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Calibration and performance of a new in situ multi-channel fluorometer for measurement of colored dissolved organic matter in the ocean

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Abstract

The development of multispectral in situ fluorescence instruments greatly enhances the study of the optical properties of Colored Organic Matter (COM). Here, we tested the inter-instrument variability of three WetLabs, Inc. SAFIres using quinine sulfate standards. As with any fluorometer, intensity and spectral biases in fluorescence output due to properties of the SAFIre's optical components necessitate corrections. Low response of the instrument to quinine sulfate and lack of an excitation/emission channel at the fluorescence maximum of this standard precluded direct spectral calibration. Calibrations conducted using seawater as a secondary standard provided an acceptable alternative. The field performance of the SAFIre from two experiments is presented here. Time series contour plots show that the instrument has the ability to detect small differences in COM optical properties, and observed fluorescence emission ratios are indicative of changes in sources of the material over the course of the study. The SAFIre was found to extend multispectral measurements to include high spatial and high temporal resolution.

Keywords: Fluorescence; CDOM; In situ instrumentation; Optical properties; SAFIre

1. Introduction

Fluorescence is an easily measured property of colored organic matter. Given the variability in spectral fluorescence of COM, measurements at several wavelength regions are required to understand the composition and sources of the material. One technique, fluorescence contouring, involves repeated emission scans concatenated over a range of excitation wavelengths. This results in three-dimensional matrices (Fig. 1), which provide a wealth of information that can be used to identify fluorescent compounds in complex mixtures (Christian et al., 1981; Lochmuller and Saavedra, 1986; Leiner and Wolfbeis, 1988) and to trace COM from different sources (Coble, 1996; De Souza Sierra et al., 1997; Del Castillo, 1999). Although useful, this method is time consuming, limiting the number of discrete samples that can be analyzed and resulting in poor spatial resolution.

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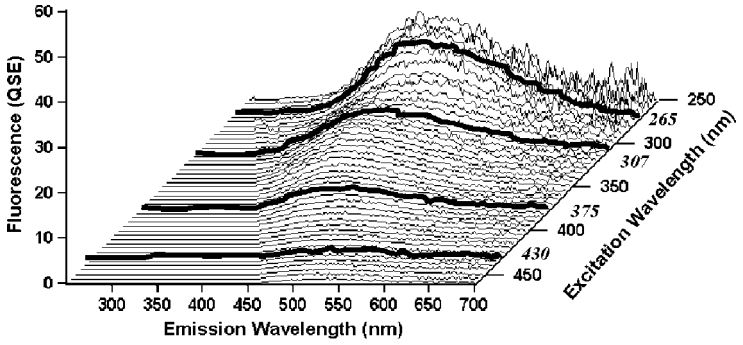

Fig. 1. Three-dimensional matrix created from the fluorescence contouring technique used to look at fluorescent compounds in seawater. The thick black lines represent four of the excitation wavelengths that the SAFIre is configured to measure. The SAFIre excitation wavelengths are italicized along y-axis.

Results from studies that have employed continuous multiple wavelength measurements have shown that unique spectral information is contained within each channel (Broenkow et al., 1985; Coble et al., 1993; Cowles et al., 1993; Battin, 1998; Del Castillo et al., 2001) but many of these studies were limited by the number of excitation and emission wavelengths available. In recent years, however, the development of new, in situ fluorescence instruments has enabled researchers to obtain real-time spectral information.

The SAFIre (Spectral Absorption and Fluorescence Instrument, WetLabs, Inc) has the ability to measure fluorescence at six excitation and 16 emission wavelengths. SAFIre filter set configuration can be completely user defined, based on availability of filters and intended use. The three instruments used in this work were configured so as to provide full coverage of the COM emission spectrum ($\sim$300 to 700 nm). Six of the emission channels correspond to the six excitation channels, for the purpose of measuring Raman Scatter, and is sometimes useful as an internal calibration. Excitation wavelengths were selected on the basis of xenon lamp intensity, COM source selectivity, and ability to stimulate chlorophyll fluorescence (Hoge et al., 1993).

The three instruments used in this work were configured as follows: excitation filters centered at 228, 265, 313, 375, 430, and 490 nm ($\pm$20 nm). In some cases, excitations 307, 350, and 487 nm were substituted in for one or more of the wavelengths previously mentioned. The emission filters are centered at 228, 265, 307, 313, 340, 350, 375, 400, 430, 463, 470, 487, 490, 500, 510, 520, 540, 565, 630, 685, 700, and 810 nm ($\pm$20 nm). This configuration has previously been shown to successfully track both COM and chlorophyll, as well as detect changes in optical properties and sources of COM in coastal regions (Del Castillo et al., 2001).

