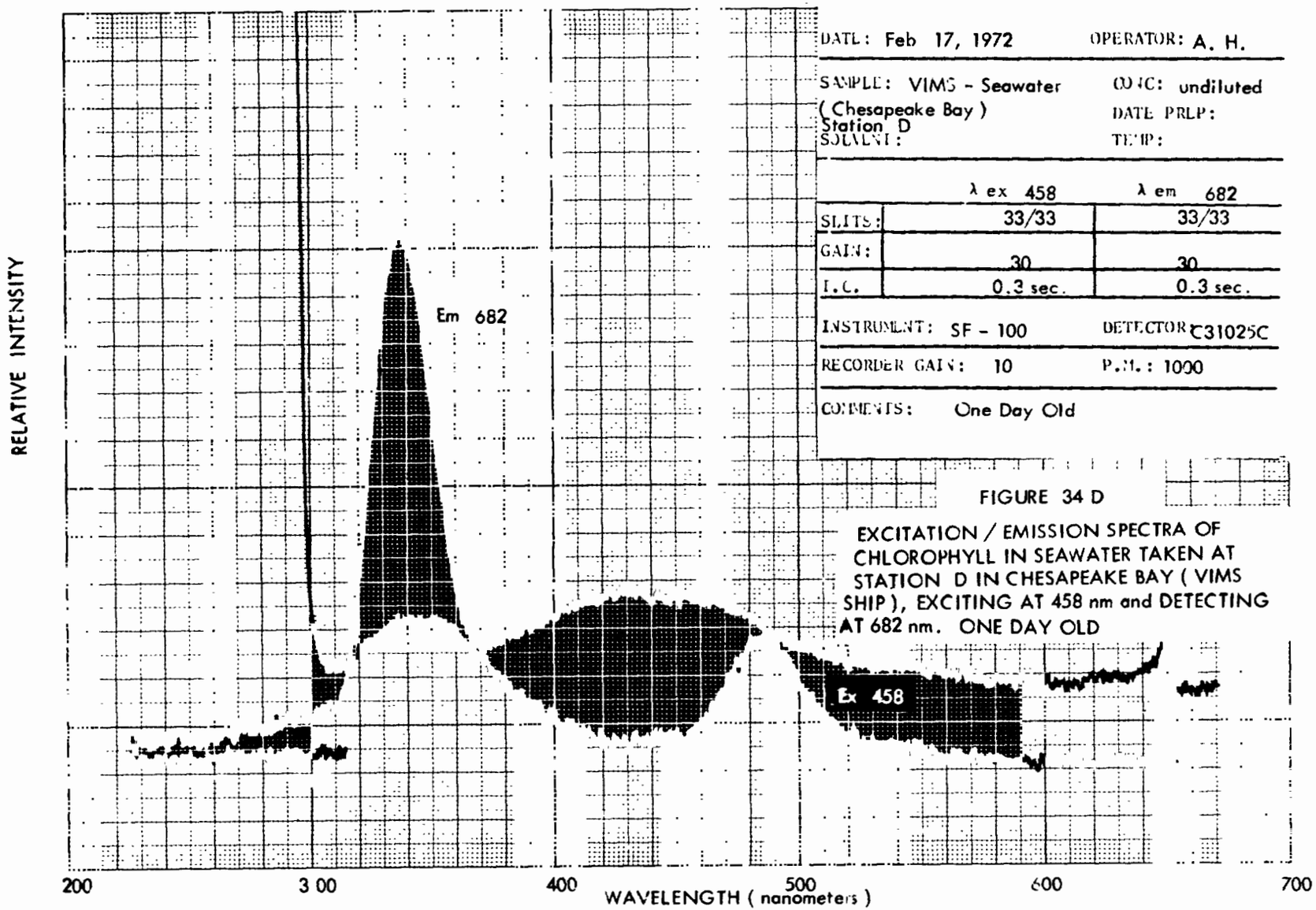
INSTRUMENT: SF- 100 DETECTOR: C31025C

RECORDER GAIN: 10 P.M.: 1000

COMMENTS: One Day Old

FIGURE 34 B

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION B IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm. ONE DAY OLD



DATE: Feb 17, 1972 OPERATOR: A. H.

SAMPLE: VIMS - Seawater (Chesapeake Bay) Station D
SOLVENT:
CONC: undiluted
DATE PREP:
TEMP:

	λ ex 458	λ em 682
SLITS:	33/33	33/33
GAIN:	30	30
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF - 100 DETECTOR C31025C

RECORDER GAIN: 10 P.M.: 1000

COMMENTS: One Day Old

FIGURE 34 D

EXCITATION / EMISSION SPECTRA OF CHLOROPHYLL IN SEAWATER TAKEN AT STATION D IN CHESAPEAKE BAY (VIMS SHIP), EXCITING AT 458 nm and DETECTING AT 682 nm. ONE DAY OLD

the sample from station E was lost before the delayed measurement was made.

All the above measurements were made without attempting to stir the sample, reflecting the settling that had occurred. Other measurements were made to determine the effect of shaking the sample just before measurement. These measurements ranged from several hours to fifteen hours. In each case, there was a measurable decrease in signal which was restored to its former strength (approximately) by shaking. The broad slits used for these measurements preclude determination of ratio of pheophytin to chlorophyll. We drew the following conclusions:

1. As the sample ages, the scattering decreases, corresponding to settling of particulates.

2. As the sample ages, the chlorophyll signal decreases (sample not shaken).

3. If a sample is agitated just before measurement, the scatter and chlorophyll signal increase again.

4. The chlorophyll signal reaches the same approximate peak height as in the fresh sample: the scattering peak is somewhat smaller (suggesting agglomeration?).

5. An older sample settles out more quickly than a fresh sample.

6. For the low resolution used (24 nm), the chlorophyll excitation spectrum is very similar for a one-day-old agitated sample and a fresh sample.

3.3.5 Conclusions

The principal results of the on-site measurements at VIMS were the more than adequate performance of the instrument on the boat with natural samples, and the collection of typical data from an important site. The detection limit for chlorophyll should be below one microgram/liter, which will be useful for most samples of interest.

The data on sample decay suggest that old samples may provide some useful information if shaken vigorously before measurement (i.e., initial chlorophyll concentration is suggested). However, it is probably true that the 24 nm bandwidth used does not allow discrimination between chlorophyll and pheophytin. The decay of a particular sample must depend strongly on the length of storage, light conditions, temperature, availability of oxygen, etc. On-site measurements of fresh samples would seem to be the only reliable recourse to obtain reliable data on identity and concentration of plankton species. On the other hand, Gelbstoff signatures changed little during storage, indicating that samples mailed at a distance would be useful for determining general levels.

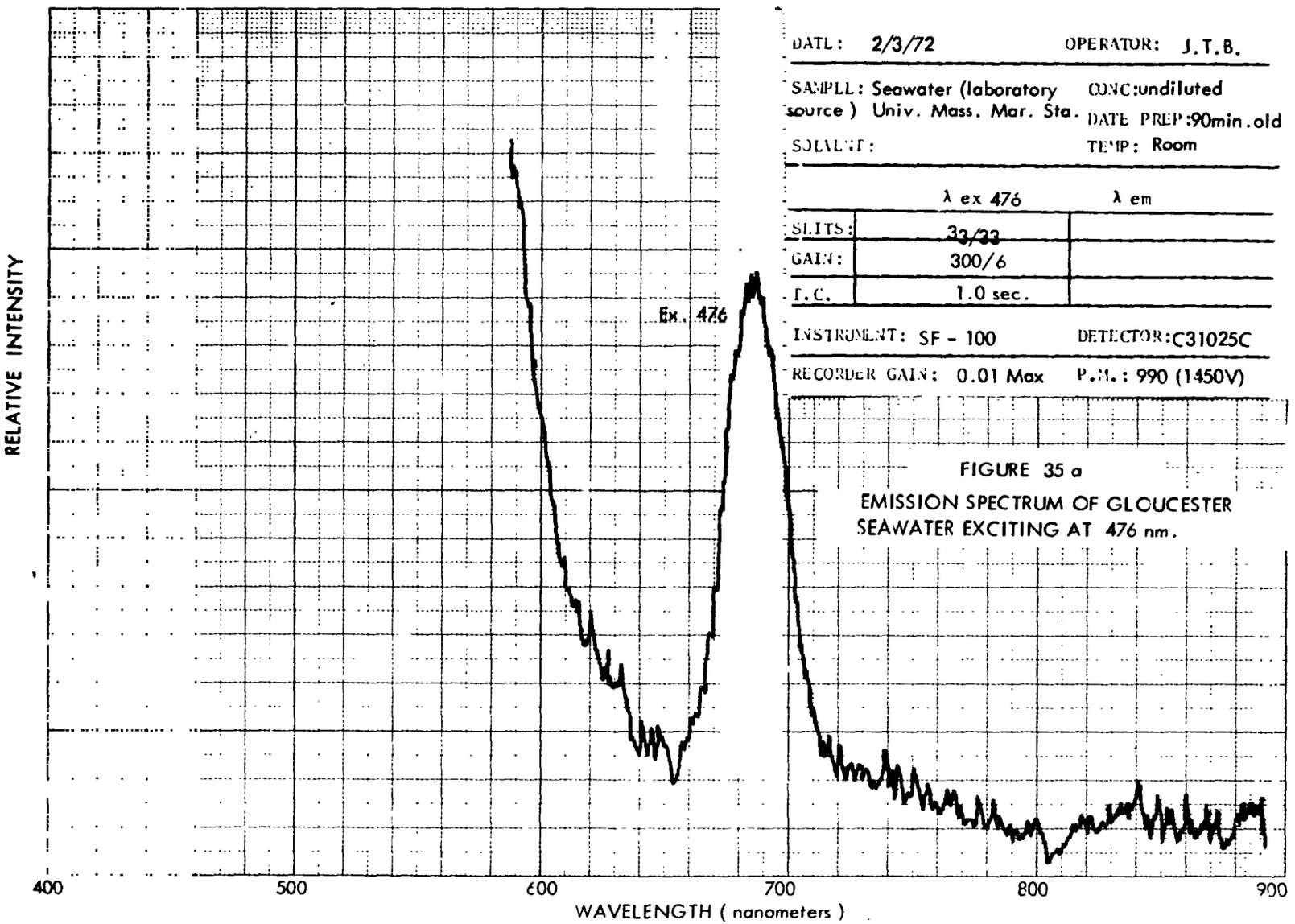
3.4 Field Testing at Cape Ann (Gloucester), Massachusetts

The proximity of the University of Massachusetts Marine Station at Hodgkins Cover in Gloucester, combined with the excellent working relations we have with its director, Dr. Charles Yentsch, have led us to make frequent use of that facility. We have used that laboratory as a test-bed for all versions of our instrument, from the unmodified SF-100 to our final two-detector version with range extended. Dr. Clarice Yentsch was also very helpful in providing a variety of algal cultures for reference data.

3.4.1 Laboratory Seawater Studies

The Marine Station is provided with an intake from Hodgkins Cove which provides fresh representative water to the laboratory. Most work was done on these samples which are not actual surface samples. Typical water is lower in temperature and chlorophyll but higher in salinity as compared to the Chesapeake Bay water at VIMS.

The data of Figures 35 and 36 are from a typical winter day with light snow. Water temperature was 0.9°C; salinity 31.6 ppth; chlorophyll content 0.5 ug/l. The emission spectrum in the chlorophyll region, Figure 35a, shows a typical peak at 683 nm, but without as large a shoulder at 740 nm as was seen in most samples from VIMS (cf. Figure 32E . The Raman



DATE: 2/3/72 OPERATOR: J.T.B.

