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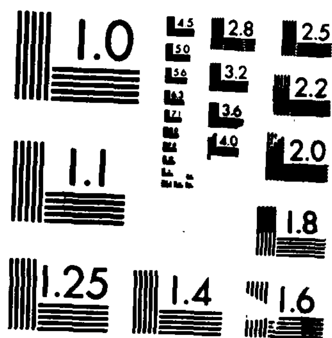

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Two-dimensional Fourier transform based pattern recognition is used to characterize natural populations of marine algae by their two-dimensional, in vivo fluorescence spectra. The two-dimensional fluorescence spectrum is called an excitation-emission matrix (EEM) and is acquired by a portable, multichannel fluorescence spectrophotometer (PMFS). Natural populations in the Gulf of Mexico south of Louisiana and the coastal area near Savannah, Georgia are characterized by their in-situ fluorescence. Characterization of unknown populations is achieved by comparing the unknown EEMs to a collection of standard EEMs acquired from 23 species (6 classes) of marine algae. Pattern recognition results from the data collected along the Georgia coast was confirmed by microscopic examination of selected samples. Keywords: -

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Analysis of Natural Phytoplankton Populations by Pattern Recognition of Two-Dimensional Fluorescence Spectra

by

P.B. Oldham and Isiah Warner

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ABSTRACT

Two-dimensional Fourier-transform-based pattern recognition is used to characterize natural populations of marine algae by their two-dimensional, *in vivo* fluorescence spectra. The two dimensional fluorescence spectrum, termed an excitation-emission matrix (EEM), is acquired using a portable, multichannel fluorescence spectrophotometer (PMFS). Natural populations in the Gulf of Mexico south of Louisiana and the coastal area near Savannah, Georgia are characterized by their measurement of *in situ* fluorescence. Characterization of unknown populations is achieved by comparing the unknown EEMs to a collection of standard EEMs acquired from 23 species (6 classes) of marine algae. Pattern recognition results from data collected along the Georgia coast indicating diatom populations were confirmed by microscopic examination of selected samples.

INTRODUCTION

Since Lorenzen (1) introduced the use of *in situ* fluorescence measurements for the purpose of monitoring natural phytoplankton populations, its popularity (2-6) has grown along with an understanding of the strengths and weaknesses of the technique (7,8). The most glaring deficiency of single wavelength pair monitoring is that the qualitative information content of the data is limited. In contrast, the use of multiple excitation and emission wavelength pairs provides a significant amount of qualitative information about the sample. The utility of the two-dimensional fluorescence spectrum, commonly termed an excitation-emission matrix (EEM), has been well described in the literature (9). Qualitative analysis or fingerprinting of fluorescent samples by EEMs has also been described (10,11).

This paper is a continuation of previous laboratory investigations (12) relating the effectiveness of Fourier transform based pattern recognition (13) of *in vivo* EEMs obtained from pure algal cultures. The previous work described the computer-assisted pattern recognition methods useful for the spectral characterization of laboratory algal cultures with EEMs. These methods were shown to be reliable for the identification of pure cultures. However, the objective in the development of these methods was for the characterization of natural phytoplankton populations from a ship-board laboratory. For this purpose, a portable, multichannel fluorescence spectrophotometer (PMFS) was developed (14).

This paper extends the work of the previous studies by presenting the use of the PMFS (14) for the acquisition of *in situ* EEMs of phytoplankton in sea water. The pattern recognition methods are used for the qualitative characterization of natural phytoplankton populations. Data sets from two separate locations are compared to a standard spectral library for identification. The locations investigated are the Gulf of Mexico, south of Louisiana and the coastal area in the vicinity of Skidaway Island, Georgia. The cruise data were collected November 27 through December 6, 1984, aboard the R.V. Gyre, and February 25-27, 1985, aboard the R.V. Blue Fin, respectively.

MATERIALS AND METHODS

Instrumentation

For each cruise, the PMFS was set up in the ship's dry laboratory. The addition of a hard disk for greater capacity and faster mass storage and a dot matrix printer for the evaluation of data sets on site were the only changes in the instrumentation since the PMFS was presented in the literature (14). A simple, two-dimensional, isometric plotting program was written for the Apple II^{+} so that EEMs could be plotted and dumped to the printer during the periods of time when new data was not being acquired. Due to the large spectral range and data acquisition speed required, low resolution ($\pm$ 5 nm) EEMs were obtained.

Samples

On both cruises, a continuous sample stream of sea water was pumped directly from the ship's sea chest through the flow cell in the PMFS. The sampling probe depth was estimated to be three or four meters below the water surface. Since the volumetric flow rate from the sea chest was too great for the flow cell, the sample stream was split prior to sampling for the PMFS. This allowed the sample stream from the sea chest to flow continuously while the sample inlet to the flow cell could be controlled independently. A small peristaltic pump was used to regulate the sampling rate through the flow cell. During the acquisition of EEMs, sample flow was stopped so that the spectrum of a single sample could be measured. Allowing the rest of the sample stream to continuously flow to waste minimized any sample hysteresis due to the dead volume in the sample hose. The flow rate from the sea chest was about five liters per minute. Since there was an estimated 10-15 min. sample turnover time associated with the sea chest itself and since the acquisition of a single EEM required at least 20 min., each spectrum was representative of a fresh sample.

Each emission spectrum was acquired as a function of a single excitation wavelength by accumulating 30 photodiode array scans at an integration time of one second per scan. This process was repeated for 32 different excitation wavelengths. The series of 32 emission spectra were then compiled into a single EEM. Each EEM was eventually reduced to a 32 x 32 matrix by averaging along

the emission dimension. Background signal was removed from the EEMs by subtracting spectra acquired of suitably filtered sea water. Glass fiber filters (GF/F) were used to remove the phytoplankton present in several discrete samples with the filtrate being used as spectral blanks. Although submicron phytoplankton can pass through the filter, no measurable fluorescence attributable to phytoplankton was found in any of the blank spectra.