Fluorescence instruments generate spectra that reflect the characteristics of both the instrument and the sample (Miller, 1981). They exhibit intensity and spectral biases in their output due to properties of the optical components. There exist fluctuations in the intensity of the light source (Roberts, 1981), changes in the excitation output due to the wavelength-dependent properties of the light source and the excitation optics (Miller, 1981), and variability in the interference filter transmission (Roberts, 1981). In addition, signal variability can be caused by stray light within the instrument (Miller, 1981), geometrical differences of the detector diode-array (Roberts, 1981), and the emission wavelength dependence of the detector and the efficiency of emission optics (Miller, 1981).

The variability in the optical components of fluorescence instruments makes corrections essential. Raw data from different instruments cannot be compared directly until appropriate correction factors are applied. In this paper we present performance data of three SAFIres, and for the

first time, a methodology for their calibration. This method, however, can be applied to any in situ fluorometer used for the analysis of COM. We also present data from two EcoHab (Ecology of Harmful Algal Blooms) cruises and the Coastal Mixing & Optics (CM&O) experiment which demonstrate the performance of the instrument and its value in field studies.

2. Methods

Discrete seawater samples were used to intercalibrate the SAFIre to a benchtop SPEX Industries Fluorolog-II spectrofluorometer. This instrument was used as a benchmark because protocols correcting spectral response have been developed (Coble, 1996). The low excitation intensity of quinine sulfate at wavelengths below 300 nm, the low response of the SAFIre, as configured in this study, to quinine sulfate at emission 375, 400, and 430 nm, and the lack of a SAFIre channel at the fluorescence maximum of quinine sulfate (Ex/Em 347.5/450 nm) precluded direct spectral calibration of the instrument using this fluorescence standard. Therefore, the calibrations were conducted using seawater as a secondary standard (Conmy, 1999). It is possible to use discrete seawater samples as a secondary standard because the emission scans obtained by the Spex Fluorolog-II can be fully corrected to eliminate instrument bias (Coble, 1996).

2.1. Sample collection

Samples were collected monthly as part of the EcoHAB project (Ecology of Harmful Algal Blooms) off the coast of Tampa Bay in the Gulf of Mexico and during the 1997 Coastal Mixing & Optics Experiment (CM&O) in the northern region of the Mid Atlantic Bight. For EcoHAB cruises, underway in situ surface data were collected continuously via a WetLabs, Inc. SAFIre connected to the flow-through system of the research vessel drawing water from 2 m. For the CM&O cruise, the SAFIre was mounted on a CTD rosette to collect depth profile data.

The internal temperature of the SAFIre was found to increase during operation, which in turn causes an increase in the fluorescence output (Conmy, 1999). Fig. 2 shows results of a laboratory evaluation of temperature effects on fluorescence response. Full equilibration took up to 2 h, and fluorescence intensities increased 5–18% during this period. Fig. 3 shows the data for 265/470 nm plotted as a function of internal temperature. This channel was less influenced by temperature than the 265/490 channel, possibly due to differences in temperature on emission filter transmissivity.

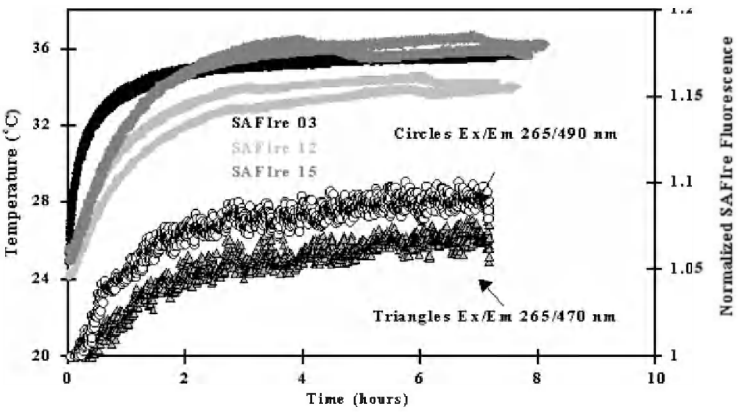

Fig. 2. SAFIre internal temperature plotted versus time. Temperature increases upon lamp initialization, then stabilizes after 1–2 h. The normalized SAFIre fluorescence also shows increase with time as temperature increases. Two fluorescence lines are shown for each SAFIre. These represent duplicate experiments.

