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FLUOROMETRY FOR CHEMICAL SENSING

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Introduction

In the context of ocean sciences, 'chemical sensing' denotes the acquisition of data on the concentration of certain chemical species (the analytes) in sea water. This acquisition process may be achieved by a 'chemical sensor' in the strict sense, i.e., a device that continuously and reversibly exhibits a change in some of its properties as a function of the concentration of a respective analyte. As an alternative, sensing may be done directly, without the detour over the properties of a sensor, by measuring some effect displayed by the analyte itself. Intermediate between these two methods is the use of an indicator that is added to a sample of sea water and changes its properties depending on the analytes' concentration. An example of a sensor is the Clark electrode, which delivers a current proportional to the concentration of oxygen. An example of direct sensing is the determination of salinity by conductivity measurements. The well-known use of litmus to monitor pH is an example for the indicator method.

'Fluorometry' refers to the measuring of fluorescence, which is the reemission of light from a compound upon exposition to light of a shorter wavelength (in this context, phosphorescence is included under the term fluorescence, although strictly speaking these are two different processes). Fluorescence occurs from a singlet excited state, while phosphorescence is originating from a triplet. This emission is characteristic for the emitting compound, but may be subject to modification by the environment. Thus, fluorometry may be applied to both sensing schemes outlined above: In a sensor device, its characteristic fluorescence properties may be modified by the concentration of the analyte. Direct sensing preferentially will be applied if the analyte itself is fluorescent.

Fluorescence

The fluorescence emission displays a number of general characteristics. Except for atoms in the vapor phase, the emission is shifted to lower wavelengths relative to the excitation. The energy loss between excitation and emission, the so-called 'Stokes shift', occurs owing to rapid decay of excitation energy to the lowest vibrational level of the excited state, as well as from subsequent decay to higher vibrational levels of the ground state. As the relaxation to the lowest vibrational level usually occurs within 10^{-12} s, the fluorescence emission spectrum does not depend on the excitation wavelength. The fluorescence can be described using the parameters spectral

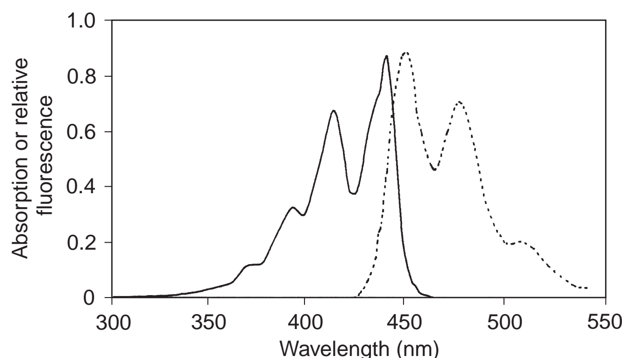

Figure 1 Typical absorption and fluorescence of an organic compound, showing 'mirror image' relationship between the absorption spectrum (——) and the fluorescence spectrum (- - - -).

distribution of the emission, quantum yield, decay time, and polarization.

If the molecule is excited to its first excited electronic state, fluorescence is usually emitted directly from this state, and the fluorescence emission spectrum appears to be a 'mirror image' of the absorption spectrum (**Figure 1**). Deviations from the mirror image rule occur if the excitation goes to higher-lying states, if the molecule undergoes major geometrical rearrangements in the excite state, or if excited-state chemical reactions occur.

Fluorescence decay times usually range from some picoseconds to nanoseconds, or up to milliseconds in the case of phosphorescence.

The fluorescence decay time τ (sometimes also called 'fluorescence lifetime') describes the average time a molecule spends in the excited state before it returns to the ground state. In the simplest case, the fluorescence intensity follows an exponential law:

$$I(t) = I_0 e^{-t/\tau} \qquad [1]$$

where τ is the time it takes for the fluorescence intensity to decrease to a value of $I_0 e^{-1}$. The decay time is connected to rate constants via

$$\tau = \frac{1}{\Gamma + k} \qquad [2]$$

and therefore depends on all processes affecting the radiative or nonradiative rate constant.

Fluorescence decay times usually range from some picoseconds to nanoseconds. If triplet states are involved in the emission process, the measured decay time can be of milliseconds to seconds, and this emission usually is called phosphorescence.