Samples were also collected along the Georgia Coast for later microscopic characterization. These were kept under refrigeration in glass bottles and examined within 48 hours of collection. A list of the standard unialgal cultures used for spectral matching is given in Table 1. These were obtained from Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine. The media used for the standard cultures was Guillard's f/2.

RESULTS

Gulf of Mexico

Although we were more interested in the water between the outer edge of the continental shelf and the coast, *in situ* EEMs were also collected at several stations outside the continental shelf for comparison. Figure 1 is an example of the quality of the data acquired in deep water where the phytoplankton density is very low. This particular EEM was acquired while stationed at the Orca Basin (26° 54' lat., 91° 23' long.) where the Gulf floor is about 2000 meters deep. The fact that fluorescence is a

TABLE 1.

List of Algal Species in Standard Library.

Class	Species
Chlorophyceae	Chlorella capsulata
	Dunaliela tertiolecta
	Chlamydomonas sp.
	Chlorosarcinopsis halophilia
Bacillariophyceae	Thalassiosira weissflogii
	Phaeodactylum tricornutum
	Chaetoceros gracilis
	Thalassiosira pseudonann
	Skeletonema costatum
Dinophyceae	Gymnodinium simplex
	Scrippsiella trochoidea
	Prorocentrum minimum
	Prorocentrum micans
	Heterocapsa triquetra
Cyanophyceae	Synechococcus sp.
	Synechococcus bacillaris
	Oscillatoria woronichini
	Phormidinium perscienium
Prymnesiophyceae	Emilianin huxleyi
	Pavlova lutheri
	Isochrysis galbana
	Hymenomonas carterae
Chloromonadophyceae	Chattonella luteus

background limited technique is very apparent from the examination of data sets such as this. Even if the large Rayleigh scattering component is carefully removed from the matrix, the Raman band for water remains a major source of interference with the fluorescence. Raman scattering is weak in comparison to Rayleigh scattering, but it is located on the longer wavelength side of the Rayleigh line. This means that in cases where the Stokes shift in fluorescence is not very large, the Raman band may overlap with the fluorescence signal. Unfortunately, such is the case in the detection of pigments in sea water. This is especially troublesome when the fluorescence signal is low. For this reason, it is crucial to correct as accurately as possible for background signal from the sea water. In fact, this is what ultimately defines the real detection limit of fluorescence measurements in marine water.

In contrast to Figure 1, the EEM in Figure 2 was acquired approximately 60 miles southwest of the Mississippi River Delta 28° 00' lat., 90° 00' long.) in about 600 meters of water. The quality of this data set is very similar to laboratory data acquired in the examination of pure cultures (12). This verifies the ability of the PMFS to obtain complete two-dimensional matrix formatted fluorescence data at the detection limits required for marine analysis in coastal areas.

To investigate the change in the composition of the phytoplankton concentration, as well as any change in the composition of the phytoplankton community, a cruise track was

selected to transect the continental shelf. The first in this series of stations was within just a few miles of the Mississippi River Delta. A series of five EEMs from various points along this route are shown in Figure 3. Although the spectral composition changes very little, there is a very obvious decrease in fluorescence intensity as the ship moved into deeper water.

Since the primary objective of these studies was the qualitative characterization of natural phytoplankton populations, the pattern recognition methods were tested on some representative EEMs, upon return to the laboratory. To illustrate the pattern recognition data analysis, the results of the spectral matching performed on the EEM in Figure 2 are listed in Table 2. Correlation functions were generated in the Fourier domain for the unknown with each standard EEM. The three mathematical parameters (12) used to indicate spectral similarity are the net negativity of real coefficients (R), the absolute sum of the imaginary coefficients (I), and the intervector distance (D) between data sets. In all three parameters, a perfect spectral match is indicated by a result equal to zero. Table 2 lists the 8 standard spectra that most closely matched the unknown spectrum. This is a prioritized list with the best match listed at the top. The standard spectra are listed by class but each also represents a particular species within that class. According to the pattern recognition results, there is little doubt as to the spectral similarity between the data sets from the Gulf of Mexico and pure

cultures of dinoflagellates. However, due to the spectral similarity between the dinoflagellates, diatoms, and golden-browns examined, there is about a 20% chance (12) that an incorrect match could be made or that a spectral mixture is present. A distinction must be made here between a spectral mixture and a physical mixture of phytoplankton. This is because it is possible to have a mixture of phytoplankton in which one type of algae dominates spectrally.

TABLE 2.

Pattern Recognition Results of Representative EEMs.

Hit #	Member I.D.	I	D	R
1	Dinoflagellate	.00683659	.12892547	-.00058790
2	Diatom	.00858582	.13121376	-.00080429
3	Dinoflagellate	.00786568	.13222932	-.00084369
4	Dinoflagellate	.00793393	.13307212	-.00082431
5	Dinoflagellate	.00893677	.13475507	-.00093936
6	Dinoflagellate	.00910403	.13491026	-.00062033
7	Golden-Brown	.00943061	.1357802	-.00091034
8	Golden-Brown	.00978840	.13890827	-.00098561

Georgia Coast

The major objective for this cruise was to determine the qualitative capabilities of using *in situ* EEMs for the gross characterization by class of natural phytoplankton populations.

For this purpose, a total of 35 EEMs were acquired from four sampling stations and various points of interest along the cruise track. The stations selected were Priest's Landing in the Wilmington River, Wausau Sound, the sea buoy, and a point five miles outside the sea buoy. Representative EEMs taken at the four stations and from the Skidaway River at the ship's dock are illustrated in Figure 4. These data sets are representative in that very little difference existed between the EEMs from the various stations. The major difference indicated by Figure 4 is a general decrease in phytoplankton density (relative fluorescence intensity) farther from the sound.