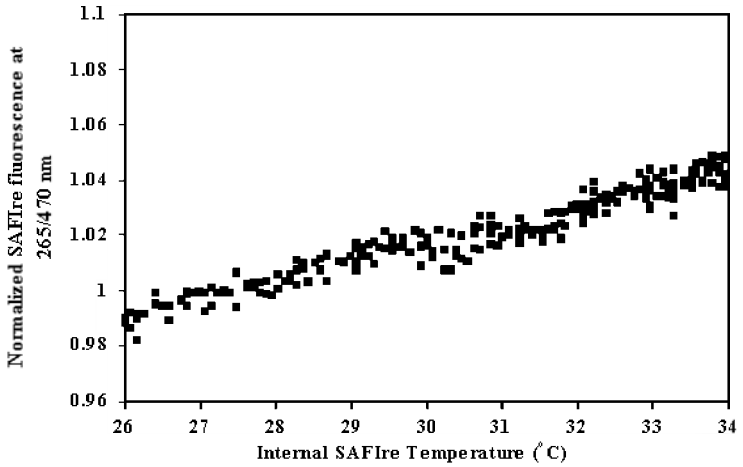

Fig. 3. Normalized SAFIre fluorescence at Ex/Em 265/470 nm versus internal temperature of the instrument during a laboratory experiment.

A 2 h warm up period was impractical for field operations, so our procedure used a warm up period of 1 h for in situ operation during underway data collection. Fig. 2 indicates that after this warm up period the maximum percent differences in fluorescence at emissions 470 and 490 nm for excitation 265 nm would be 4.76% and 2.79%, respectively.

A 15 min warm up period was used for profiling mode during the CM&O cruise. This was considered adequate because the water temperature was low, helping to maintain a stable instrument temperature. SAFIre internal temperature varied between 13°C and 17°C for the entire 3-week study period, with variation during a single cast not exceeding 2°C. Internal temperature was not dependent on the ambient water temperatures, which ranged from 5°C to 9°C. Likewise, there was no apparent dependence of COM fluorescence on either internal or ambient temperature. These results suggest that effects of temperature on fluorescence intensity are not as extreme in the field as in the laboratory, especially when water temperatures are low. Temperature effects can be mitigated by immersing the instrument in flowing seawater during underway mapping operations, and this procedure has become our standard practice.

2.2. Spectral calibration of SAFIre

Data for the calibration of the SAFIre were collected at pre-selected sampling stations encompassing various water types, from river-influenced coastal waters to high salinity open-ocean waters. For the EcoHab cruises, the instrument was allowed to collect data continuously for five minutes while the vessel was stationary, during which time discrete water samples for intercalibration with the Spex Fluorolog-II were collected from the outflow of the SAFIre. EcoHab samples were filtered through pre-combusted GF/F filters. CM&O samples were collected through 0.45 μm membrane filters from a rosette. Both were stored frozen in pre-combusted amber-colored glass bottles until analysis.

2.3. Spectral calibration of fluorolog-II

High resolution fluorescence spectroscopy was performed on the calibration samples following the method described by Coble, 1996. Briefly, 48 individual emission scans were collected at excitation wavelengths 5 nm apart between 220 and 455 nm. Emission wavelengths ranged between 250 and 700 nm, with data collected every 2 nm over an integration time of 0.5 s. Bandwidths were 5 nm for

both excitation and emission. Three-dimensional excitation–emission matrices (EEMs) were generated by conjoining the individual spectra. The data were then normalized to the water Raman scatter peak at Ex/Em = 275/303 nm, which was experimentally determined using daily MilliQ water scans. Water blank matrices were subtracted from the sample matrices to eliminate Raman peaks. Corrections for instrumental artifacts were made by multiplying the EEMs by a spectral correction matrix which contains both excitation and emission correction factors for the entire range of observations (Coble et al., 1993). Fluorescence intensity was converted to equivalents of quinine sulfate dihydrate in parts per billion (QSE) by multiplying the EEMS by a calibration factor derived from the fluorescence intensity at Ex/Em = 350/450 nm of a dilution series of quinine sulfate in 0.05 M sulfuric acid (Velapoldi and Mielenz, 1980). All data processing was performed using Galactic Industries GRAMS-V5 software.

2.4. Generation of correction factors

The spectral and fluorescence intensity correction factors were determined from the linear-least-square regression of SPEX Fluorolog-II corrected discrete sample values versus uncorrected SAFIre fluorescence values for each excitation and emission channel. The equations were then applied to all raw SAFIre data to yield actual in situ concentrations.

3. Results

3.1. Inter-instrument variability

Plotted in Fig. 4 are uncorrected excitation and emission spectra for quinine sulfate from three different SAFIres along with the corrected spectra obtained with a SPEX Fluorolog-II spectrofluorometer. The spectra were normalized to each instruments' maximum intensity to emphasize the spectral differences. The variation in spectral shape occurs due to differences in the instrument set up, most notably, differences in band-pass filter transmissivity (used for both excitation and emission), flash lamp spectral output and photodiode sensitivity.