SAMPLE: Seawater (laboratory source) Univ. Mass. Mar. Sta. CONC: undiluted DATE PREP: 90min. old

SOLVENT: TEMP: Room

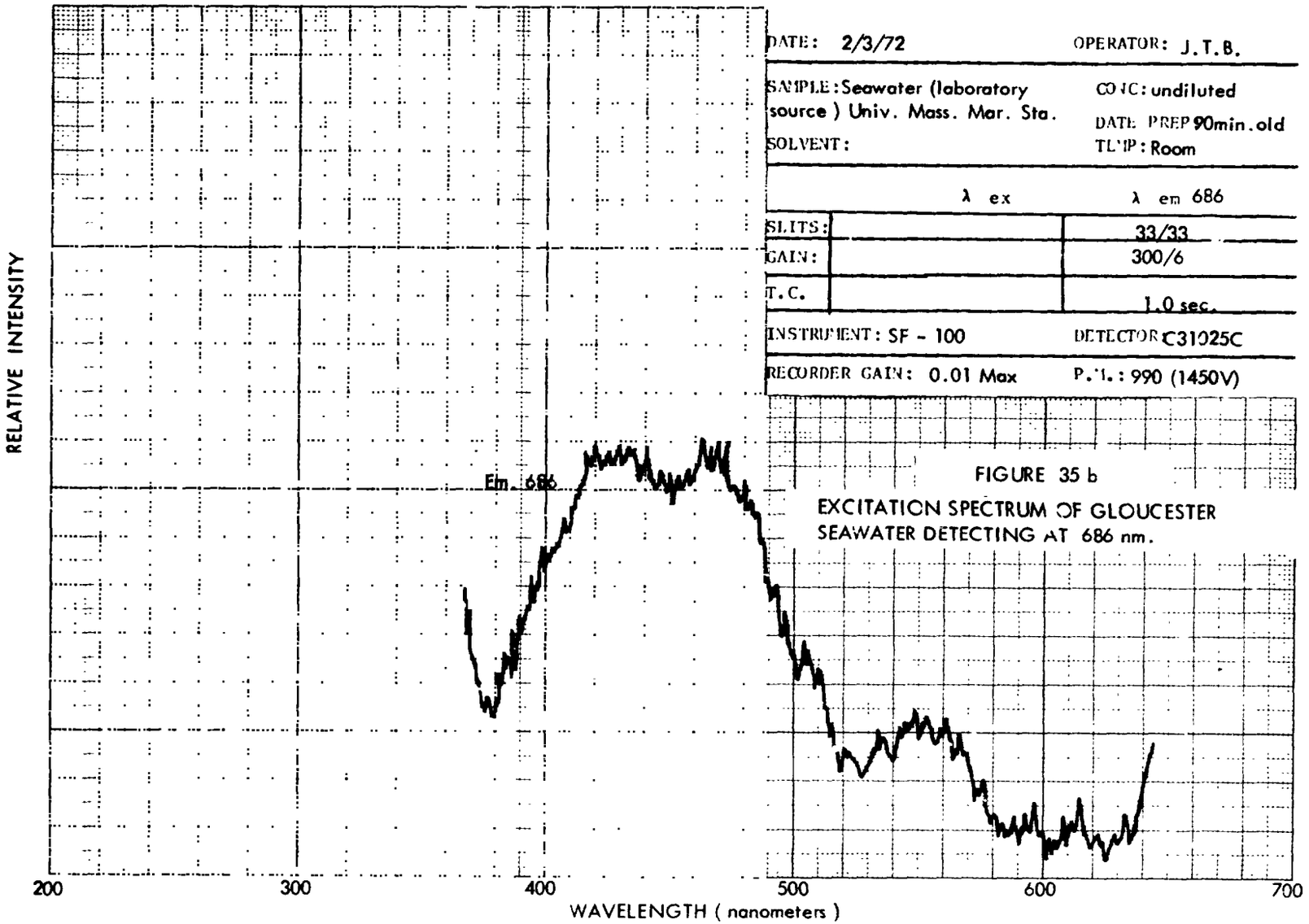
	λ ex 476	λ em
SLITS:	33/33	
GAIN:	300/6	
T.C.	1.0 sec.	

INSTRUMENT: SF - 100 DETECTOR: C31025C

RECORDER GAIN: 0.01 Max P.M.: 990 (1450V)

FIGURE 35 a

EMISSION SPECTRUM OF GLOUCESTER SEAWATER EXCITING AT 476 nm.



DATE: 2/3/72 OPERATOR: J.T.B.

SAMPLE: Seawater (laboratory source) Univ. Mass. Mar. Sta. CONC: undiluted

SOLVENT: DATE PREP 90min. old

TEMP: Room

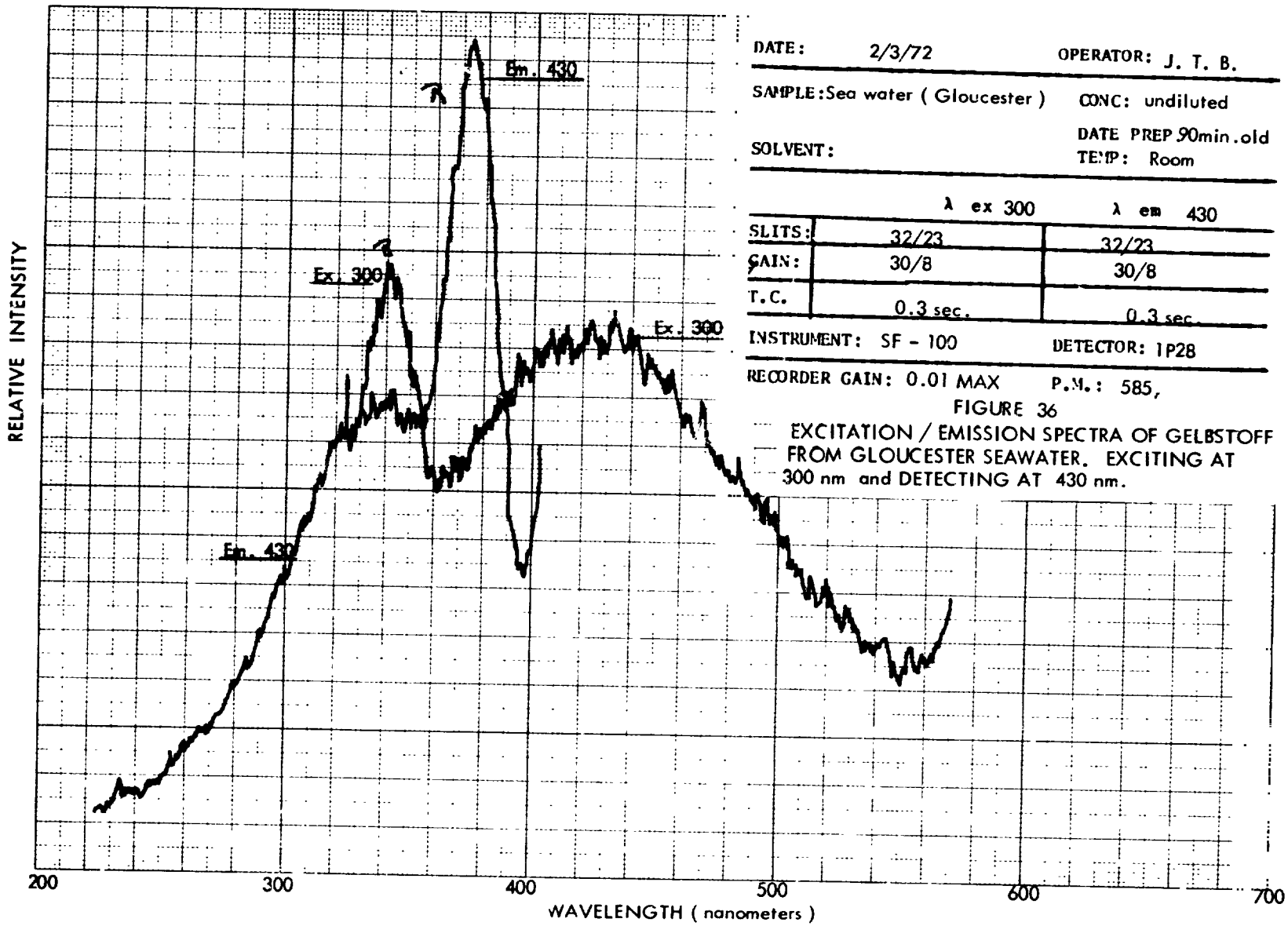
	λ ex	λ em 686
SLITS:		33/33
GAIN:		300/6
T.C.		1.0 sec.

INSTRUMENT: SF - 100 DETECTOR C31025C

RECORDER GAIN: 0.01 Max P.M.: 990 (1450V)

FIGURE 35 b

EXCITATION SPECTRUM OF GLOUCESTER SEAWATER DETECTING AT 686 nm.



DATE: 2/3/72 OPERATOR: J. T. B.

SAMPLE: Sea water (Gloucester) CONC: undiluted

SOLVENT: DATE PREP 90min. old
TEMP: Room

	λ ex 300	λ em 430
SLITS:	32/23	32/23
GAIN:	30/8	30/8
T.C.	0.3 sec.	0.3 sec.

INSTRUMENT: SF - 100 DETECTOR: 1P28

RECORDER GAIN: 0.01 MAX P.M.: 585,

FIGURE 36
EXCITATION / EMISSION SPECTRA OF GELBSTOFF FROM GLOUCESTER SEAWATER. EXCITING AT 300 nm and DETECTING AT 430 nm.

scatter rises off-scale on the left side at this gain. The corresponding excitation spectrum in Figure 35b is unlike the Chesapeake Bay samples in the size of the long wavelength shoulder at 550 nm, and the appearance of an excitation peak at 430 nm.

The instrument gain for these traces was approximately seventeen times the standard gain used at VIMS: the time constant was increased to one second. Assuming basic instrument performance had not changed, the relative chlorophyll fluorescence for this sample would be 6.6 for comparison with Table 2. From Table 2, we would expect a much lower fluorescence reading for such a low chlorophyll content. Either the VIMS chlorophyll values are too high, or Yentsch's is too low. We tend to believe the Yentsch value. (Note in Table 2 that chlorophyll values at 1.' meter are individually less than at the surface.) In this case, it is clear that the sensitivity of our instrument is such that we see 1/2 microgram of chlorophyll per liter of seawater with a signal-to-noise of approximately forty to one.

In Figure 36, we show excitation/emission spectra for Gelbstoff emission from the same sample. The Raman peaks have been labeled with an R. Peak excitation and emission wavelengths are very similar to those for Chesapeake Bay waters. Using the peak Gelbstoff luminescence intensity as a rough index of the amount present and adjusting gains to match those used at VIMS, the relative value to be compared to line two of Table 2 is unity. This suggests a Gelbstoff level less than 1/80th that found in Chesapeake Bay.

3.4.2 Sample Decay Studies

The principal reason for studying decay measurements was to determine the utility of studying samples mailed from distant sources. For this purpose, studies were carried out up to a week. Very short-term studies (less than one hour) were also made to determine whether samples could be brought to a nearby laboratory for study. For decay studies, only peak emission intensities were monitored.

With the instrument set up for peak chlorophyll emission, no noticeable decay was noticed during the first minute. The emission peak decayed to about 2/3 of its initial value in about fifteen minutes, while being irradiated in the instrument. While it is now assumed that this decay is largely due to settling of particulates, it is also possible that photochemical decay occurred.

For the longer term measurement, a freshwater sample was stored in a clear glass bottle and kept tightly shut for the period of a week. The bottle was stored in the dark between sampling periods to simulate mailing conditions. Both the Gelbstoff and chlorophyll emissions were monitored periodically. After four days, the chlorophyll emission intensity drops to about one-third of its initial value (with an agitated sample), after which it remains essentially constant for the remainder of the week. Similar experiments on Gelbstoff showed a much smaller time dependence, resulting in an actual increase of about twenty percent. These data are plotted in Figure 37.

The apparent longevity of the chlorophyll signal is probably misleading. For example, alteration products of chlorophyll in dying plankton may include pheophytins. According to French et al. (A1956), the fluorescence spectrum of pheophytin a is displaced about 10 nm to the red as compared to chlorophyll a when in an ether solution. If this be true also for the pigments in vivo, a monochromator set to peak the chlorophyll emission would see the pheophytin off its peak and so read less. The same type of argument holds for the absorption spectra. Further, Goedheer (A1968) quotes quantum yields of fluorescence as 0.22 for chlorophyll a and only 0.14 for pheophytin a. Hence, if every chlorophyll molecule became a pheophytin molecule, we would expect to see a time decrease in the "chlorophyll emission" which leveled out at some non-zero level corresponding to detected pheophytin emission. With this sort of possible mechanism in mind, the reduced emission in the chlorophyll region may not be chlorophyll; hence the use of old samples to determine chlorophyll and pigment concentrations is quite suspect.