As far as spectral changes, the only variation shows up in the data set acquired at the dock. In this EEM, the peak ratio between the chlorophyll peak (largest peak, located at approximately λ_{ex} = 450 nm and λ_{em} = 680 nm) and the carotenoid peak (located at approximately λ_{ex} = 530 nm and λ_{em} = 680 nm) is larger than that of the other data sets. This could possibly be caused by the presence of a different algal species in this sample, or it could be caused by a mixture of two or more different species. A mixture of a carotenoid containing diatom with a green algae could very easily account for such a spectral pattern.

The results of the pattern recognition performed on these 35 data sets are illustrated by bar charts in Figure 5. This is a histogram representation of the number of matches or hits by type of standard. Figure 5 illustrates the cumulative results of the

best 3 matches. Twenty-two of the data sets indicated by hit #1 most closely matched a particular diatom (Chaetoceros gracilis) standard. The second closest match or hit indicated was Pavlova lutheri, which is a golden-brown. A dinoflagellate, Prorocentrum minimum, placed a distant third in spectral similarity. As indicated previously, most diatoms and golden-browns are spectrally very similar so that it is not surprising that these two are closely ranked as the most probable match in this case. However, these results appear to leave little doubt that the majority of the samples most closely resemble diatom populations. The fact that an overwhelming number of these 35 EEMs match the same standard also verifies the preliminary visual interpretation of the data sets, i.e., the samples were all spectrally similar. Thus, it must be concluded that the entire coastal area investigated during this cruise contained a very homogeneous population of diatoms.

Certainly it is easy to make such a claim when the results are not subject to verification by other characterization techniques. For this reason, samples were collected for microscopic characterization. Since the in situ method of characterization is a bulk method in which the fluorescence fingerprint is characteristic of all fluorescing components in the sample, it is not sufficient to simply identify certain individual species in the sample. Therefore, the samples were characterized by identifying the species present and determining their relative abundance.

A water sample was collected at each of the four stations for microscopic examination so that each of the primary sampling sites were represented. The results of this examination indicated a predominance of the diatom *Skeletonema costatum* in all of the samples. The samples also contained lesser and varying amounts of small flagellates, many of which were not pigmented. Thus, the *in situ* EEMs proved to be an accurate technique for the bulk characterization of natural phytoplankton populations. However, it is reasonable to ask why the standard spectrum of *Skeletonema costatum* that is in the spectral library was not correctly matched with the unknown samples. The most likely reason why they did not match is in the inclusion of a poor standard spectrum in the spectral library. It was difficult to obtain characteristic spectra of the laboratory culture of *S. costatum* due to its particular growth curve. The spectral features of *S. costatum* were also those most dramatically affected by cell physiology. Thus, at the present time the spectral library does not contain a good characteristic *in vivo* EEM of *S. costatum*.

A second possible explanation of this slight mismatch (correct match by class but not species) involves the flagellates contained in the samples. Even though these flagellates were small in number relative to the diatoms, and many were without pigments, they may have provided a sufficient spectral contribution to skew the pattern recognition results.

The return trip up the river between Priest's Landing and the ship's dock provided an opportunity for establishing the versatility of the PMFS by acquiring data in a time-emission matrix (TEM). This data set is shown both as an isometric projection and a contour plot in Figure 6. The one-dimensional emission spectra that make up this matrix were obtained at one minute intervals for a total time frame of 32 minutes. Samples were excited at 450 nm. The most interesting feature of this data set is the dramatic increase in fluorescence signal at approximately the midpoint of the time series and the subsequent decrease in signal shortly thereafter. At the intersection of the Skidaway and Wilmington Rivers, the fluorescence signal reached a maximum. Since the emission wavelength features of the one-dimensional spectra remain similar, it is impossible to determine the underlying cause of such a dramatic increase in signal with the TEM alone. However, the versatility of the PMFS allowed a phenomenon to be detected that could have easily gone unnoticed with a filter fluorometer had the proper filters not been selected. It also indicates the potential for monitoring dynamic changes in phytoplankton populations as a function of emission profile.

DISCUSSION

The data presented indicate the feasibility of qualitatively characterizing natural phytoplankton populations by their *in vivo* fluorescence fingerprints. The pattern recognition methods developed previously are used to aid in the objective matching of

unknown and standard EEMs. Although the overall reliability of these techniques has proven acceptable, even better accuracy and selectivity could be achieved with a more complete collection of highly characteristic spectral standards. Further studies of the effects of mixtures on algal recognition could also provide similar improvements in accuracy. It is not yet possible to fully determine the reproducibility of results from different cruises. However, the pattern recognition results previously reported (11) for laboratory cultures indicates that the analytical method described is highly reproducible.

Aside from the qualitative characterization experiments with *in situ* EEMs, the potential and versatility of the PMFS were also demonstrated. The ability to selectively acquire emission wavelength information rapidly as a function of excitation wavelength, sample probe depth, surface position, or any other time-dependent parameter renders the PMFS a potentially powerful tool for the marine scientist.

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FIGURE CAPTIONS

FIG 1 *In situ* EEM acquired at the Orca Basin in the Gulf of Mexico. a) background subtracted EEM; b) renormalized residual EEM following removal of Rayleigh scattering.

FIG 2 EEM from the Gulf of Mexico.