Response factors were calculated by dividing the normalized uncorrected SAFIre values by the normalized corrected SPEX (Table 1). Multiplying the response factors by 100 is an indication of how much correction is needed at each wavelength pair where the values range between 17% and 220%.

To check instrument performance and stability over time, quinine sulfate standards were pumped through the instrument before each use. Temporal stability of both intensity and spectral response are

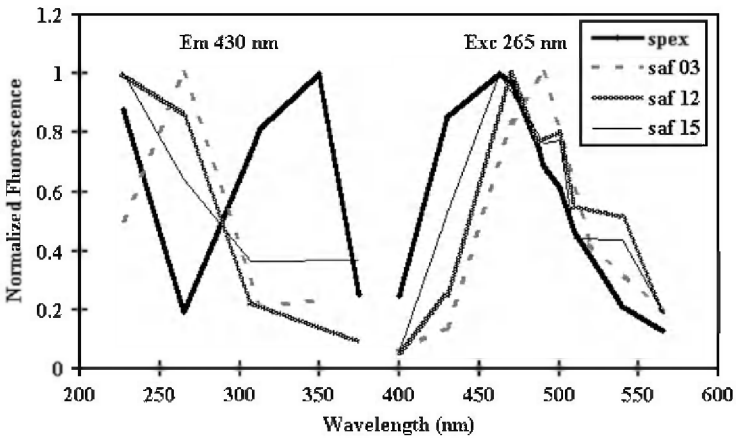

Fig. 4. Normalized raw excitation and emission spectra from three SAFIres and normalized corrected SPEX spectra for the quinine sulfate standard. The raw spectra show intensity and spectral differences amongst the similarly configured SAFIres.

Table 1
Response factors for SAFIres to quinine sulfate at excitation 265 nm

SAFIre	Emission (nm)	Normalized SPEX	Normalized SAF	Response factor
Saf 103	400	0.211	0.062	0.2943
	430	0.830	0.141	0.1700
	470	0.925	0.835	0.9027
	490	0.701	1.000	1.4270
	520	0.389	0.427	1.0997
	565	0.115	0.196	1.6984
Saf 112	400	0.211	0.055	0.2600
	430	0.830	0.250	0.3014
	470	0.925	1.000	1.0809
	487	0.743	0.770	1.0360
	500	0.585	0.799	1.3660
	510	0.499	0.554	1.1115
	540	0.237	0.518	2.1816
	565	0.115	0.195	1.6857
Saf 115	400	0.211	0.059	0.2779
	430	0.830	0.529	0.6365
	463	0.981	1.000	1.0191
	490	0.701	0.765	1.0917
	500	0.585	0.773	1.3217
	510	0.499	0.445	0.8930
	540	0.237	0.440	1.8534
	565	0.115	0.192	1.6668

The SPEX data were normalized to the quinine sulfate peak at 350/450 nm. The SAFIres are normalized to the emission maxima.

good, with only an 8.7% change in intensity over the course of 2 years of use and no detectable change in spectral bias (Fig. 5). In general practice, however, the standard curves were run on the SAFIres before each field use and again if the instrument was in use for more than a week.

3.2. Field performance

Instrument field performance was examined for three cruises. Data used to generate correction factors using discrete seawater samples are shown for August 1998 and July 1999 EcoHAB cruises in Figs. 6 and 7, respectively. Samples were taken at various places along the cruise track over a wide salinity range. The channels shown exhibit strong correlations with the discrete samples. The highest SAFIre112 response was observed at emission 470 nm for excitations 265 (Figs. 6A & 7A) and 307 (Figs. 6B & 7B) nm, due to high sensitivity of the SAFIre at this emission. The output at excitation 265 nm was higher than 307 nm for both years. The response factors in 1998 were found to be approximately one half that of 1999. This is the result of instrument servicing during that year, thereby changing the signal output. Upon applying correction factors to the raw

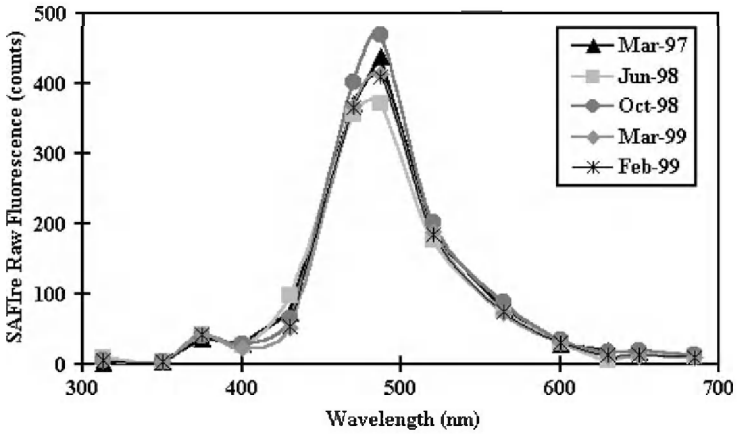

Fig. 5. Time series of raw emission spectra for SAF 103 at excitation 265 nm for a quinine sulfate standard. The peak was located at 487 nm and displayed a coefficient of variation of 8.7% over 2 year period.

