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Review Article

Development of *In Situ* Sensors for Chlorophyll Concentration Measurement

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Chlorophyll fluorescence measurement is a sensitive and effective method to quantify and analyze freshwater and sea water phytoplankton *in situ*. Major improvements in optical design, electronic technology, and calibration protocol have increased the accuracy and reliability of the fluorometer. This review briefly describes the improvement of probe design, excitation light sources, detectors, and calibrations of *in situ* fluorometers. Firstly, various optical designs for increasing the efficiency of fluorescence measurement are discussed. Next, the development of electronic technology to meet and improve *in situ* measurement, including various light sources, detectors, and corresponding measurement protocols, is described. In addition, various calibration materials, procedures, and methods are recommended for different kinds of water. The conclusion discusses key trends and future perspectives for *in situ* fluorescence sensors.

1. Introduction

Chlorophyll fluorescence is the red light re-emitted by chlorophyll molecules when excited by a light source. Chlorophyll fluorescence is a noninvasive method for analyzing photosynthetic energy conversion of higher plants, algae, and bacteria. Fluorometry has long been used to study phytoplankton in natural aquatic environments. Typical applications include estimation of primary productivity [1–3] and phytoplankton distribution [4–6], understanding photosynthetic characteristics [7–11] and taxonomic discrimination [12, 13], and assessment of nutrient status [14–16] and toxins sensitivity [17–19]. Due to the unique fluorescent properties of chlorophyll (Chl) and accessory phycobiliprotein (PBP) pigments contained within phytoplankton cells, the fluorometer is regarded as a highly sensitive tool for the quantification and analysis of phytoplankton [20]. Spectrophotometry, high-performance liquid chromatography (HPLC), and fluorometry have been routinely used in laboratories for decades, but none of them can be used in the field, and thus require sampling and sample transportation. The sampling procedure is typically time consuming, and the samples to be measured have the potential to change during transportation. Chlorophyll concentration and harmful algal blooms over very large areas can be detected by remote monitoring of ocean color. However, small variations of Chl cannot be identified by such means. In contrast, *in situ* fluorometers offer continuous measurement of chlorophyll concentrations in the field. Furthermore, *in situ* fluorometers do not require pretreatment or a large sample volume; the method is simple, nondestructive, selective, sensitive, and rapid [21]. On the other hand, fluorescence is weak, unstable, and easily influenced by environment.

A variety of methods and technologies have contributed to the design, construction, and application of *in situ* fluorometers. The first recorded use of a fluorometer was for continuous measurement of *in vivo* chlorophyll concentration by a modified model III Turner fluorometer in 1966 [22]. This method was valid and proved to be an extremely useful tool in broad ecological programs carried out at sea. However, the *in vivo* Chl *a* fluorescence technique for phytoplankton depends on the effective absorption and fluorescence quantum yield

of Chl *a*. Loftus and Seliger [23] reported that the range of variation in the ratio of *in vivo* fluorescence to extractable Chl *a* reached nearly 10-fold with significant areal and seasonal differences in addition to ambient light dependent fluorescence inhibition. Thus, the major challenge of *in situ* fluorometers is the accuracy and stability.

Given that the accuracy and stability of *in vivo* and *in situ* fluorescence are influenced by the environment in which phytoplankton lives, much literature has reported the different fluorescence values of phytoplankton community structure [24], temperature, turbidity, nutrient limitation, and irradiance [25, 26]. Moreover, the value of Chl *a* fluorescence is affected by yellow substances [27, 28], chromophoric dissolved organic matter (CDOM) [29], and biofouling [30, 31]. All of these factors can be regarded as objective factors. At the same time, some active factors such as design and operation may also influence fluorescence measurement. Thus, better designs and methods of operation can improve accuracy, limit of detection (LOD), resolution, and stability. To date, numerous types of commercial *in situ* chlorophyll fluorometers are available, some of which were surveyed and evaluated by the Alliance for Coastal Technologies [32] in 2005. Generally, almost any instrument based on optical fluorescence detection contains the following basic subsystems (Figure 1): light source, detector, light guide, wavelength-selection device, and signal processing electronics [33]. With the development of optics and electronics, various high-performance fluorometers with a combination of those subsystems have been developed and put into use. Moreover, noteworthy advances have been made in selecting excitation light sources, excitation protocol, detectors, and calibration methods, each of which has helped improve accuracy or stability.

The theory, measurement protocols, and application for the *in situ* measurement of phytoplankton fluorescence were reviewed by Babin in 2008 [34]. The present review focuses on developments of the accuracy and stability of Chl *a* measurement based on fluorometers, with primary emphasis on literature published in journals and proceedings rather than on information from manufacturers. Recent novel developments are highlighted, and anticipated future trends are discussed.

2. Probe Design

Probe design, referring to the coupling of the light source and detector, is of great importance for optical sensors. The most popular structure of the benchtop uses a cuvette, which is the cell of water sample. For *in situ* measurement, good probe structure can enhance not only the efficiency of coupling but also the signal stability. In order to reduce the omnipresent interference signal from excitation or ambient light, and to improve signal-to-noise ratio (SNR), filtering is a common method. Moreover, with optical fibers transmitting the excited light and emission fluorescence, remote and microscale measurements can be carried out [35]. Following are some special designs for improving the signal collection with regard to optical design and detecting angle.