FIG 3 Representative EEMs acquired in the Gulf of Mexico.

a) 29° 00' lat, 89° 28' long

b) 28° 50' lat, 89° 38' long

c) 28° 42' lat, 89° 40' long

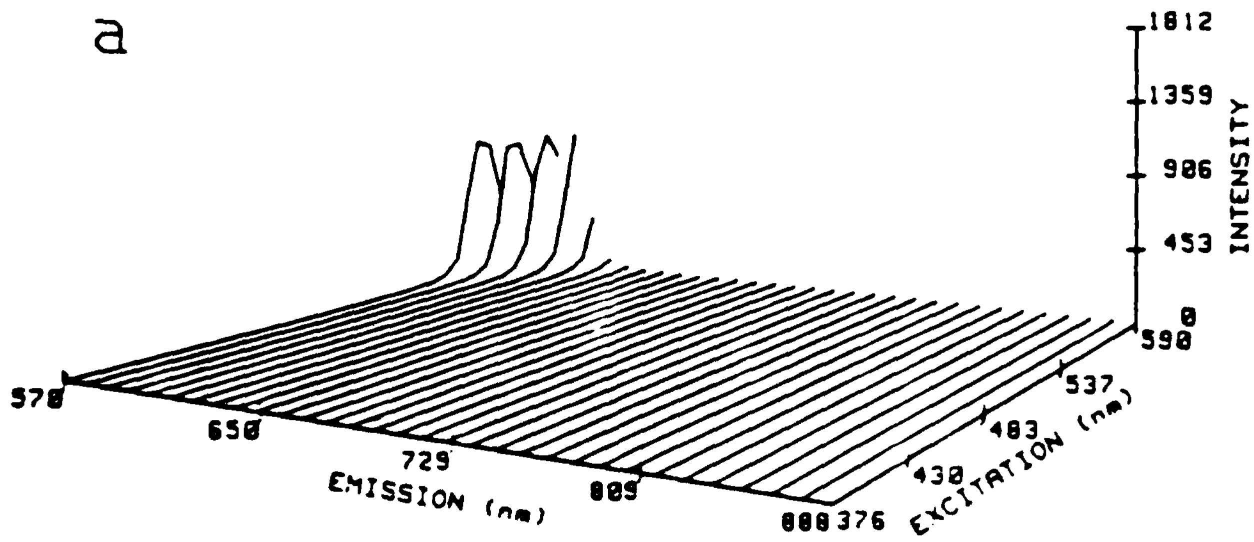
d) 28° 38' lat, 89° 49' long

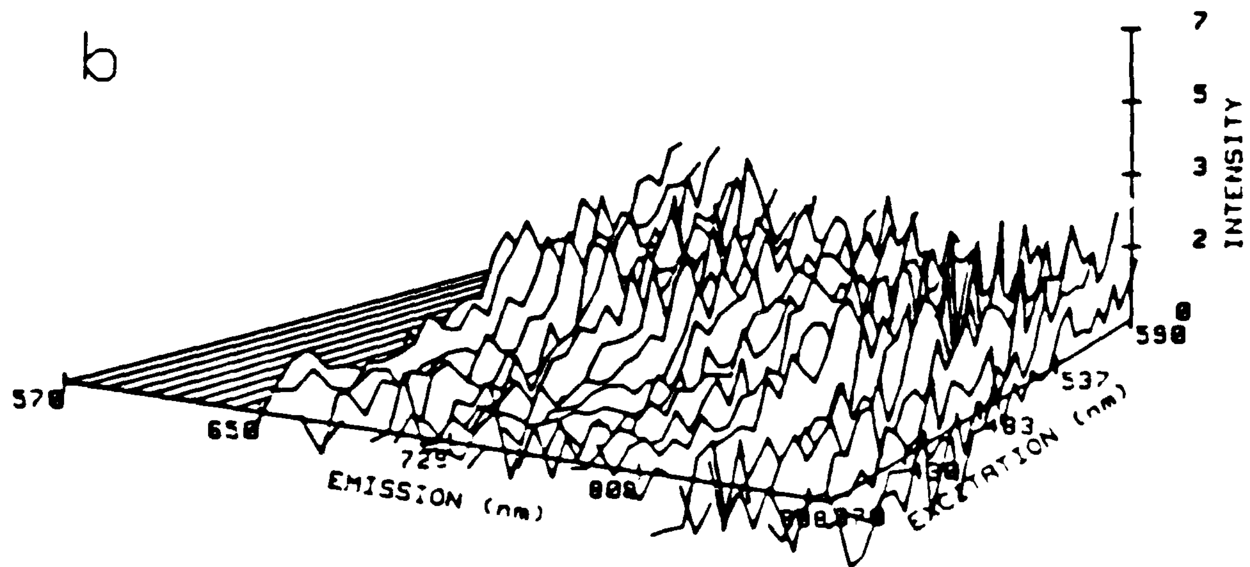
e) 28° 32' lat, 89° 48' long

FIG 4 Representative EEMs acquired along the Georgia coast. a) Skidaway River next to the dock; b) Priest's Landing in the Wilmington River; c) Wausau Sound; d) sea buoy; e) 5 miles southeast of the sea buoy.

FIG 5 Results of pattern recognition study.

FIG 6 Time-emission matrix (TEM) acquired along the Wilmington and Skidaway Rivers.