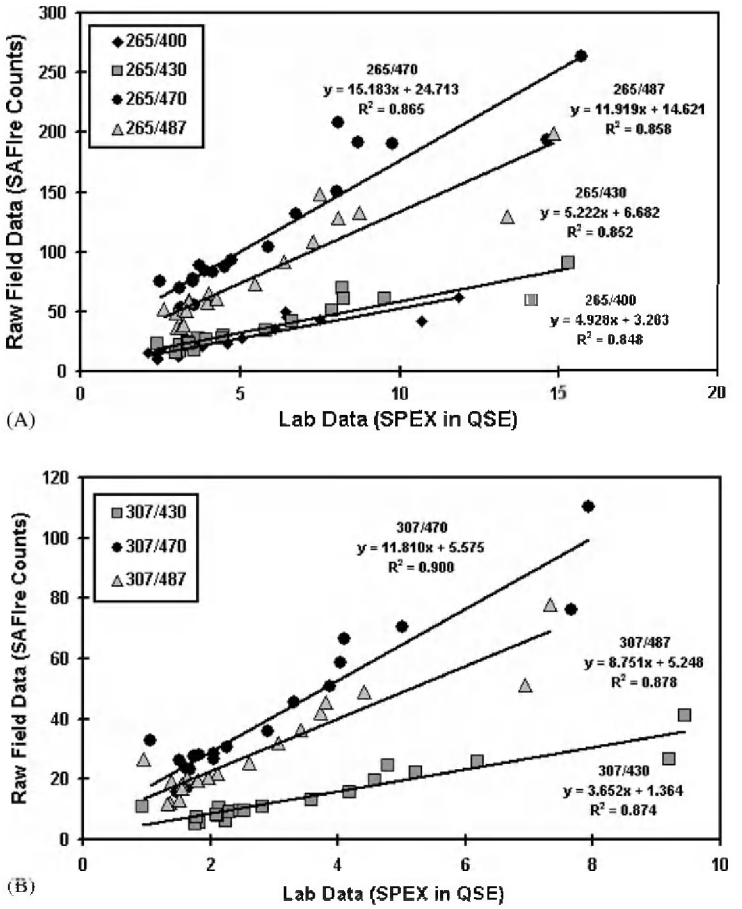

Fig. 6. Calibration curves and r^2 values for excitations 265 (A) and 307 (B) nm from the August 1998 cruise. Curves generated using seawater samples.

SAFIre data, the intensity and spectral differences found to exist between the corrected data of the two instruments were negligible. Fig. 8 shows a comparison of corrected emission spectra of a seawater sample (July 1999) at excitation 265 nm for SAFIre112 and SPEX. The corrected SAFIre data show percent differences ranging between 0.11% at 487 nm to 2.5% at 400 nm.

SAFIre103 calibration curves for the 1997 CM&O field experiment are shown in Fig. 9. The response factors were lower than during the EcoHAB cruises and maximum response was slightly red shifted from 470 to 490 nm for both excitations 265 (Fig. 9A) and 313 (Fig. 9B) nm. Differences in response factors for the two instruments are not unexpected as filter sets, even if nominally the same are not matched. The r^2 values were also lower, but this may be attributed to both lower COM concentrations and salinity range, and also because of weaker lamp output of SAFIre103, which was a prototype instrument. Although the concentrations were low, the SAFIre was able to show distinct trends in the COM values.

