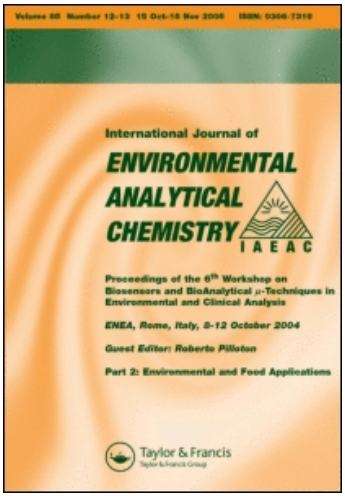

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PRELIMINARY INVESTIGATION OF THE EFFECT OF SELECT POLLUTANTS ON MARINE PHYTOPLANKTON USING MULTIDIMENSIONAL FLUORESCENCE MEASUREMENTS

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The use of multidimensional fluorescence for detecting the effects of select pollutants on algal fluorescence and production is demonstrated. Multidimensional fluorescence is ideally suited to rapidly measure algal fluorescence generated by both *chlorophyll a* and accessory pigments, as well as any changes induced by pollutants. Laboratory cultured and natural algae samples from classes *Chlorophyceae*, *Bacillariophyceae*, and *Cyanophyceae* were exposed to substituted nitroaromatics and fluorescence spectra of the algae recorded. Notable spectroscopic changes and fluorescence quenching were observed. In addition, a novel method for rapidly preconcentrating dilute natural marine samples is described.

KEY WORDS: Multidimensional fluorescence, phytoplankton, pollutants, preconcentration.

INTRODUCTION

Marine phytoplankton are essential components of the aquatic environment and have been the focus of many research efforts.[1–5] With the growing concern for the environment, the adverse effects of many chemicals on algal growth and production have received a great deal of attention.[6–13] Chemicals that have invaded the marine environment such as substituted nitroaromatics,[6] herbicides,[9,12,13] heavy metals,[7,8] and polynuclear aromatics[10,11] have all been shown to have detrimental effects on phytoplankton production.

Various methods to detect algal growth and production are available including cell counting,[10] monitoring the rate of biological nitrogen fixation or oxygen production[11,14] and monitoring algal fluorescence.[12,13,15–17] By measuring *in vivo* pigment fluorescence, more specifically fluorescence quenching, chemically induced photosynthesis inhibition can be detected.

Pigment composition of algae has been shown to vary with classification.[1–3] Pigments present most frequently are classified as either a type of chlorophyll, carotenoid, or phycobilin. *Chlorophyll a* serves as a universal link between the algal classification by being the only pigment present in all species and is usually the fluorescent species monitored. In species containing multiple pigments, each

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pigment interacts to facilitate photosynthesis. The accessory pigments (carotenoids and phycobilins) channel energy to *chlorophyll a* for photosynthesis through a biological energy transfer mechanism. This allows photosynthesis to continue over a broad range of natural light wavelengths and to more efficiently utilize available energy.

Chemicals such as substituted nitroaromatics, herbicides, heavy metals, and polynuclear aromatics (PNAs) have been shown to disrupt the photosynthetic pathway and possibly the energy transfer mechanism.[7,8,10–12] *Chlorophyll a* fluorescence quenching is frequently used as evidence for such phenomena. In most studies only *chlorophyll a* fluorescence is monitored as a function of both time and pollutant concentration. However, with the presence of multiple pigments and energy transfer occurring between pigments, the effects on those systems by pollutants should be reflected spectroscopically as fluorescence quenching of specific pigments. Unfortunately, a great deal of this information is lost by monitoring only one emission wavelength with conventional fluorometers. Spectroscopic methods, capable of monitoring a range of excitation and emission wavelengths, could acquire this additional data in relatively short periods of time. A portable multidimensional fluorometer capable of such measurements had been developed.[18] This instrument can rapidly acquire data in the form of Excitation–Emission Matrices (EEM), which are used in classifying algae according to their characteristic pigment composition.[5]

A major problem encountered with measurement of *in vivo* algae fluorescence are small or undetectable signals resulting from low cell concentrations. Low cell concentration is a condition frequently encountered when samples from extreme depths or areas of low productivity are analyzed. In order to make a reasonable fluorescent measurement, a sufficient concentration of algal cells is necessary. In some cases, preconcentration of cells is achieved simply by centrifuging, collecting, and resuspending the sample of algal cells in a smaller volume. Unfortunately, this is a cumbersome and tedious process when cell concentrations are low enough such that large sample volumes are required to collect a sufficient number of cells. We have developed a method of specifically designed to rapidly concentrate algal cells from large sample volumes. This method uses asymmetric, porous, cylindrical, ceramic filters in which the sample is recycled internally while a mild external vacuum removes the filtrate and reduces the total volume.