Excitation with polarized light results in polarized emission, since fluorophores preferentially absorb photons whose electric vectors are parallel to the transition dipole moment of the fluorophores. The fluorescence polarization P after excitation with vertically polarized light is defined as

$$P = \frac{I_n - I_p}{I_n + I_p} \qquad [3]$$

where I_p and I_n are the fluorescence intensities of the vertically (n) and horizontally (p) polarized emission. In a solid environment at low fluorophore concentrations, the polarization is determined only by the relative directions between absorption and emission dipole moment in the fluorophore. In liquids, where the fluorophore molecules may move freely, a decrease in polarization can be observed owing to rotational diffusion or excitation transfer between fluorophores.

Techniques

Direct Sensing

Direct measurement of the concentration of fluorescent materials in sea water is usually done with samples taken in the field and transported to a laboratory, either on board a research ship or on land. The procedure of measuring involves three steps: excitation of the analyte, detection and identification of fluorescent light, and determination of concentration of fluorescent analyte.

To excite a given analyte a source emitting light within the analyte's absorption band is necessary. A monochromatic or at least narrowband source serves to discriminate the analyte against all compounds with different absorption. In any case, the detected fluorescence may be the superposition of the emissions from various different analytes, and to add further specificity, the fluorescence light has to be spectrally analyzed. Most analytes have a characteristic 'fingerprint' spectrum; thus, it is possible to identify the analyte from its spectrum provided a limited choice of analytes is known to be present. The main difficulty is in extracting from the intensity of the detected fluorescence the concentration of the respective analyte. This can only be done with a certain amount of reliability if an 'internal standard' can be used for comparison. If water samples are analyzed in the laboratory, a fluorophore (e.g. quinine sulfate) added in a well-defined concentration can serve as a standard. This is not possible for *in situ* measurements. The only substance available as a standard in any case is water itself, but water is

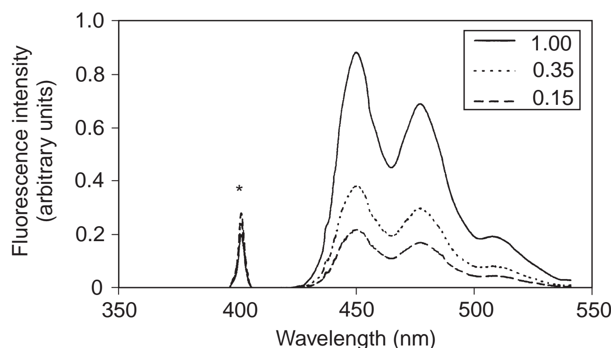

Figure 2 Fluorescence spectra of perylene at three different concentrations with the water Raman line (*) as the reference (maximum concentration 100 ng/l).

nonfluorescent. Also, scattered light cannot be used, since scattering strongly depends on the amount of small particles (dust, microorganisms, etc.) in the sample.

There is, however, a weak wavelength-shifted component, called Raman scattering, that is useful as a standard. Raman scattering does not, like fluorescence, produce a specific wavelength but rather a difference in wavelengths between excitation and emission. Thus, in shifting the excitation wavelength the scattered wavelength is also shifted. The intensity of the Raman band of water may be used as the standard against which the fluorescence intensity of the analyte is calibrated (**Figure 2**). The quinine sulfate standard is related to the Raman standard by defining the ratio of the integrated fluorescence of a $1\,\mu g\,l^{-1}$ quinine sulfate solution to the integrated H_2O Raman band as the 'quinine sulfate unit' (QSU). Results in QSUs should be directly comparable among different instruments and different laboratories.

A much more refined method is three-dimensional fluorescence (or excitation–emission matrix spectroscopy, EEM). The excitation as well as the emission wavelengths are scanned over a board range and the fluorescence intensity is plotted as a function of both (**Figure 3**). Sophisticated mathematical evaluation allows identification and relative quantification of multiple analytes.

Another technique for distinguishing between multiple analytes is time-resolved fluorometry. Excitation is done with very short light pulses (duration < 1 ns), and the fluorescence spectrum is recorded after a certain delay. Since the fluorescence decay times of various compounds are different, the spectrum will change with increasing delay. With short delays, all excited compounds contribute to the spectrum. After a short while, the fluorescence of some of the compounds has vanished while that of others persists (**Figure 4**). A careful analysis of a sequence of successively delayed spectra provides the relative concentration of the various fluorescent compounds contained in the sample.