Time series contour plots of temperature, salinity and chlorophyll *a* from the CM&O experiment are shown in Fig. 10. A contour plot of COM fluorescence at Ex/Em =265/490 nm from the SAFIre is presented in Fig. 11A. A ratio of corrected SAFIre emissions 400 and 490 nm at

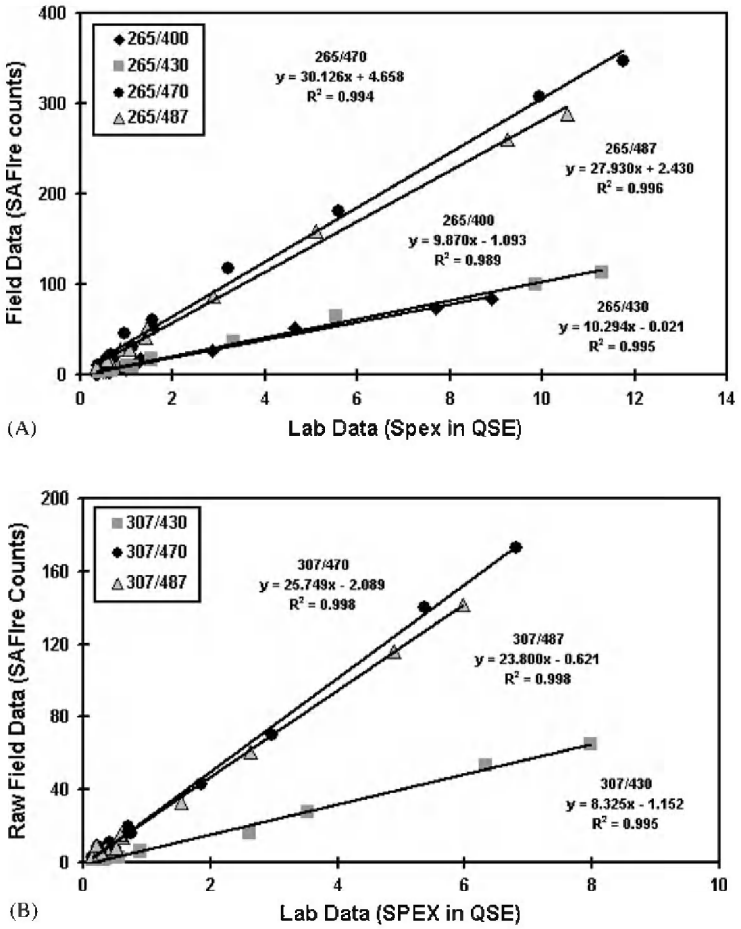

Fig. 7. Calibration curves and r^2 values for excitations 265 (A) and 307 (B) nm from the July 1999 cruise. Curves generated using seawater samples.

excitation 265 nm is plotted in Fig. 11B. The white blocks are where data were missing due to equipment failure or no sampling days. These contour plots are discussed in detail in the next section.

4. Discussion

Like all fluorometers, the SAFIre needs to be spectrally and intensity corrected. As shown in Fig. 4, without spectral compensation, erroneous conclusions could be made that more than one fluorophore was present in the standard solution due to the multiple peaks of the emission spectra. This is certainly not the case, since the sole emission peak of quinine sulfate is located at 450 nm. Table 1 also shows that without corrections, the SAFIre fluorescence is five times too low at one channel and more than double the correct value at another. Note that this is only taking into account the spectral biases. When working with raw data, the intensity variations add to these differences.

When collecting field data, the SAFIre and discrete samples were strongly correlated. The higher response at excitation 265 nm as compared to 307 or 313 nm for EcoHAB and CM&O

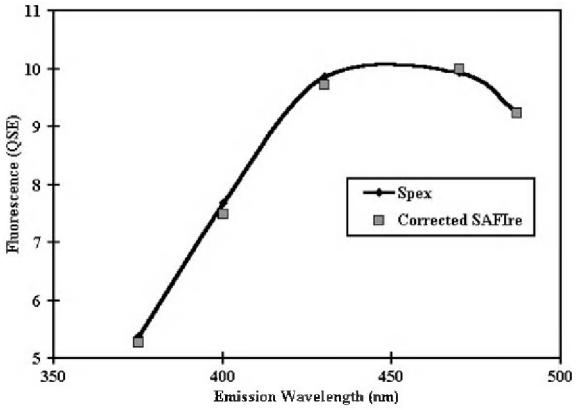

Fig. 8. Comparison of corrected SAFIre and SPEX emission spectra at excitation 265 nm for a July 1999 sample. Symbols correspond to emission bands on the SAFIre.

experiments is due to both higher humic fluorescence at this wavelength and also instrument configuration. The large change in response observed in SAFIre112 between 1998 and 1999 due to instrument servicing, highlights the need for recalibration before each use.