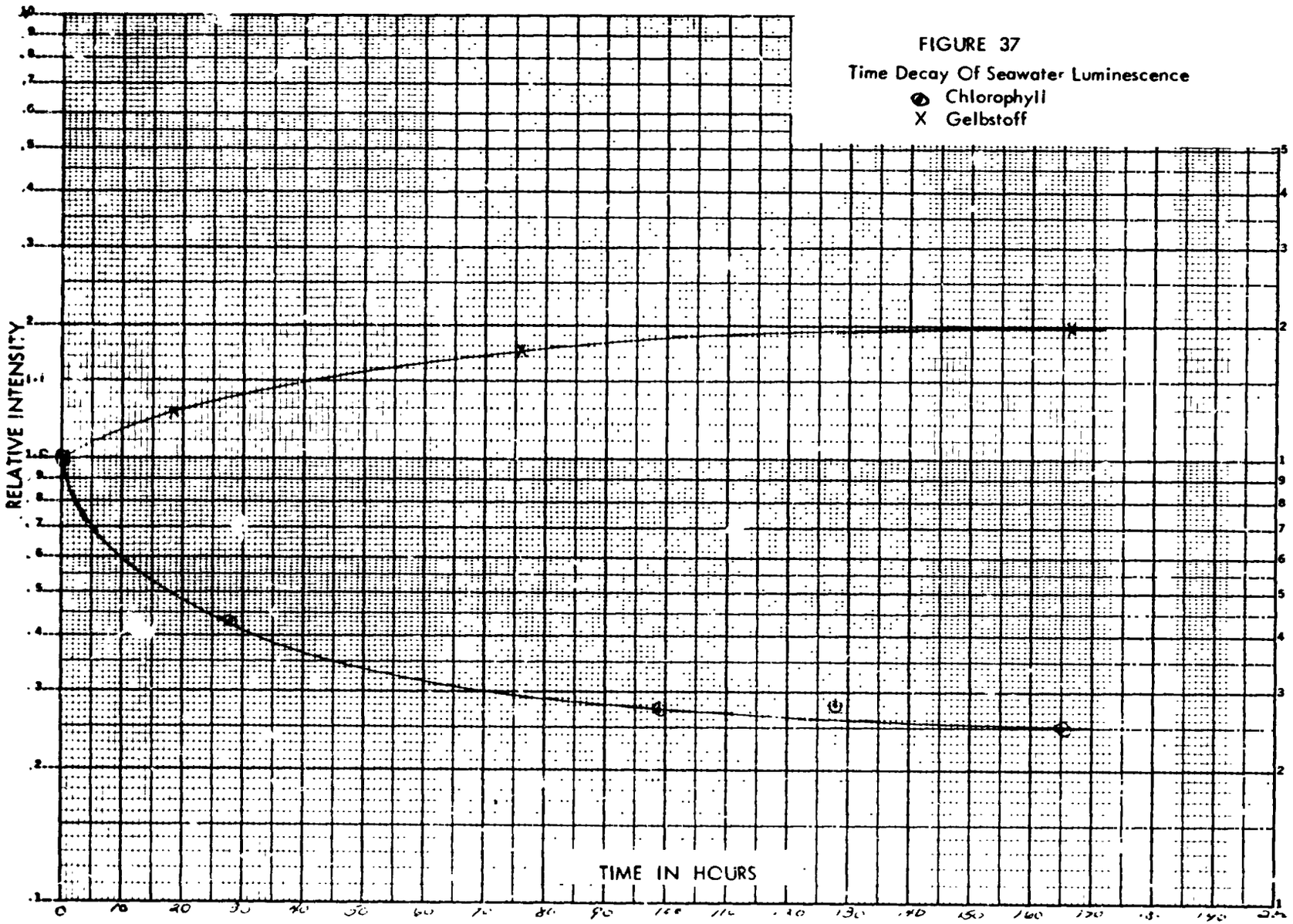


FIGURE 37
Time Decay Of Seawater Luminescence
● Chlorophyll
X Gelbstoff

3.4.3 Filter Studies

Yentsch has shown that the major emission in the chlorophyll region is due to particulates whereas the Gelbstoff emission is due to solute in the marine water. Filtering samples to separate these components may have several advantages. First, filtering is a concentration step which will allow an effective increase in sensitivity. Second, having more concentrated samples will allow use of higher spectral resolution which should allow better determination of similar pigments. Finally, dry filtered samples are known to decay more slowly than solutions.

We therefore began experiments to compare spectra from filtered plankton and original water samples. A goal was to develop a procedure which will allow direct front surface luminescence examination of the damp filter paper yielding semiquantitative concentration information. A requirement is that the volume of original water and the size of the filter paper be so chosen as to produce a uniform particle distribution which does not cover the surface. In this case, increasing or decreasing particulate concentration will have a corresponding effect on the area of the filter paper covered, and the luminescence signal should be a rough measure of original concentration. If too great an initial water volume is used, the filter paper will be more than completely covered. Since only the top layer is viewed, concentration information is lost. For too small volumes of water, sensitivity is lost.

Using Whatman GF/C glass fiber filters (as suggested by E. Weaver) supported on a glass frit, we have demonstrated that it is feasible to filter to dryness and then examine the residue front surface on our standard instrumentation. The residual dampness of the paper is sufficient to make it adhere to a flat card placed in the sample holder. A difficulty arises with uniformity of particulate deposition. Thus far, we have been unable to assure uniform deposition of material which is necessary for quantitative work, since we only sample part of the filter paper area. Quite often the deposit would form an annular ring with the center of the filter paper quite free of deposit. While we have not determined the reason for this, we feel this problem could be

overcome. By carrying out a series of filtrations using volumes differing by an order of magnitude, and selecting only those papers which appeared uniformly covered to the eye, we found the "chlorophyll" emission is approximately linear with concentration of deposit (or volume of sample).

For the numerous samples examined at other sites, the primary approach has always been to look directly at water samples. In one case, at the Florida State University Marine Station near Carrabelle, Florida, there was an instrumental instability problem which did not allow reproducible measurements at high gain. In this case, we did use the filtering technique to obtain data on the particulate emission.

3.4.4 Time Variation in Chlorophyll Fluorescence

In addition to the measurements described above, another interesting experiment was performed which indicates that algal concentrations fluctuate sizeably due to water movement. The instrument was equipped with a flow cell which allowed continuous monitoring of the water piped in from Hodgkins Cove. Exciting at 470 nm, the chlorophyll emission was monitored at 678 nm as the water flowed through at 3 liters per minute. The results of a typical scan are given in Figure 38. The time base (x-axis) is 25 sec/cm. The sharp negative signals are zero checks to assure the observed variations were not due to amplifier drift. The maximum variation is about 20% of the average.

These results are assumed to be related to wave motion in Hodgkins Cove. They also indicate that it is not necessary to take very accurate data on intensity at a given site at a particular time, since significant variations occur over short times.

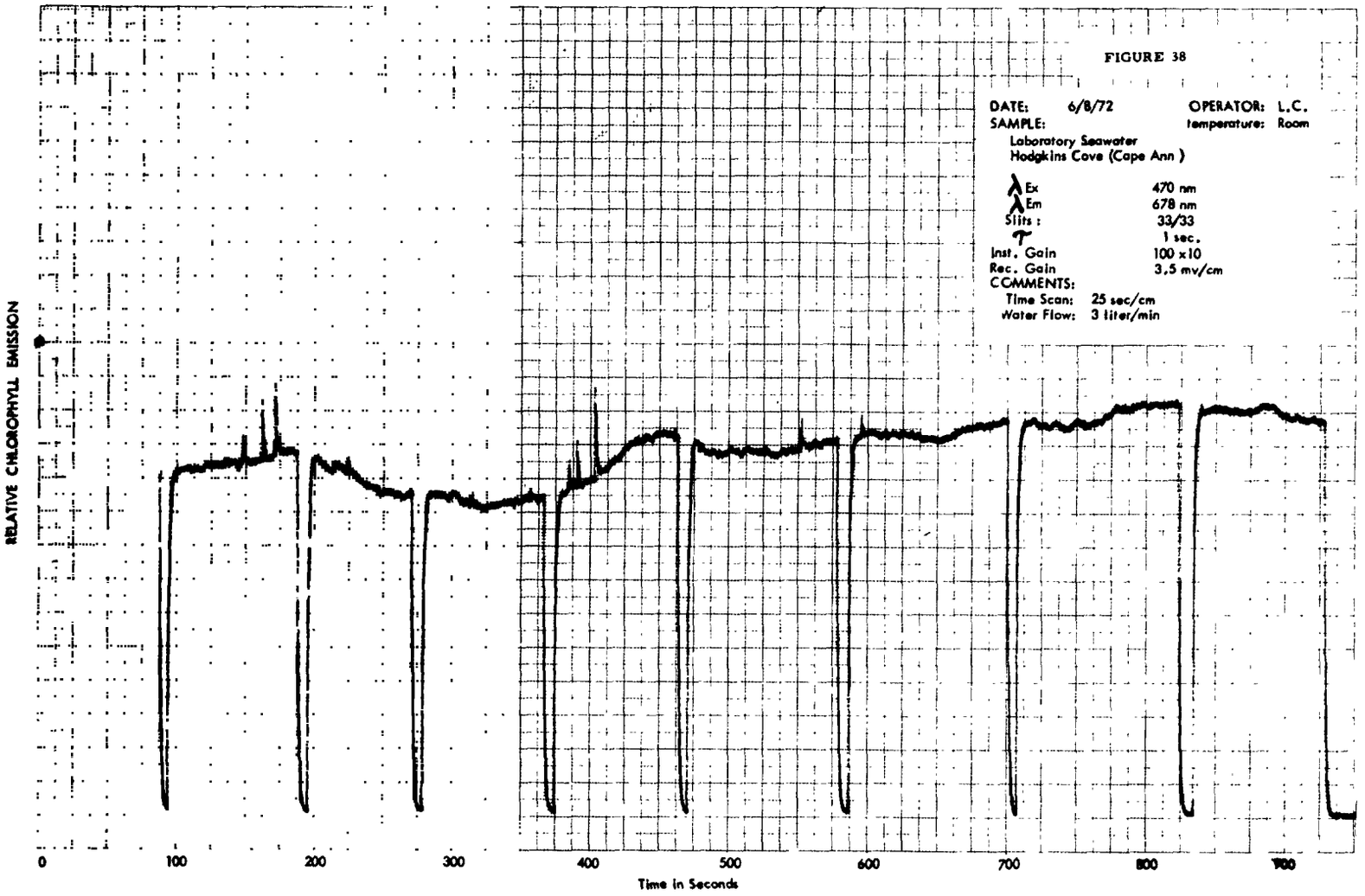


FIGURE 38
DATE: 6/8/72
OPERATOR: L.C.
SAMPLE:
Temperature: Room
Laboratory Seawater
Hodgkins Cove (Cape Ann)
λEx 470 nm
λEm 678 nm
Slits: 33/33
τ 1 sec.
Inst. Gain 100 x10
Rec. Gain 3,5 mv/cm
COMMENTS:
Time Scan: 25 sec/cm
Water Flow: 3 liter/min
RELATIVE CHLOROPHYLL EMISSION
0
100
200
300
400
500
600
700
800
900
Time in Seconds

4. EXPERIMENTAL RESULTS

4.1 Compendium of Spectral Data

One of the principal purposes of this project was to assemble a Compendium of Spectral Data containing comparative traces of marine luminescence from a large variety of sites and at various times. This objective has been modified slightly by the change to on-site measurement, resulting in somewhat less data than anticipated. Nevertheless, a considerable body of data has been assembled and published separately in the Compendium of Spectral Data, which comprises Appendix C of this Final Report. The spectral data in the Compendium are traced from the originals onto a standard grid for easier comparison.

Principal data include excitation/emission spectra of chlorophyll and Gelbstoff in natural waters taken on-site on fresh samples. Where on-site measurements were impossible (e.g., West Coast sites) only Gelbstoff measurements were made on mailed samples. In one instance (Carrabelle, Florida) where instrumental problems made water measurements untrustworthy, samples were filtered, and chlorophyll measurements were made front-surface on the particulates.