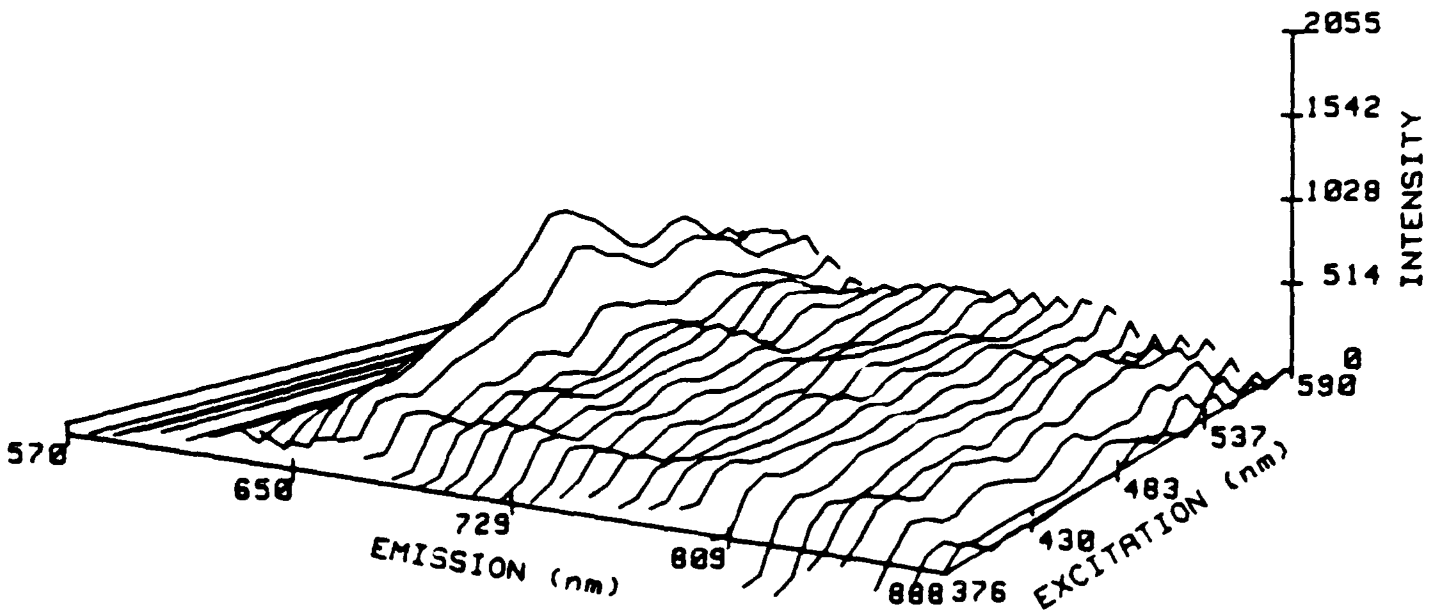


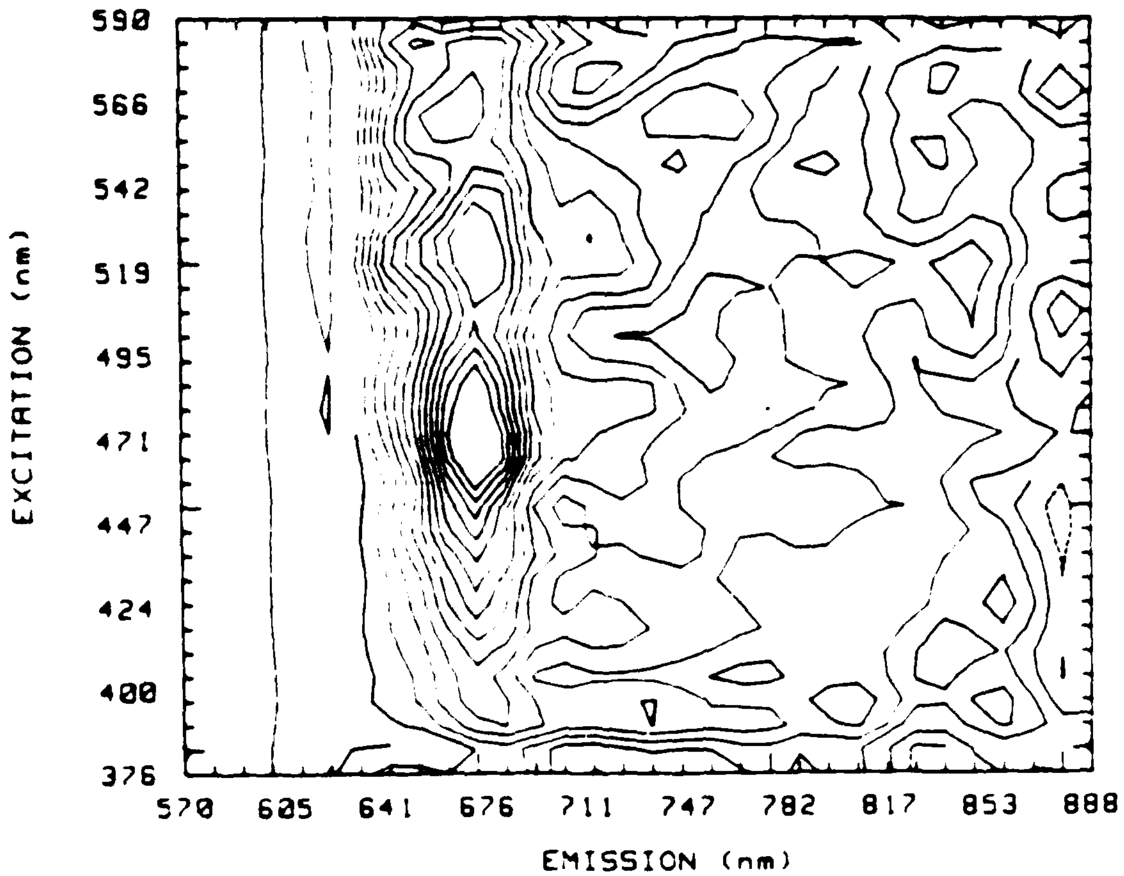


In situ EEM acquired at the Orca Basin in the Gulf of Mexico.

(a) background subtracted EEM; (b) renormalized residual [illegible] with removal of Rayleigh scattering.