After the response factors are applied to the raw data, spectral and intensity differences are negligible between the instruments (Fig. 8). Often, differences in sampling techniques can prove problematic when comparing in situ data obtained from unfiltered seawater (COM) to discrete filtered samples (CDOM), but within our study area, the concentration of organic particles tended to be lower than the dissolved fraction, so little variation is found. Gilbes (1996) showed that the dissolved

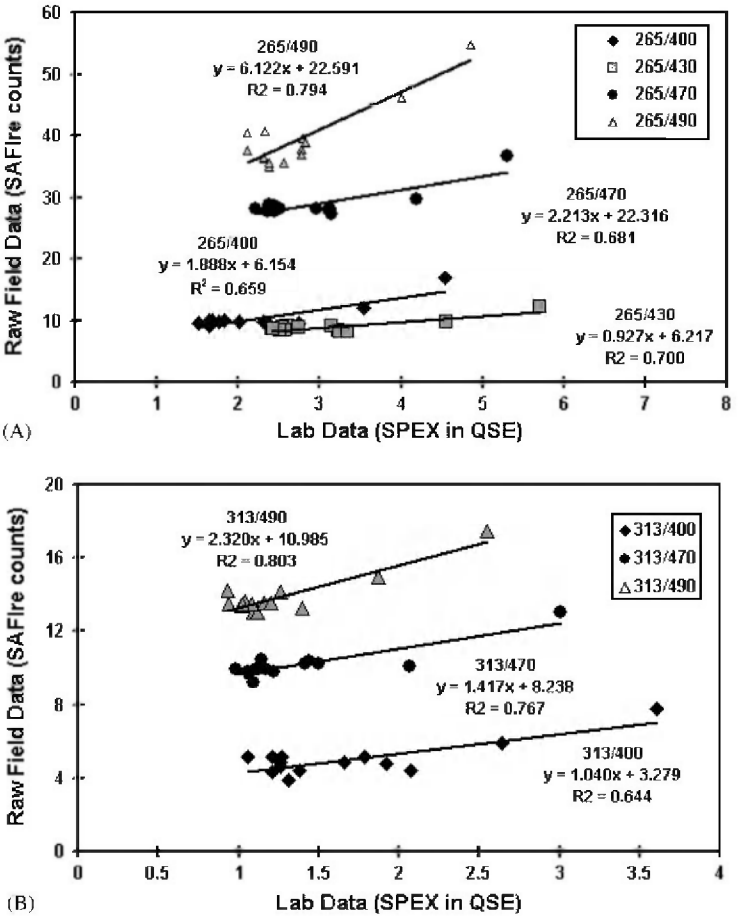

Fig. 9. Calibration curves and r^2 values for excitations 265 (A) and 313 (B) nm from the Spring 1997 CM&O cruise. Curves generated using seawater samples.

organic carbon off of Tampa Bay was approximately 18 times higher than the particulate portion.

During the CM&O experiment, SAFIre103 was used to generate a time series of COM fluorescence to be used in conjunction with temperature, salinity and chlorophyll *a* (Figs. 10 and 11). It was found that the beginning of the cruise was marked by well-mixed cooler water with higher salinities and lower COM fluorescence. On Julian Day 118, a small parcel of warmer and slightly more saline surface water appeared. Here the SAFIre measured lower surface COM values than the surrounding days. This was further supported by the discrete samples that showed fingerprints of Excitation-Emission Matrices (EEMs) similar to photobleached water (Conmy, 1999). During the latter part of the cruise, the water became strongly

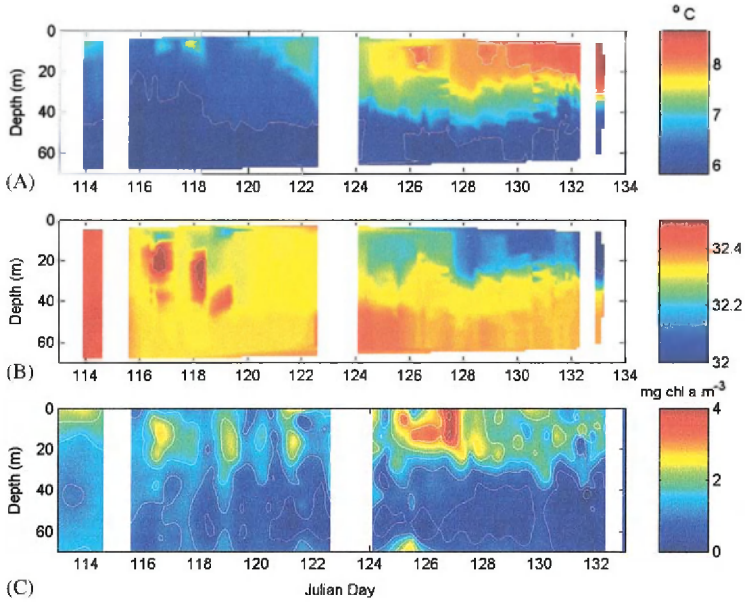

Fig. 10. Time series contours of temperature (A), salinity (B) and chlorophyll *a* (C) during the CM&O experiment.