Also included in the Compendium are excitation/emission spectra of a number of algal cultures (reference spectra) for comparison with natural water spectra.

4.1.1 Instrument Settings

As was pointed out earlier, time was often at a premium during on-site measurements. Therefore, data were usually taken at set excitation and emission wavelengths to allow intercomparison. Multiple excitation/emission data were taken on samples from VIMS and Cape Ann, Massachusetts, as discussed previously.

For "chlorophyll" data the bandpass of both excitation and emission monochromators was usually set wide open, or about 24 nm. This was necessary for sensitivity for the weakest samples. The excitation wavelength

was usually set about 460 nm, although this was sometimes varied if it was far from the peak excitation. The monitoring wavelength was set on the peak chlorophyll emission which occurs about 680 nm.

For "Gelbstoff" spectra the excitation was set at 280 or 290 nm and the monitoring wavelength at 440 nm in most cases. Bandpass on both excitation and emission monochromators was kept at about 17 nm. (There was less question of detectability with Gelbstoff.)

The reference algae were usually monitored with a bandpass of 6 nm since there was ample signal. The smaller bandpass allows development of greater detail in the spectra. Excitation was usually set to give the greatest chlorophyll signal. The monitoring wavelength was usually the peak of chlorophyll emission. This peak often occurred at wavelengths slightly longer than 680 nm, probably because of the effects of reabsorption of the chlorophyll emission in the strong samples.

4.1.2 Relative Intensity

In the early stages of the project, an attempt was made to calibrate the instrument before each measurement using a ruby rod or water Raman as reference. Consideration of the variation of chlorophyll emission at a given site, not only with season, but even during days and hours, as shown by the continuous monitoring experiments at Cape Ann, make precise measurements meaningless. Therefore we have defined a "Relative Intensity" for each chlorophyll curve which is proportional to the ratio of the peak height and instrument gain. Since instrument gain probably varies much less than chlorophyll emission at a given site, this factor can be used as a guide to relate measurements made in different places at different times. The Relative Intensity is given on each chlorophyll trace for actual marine waters in the Compendium. The range is from 99 for a Chesapeake Bay sample to much less than 1 for a sample from Hodgkins Cove at Cape Ann, Massachusetts.

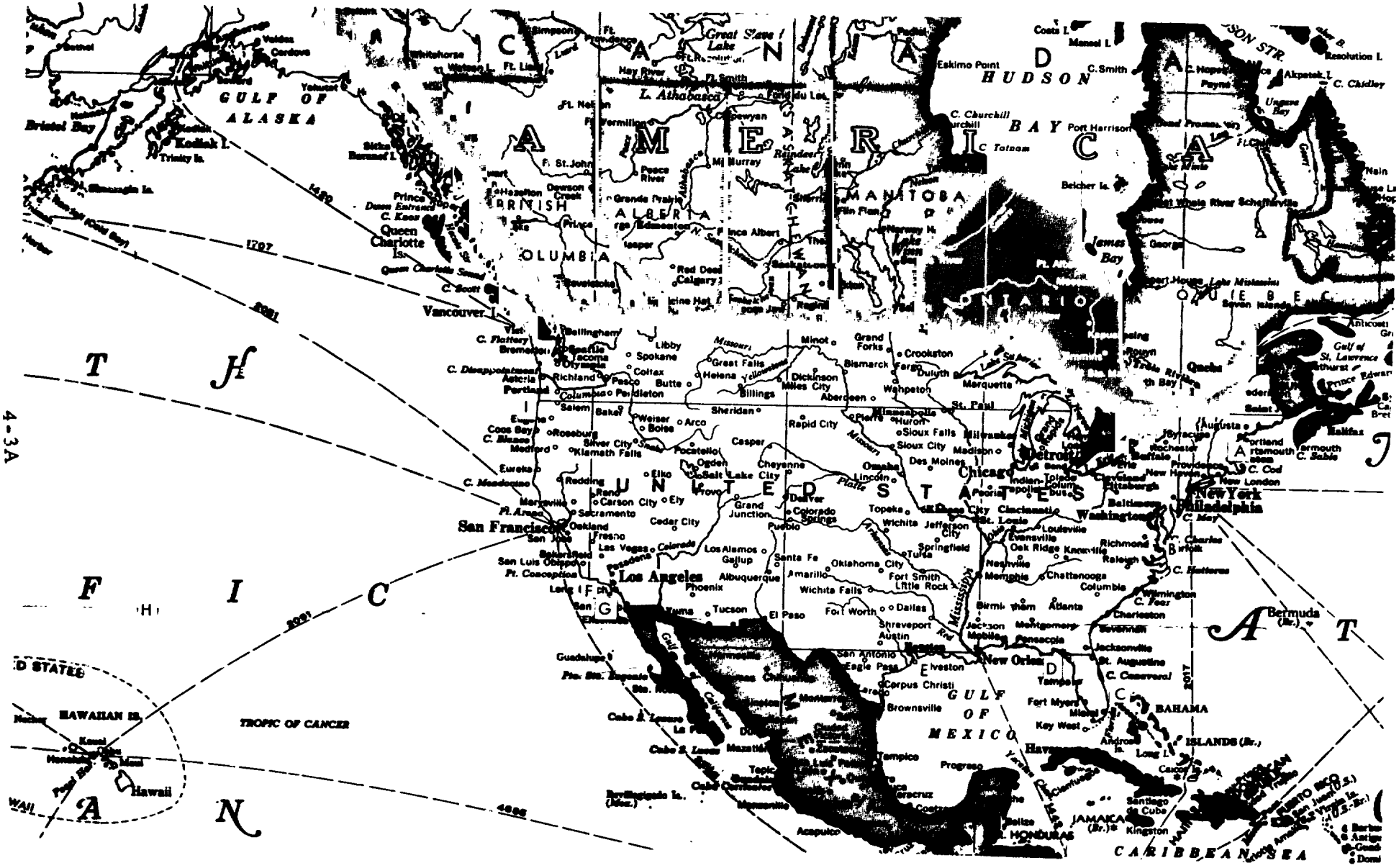
4.2 Site Descriptions

For typical on-site measurements, standard instrumentation was sent by air to the vicinity of the selected laboratory site, and transported by station wagon to the laboratory. Standard equipment consisted of the Fluorescence Spectrophotometer, lamp power supply, x-y recorder, cuvettes and filtering apparatus for particulates. Usually laboratory bench space and facilities had been prearranged, allowing quick set-up for measurement. After instrument checkout on standard samples or water Raman, local water samples were examined. This would be followed by study of samples obtained by short boat trips to selected water sites. Often the water samples were measured within hours of collection. Where possible, the water samples were subjected to standard laboratory analysis for chlorophyll, salinity, etc., by the host laboratory. In the following sub-sections, we shall discuss each of the sites selected and the method of collecting and handling samples.

Samples from nine different geographic sites were measured and included in the Compendium of Data. The first five of these, covering the Atlantic and Gulf coasts, were covered on-site. Here measurements were made on chlorophyll and Gelbstoff, and on several algal cultures supplied by laboratories. The remaining four sites included three off the West Coast and one several hundred miles north of Hawaii. Lack of time and funding made it impossible to monitor these on-site; therefore samples were mailed to Bedford, and only Gelbstoff was monitored. The sites will be described in some detail in the following subsections. They are indicated on Map A. Further details will be found in section 2.1 of the Compendium of Spectral Data.

4.2.1 Site A: Cape Ann, Massachusetts (University of Massachusetts Marine Station

The laboratory is located on the western side of Cape Ann at Hodgkins Cove. Numerous measurements were conducted at the laboratory through-



Map A: Survey of Sampling Sites

out the project. Representative data from several dates are included in the report.

Typical results were obtained on both chlorophyll and Gelbstoff, as reported in Section 3.4 and in the Compendium of Spectral Data. Dr. Charles Yentsch, Director of the Marine Station, provided assistance in all phases of this project. The laboratory provided independent data on other water variables, including chlorophyll content, whenever desired.

4.2.2 Site B: Gloucester Point, Virginia (Virginia Institute of Marine Science)

The Virginia Institute of Marine Science (VIMS) is located at Gloucester Point on the York estuary of Chesapeake Bay. Measurements here were conducted on 15 and 16 February 1972. These measurements included laboratory water samples (off the VIMS pier), on ship measurements at four sites ranging from the mouth of the York River up into Mobjack Bay, and some algal culture measurements. This laboratory also provided independent measurement of water parameters.

The measurements aboard ship were an important step in checking our field instrumentation and are clear proof that this type instrumentation can be made to function well aboard a small boat with simple motor-generator power. A more detailed account is given in Section 3.3, as well as in the Compendium.

The on-site measurements were arranged through the cooperation of Dr. Paul Zubkoff, Chairman of the Department of Physiology. Mr. J. Ernest Warriner III assisted in collecting samples, piloting the boat, and setting up laboratory facilities. He also provided samples for our abortive attempt at mailing samples.

4.2.3 Site C: Fort Lauderdale, Florida (Nova University Physical Oceanographic Laboratory)

This laboratory is located just south of Fort Lauderdale near the Atlantic Ocean. Samples were gathered 3 and 4 April 1972 and measured

at the Oceanographic Laboratory. This site is of particular interest because of the close proximity of the Gulf Stream offshore. Gulf Stream water has low productivity and high clarity as contrasted with inshore waters having high turbidity due to yellow humic acids draining out of Port Everglades via New River. The source of these acids is the Everglades, through the drainage system. Productivity of inshore regions is high as a result of domestic pollution along the drainage basin.

Arrangements for these measurements were made by C. Yentsch, with the kind assistance of Dr. W. Richardson, the Laboratory Director. Yentsch assisted in collecting samples for immediate measurement.

4.2.4 Site D: Carrabelle, Florida (Florida State University Marine Station)

The marine station is located in the panhandle of Florida on the Gulf of Mexico at Turkey Point, near Carrabelle. The water is sedimentary and shallow. Measurements were made in mid-April 1972.

This site produced the fewest good measurements because of the onset of instrument instability problems. As a result, it was necessary to discontinue direct measurement of chlorophyll in water samples and turn to filtering methods, using elementary apparatus we had brought with us. These filtered particles were then studied by front-surface methods. The experience shows that this method is also viable, although less quantitative

Following these measurements, the field instrument was returned to our Bedford laboratory where an improved feedback circuit in the photomultiplier high voltage supply was added. This made the instrument perform stably in all future measurements.

Arrangements to use the laboratory facilities were made through Dr. Jack Winchester, Head of the Department of Oceanography at Florida State University in Tallahassee.

4.2.5 Site E: Galveston Bay (National Marine Fisheries Laboratory)

The laboratory is located near Galveston Bay. Measurements were made on 20 June 1972 on samples from nine bay sites chosen to represent typical areas. Some areas were ideal nurseries: others were possibly polluted by nearby plants.

Arrangements for the use of the laboratory facilities and a boat to collect samples were made by Mr. Robert Temple, Assistant Director of the Laboratory. Mr. Frank Marullo collected samples, and Mr. Neil Baxter provided the data on temperature and salinity.

4.2.6 Site F: Pacific Ocean--Southern California (University of California at Santa Barbara Marine Science Institute)

For this and the following three sites the samples were mailed to Bedford for delayed examination. Because we feel such measurements on chlorophyll are invalid, only Gelbstoff measurements were made.

This site is of particular interest because it has an abundance of kelp beds and natural oil seepage. Five samples were provided, collected on 12 September 1972. These ranged from three miles off-shore to directly off the beach. They include water from kelp beds and in an oil slick region.

Sample collection was arranged by Dr. Robert Holmes, Director of the Institute.

4.3 Discussion of Chlorophyll Data

Before directing our attention to the data themselves, it is worthwhile to review the limitations of the data. First, there is the question of absolute intensity. Because of the evolutionary nature of the field instrument, the sensitivity varied from site to site. Thus, as power supply problems grew (culminating in unacceptable behavior at site D in Carrabelle, Florida), it was necessary to modify the photomultiplier voltage. The non-linear gain corrections for this are not known and not incorporated in our Relative Intensity figures. The power supply problems also caused apparent drift in

signal which was most apparent in high gain situations such as was necessary in Gloucester, Massachusetts, where chlorophyll content was less than one milligram per liter. The resulting background variation could be interpreted as excitation peaks and valleys. See, for example, Figure C-8, where the observed variations may not be real. (Note that we shall refer to figures in the Compendium with the prefix C-.)