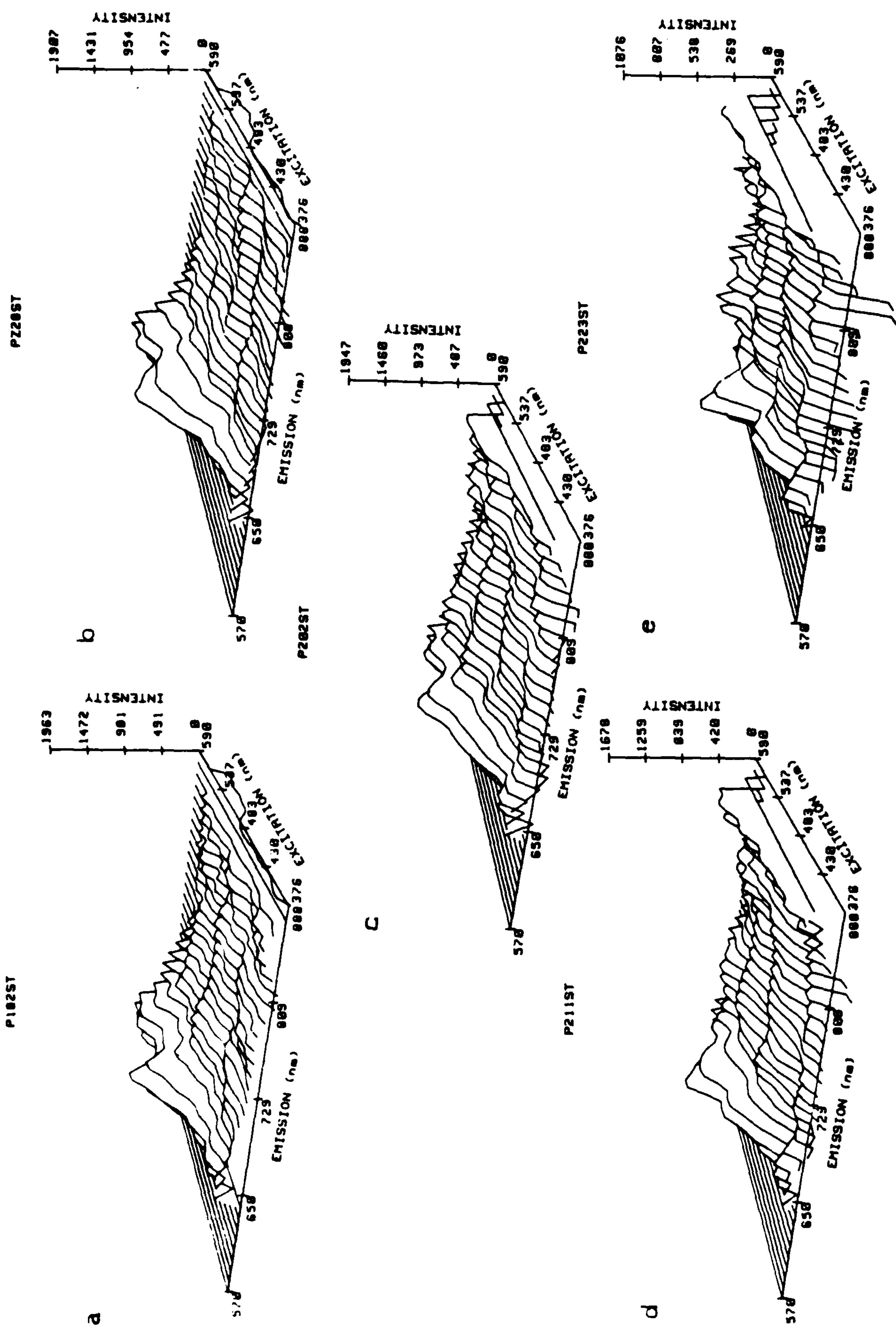
Figure 1





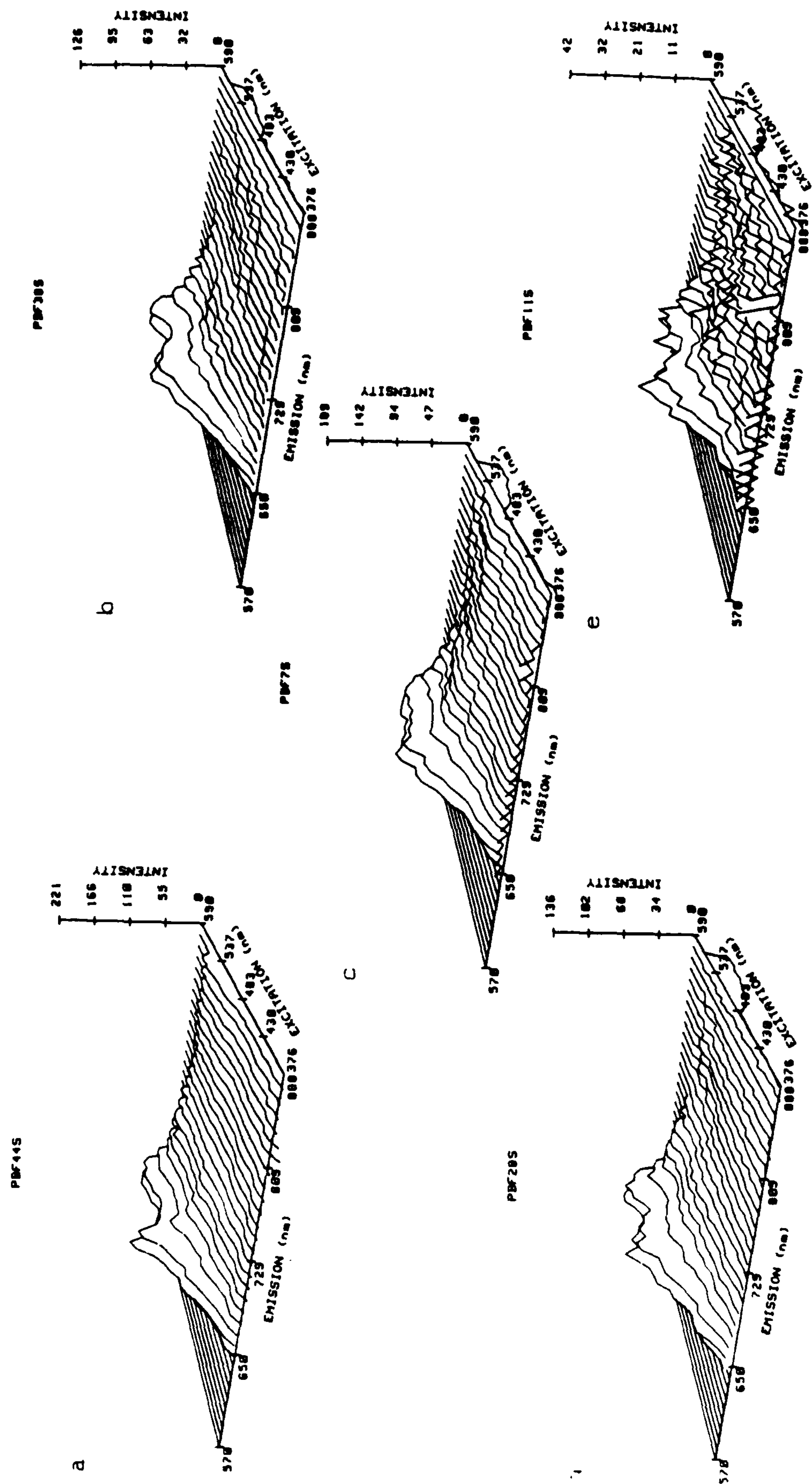
EEM from the Gulf of Mexico.