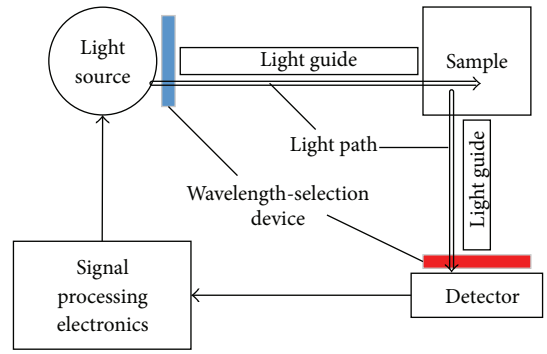

Figure 1: Components of a fluorometer.

2.1. Optical Design. The purpose of optical design is to receive as much useful signal and as few interferences as possible where the fluorescence emission of *in situ* chlorophyll is very weak. Optical fiber, which is favored for its well-known advantages of being lightweight, flexible, low-loss, and cost-effective and having remote-monitoring capability, can be used both to transmit light signals and as a probe. Both the exciting light and the fluorescence from algae chlorophyll can be transmitted along a fiber bundle, which provides a very versatile means for *in situ* fluorescence of natural water [36]. By changing variable source/receiver combinations, this method is also applicable to other *in situ* natural targets. Optical fiber probes can be designed in various types. Most of the optical fiber probes are flat face design, which consist of both excitation source and receiver on a single flat surface. Some other structures have emerged in recent years, such as microscale probe, optical fiber probe, and some special structures. In addition, light baffle is key to reducing interference from ambient light. Following are some special structure designs developed to improve measurement.

In order to reduce interference from ambient red light, especially during the daytime, a series of light baffles designs were constructed and tested by D'Sa et al. [37]. The selected design of the light baffle was constructed out of PVC and composed of three cylinders. Two inverted V-shaped 360° openings around the baffle allowed water to flow through the baffle and the dual fiber sampling volume. The measurements indicated that the baffle was very efficient at minimizing any contributions of diel ambient light variations to the fluorescence signal.

In order to achieve a stable fluorescent and absorbing measurement in the water column, Barth et al. [38] developed two instruments. One was a multichannel fluorometer which was emphasized by long-term stability. A short-arc flash lamp and bandwidth interference filters (10 nm) were used to achieve 420 nm (to excite fluorescence), 270 nm, and 530 nm (to excite Raman) excitation light. The optical fibers from the light source assembly were fed to two probing heads for 270/530 nm and 420 nm excitation, respectively, positioned above two fused silica windows. Six detection fibers surrounded the excitation fiber in a conical position with the tilt angle such that the fields of view coincided well outside the probe housing. The two probes had a common

ray path of excitation and emission. The Raman scattering was used to calibrate sensitivity and to correct the data of the measuring volume due to variable turbidity of the water, variations of the lamp intensity, and weak contaminations of window surfaces.

In order to perform high-resolution measurements of *in situ* chlorophyll and turbidity, a microscale optical probe was developed to sample the biooptical properties of undisturbed water by Wolk et al. [39]. The combined chlorophyll/turbidity sensor had six Light Emitting Diodes (LEDs) arranged on a circumference with a diameter of 20 mm and canted in such a way that their collimated beams were mutually orthogonal. The beams intersected at one focal point, the sampling volume, located 15 mm above the center of the LED circle and in front of the fluorescent receiver diode. The second receiver diode calculated turbidity by measuring the intensity of backscatter light from suspended particles. Because of the small size, rounded shape, and "sideways look," the small scale structures of fluorescence and turbidity were not destroyed by flow deformation. The sensitivity and spatial resolution of the probe were corroborated by testing in field and laboratory experiments.

A simple, compact, and highly sensitive capillary-based probe for the *in situ* detection of fluorescence signals with high sensitivity was demonstrated by Long et al. [40]. They proposed a conceptually straightforward theoretical model to optimize the factors affecting the fluorescence-capture capability of the capillary-based probe. By measuring the fluorescence spectra of Cy5.5 dye and blue-green algae, the proposed probe provided more than a tenfold increase in the fluorescence signal detected compared with direct measurements by a flat-tipped multimode fiber (MMF) probe.

A new "T" optical design was used to improve the efficiency of fluorescence excitation and collection by Chekalyuk and Hafez [41]. The "T" optical configuration included two emission collection-filtration optical units, ECF1 and ECF2. The "T" optical scheme was designed to direct this reflected emission for analysis by the sensor associated with the ECF unit located in the opposite shoulder of the "T" scheme. In comparison to a conventional 90-degree optical scheme, this setup provided up to a fourfold increase in overall laser-stimulated emission (LSE) due to the doubling of excitation intensity via reflection of the excitation beam into the sample by a 100% mirror. Meanwhile, the intensity of collected LSE signal was doubled by reflection of interference filters F1 and F2. A commercial version named Aquatic Laser Fluorescence Analyzer [42] has been used in the field, but only in flow-through measurement.