Another important limitation is that the data are not corrected for instrument response. The data of Figure 31 show that the lamp output varies with wavelength. Excitation peaks below about 380 nm will appear too weak, and there will always be a spurious rise at 470 nm where the xenon lamp has its largest peak. The data of Figure 31 are based on Rhodamine B as a quantum counter. Further work has indicated that the output is underestimated at valleys in the Rhodamine absorption in the vicinity of 450 nm and 380 nm, and overestimated at the peak absorption at 560 nm. It is unfortunate that our data correction scheme was not completely operative in time to correct traces for the compendium.

While lack of correction makes reference to absorption data difficult, relative differences between excitation curves can be interpreted meaningfully. Thus, when we examine the traces of the Compendium, we shall look for differences first--then relate them to possible components.

Another limitation is the necessity for using rather large bandpass (approximately 24 nm) in order to achieve our good sensitivty for low level chlorophyll measurements. This precludes monitoring of excitation very close to emission and also limits accurate delineation of curve profiles. On the other hand, this bandpass has relevance to potential remote sensing application.

4.3.1 Chlorophyll Emission

If we page through the Compendium, concentrating on chlorophyll emission data, three facts emerge. First, there is a large variation in relative intensity which corresponds roughly with chlorophyll concentration

as measured by standard laboratory procedures. (See Section 3. 3. 3.) Second, the chlorophyll emission occurs with slight variation at about 680 nm. Part of this variation may be due to slightly inaccurate setting up of the wavelengths on the x-y recorder in the field. Part is due to concentration effects (self-absorption) which can shift emission to slightly longer wavelengths. In any case, the bandwidth of 24 nm does not allow any certain statements to be made about significant variation in emission peak position. A third interesting observation concerns a shoulder which appears on most emission traces at about 740 nm. (See, for example, Figures 32_B, 32_C, 32_E.)

There is no agreement as to the source of the 740 nm shoulder. Some believe it is part of the emission spectrum of chlorophyll A. Others believe it is one of the many sub-types of chlorophyll being discovered regularly. The variation in intensity of the shoulder is also well documented. (See Figure 3.) In the Compendium approximately one-third of the chlorophyll emission traces from on-site water samples did not show evidence of this shoulder. There is certainly a great variation, as evidenced for example by Figures C-57 and C-63, both from the Galveston Bay area.

From a purely instrumental point of view, the data allow explanation of the 740 nm shoulder as either a separate moiety which occurs in company with chlorophyll a, or as a band of chlorophyll a which suffers less reabsorption than the major peak. Because of our observations on excitation spectra, we tend to believe the latter explanation.

4. 3. 2 Chlorophyll Excitation

It is well known that the auxiliary pigments (carotenoids, phycobilins, etc.) transfer energy very efficiently to chlorophyll. Therefore, the excitation spectrum of chlorophyll in algae may show peaks not associated with chlorophyll itself but with auxiliary pigments. If this be so, it should be possible to make rough determinations of the type of alga from the excitation spectrum.

Looking through the compendium at excitation spectra, it is clear there are variations in the spectra. Yet there are also very great similarities. We shall consider the form shown in Figure 32_E as "typical." This form is marked by its xenon-accentuated peak at 470 nm, a shoulder at 550 nm, and a shoulder at 440 nm. (The second order scatter peak at 340 nm is to be neglected.) In the Compendium, the following on-site water traces exhibit this typical form: C-6, 12, 16, 18, 20, 26, 28, 30, 34, 36, 38, 40, 42, 56, 60. One variant from this form has a double peak occurring at 430 and 470 nm, e.g., C-68 from Galveston Bay. Others showing this variation include C-2, 4, 8, 24, 32, 58, 62, 66, 68, 70. In some cases, e.g., C-32, 64, 66, 70, the 430 nm peak equals or exceeds the 470 nm peak. Another variant concerns the size of the peak at 550 nm. Traces C-2, 4, 10, 22, 24, 26, 34, 40 have well developed peaks in this region. This peak may be associated with the pigment fucoxanthin in diatoms, or with phycoerythrin in other algae.

At this point, we note that according to Yentsch all the natural waters sampled should have diatoms and dinoflagellates as their principal algal components. Spectra C-81, 83 and 85 are all diatom excitation spectra showing the form of our "typical" spectrum. Usually the 430 nm peak is slightly less than the 470 nm peak. Figure C-87 of a dinoflagellate also shows the "typical" form, except [illegible] 430 nm peak is much less than the 470 nm peak. (Note that the [illegible] spectra mentioned in this paragraph was taken with [illegible] nm resolution, s[illegible] peaks considerably.) We conclude that it is reasonable to ass[illegible] we are seeing the major component of the waters in each case.

Presumably the double peaked spectra could be interpreted as due to the presence of another algal form, for example NANNOCHLORIS ATOMUS of Figure C-72.

4.3.3 Quantum Counting

Examining a "typical" excitation trace of any of the diatom cultures, we are struck by the similarity with quantum counter curves for determining the intensity of excitation light from our source as a function of wavelength. We have already exhibited such a curve in Figure 31, using Rhodamine B. In this case, all of the energy is absorbed by the dye at every wavelength, due to the high concentration. The fluorescence is monitored at a much longer wavelength. If, as is usually the case with aromatics, the quantum yield is independent of wavelength, the relative emission is a measure of the number of photons incident on the Rhodamine B, thus acting as a "quantum counter." We have already noted that Rhodamine B is not the ideal quantum counter since it has very weak absorption in some regions, and the fluorescence can be partially self-absorbed. We have found other superior materials which do not show this effect, though they do not cover as much of the spectrum as does Rhodamine B.

In Figure 38A, we have superposed the "excitation spectrum" of a concentrated dye which emits at 572 nm and the excitation spectrum of the diatom PHAEODACTYLUM TRICORNATUM of Figure C-60. The dye spectrum, in a heavy line, ceases to absorb sufficiently past about 480 nm to act as a quantum counter. However, at shorter wavelengths, the agreement is surprisingly good. Both spectra were taken at 6 nm resolution. If we examine the upper curve of Figure C-50, taken on particulates from seawater in the Gulf of Mexico, we also find surprising agreement. We form the tentative conslusion that this diatom is acting as a quantum counter, although it is perhaps imperfect in the region from 340-430 nm.

This conclusion, which should apply to the water samples also, has far-reaching effects on how these spectra may be used to identify algae, and how the spectra are to be interpreted. The basis of the interpretation depends on the detailed analysis of fluorescence from particulates. Essentially, it assumes that the surface layer which absorbs the light is optically dense, i.e., all the light is absorbed. Further, almost all of this energy

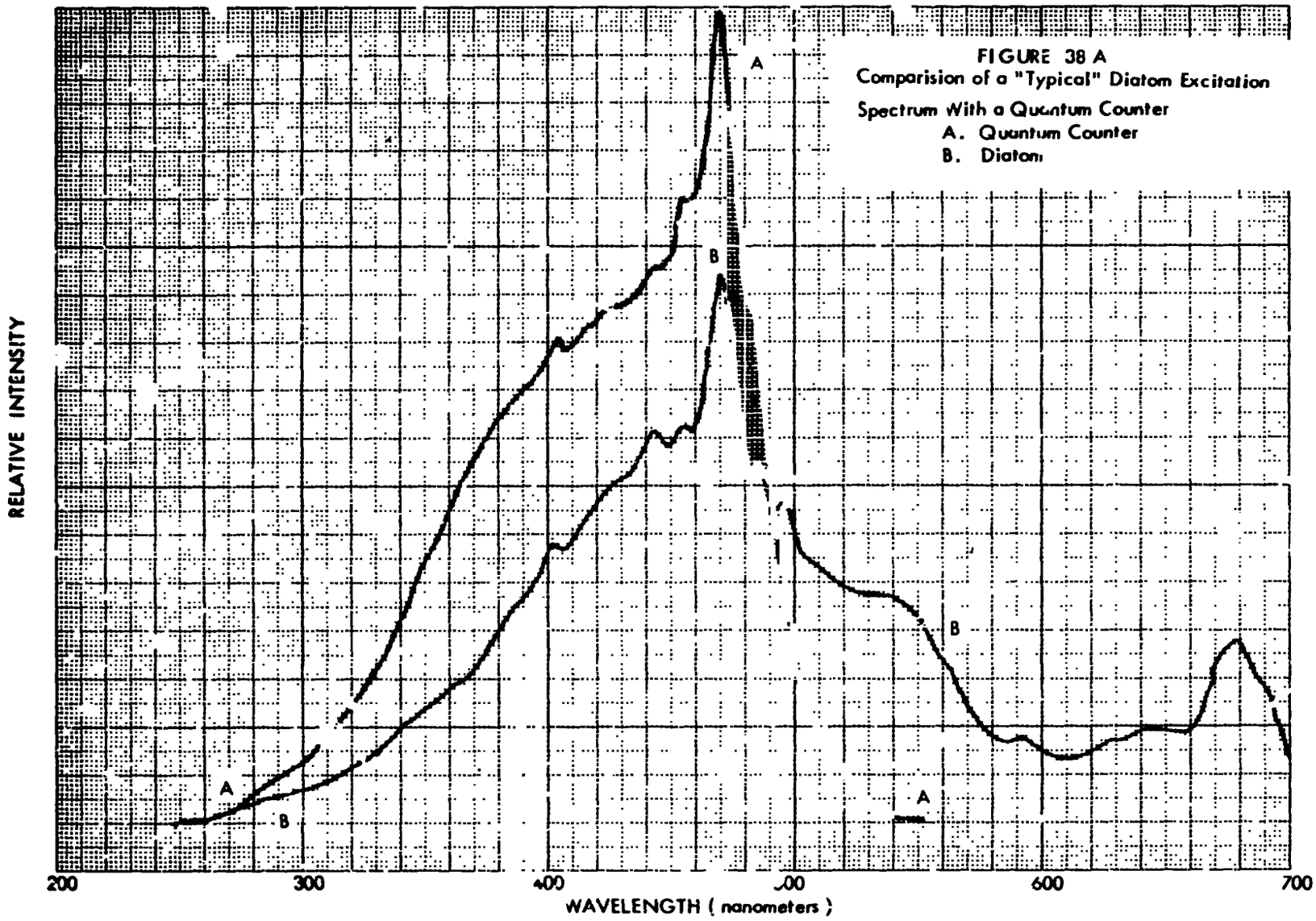


FIGURE 38 A
Comparision of a "Typical" Diatom Excitation Spectrum With a Quantum Counter
A. Quantum Counter
B. Diatom

is transferred to the chlorophyll. Thus, the excitation spectrum, even of a very dilute suspension, is the spectrum of a concentrated sample. (We have also met this situation in our study of oils where very dilute emulsions have spectral properties of concentrated oils.) We shall return to this subject in our conclusions.

4.3.4 Algal Cultures

Most algal cultures exhibited chlorophyll emission at 680 nm when excited in the chlorophyll excitation band near 430 nm. Many also exhibited a variety of shorter wavelength emissions when excited at shorter wavelengths. Thus, the diatom THALASSIOSIRA FLUVIATILUS (Figure C-191) exhibits emission at 510 nm in addition to 680 nm when excited at 354 nm, and emission at 350 nm when excited at 290 nm. In Figure C-192, this same diatom exhibits emission at 450 nm when excited at 350 nm. (This last could contribute to Gelbstoff spectra of unfiltered waters.) SKELETONEMA COSTATUM (Figures C-189, 190) is another diatom with similar secondary emissions. The dinoflagellate GYMNODIUM NELSONI of Figure 196 also has a 450 nm emission when excited at 350 nm. The blue-green alga, SCHIZOTHRIX (Figure C-90) exhibits a secondary emission at 610 nm when excited at 400 nm. The green algae NANNOCHLORIS ATOMUS (Figures 183, 185 and 186) and DUNALIELLA (Figures 187 and 188) exhibit fluorescence at 340 nm and 450 nm when excited at short wavelengths.