This paper describes preliminary findings using multidimensional fluorescence measurements to monitor the fluorescence of algae in the presence of a series of substituted nitroaromatics. Cultured algae from the classes *Chlorophyceae* (green) and *Cyanophyceae* (blue-green) along with natural marine samples were monitored after exposure to substituted nitroaromatics. Fluorescence was monitored as a function of both exposure time and concentration of the nitroaromatic. In addition, a description of the preconcentration apparatus and method for preconcentrating natural marine samples is presented.

EXPERIMENTAL

Unialgal cultures of *Chlorella vulgaris* (*C. vulgaris*) of class *Chlorophyceae* (green)

Table 1 Pigment composition of algae

Class	*Common name*	*Pigment*				
		Chlorophyll			*Carotenoids*	*Phycobilins*
		a	*b*	*c*		
Chlorophyceae	Green	+	+			
Cyanophyceae	Blue-green	+				+
Bacillariophyceae	Diatom	+		+	+	

and *Phormidinium perscienium* (*P. perscienium*) of class *Cyanophyceae* (blue-green) were selected for this study to represent two different pigment compositions. Natural samples collected ten miles off-shore from Savannah, Georgia containing primarily algae of class *Bacillariophyceae* (diatoms) were used to represent a third algal pigment composition. General pigment composition of these algae is listed in Table 1. Both unialgal cultures were grown with a 12 hr/12 hr, light/dark cycle in Provasoli's ASP 6 artificial sea water maintained at 20–25 °C. Light at 500 lux was supplied by cool white fluorescent lamps. Cells in the log phase of growth were used at a concentration of 10^6 cells/mL. Natural diatom samples were preconcentrated to 10^6 cells/mL by a method to be described later.

Exposure of the algae to substituted nitroaromatics was achieved by centrifuging the algae and removing the supernatant, and then resuspending the cells in Provasoli's ASP 6 sea water containing the appropriate substituted nitroaromatic. Each algae sample was exposed to varying concentrations of the nitroaromatics. Fluorescence spectra were then recorded at intervals of one, four, and seven hours and again after five days.

Data were collected with a portable multidimensional fluorometer previously described.[5,18] Briefly, the instrument simultaneously collects an entire fluorescence emission spectra at each excitation wavelength by using a flat field spectrograph coupled to a 512-element intensified diode array. In this study, 32 individual emission spectra were obtained at excitation wavelengths ranging from 325 to 592 nm. The 32 emission spectra were plotted sequentially to form an isometric projection of fluorescence over a range of excitation and emission wavelengths which is commonly referred to as an excitation–emission matrix (EEM).[19]

Preconcentration

The natural samples collected offshore from Savannah, Georgia were too dilute for direct analysis by multidimensional fluorescence. Therefore, preconcentration of algae to achieve sufficient concentrations was required for reliable fluorescence measurements. A diagram of the apparatus used to concentrate algal cells is presented in Figure 1. The dilute sample is retained in the reservoir and pumped through the cylindrical ceramic filters. Excess volume is reduced through 0.1 micron pores by applying a mild vacuum outside the filter through the stainless steel case. The filtrate is collected with an in-line trap. Algal cells are gently retained within the filter using a vacuum and collected by backflushing the filter