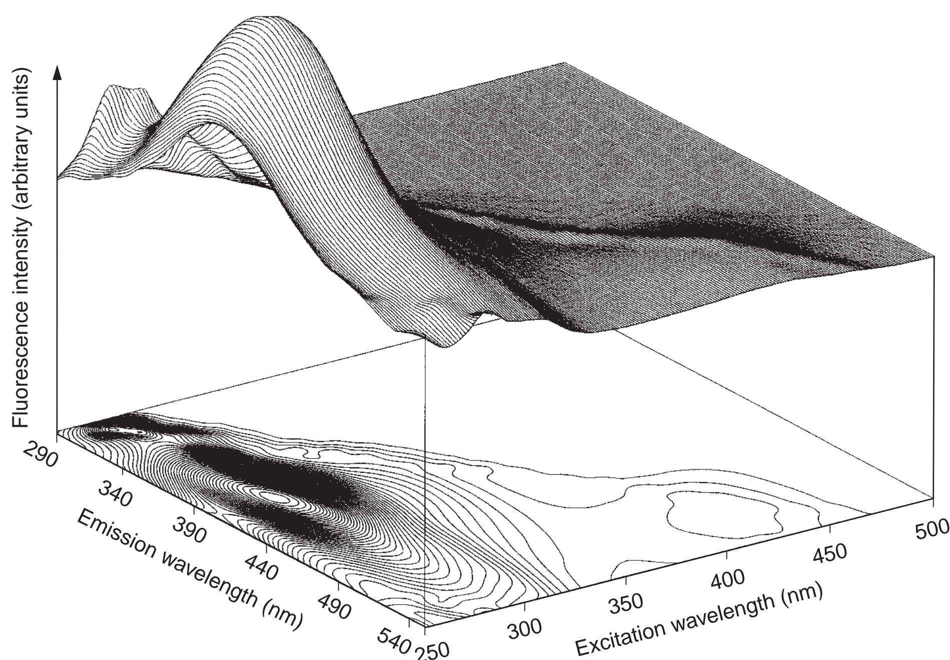

Figure 3 Three-dimensional or excitation-emission matrix spectrum. The small ridge running across the spectrum is the water Raman line. (Courtesy of M.J.P. Leiner and K. Kniely.)

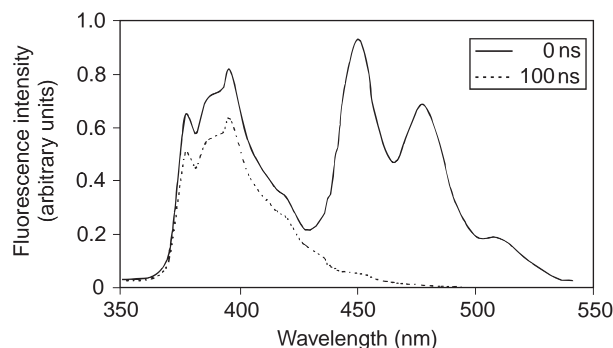

Figure 4 Time-resolved fluorometry of a mixture of two compounds with decay times 4 ns and 300 ns. Spectra taken immediately after excitation (0 ns) and with a considerable delay (100 ns).

Fluorescence polarization measurement is a rather unusual technique in ocean sciences. The only experimental difference from simple fluorometry is that excitation is done with polarized light and the emission is recorded separately for two perpendicular directions of polarization.

Indicators and Sensors

Indicators and sensors have in common that the fluorescent compound is not a constituent of the sea water under investigation but is added deliberately during the measuring process. From the standpoint of fluorometry it makes no difference whether the fluorescent indicator is dissolved in the sea water sample or is incorporated in a sensor element that is brought into contact with the water. Therefore, the following technical description applies to indicators and sensors as well.

Fluorescent chemical sensor devices consist essentially of three main building blocks: an illumination device, the sensor element proper, and a detecting apparatus. Since the fluorescence to be excited and detected is that of the sensor element alone, much less effort needs to be made to discriminate between various sources of fluorescence. This opens the possibility of using small and cheap light sources such as light-emitting diodes. Usually the sensor is used *in situ* rather than in a laboratory and the sensor element is dipped directly into the sea water. However, this implies that the excitation light has to be brought to the sensor element. This can be done by implementing a submersible sensor unit consisting of the light source, the sensor element, and even the detector. Alternatively, only the sensor element may be submersed and light can be transported via optical fiber to and from the element. In some cases even the sensor element may be part of an optical fiber.