In general, these secondary emissions are much weaker than the chlorophyll emission (as can be seen by the appearance of Raman peaks on the spectra). Their existence can presumably be traced to the emission of individual pigments as discussed earlier. This would indicate that not all energy is transferred to chlorophyll. On the chlorophyll excitation spectra, it would cause low intensities in some absorption regions as compared to an ideal quantum counter curve. In Figure 38A, we have noted that the excitation trace of the diatom is low in the region 340-430 nm. Thus, we can maintain a consistent picture allowing for some individual pigment emission while maintaining the idea of quantum counting.

In most water samples, we did not detect emissions other than at 680 nm and in the Gelbstoff region. It now appears that some of what we have termed Gelbstoff emission may in fact be due to algae. (A simple experiment on filtered and unfiltered samples is possible.) Because of difficulties associated with on-site measurements and lack of time, we did not always look in all spectral regions and so may have missed secondary emissions.

4.3.5 Inter- and Intra-Site Variations

In the previous discussion, we have viewed the Compendium as a whole in order to note similarities and differences. We now comment on spectra by site in order to determine where differences occur.

It is hard to classify the spectra from Cape Ann, Massachusetts (Site A) because the chlorophyll level was so low and because our instrument was not so stable as after power supply improvements.

The spectra from Site B (Chesapeake Bay) are all "typical", differing only in intensity.

The spectra from the Atlantic Ocean off Fort Lauderdale, Florida, showed significant variation, presumably reflecting the great change in passing from the Gulf Stream into coastal waters. Station 1 (Figure C-22) has a "typical" spectrum except that the 550 nm peak is accentuated. Station 2 (Figure C-24) has a less accentuated peak at 550 nm, but now has a double peak at 430 and 470 nm. Station 3 (Figure C-26) is more typical with the double peak minimized and the 550 nm peak smaller. Station 4 (Figure C-28) is very typical, while Station 5 (Figure C-30) has a larger 430 nm peak, though not split. Station 6 (Figure C-32), which is in the harbor, is "typical", except that the 430 nm peak now is larger than the 470 nm peak.

The few unfiltered water samples from Site C, near Carrabelle, Florida, are all fairly "typical" although the 550 nm peak is accentuated in Figure C-34.

The data from Site D in Galveston Bay (Figures C-54, 56, 58, 60, 62, 64, 66, 68, 70) showed the greatest variation among themselves and relative to the other sites. The first three stations show an unusual sizeable unstructured excitation extending from 520 nm to the long wavelength chlorophyll absorption band. This is not seen on any other traces. Otherwise, these spectra are fairly "typical", although Figure C-58 exhibits well-split 430-470 nm peaks. Station 4 (Figure C-60) is "typical" except for some remaining long wavelength excitation. Figure C-64 of Station 6 shows a structured excitation with a third peak at 410 nm. Stations 7-9 all show the split peak and a considerable long wavelength excitation.

Thus, there is considerable variation within some sites, as well as between sites. We can only conjecture what the Pacific samples would have shown.

4.4 Gelbstoff Excitation and Emission

Gelbstoff spectra have already been discussed in some detail in Section 3.1.3. They are characterized by being a mixture of many materials. This is most easily seen when the excitation spectra shift as monitoring wavelength is changed (Figure 23) and emission spectra shift as excitation wavelength is changed (Figure 23). The data taken at most sites were restricted to a diagnostic excitation at 350 nm and monitoring wavelength of 440 nm. In general, the largest difference in spectra was in intensity.

At several sites, an excitation wavelength of 280 or 290 nm was used in addition to the 350 nm excitation. The shorter excitation proved to be interesting because it tended to excite a larger number of emitting moieties. The press of time on-site unfortunately did not permit taking data at many excitation and monitoring wavelengths. All data were taken on unfiltered samples except at Carrabelle. Thus, the particulates may have contributed to some of the spectra as discussed in 4.3.4.

There are hints of structure in some emission traces such as C-111 and C-115, both at Fort Lauderdale. The former shows a shoulder on the

long wavelength side of the peak emission. This sample came from Port Everglades harbor. The latter trace shows an emission shoulder at even longer wavelengths, about 500 nm. This sample was taken close to the coast in humic waters. It is quite probable that more multicomponent spectra would have revealed more specific signatures related to waste materials, etc.

The emission spectra excited at 280 nm in Carrabelle waters were similar to those excited at 350 nm, except for lower intensity and descending monotonically to shorter wavelengths. (See, for example, Figure C-127.) In Galveston Bay, on the other hand, many emission spectra for excitation at 280 nm have their peaks shifted down to below 400 nm, as for example in Figure C-140. This is true only for stations 1-5: the balance have spectra more like those of Carrabelle.

The most unusual excitation trace occurs in Figure C-151 off the coast from Santa Barbara at Station D. This sample is taken 100 m offshore from the mouth of Goleta Slough, which is probably polluted. The excitation maximum has shifted to approximately 315 nm.

Several samples from the E. B. Scripps cruise off Southern California show some enhanced emission below 400 nm, e.g., Figure C-156, whereas others do not, as in Figure C-160. The greatest enhancement occurs in Figure C-162 at Station 8. This peak is augmented by Raman, but yet is unusually large, being peaked close to 350 nm.

5. BIBLIOGRAPHY AND RELATED WORK

5.1 Bibliography

A second principal objective of this program was to assemble a bibliography of related work, with particular emphasis on recent luminescence studies of water samples in situ. As expected, there is a large amount of published work on samples which have been manipulated in the laboratory, or on cultures. Also as expected, there is a paucity of luminescence data on marine samples in situ.

The Bibliography will be found at the end of this report as Appendix B. It is organized under the following subheadings:

A. Chlorophyll and Other Plant Pigments: Photosynthesis

B. Gelbstoff

C. Bioluminescence

D. General Marine Luminescence

E. Related Marine Biology

F. Related Marine Chemistry

G. Fish Pigments and Oils

H. Pollution

I. Optical Properties of Seawater

J. Miscellaneous

The Bibliography does not pretend to be exhaustive. We have reviewed much more material but have selected items which were useful to the project or which we wished to refer to in our reports. In the following section we shall discuss published work on marine luminescence in situ.

5.2 Related Work

As we anticipated at the inception of this program, very few luminescence measurements have been made in situ on surface waters. On the other

hand, considerable numbers of papers have appeared on the luminescence of extracts, or laboratory cultures. Quite commonly fluorescence measurements utilizing simple filter instruments are made in situ. Most measurements are made with fluorimeters which lack specificity. Duursma and Rommets (B1961) adapted a Zeiss spectrophotometer, giving them higher specificity, and Traganza (B1969) used a Baird-Atomic instrument aboard ship. According to Duursma, Traganza used "the most developed laboratory equipment", since he could investigate both the excitation and emission spectra. Also mentioned is the Fraunhofer Line Discriminator developed under the aegis of the Geological Survey by Perkin Elmer Corp. This last device examines sun-stimulated luminescence and demands detection only in Fraunhofer lines.

Traganza's work seems to be the only attempt at full excitation/emission signatures before the present work. Traganza used an earlier model Baird instrument which certainly lacked the sensitivity of our present model for chlorophyll, and probably also for Gelbstoff. His results are reproduced in Figures 39-41, for comparison with the results of our study.

Kullenberg and Nygård (B1971) describe an advanced version of an instrument designed by Jerlov (I1968); however, it is still a fluorimeter. This particular instrument employs a modulated excitation source to allow easy automatic background subtract of sun scatter. The authors' results from measurements in the Baltic suggest a relation between particle content and fluorescence, except near the surface layer.

Zarabashev and Zangalis (A1970) have written on the "Fluorometric Determination of Chlorophyll In Vivo" and noted that the Lorenzen method of exciting in the 430 nm excitation band of chlorophyll also excited other materials (including Gelbstoff) which cause an error in fluorimetric methods. Their instrumentation consists of a line source (the Hg line at 436 nm) and a monochromator detector. Figure 42, from this paper, shows their results.

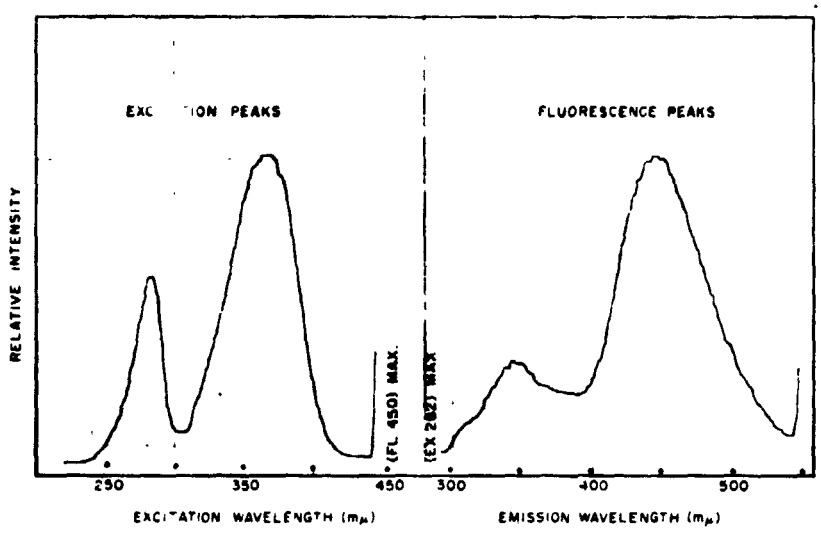


FIGURE 39. Left, excitation spectrum of sea water collected in a surface concentration of Trichodesmium sp. near 35°25.8'N. 67°19'W; right, fluorescence spectrum of same. (The irregular traces were caused by the roll of the ship.) (Yentsch, B1969.)

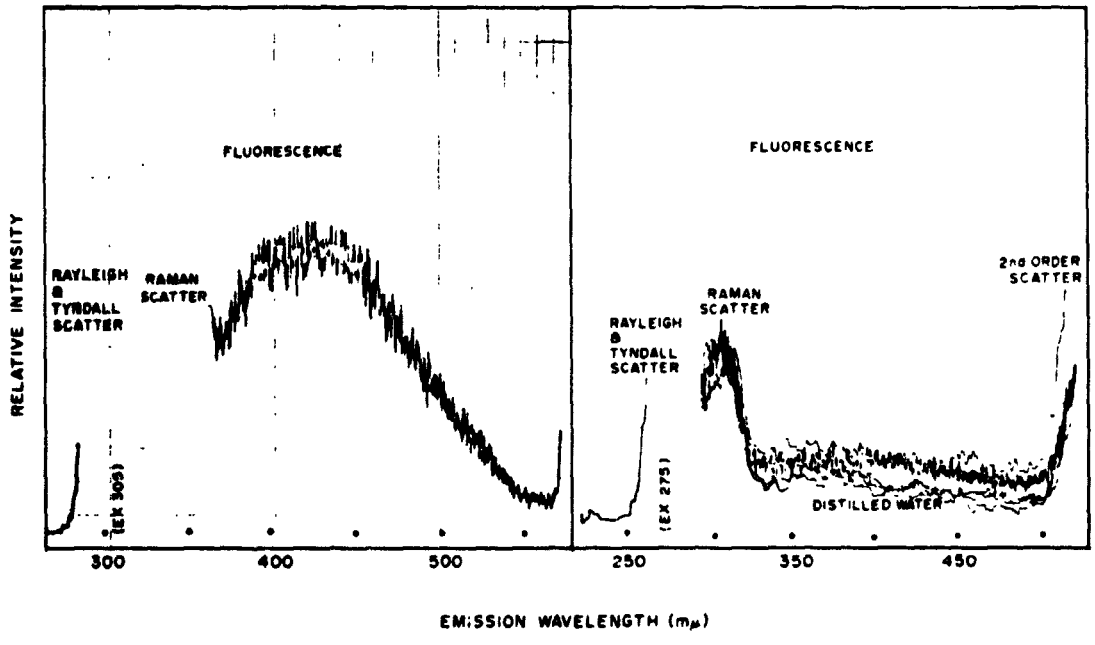


FIGURE 40. Left, moderate, broad-peaked fluorescence spectrum of Atlantic Shelf water collected at 30 m near Nantucket Shoals (40°05'N, 69°37'W); right, weak, broad-banded fluorescence spectra of Sargasso Sea water (35°25.8'N, 67°19'W) collected at depths of 1, 8, 17, 32, 57, 82, 107, 132, 157, and 206 m. (Yentsch, B1969.)