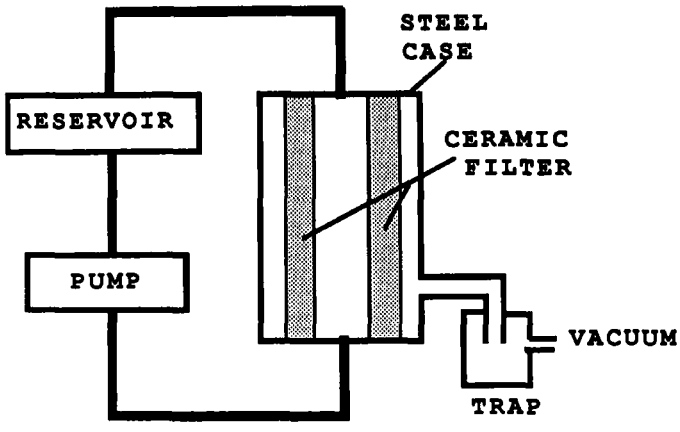

Figure 1 Diagram of preconcentration apparatus

after the vacuum is removed. The method and apparatus was designed for large dilute volumes (>250 mL). In this study, a one liter volume of sample, reduced to 30 mL, provided sufficient concentration of cells to yield a measurable fluorescence signal.

HPLC Uptake Data

The rate of uptake for 1,3-dinitrobenzene was monitored indirectly using high performance liquid chromatographic measurements. After exposure of the pollutant to the three algal cultures, the cells were centrifuged and the supernatant analyzed for pollutant or metabolites. A Perkin-Elmer model Series 4 liquid chromatograph with a C-18 reverse phase column and UV-Vis detector was employed for analysis. The mobile phase was a 1:1 mixture of methanol and water at a flow rate of 2 mL/min.

RESULTS

Representative EEM spectra of *C. vulgaris* and *P. perscienium* are presented in Figures 2a and 2b. In Figure 2a, the EEM of *C. vulgaris* reveals only *in vivo chlorophyll a* fluorescence with sole emission at approximately 667 nm. This is typical of *in vivo chlorophyll a* fluorescence. The spectral detail occurs in the excitation region which reveals absorption in two wavelength regions, at approximately 471 and 592 nm. The representative EEM of *P. perscienium* in Figure 2b indicates that the pigment composition is different than *C. vulgaris*. Fluorescence in the 667 nm region, characteristic of *chlorophyll a*, is present at the excitation wavelength of 566 nm and not at the typical excitation wavelengths found in Figure 2a. Also appearing in the spectrum is fluorescence at wavelengths lower than 667 nm. French and Young have attributed this fluorescence to the pigment