The sensor element in most cases is a polymer membrane containing a fluorescent indicator dye. The fluorescence of the indicator can be altered by the presence of the analyte in several different ways: The analyte may interact with the excited molecule in a way that brings about changes in the fluorescence intensity. On the other hand, it binds to the indicator and thus alters the molecular properties in such a way that the absorption and/or emission are shifted spectrally. And finally, binding of the analyte may not alter the spectral properties of an indicator molecule but may change its mass and size. This yields a different moment of inertia and hence a different rotational diffusion and a change in fluorescence depolarization.

A reduction in fluorescence intensity is called quenching. Two types of quenching can be distinguished. Quenching is called dynamic if the interaction between the quencher (analyte) and the luminophore (indicator) occurs while the latter is in the excited state. In this case the rate constant of the nonradiative decay is increased, shortening the decay time and diminishing the quantum efficiency. Dynamic quenching is not associated with a chemical reaction between indicator and analyte. If, on the other hand, the interaction occurs while the indicator is in its electronic ground state, the quenching is called static. Static quenching may or may not include a chemical reaction.

In the simplest case, the reduction of the fluorescence intensity or the decay time is described by a linear function, the so called Stern–Volmer equation,

$$\frac{I_0}{I} = 1 - k_{SV}[A] \qquad [4]$$

where I_0 and I are the unquenched and quenched intensity, k_{SV} is the Stern–Volmer constant (a quantity characteristic of a certain combination of indicator and quencher), and [A] is the concentration of the quenching analyte (**Figure 5**).

The Stern–Volmer equation shows that we have to measure two quantities, I and I_0, to extract the analyte's concentration. Because of the more reproducible conditions in a sensor membrane, instead of using an internal standard it is sufficient to recalibrate the sensor from time to time by applying a standard solution of the respective analyte. This is rather easy if the sensor is used in a laboratory environment, but presents difficulties for *in situ* measurements, especially when the sensor equipment is intended to measure autonomously over an extended time. This difficulty can be overcome by

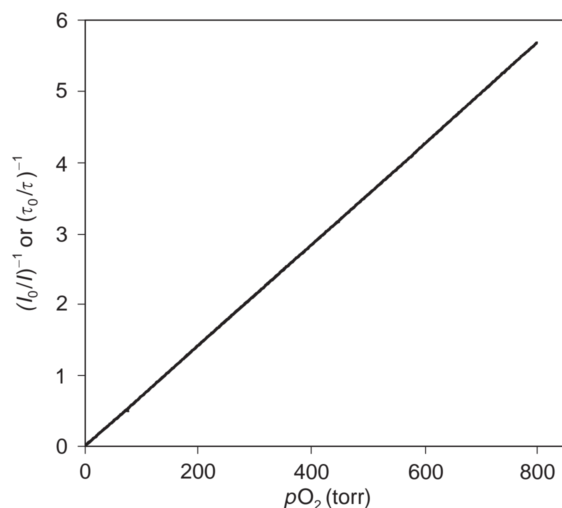

Figure 5 Stern–Volmer plot (see text) of fluorescence quenching by oxygen.

measuring the fluorescence decay time rather than the intensity. The decay time also obeys a Stern–Volmer law but it is independent of drifts in fluorescence intensity that may be brought about by leaching or bleaching of the indicator dye or by aging effects in the light source or the detector. Thus, decay time-based sensors, despite requiring a more sophisticated optoelectronics, have much superior long-term stability and, in fact, are the only promising candidates for unattended monitoring purposes.

Static quenching implies some close association between the indicator molecule in its ground state and the analyte. Usually an equilibrium is reached between associated indicator–analyte pairs and free indicator, which is determined by the association constant characteristic for the partners. The higher the concentration of the analyte, the more the equilibrium is shifted toward the association.

The associated and the free indicator may have distinct fluorescence spectra or distinct decay times. The spectra of the two forms intersect at a certain wavelength, the so-called isosbestic point. The total fluorescence intensity at that wavelength is independent of the relative concentrations and hence independent of the analyte. Thus, this intensity can be conveniently used as an internal standard. For two distinct decay times, the decay function becomes the sum of two exponentials

$$I(t) = I_0(a\mathrm{e}^{-t/\tau_a} + b\mathrm{e}^{-t/\tau_b}) \quad [5]$$

where a and b represent the relative concentrations and τ_a and τ_b the respective decay times of the associated and the free indicator. The concentration of the analyte can be extracted from the decay curve by a fitting algorithm.