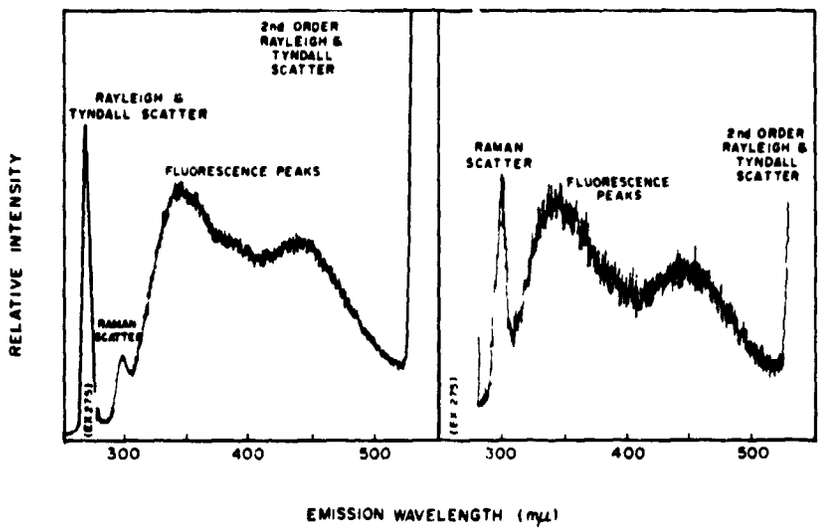


FIGURE 41. Left, fluorescence spectrum of a 7-day culture of concentrated suspended matter, incubated at sea. The sample consisted of surface water from the Continental Shelf near 40°43'N, 69°11'W. Right, fluorescence spectrum of a 6-day culture of Skeletonema costatum at the Woods Hole Oceanographic Institution. (Yentsch, B1969.)

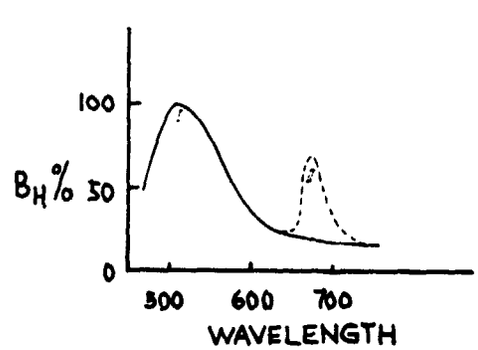


FIGURE 42. Normalized spectral distribution of sea water luminescence (B_H) after excitation by the 436 nm mercury line:

I) luminescence spectrum of the dissolved substances; II) luminescence band of chlorophyll. Further explanation in the text. (Karabashev and Zangalis, A1970.)

They advocate getting a baseline from filtered seawater in order to be able to determine actual chlorophyll signal.

In a more recent paper, the same authors (Karabashev and Zangalis, B1971) have "discovered that the photoluminescence spectrum of seawater depends on the spectral composition of exciting radiation." Their instrument had now been modified to allow excitation by the 313, 365, 436 and 546 lines of mercury. The results of this study are given in Figure 43, where changes in the emission are noted as a function of excitation wavelength.

In yet another paper, titled "New Data on Sea Water Photoluminescence," Karabashev, Zangalis, Solov'yev and Yakubovich (I1971) discuss results of the measurement of what we call Gelbstoff fluorescence when excited with the 365 and 436 nm lines of mercury. These results are given in Figure 44. As if to confirm our statement on the paucity of in situ luminescence data on Gelbstoff (or chlorophyll), the authors state, ". . . neither in this nor in any other work published abroad have we found any information about the spectral distribution of SWP." (SWP is their term for seawater photoluminescence.) A very important practical result of their measurements is that the luminescence spectrum of SWP is independent of salinity, oxygen concentration, and pH in the limits 0-13%, 0-8 ml/l, and 7.0-8.5 respectively.

Ivanoff and Morel (I1971) have also written on the "Spectral Distribution of the Natural Fluorescence of Sea-Waters." Again, they are primarily interested in what we term Gelbstoff emission. The spectral data published in this paper are reproduced as Figure 45. (Professor Jerlov informs me that the labeling on curves 2 and 3 is reversed.) These authors also use mercury lines for excitation. Their results are certainly less complete than those of Karabashev et al. since they do not show the decline in the short wavelength region. Jerlov has also informed me that Ivanoff and Morel have more recent and better results which are not yet published.

During a trip to Europe in the Fall of 1972, it was possible to discuss our work and that of others with Professor Jerlov in Copenhagen. It was

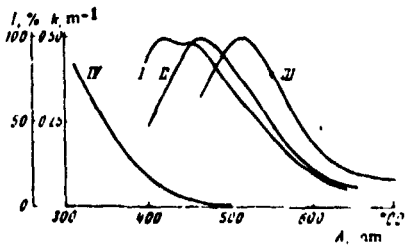


FIGURE 43: Mean normalized spectral distribution of photoluminescence of sea water for excitation by the 313 nm (curve I), 365 nm (curve II), and 436 nm (curve III) lines of mercury. IV) Absorption spectrum of "yellow substance" in water sample from Gulf of Riga. I is the relative spectral spectral intensity, k is the absorption coeffieient of substances dissolved in the sea water. (Karabashev and Zangalis, B1971.)

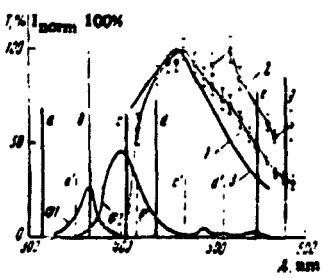


FIGURE 44: Results and measurement conditions for the SWP spectra:

1) average over all stations, excitation using filter with transmission F1; 2) the same using filter with transmission F2; 3) SWP spectrum from (4) a through d are the positions and relative intensities of Hg lines in the spectrum of the exciting source, and a' through d' are the positions of the Raman lines from water for the Hg lines a through d, respectively (from (19); I_{norm} is the normalized spectral intensity of SWP and T is the transmission of the filters in the exciting beam. (Karabashev et al., I1971.)

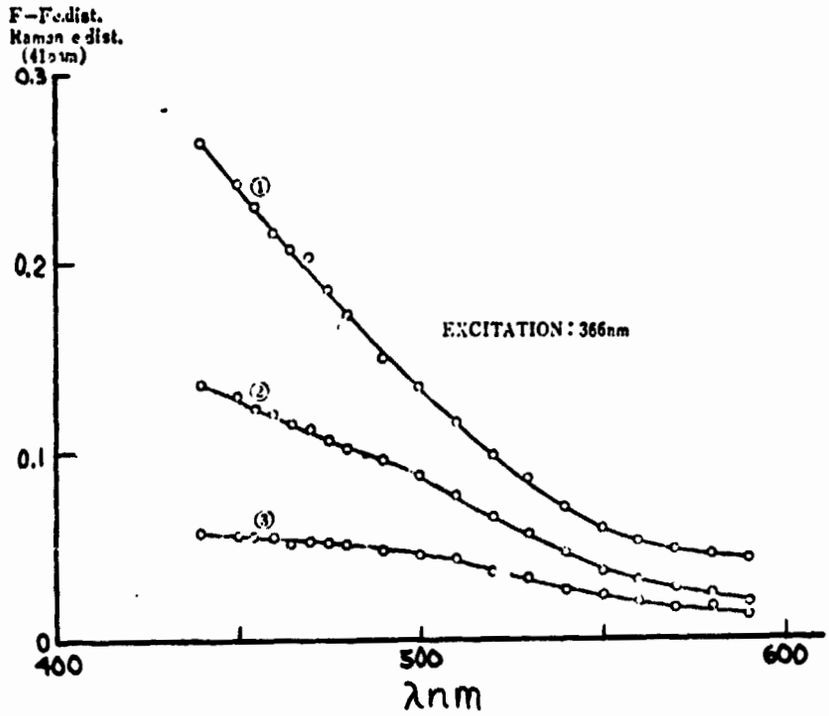


FIGURE 45: Spectral distribution of the fluorescence of seawater (the fluorescence Fe. dist. of pure water has been subtracted from that, F, of the considered seawater, and the difference F-Fe. dist. is compared to the Raman effect of pure water at 418 nm).

Curves n°1, 2, 3 correspond respectively to coastal water, to surface Mediterranean water, and to deep 500 m Mediterranean water. (Ivanoff and Morel, 1971.)

Jerlov's impression that there was no work in progress anywhere which had proceeded so far as ours in collection of specific data. Jerlov also indicated that his group was about to purchase instrumentation which would allow scanning of both excitation and emission in order to further investigate the extraordinary blue fluorescence in the Baltic. This fluorescence is noticed as a peak in the upwelling light. It has a narrower bandwidth than normal Gelbstoff emission. (It is quite possible he was seeing an oil such as we saw in Figure 23.)

We are also aware of current work being performed by SPARCOM, Inc., on "Laser Induced Fluorescence of Algae" with the support of NASA/Wallops Island. This work has used a tunable laser to examine algal cultures. For eventual airborne detection no doubt lasers will be extremely useful; however, it seems cumbersome to do point by point measurements on laboratory cultures which are very easy to scan automatically with more than adequate sensitivity with continuous instruments. This work, when available, should be compared with the data of the second and fourth sections of our Compendium of Spectral Data.

6. DISCUSSION AND RECOMMENDATIONS

6.1 Interpretation of Data

In Section 4.3.3, we formed the tentative conclusion that algae particulates were totally absorbing over much of the near ultraviolet and visible spectrum and acted approximately as quantum counters. This conclusion would have a far-reaching effect on how spectra are to be interpreted and how algae are to be identified.

In most cases, plant pigments absorb energy and transfer a large fraction to chlorophyll. Chlorophyll is also excited by direct absorption. The energy is now used for photosynthesis, but some fraction is emitted as chlorophyll fluorescence. If the same fraction is emitted, regardless of whether excitation was direct or came from energy transferred from other then quantum counting may be observed, resulting in the "typical" excitation spectrum of Section 4. Further, this would be a maximum spectrum: any deviations would be expected to fall below this curve.

If a particulate is not optically dense in some spectral region because of the lack of a pigment, this will cause a relative dip in the "typical" spectrum. If the absorption in a spectral region is caused by the presence of an absorbing pigment which does not transfer energy to chlorophyll or any other pigment--an internal filter effect--then this too will cause a dip in the "typical" excitation spectrum for chlorophyll fluorescence.

As a result of these conclusions, the interpretation of excitation spectra for identification must depend on negative deviations from a "typical" spectrum and the absence of certain pigments, rather than their presence. Also important may be the presence of pigments which cause total absorption in a spectral region, but which do not contribute to chlorophyll emission.

We note that the data of Figure 38A only suggest quantum counter action of algal particulates to about 480 nm, due to a limitation of the comparison dye. The Rhodamine data of Figure 31, which extend to 580 nm,

suggest that in many cases quantum counter action may extend past 550 nm, where there is a characteristic peak. It is quite possible that in diatoms and dinoflagellates which contain no phycobilins quantum counting may cease at longer wavelengths. Thus, it may be useful to examine excitation spectra between 550 nm and the chlorophyll absorption at 670 nm.

Gelbstoff data are not expected to exhibit quantum counter action because of the low concentration and because they do not arise from particulates.

6.2 General Conclusions

Surveying the results of this project, we can make the following general statements:

Luminescence data on natural waters can be useful in roughly quantitating and identifying algal concentrations.

Luminescence data on natural waters can also be useful in determining Gelbstoff concentrations and establishing the existence and identity of pollutants such as oil.

The sensitivity of luminescence methods suggests that chlorophyll can be monitored remotely from an aircraft, particularly at night.

The interpretation of chlorophyll excitation spectra should be based on a quantum counting scheme based on total absorption throughout most of the ultraviolet and visible spectrum by each particulate.