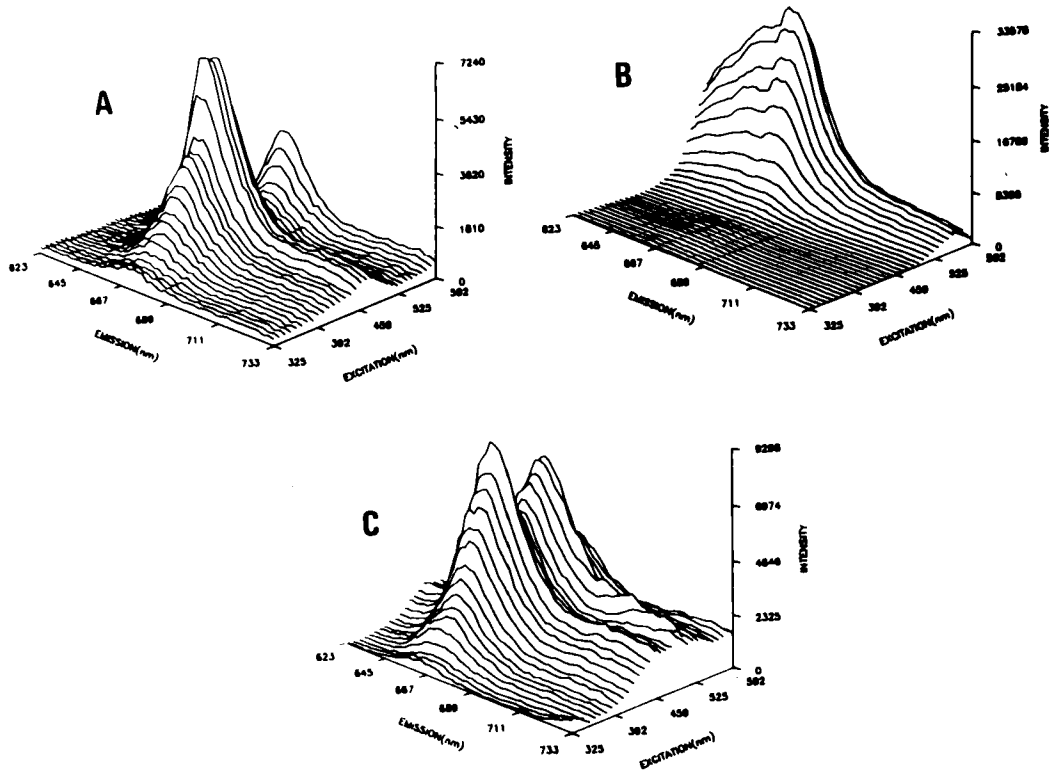

Figure 2 Representative EEMs of (a) *Chlorella vulgaris*, (b) *Phormadinium perscienium*, and (c) natural sample containing predominantly algae of class *Bacillariophyceae* (diatoms).

phycocyanin and suggest that the *chlorophyll a* fluorescence is a result of energy transfer from phycocyanin.[1]

A representative EEM of the natural sample after preconcentration is presented in Figure 2c. The EEM, as well as physical examination of the algal cells, indicates the predominant algae present belong to class *Bacillariophyceae* (diatoms). As expected, the characteristic fluorescence of *chlorophyll a* in the 667 region is present and excitation occurs in the expected wavelength regions for *chlorophyll a*. *Chlorophyll a* fluorescence is also observed in the excitation wavelength region of 523 nm. This can be explained by the absorption of light at this wavelength by non-fluorescent carotenes (characteristic of diatoms) and subsequent energy transfer to *chlorophyll a*.

Exposure of *C. vulgaris* to the three substituted nitroaromatics and subsequent measurements of fluorescence intensity suggests that the change in fluorescence intensity is dependent on the particular substituent on the aromatic ring. Papageorgiou and Arondelis have suggested that nitroaromatics with less ionizable substituents are more effective quenchers of *in vivo chlorophyll a* than more ionizable groups.[6] Thus, it appears that the relative toxicity of the substituted nitroaromatics is dependent upon the particular substituent on the aromatic ring.

Table 2

Condition	*Fluorescence intensity*	
	C. vulgaris	*P. perscienium*
Control (no nitroaromatics)	3741	33578
10^{-3} M DNB, 4 hr exposure	651	9707
10^{-3} M NBA, 4 hr exposure	2728	25696
10^{-3} M NP, 4 hr exposure	3544	25978

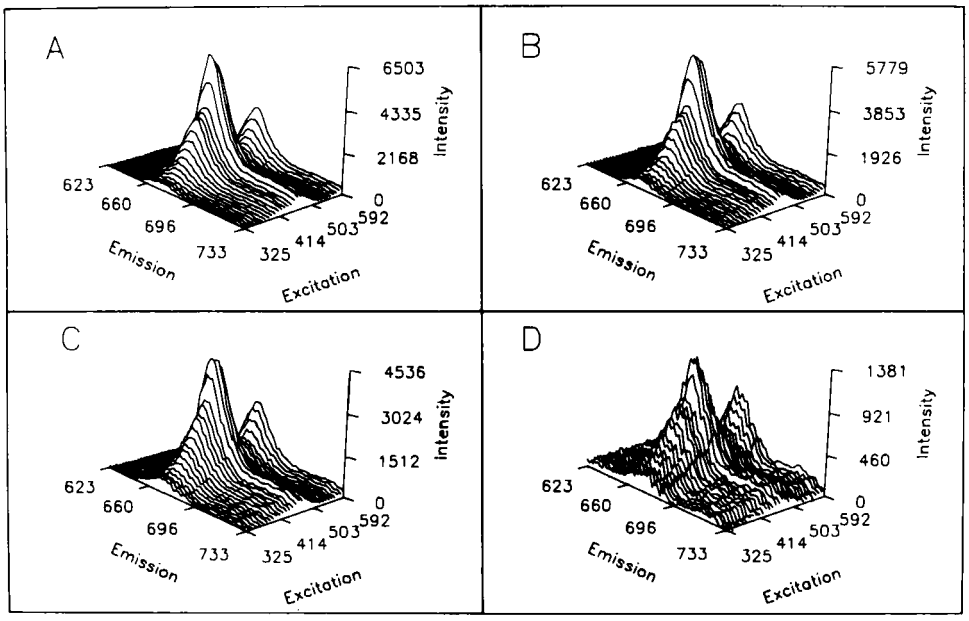

Figure 3 EEMs of *C. vulgaris* exposed to 1.3-dinitrobenzene (DNB) at concentrations of (a) 10^{-6} M, (b) 10^{-5} M, (c) 10^{-4} M and (d) 10^{-3} M.