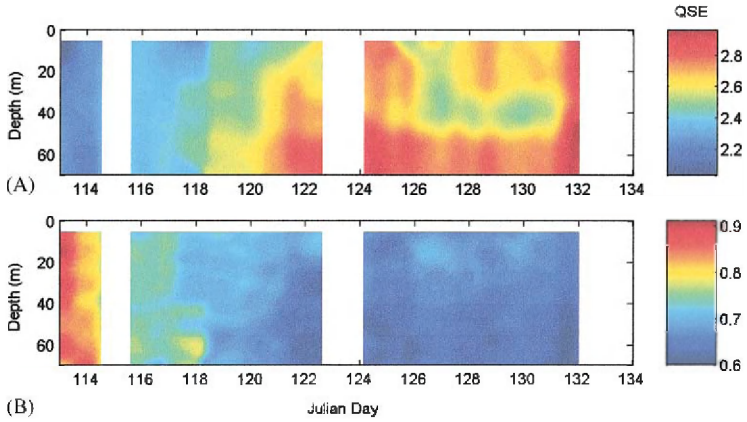

Fig. 11. SAFIre COM fluorescence at Ex/Em 265/490 nm (A) and emission ratio of 400:490 nm at excitation 265 nm (B) during the CM&O experiment.

stratified. A chlorophyll *a* max was observed around Julian Day 126, which occurred where high COM values were observed with the SAFIre. Discrete samples from this section showed EEMs indicative of a new production fingerprint, where humic and pigment fluorescence were higher (Conmy, 1999). During the last few days of the experiment, a warm, lower salinity, freshwater lens appeared at the surface. The SAFIre measured high COM fluorescence in this section and the discrete samples supported this, as well (Conmy, 1999). Chlorophyll *a* values were low, suggesting a different source of the material, as compared to Julian Days 125–127. Given the lower salinities, a riverine source is most plausible.

To further distinguish marine from terrestrial sources of COM, a technique of ratioing fluorescence emission at two wavelengths was used. In Del Castillo et al. 2001, fluorescence ratios were used to observe spectral differences in surface waters. Here, we apply this technique to depth profiles for the first time. The ratio of corrected SAFIre emissions 400 and 490 nm at excitation 265 nm is plotted in Fig. 11B. All values are lower than one due to higher fluorescence at 490 nm. A higher ratio means the fluorescence is blue shifted away from 490 nm. This is indicative of either a marine source or photobleaching of the material. In the early part of the cruise, low COM concentrations plus high ratio values suggests photobleaching may have occurred. Conversely, when ratios are lower, a terrestrial or deepwater source source is suggested. The contour plot shows this during the latter part of the cruise. It is important to note that the ratio is independent of concentration, so that differences may be attributed to the composition of the material and not dilution.

These plots adequately show that the SAFIre was capable of detecting small changes in COM optical properties and that the data are consistent with the temperature, salinity and chlorophyll *a* values.

5. Conclusions

Overall response of SAFIre to quinine sulfate was surprisingly stable and linear in all our experiments, even at excitation and emission wavelengths where this substance has extremely low absorption or fluorescence. As expected, all three SAFIre instruments displayed spectral variations due to differences in the optical components. Discrete seawater samples served as an acceptable standard for the generation of corrected CDOM fluorescence spectra, however it was necessary to use a fully calibrated spectrofluorometer to obtain accurate results and to permit comparison with other studies. This was necessitated by a combination of mismatch between fluorescence spectra of quinine sulfate and seawater, as well as by the spectral response of our SAFIres.

One way to improve results and simplify calibration would be to include Ex/Em channels optimized for quinine sulfate, i.e. at 350/450 nm. Instrument spectral response could then be compared to published spectra for quinine sulfate (Velapoldi and Mielenz, 1980), allowing a reference spectrum to be generated as described by Roberts (1981). We have reconfigured our instruments and work is in progress on resolving spectral calibration issues with the goal of eliminating the need for intercalibration with a spectrofluorometer.

Further assessment of the internal temperature influence on fluorescence must also be conducted, specifically looking at the instrument behavior under different field conditions.

Although the calibration process of the SAFIre can be time consuming, the data collected with this instrument are very valuable. As seen with the CM&O time series, the SAFIre extends multispectral measurements to include high spatial, as well as high temporal resolution. The WetLabs, Inc. SAFIre has the ability to detect small differences in optical properties. The measurements provide an enhanced understanding of in situ processes on COM composition, improved mixing models and use of CDOM as a water mass tracer. Overall, the SAFIre has proven useful in allowing speedy collection of sensitive in situ fluorescence measurements.

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