If the associated and free indicator have different absorption spectra, the result in fluorometry is simply a different fluorescence intensity because of a different probability of excitation. If, on the other hand, the species have different emission spectra, the result will be the superposition of the two spectra. In many cases there exists an isosbestic point, that is, a wavelength where the two emission spectra cross and where the emission intensity is independent of the relative concentrations of two species. This point may be used for internal referencing. The concentration of the analyte can be deduced from the ratio of emission intensities at two wavelengths that are outside the overlapping region of the two spectra.

Applications

Direct Measurement of Fluorescent Species in Sea Water

The most important marine fluorophore is chlorophyll. However, this article is devoted only to sensing of dissolved compounds (see **Fluorometry for Biological Sensing**) for fluorophores in living organisms). **Table 1** summarizes the most commonly measured substances as well as their excitation and emission wavelengths.

Dissolved organic matter (DOM) is the catch-all denotation for all sorts of organic products from metabolism and biological decomposition. The term dissolved organic carbon (DOC) is more specific and quantitative as it is related to the carbon content in the dissolved organic matter. Consequently, DOM

Table 1 Dissolved chemical species in sea water measurable directly via fluorescence

Analyte	$\lambda_{ex}/\lambda_{em}$ (*nm*)	*Decay time* (*ns*)
Dissolved organic matter (DOM/DOC)	220–390/300–500	< 2
Dissolved proteinaceous materials	220–280/300–350	< 2
Humic acids	230, 300–350/420–450	< 2
Polycyclic aromatic hydrocarbons	300–450/400–550	4–130
Trace plastic and epoxy compounds	UV/UV	< 2
Heavy metals	X-ray	—

λ_{ex}, excitation wavelength; λ_{em}, emission (fluorescence) wavelength.

can be quantified only in mass per unit water volume, while DOC can be specified in moles of carbon per unit volume. DOC nevertheless subsumes a multitude of compounds. They are important components of the global carbon cycle. Little is known about the detailed chemical composition of DOM, but recent investigations have shown that acyl oligosaccharides may form a significant fraction.

A large part of DOM is formed by components that can be detected via fluorescence (fluorescent organic matter, FDOM). The ratio FDOM/DOM seems to be rather constant in surface waters; accordingly, the correlation between DOC and fluorescence intensity is fairly robust. However, compounds of low molecular weight essentially lack fluorescence, at least when excited in the usual wavelength range for DOC (250–350 nm). This low-fluorescent DOC was determined at about 150 μmol in the Mid-Atlantic Bight. DOC from rivers seems to be more strongly fluorescent than DOC from marine systems. Fluorescence intensity is 2–2.5 times higher in deep waters, where photodecomposition and oxidation of high-molecular-weight matter is low. There may, in certain extreme cases, even be a negative correlation between DOC and fluorescence intensity. This shows that the use of fluorescence data to determine DOC quantitatively is not straightforward. Recalibration by comparison with chemical DOC quantification is necessary if water conditions change significantly.

Among the components of DOM, the nutrients (nitrate and phosphate) play a major role because of their importance for metabolism. They are usually nonfluorescent, but their concentrations often correlate well with overall DOC and thus also with fluorescence intensity under equivalent conditions. However, the ratio of carbon to nitrogen or phosphorus may vary in a wide range (observed C : N ratios are in the range 16–38). Specific detection of nutrients is usually impossible with direct fluorescence methods.

The situation is more favorable for proteinaceous material. The characteristic absorption and emission wavelengths (λ_{ex} 220–270 nm, λ_{em} 300–350 nm, respectively) allow differentiation from other compounds. The same is true for humic acids, which can be distinguished by their unusually long-wavelength emission (420–450 nm).

The second large bulk of fluorescent compounds in sea water is made up of pollution products. The main sources are sewage, oil spills, runoff, and atmospheric deposition. A class of special interest is that of polycyclic aromatic hydrocarbons (PAHs), which are released into the environment in large quantities. They are quite persistent, and some of them are potent carcinogens. Their fluorescence spectra are not significantly different from those of other organic material, but they are outstanding by their long fluorescence decay times. Thus they can be distinguished by time-resolved spectroscopy. The detection limit for PAHs by time-resolved fluorometry has been shown of the order of nanograms per liter in the presence of DOC at milligrams per liter.