Table 2 lists relative intensities after exposure to each nitroaromatic. From these data, it is seen that 1,3-dinitrobenzene, with less ionizable substituents is a more effective quencher than 3-nitrobenzoic acid or 2-nitrophenol which have more ionizable groups.

By comparing the control EEMs in Figures 2a–b to the EEMs in Figures 3a–d and 4a–d, the effectiveness of multidimensional fluorescence to observe the sensitivity of the concentration of toxic pollutants on algae is seen. In Figures 3a–d, *C. vulgaris* fluorescence after one hour of exposure to 1,3-dinitrobenzene (DNB) at concentrations ranging from 10^{-6} M to 10^{-3} M is presented. Maximum fluorescence is obviously being quenched in the presence of DNB. In Figures 4a–d, *P. perscienium* fluorescence is presented after exposure to DNB under the same conditions. In these spectra, not only a change in intensity is observed, but also a change in the ratio of intensity of *chlorophyll a* fluorescence to phycocyanin

fluorescence is observed. From these spectra, it appears that *chlorophyll a* fluorescence is decreasing at a much greater rate than phycocyanin fluorescence.

Diatoms collected from the sea water exhibited quenching comparable to *C. vulgaris*. That is, overall intensity was quenched and no changes in peak ratios were observed indicating only *chlorophyll a* fluorescence is being observed.

The effects of exposure over a period of time can also be monitored using multidimensional fluorescence. In Figures 5a–d, EEMs are presented for *P. perscienium* after exposure to 10^{-5} M DNB at one, four, and seven hours with one additional EEM taken after five days' exposure to DNB. It is seen from these spectra that there appears to be an increased effect of pollutant with exposure time. However, after five days, it appears as if the culture is beginning to recover. This recovery is indicated by the increased intensity of *chlorophyll a* fluorescence relative to phycocyanin fluorescence.

HPLC Uptake

From the quenching observed in the present work and data presented from previous research efforts,[6] it appears that 1,3-dinitrobenzene is accumulated by the algal cells. Analysis of the growth media by HPLC reveals that the accumulation continues until either death of the culture or complete consumption of the pollutant occurs. Cultures of *C. vulgaris* containing 10^6 cells/mL exposed to 10^{-5} M DNB initially accumulated the pollutant at a rate of 5.0×10^{-7} mole/L/hr. After 20 hours, an uptake rate of 3.8×10^{-7} mole/L/hr was measured. After 24 hours, a DNB concentration of 6.3×10^{-6} mole/L remained indicating an average uptake rate of 1.5×10^{-7} mole/L/hr over 24 hours. This is an indication that uptake during the dark cycle is extremely diminished. This is in contrast to cultures of *P. perscienium* (10^{-6} cells/mL) which accumulated DNB initially at 3.0×10^{-7} mole/L/hr and contained concentrations which were lower than the detection limits of the HPLC method (1×10^{-7} mole/L) after 24 hours. Natural diatom samples concentrated to 10^6 cells/mL initially accumulated DNB at 1.3×10^{-6} mole/L/hr. After 20 hours, the uptake rate slowed to 3.5×10^{-7} mole/L/hr. After 24 hours, a DNB concentration of 5.5×10^{-6} mole/L remained. This indicates that the initial rate was not maintained and uptake during the dark cycle is limited.

Preconcentration

Collection efficiency of the preconcentration apparatus and method was evaluated with unialgal cultures of *C. vulgaris* and *P. perscienium*. After 30 mL of a 10^6 cells/mL *C. vulgaris* culture were diluted to one liter, the resulting solution was then circulated through the cylindrical filter while a vacuum was applied to the outer region of the filter. The excess volume was removed in fifteen minutes. The vacuum was removed and cells collected from the filter by backflushing with 30 mL of ASP 6 media. A cell concentration of 10^6 cells/mL was achieved in the backflushed solution indicating approximately 100% collection efficiency. A similar collection efficiency was achieved using 10^6 cells/mL of the *P. perscienium* culture.