Since absorption throughout the spectrum contributes to chlorophyll emission, maximum sensitivity for chlorophyll detection will be obtained by wideband excitation up to and including the last chlorophyll absorption at 670 nm. This may be more sensitive than narrow-band laser excitation.

6.3 Recommendations for Future Work

The reorientation of the approach to on-site measurements led to certain incompleteness of data, due to shortage of time and funds, and field instrument evolution. The concept of particulate quantum counting redirects the approach to data interpretation. The following items are recommended for further study and measurement:

1. A dedicated field instrument, essentially similar to the final instrument used in this project, but equipped with a continuous on-stream monitoring system, should be built for field measurements.

2. Laboratory measurements on a variety of algal cultures should be carried out to verify the quantum counting concept and establish limitations of applicability.

3. On-site measurements should be conducted at a few carefully selected sites with sufficient time to carry out a considered program of experiments, and where auxiliary and independent measurements of important parameters can be carried out.

4. More detailed multicomponent spectra should be taken at selected sites, both to seek evidence of other non-chlorophyll algal fluorescence, and to develop more detailed Gelbstoff spectra. Measurements of both sorts should be made on natural waters, filtered particulates and filtrates.

5. Calculations should be performed comparing the utility of broad-band arc excitation versus narrow-band laser excitation for remote sensing.

7. ACKNOWLEDGEMENTS

The authors wish to thank the numerous marine scientists who contributed to this program by means of letters and personal conversations. Special thanks go to those responsible for arranging on-site measurements who are acknowledged elsewhere. Dr. Charles Yentsch was a consultant to this program and provided assistance both in laboratory facilities and in interpretation. Mr. Luther Campbell was responsible for the successful modificiation of a laboratory instrument for field use. Finally, we wish to thank Mrs. Geraldine Garnick for aid in taking laboratory data and in organizing the large amount of data accumulated.

Appendix A

General Principles of Fluorescence Analysis

In figure A-1 we relate and define absorption and fluorescence by means of a generalized energy level diagram which would apply to a typical aromatic organic molecule in dilute solution. Light is absorbed by the molecule in its ground state -- usually a singlet -- here designated by S_o. The absorption of energy raises it to one of a number of higher electronic singlet levels, designated S_1, S_2, etc. These levels are further split by small vibrational differences into sublevels, indicated by fine lines in the diagram. Further rotational splitting is usually masked by the broadening caused by molecular collisions in the matrix which may be solid or liquid.

The population of any level depends on the Boltzman factor, $e^{-E/kT}$, where E is the energy of a particular level, k is the Boltzman constant and T is the absolute temperature. The vibrational spacing in organic aromatic molecules is typically 700 cm^{-1}, whereas the thermal energy at room temperature (300° K) is only about 210 cm^{-1}. Thus the ratio of molecules in the first vibrational state to those in the zero vibrational state of S_o will be given by

$$N_{o1}/N_{oo} = e^{-\Delta E/kT} = e^{-700/210} = 0.04 \text{ at room temperature,}$$

Thus about four percent of the molecules will be in the first vibrational state and about ninety-six percent in the ground state. (Higher states have negligible populations.) Absorption then occurs predominantly from the zero vibrational of S_o to various vibrationals of the higher singlets. (Absorption from the poorly populated higher ground vibrational give rise to "hot bands.") This selective absorption is the basis of identification by absorption spectroscopy.

Once excited, a molecule may return to the ground state by radiating light, or by radiationless transitions (aided by molecular collisions) which result in dissipation of energy in the form of heat. In dilute solution (or solid) the populations of the vibrationals of S_1 are also determined by a Boltzman factor. As a result the large majority of transitions from S_1 to S_o occurs from the zero

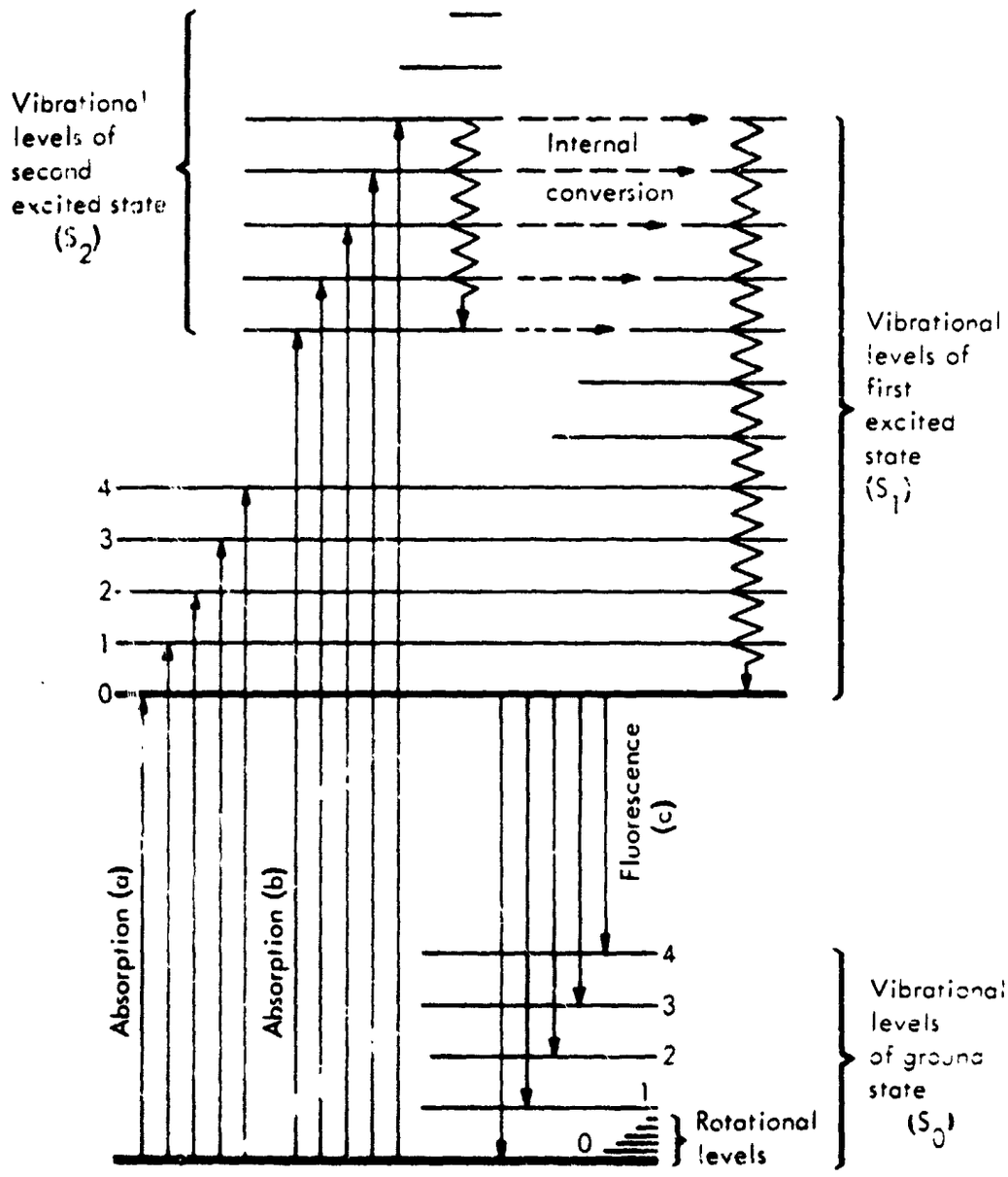


Figure A-1. Transitions Giving Rise to Absorption and Fluorescence Emission Spectra

vibrational state of S_1 to various ground vibrationals. These processes are indicated in figure A-1 where radiationless processes are designated by zigzag lines and absorption and emission processes by solid lines.

Because fluorescence, originating in the zero-vibrational of S_1, may terminate in any of the ground vibrational states, the fluorescence often displays vibrational structure which is a mirror image of absorption.

Comparison of possible absorption and fluorescence transitions reveals that the transition between zero-vibrational levels, the so-called 0-0 transition, is common to absorption and fluorescence, resulting in self-absorption at the common wavelength. All other fluorescence transitions occur at lower energies (and longer wavelengths) than the corresponding absorption. As a result, fluorescence is found to occur at wavelengths just greater than the longest wavelength absorption.

A third possibility exists for a molecule in the S_1 state -- and this is not depicted in figure A-1. It may undergo a radiationless intersystem crossing to an excited triplet level, T_1, lying below S_1. Such a triplet may also radiate, resulting in phosphorescence at even longer wavelengths. Because the radiative lifetime of the triplet is so much longer than the singlets (typically one second compared to 10^{-8} seconds), non-radiative processes usually dominate over emission at room temperature and phosphorescence is not observed. Since we are interested in emission at ambient temperatures, we shall disregard triplets except as further non-radiative paths for deexcitation from S_1.

To continue our contrast of absorption and emission processes, note that in an absorption experiment one measures an incident intensity and a transmitted intensity, both at the same wavelength, which are almost equal in magnitude. The small intensity difference is the desired information about the sample. In a fluorescence experiment the incident (exciting) light is at one wavelength and the emitted light (fluorescence) is at another wavelength. Since the incident light can be made monochromatic, its contribution to background at the fluorescence wavelength can be made very small. As a result, the

fluorescence, which contains the desired information, is measured against an almost zero background. It is this difference which makes fluorescence methods so much more sensitive than absorption for materials which fluoresce with reasonable efficiency. Considering a typical organic having an extinction coefficient of 10^4 cm^2/m-mole and a fluorescence quantum yield of 0.4, Förster[1] calculates a detection limit of 10^{-12} mole liter or 5×10^{-13} g for low spectral resolution. With the newer high intensity light sources available this could be exceeded. The practical limit to sensitivity is scattered light and background emission. Standard laboratory instrumentation, such as the Baird-Atomic "Fluorispec" Fluorescence Spectrophotometer allows measurement of sub-nanogram quantities of material corresponding to concentrations of less than one part per billion.

Fluorescence spectroscopy has another advantage, in addition to great sensitivity. This results from the double specificity of excitation and fluorescence wavelengths. In absorption spectroscopy a mixture will have a unique absorption spectrum which is the simple sum of the absorbances of the components. In fluorescence spectroscopy a single organic compound, in dilute solution in a non-absorbing, non-interacting matrix, will have a unique fluorescence spectrum and a unique excitation spectrum. A mixture of fluorescing compounds, possibly concentrated, in a matrix which may be absorbing (but not fluorescent) will no longer have a unique fluorescence spectrum or a unique excitation spectrum. (Seawater is just such a mixture.) Rather a particular fluorescence spectrum will depend on the wavelength and spectral bandwidth of the excitation and a particular excitation spectrum will depend on the wavelength and bandwidth of the observed fluorescence band. Thus it is necessary to take care in interpreting observed results.

This complicated interrelationship between excitation and emission is caused first because of the overlapping of the simple excitation and fluorescence spectra of the individual components. It is further complicated because of the possibility that one type of excited molecule may transfer its energy to a second type, resulting in absorption by the first and fluorescence by the second. The

matrix (solvent) may itself absorb without fluorescing, thereby acting as an internal filter to reduce excitation of fluorescent species. The matrix or a non-fluorescing component may act to quench normal fluorescence of a species, i.e., deexcite by changing energy into heat without radiation. Finally, high concentrations of the same or differing species may result in quenching of emission. High concentrations of one species may result in formation of dimers which may or may not fluoresce. Dimer fluorescence usually has its fluorescence shifted to longer wavelengths than the monomer.

In addition to the effects of interactions between fluorescing components and the matrix, observed excitation and fluorescence may be affected by the geometry and dimensions of the sample. If the sample is an optically thick layer which absorbs all of the incident radiation over a broad excitation region, the excitation spectrum of the fluorescence may mimic the photon flux versus wavelength distribution of the source and act as a quantum counter. A film may be optically thick for a strong absorber and optically thin for a weak absorber. In an emulsion a large droplet may be optically thick for all excitation wavelengths resulting in only surface fluorescence which does not reflect the true volume.

The great possible complexity of observed excitation and fluorescence from such a complex sample prevents a priori prediction of results. Nevertheless, the very complexity of results implies high information content in the method, allowing a skilled experimenter not only to detect but to identify components.

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Appendix B

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