Three-dimensional fluorometry has also been used to differentiate between various constituents of fluorescent matter in sea water. For humic acids, different excitation emission maxima were found for different types of water: coastal waters peaked at 340/440 nm, shallow transitional waters at 310/420 nm, eutrophic waters near 300/390 nm, and deep-sea samples at 340/440 nm (exitation/emission wavelengths). Although the chemical nature of the different humic species is not yet clear, these results provide means of distinguishing between water mass sources in the ocean. Three-dimensional fluorometry was found to be advantageous for identifying pollutants. It was used, for example, to detect trace plastic and epoxy compounds in the presence of other organic materials.

X-ray fluorescence is the method of choice for measuring metal contaminants. Excitation as well as emission wavelengths are in the X-ray region. The spectra obtained are rather specific for certain metals. Portable X-ray fluorescence spectrometers are available for measurement in the field.

Indicators and Sensors

Table 2 summarizes the sensor type, the range covered, and the sensitivity obtained for several analytes.

Dissolved oxygen is the analyte most readily measured using a fluorescent optical chemical sensor. Oxygen, because of its triplet ground state, is a notorious fluorescence quencher. Various organic fluorescence indicators have been proposed for measuring oxygen, but in recent years metallo-

Table 2 Dissolved chemical species in sea water measurable via fluorescence sensor devices

Analyte	*Sensor type*	*Range, sensitivity*
Oxygen	Decay time	0–60 ppm, 1 ppb
pH	Decay time	6–10, < 0.1
Carbon dioxide	Intensity, decay time	200–1000 ppm, 1 ppm
Aluminum	Intensity	10–1000 nmol l^{-1}, 10 nmol l^{-1}

organic complexes have been found to be most suitable, especially ruthenium diimine complexes and platinum or palladium porphyrins. These indicators are outstanding because of their long fluorescence lifetimes and good quenchability by oxygen. The sensors cover the whole range of dissolved oxygen concentration encountered in aquatic environments. Compared to electrochemical sensors (Clark electrode) they have the advantage that they do not consume oxygen and hence may be used even in stagnant water. The technique of lifetime imaging has been applied to the study of spatial oxygen distribution in sediments.

Fluorescent sensors for other analytes have been made commercially available only in the field of medical diagnostics. Fluorescent optical sensors have been developed by several laboratories for marine research, but these have not reached the market yet. A sensor system for CO_2 was developed by C. Goyet and colleagues. Using a combination of fluorescent and absorptive indicator dyes, they succeeded in measuring pCO_2 in sea water with a mean relative error of about 2% compared to results from a gas chromatograph.

Luminescent pH sensing has also been reported. In the range from pH 6 to 9, a resolution of better than 0.1 pH is readily achieved.

A fluorescent indicator rather than a true sensor has been used to determine dissolved aluminum in sea water at the nanomolar level. The indicator salicylaldehyde picolinoylhydrazone complexed with aluminum gives a fluorescence peaking at 486 nm when excited at 384 nm. In the concentration range from 38 to 930 $\mathrm{nmol\,l^{-1}}$, a detection limit of 9.8 $\mathrm{nmol\,l^{-1}}$ and a precision of about 2% were achieved.

Numerous other sensors have been tested in the laboratory, but no proof of suitability for marine applications has been given. There are remarkable new approaches to the detection of nutrients (nitrate, phosphate), but in these cases the specifications needed for marine research have not yet been accomplished.

Conclusion

Numerous fluorescent sensing methods have been developed, but only a limited number have become standard methods in marine research. The most recent developments (decay-time sensors, three-dimensional fluorescence, lifetime imaging) offer up interesting prospects. In particular, the high long-term stability of decay time-based sensors make them prime candidates for instrumentation on unattended measuring stations needed for global ocean observing systems.

Symbols used

[A]	analyte concentration
Γ	radiative rate constant
I	fluorescence intensity
I_0	fluorescence intensity immediately after excitation
I_n	fluorescence intensity polarized perpendicular to the plane of incidence
I_p	fluorescence intensity polarized parallel to the plane of incidence
k	radiationless rate constant
k_{SV}	Stern–Volmer constant
λ_{ex}	excitation wavelength
λ_{em}	emission wavelength
Q	fluorescence quantum yield
P	fluorescence polarization
τ	fluorescence decay time
t	time

See also

Carbon Cycle. Fluorometry for Biological Sensing. Pollution: Effects on Marine Communities. Refractory Metals. Transition Metals and Heavy Metal Speciation.

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