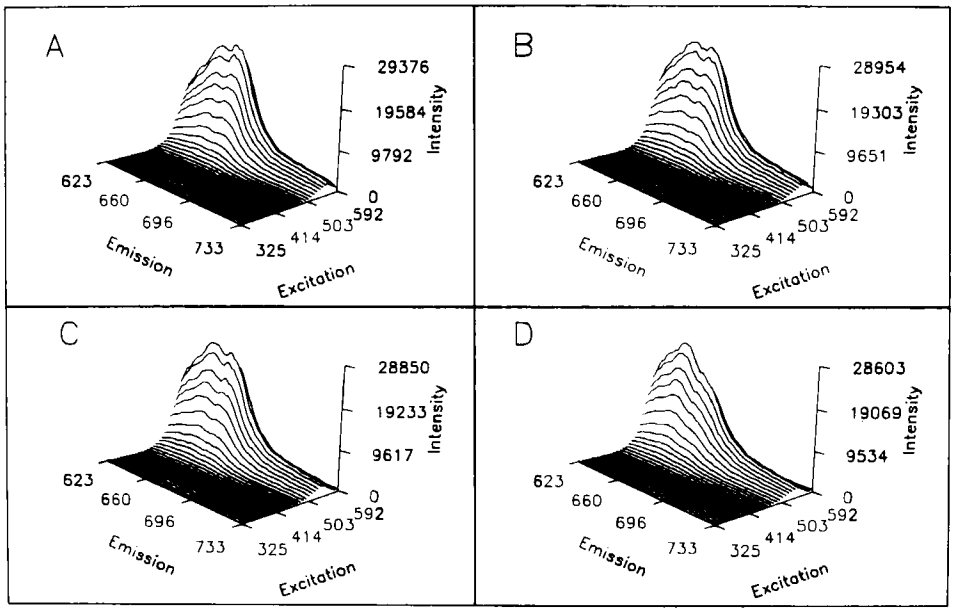

Figure 4 EEMs of *P. perscienium* exposed to 1,3-dinitrobenzene (DNB) at concentrations of (a) 10^{-6} M, (b) 10^{-5} M, (c) 10^{-4} M and (d) 10^{-3} M.

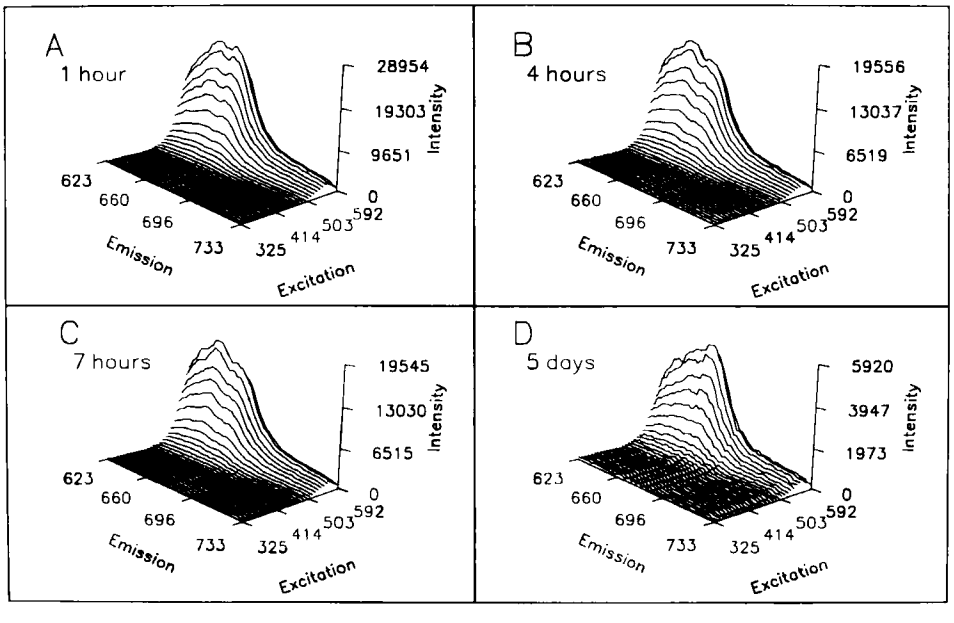

Figure 5 EEMs of *P. perscienium* exposed to 10^{-5} M, 1,3-dinitrobenzene measured at (a) 1 hour, (b) 4 hours, (c) 7 hours and (d) 5 days.