Figure 2



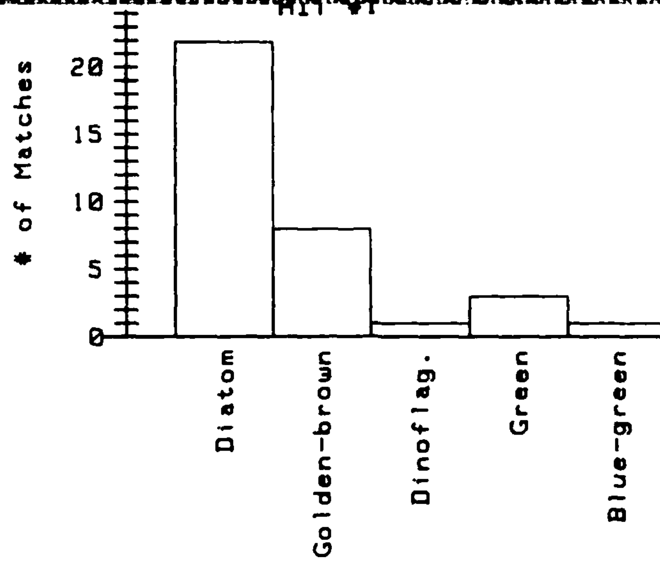
Representative EEMs acquired in the Gulf of Mexico.

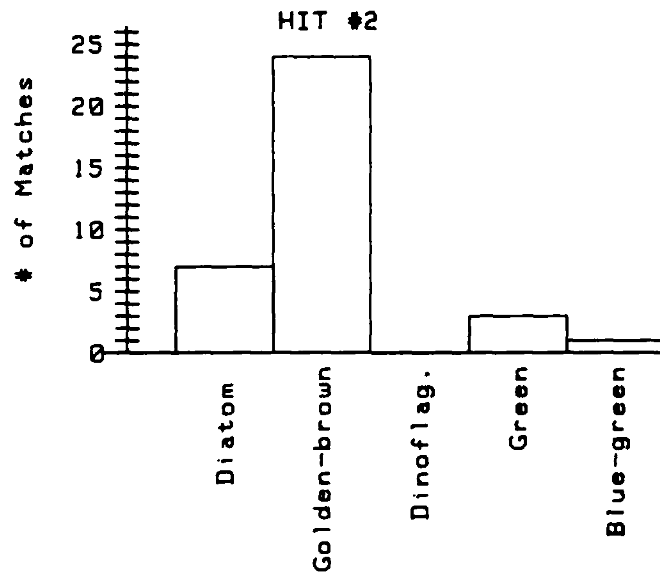
Figure 3

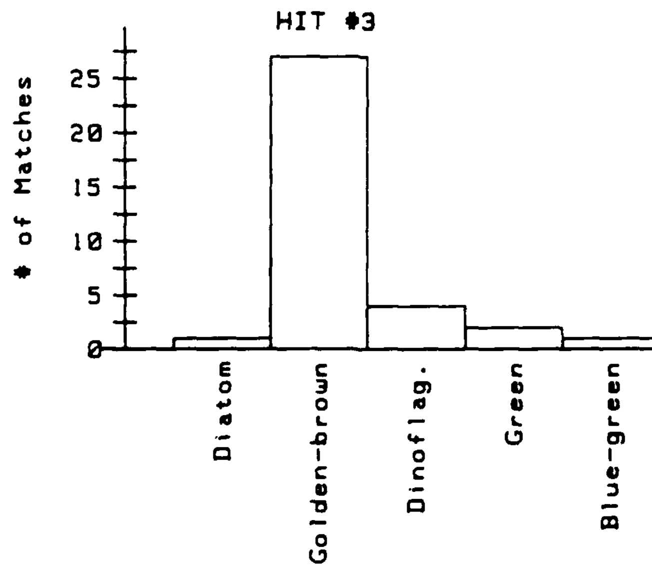


Representative EEMs acquired along the Georgia coast.

Figure 4







Results of pattern recognition study.