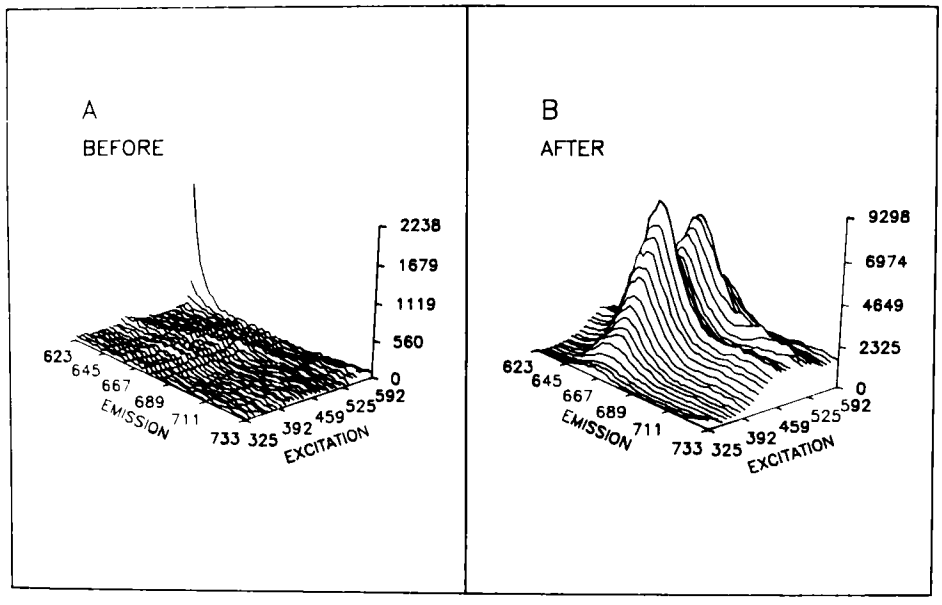

Figure 6 EEMs of natural sea water samples (a) before and (b) after preconcentration.

A one liter sample of natural sea water was examined using the preconcentration method. Figures 6a and 6b represent EEMs of sea water before and after preconcentration. It is clearly seen the the preconcentration procedure is effective in concentrating the dilute natural sample. Sufficient concentration of cells is essential for reliable analysis and the concentration method proves exceedingly successful in providing adequate cell concentrations.

DISCUSSION

Multidimensional fluorescence, which is well suited for fingerprinting algae, also appears to be a useful tool for monitoring *in vivo* algal fluorescence and observing spectroscopic changes due to pollutants. Although conventional fluorescence measurements have been used to observe the quenching effects of pollutants on *in vivo* fluorescence, all available information is often not used. By rapidly monitoring a range of excitation and emission wavelengths, multidimensional fluorescence measurements can be used for monitoring the inhibitive effects on the fluorescent pigments as well as for identifying pigment composition.

Although multidimensional fluorescence methods easily monitor pigment quenching, such as the quenching of *in vivo C. vulgaris chlorophyll a* fluorescence, that is not the strength and utility of the approach. By monitoring a wavelength range, fluorescence from two algal pigments can be simultaneously observed allowing insight into the effects of pollutants on the photosynthetic pathway. For example, multidimensional fluorescence measurements in the present study indicate that DNB accumulated by *P. perscienium* initially affects *chlorophyll a* fluorescence

but does not readily quench phycocyanin fluorescence. Thus, the mechanism of toxicity by pollutants is better illuminated. In this regard, further fluorescence studies are underway to examine the effect of nitroaromatics using different classes of marine algae. These measurements will be combined with HPLC studies to provide more information on the role of the nitroaromatics in altering the *in vivo* algal fluorescence, which should provide more information on the mechanism of toxicity. Multidimensional fluorescence analysis also appears to have potential for indirectly monitoring the presence of pollutants in marine environments using algae as a bioindicator.

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