Figure 5

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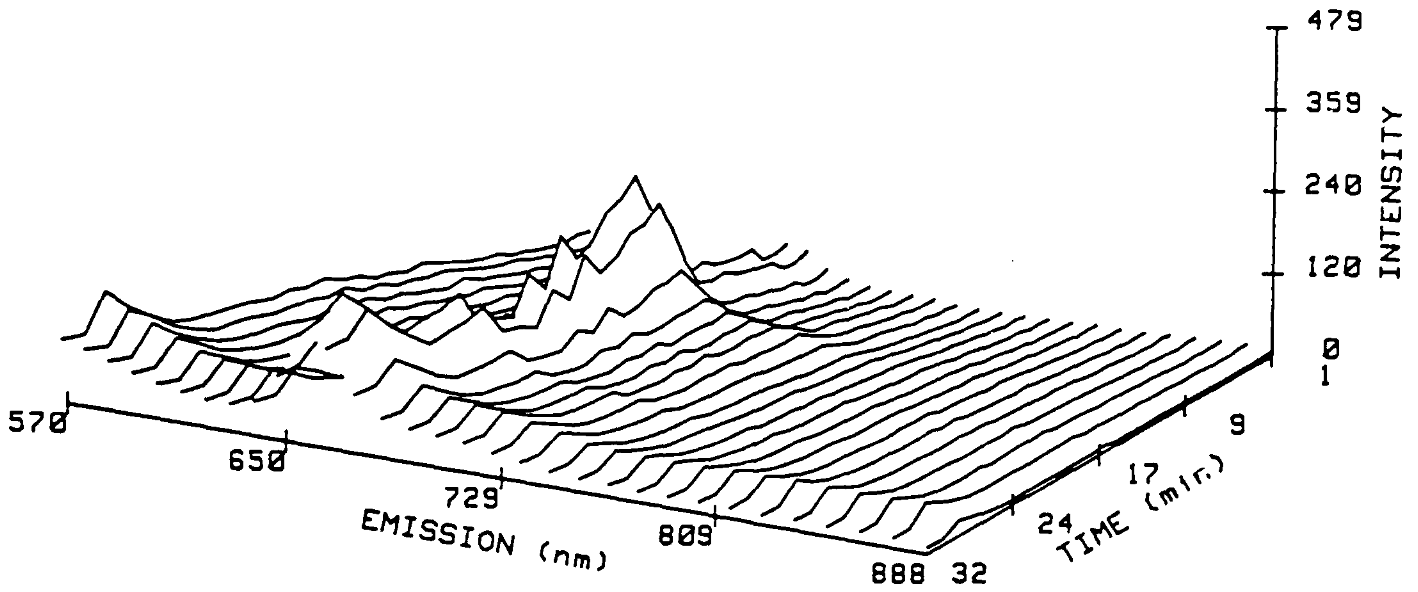

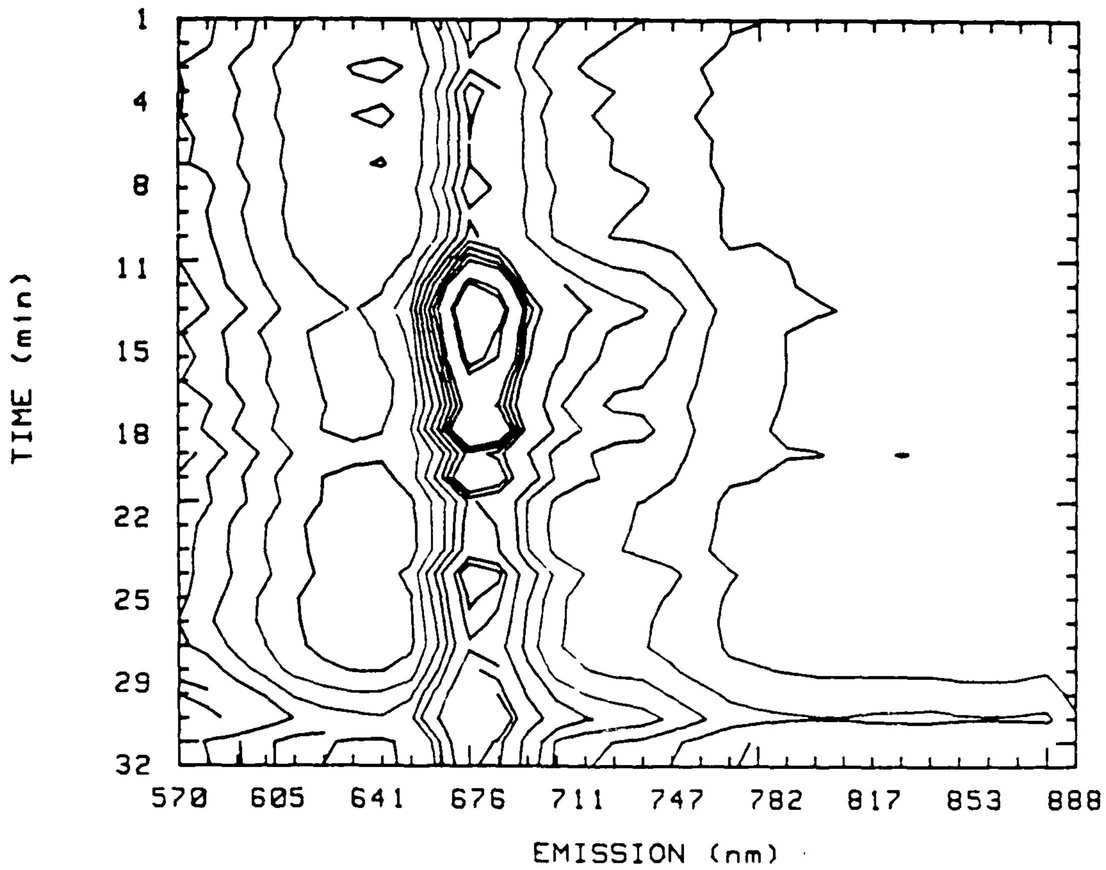

Time-emission matrix (TEM) acquired along the Wilmington and Skidaway Rivers.

Figure 6

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