Very recently, a lensless miniature portable fluorometer [43] was designed to measure chlorophyll and CDOM concentration in aquatic environments. In order to obtain better spatial resolution and light collection efficiency as well as smaller size, power, and cost, contact fluorescent imaging method was used. This method utilized LEDs for fluorescence excitation and a single contact fluorescent imaging pixel array for fluorescence collection. Considering the low concentration of chlorophyll and overlap of excitation and emission light, researchers used absorption filters with ultrathin glass strips to attenuate excitation light. Although the limit of detection was 0.7 nM (0.625 μg/L), the price was below $500.

It has been concluded that theoretical models and numerical simulations are necessary to improve the design of fiber probes. However, because of the small numerical aperture, variability, and low quantum yield, detection of fluorescence is challenging for a fiber-optic probe.

2.2. Detecting Angle. The angle between excitation and emission light is important for collection efficiency and minimizing interference. As is known, the beams of excitation and emission are orthogonal in most benchtop and *in situ* fluorometers. However, for optimizing the structure and improving SNR of *in situ* instruments, other angles have also been reported as improving SNR. Ma et al. [44] proposed a conceptually straightforward theoretical model to optimize the factors affecting the fluorescence-capture capability of a bifurcated/coaxial fiber-optic probe.

Doubell et al. [45] described a laser fluorescence-based sensor to measure *in situ* chlorophyll fluorescence distribution to millimeter scales. They used a blue diode laser for chlorophyll excitation (peak 410 nm). The beam was projected at 45° outward and into the oncoming flow. The positioning of both the excitation and the receiver diode on a single flat surface further reduced the possibility of recirculation within the sample volume due to mixing caused by irregularities in the probe shape.

In order to characterize a dual-fiber-optic sensor for measurement of Chl fluorescence in aquatic environments, D'Sa and Lohrenz [46] described a theoretical model. A numerical simulation was developed to approximate the optical geometry of the dual-fiber-optic sensor. This permitted a visual representation of the fluorescence distribution within the sensor sampling volume. A Monte Carlo simulation was used to evaluate sampling variability associated with the number and distribution of particles within the sampling volume. It was proved that laboratory observations and previously published results were generally consistent with model predictions.

Kulchin et al. [47] designed a special immersible module. The exciting and measuring channels were of separate design, and the light beams were not orthometric. The emitting optical fiber was perpendicular to the flat glass surface, while the receiving fiber was disposed at an angle of 26° to the normal. In this case, the fluorescence zone grew in size; meanwhile, it reduced the influence of laser radiation propagating inside the glass and penetrating into the receiving fiber. In order to protect the photosensitive receiving part from direct and scattered sunlight, a metal construction was designed. The design as a whole improved reliability and reduced background illumination.

An opposite structure was used in a polydimethylsiloxane-based microfluidic chip, which integrated filters, source, detector, and electronically controlled valves [48]. A very compact low-cost LED with a peak emission wavelength of 430 nm served as the excitation source. A silicon detector, located on the side of the microchannel opposite the source, was placed as close as possible to the channel to maximize the

captured fluorescence signal. To achieve the highest possible detection sensitivity from the opposite structure, two filters were used for detector shielding from the excitation source signal. One filter was the excitation source filter, which passed the low wavelength signal (430 nm) while attenuating any signal the source produced at the fluorescence signal wavelengths (662 nm). The second filter was a metal/dielectric detector filter, which blocked the excitation source while passing the fluorescence source.

Detecting angle is related to optical structure and is crucial to receiving maximum fluorescence. By combining an appropriate detecting angle with a highly selective filter, interference signals can be minimized. As a result, the signal-to-noise ratio (SNR) can be maximized to improve the accuracy and stability.

3. Excitation Source

The excitation source can greatly affect the quality of fluorescence signal because of its importance as a prerequisite of light-induced fluorescence measurement. Various light sources can be used to stimulate fluorescence of Chl *a*, such as lamps, lasers, and LED. Different light sources have their own advantages and disadvantages. Thus, a well-selected light source and good excitation protocol can improve accuracy and reduce interference, size, and cost. Following are the most relevant achievements related to the scope of this paper.

3.1. Various Light Sources

3.1.1. Lamps. Broadband lamps, which are bulky and have high power requirements, were used in initial benchtop fluorometers. In order to minimize power and optimize the excitation of fluorescence, a flashing xenon lamp was chosen as the radiation source [49, 50]. However, broadband radiation has the potential to cause overlap with fluorescence. Consequently, one or more blue optical filters were typically used to reduce interference from other spectra in the region.

Desiderio et al. [51] designed a multiple excitation fluorometer with quartz-tungsten-halogen lamp, which was filtered by three interference filters in a filter wheel. With the forward-scatter fluorescence geometry and Spectralon as the sample cell material, the sensitivity was increased. The data show that as little as 0.1 μg chlorophyll/L could be detected in a single shot (averaging 100 spectra per 10 s data-acquisition time).

Broadband lamps are also used in multiparameter instruments. McKee et al. [52] used a pulsed xenon flash lamp filtered to provide wavelengths between 400 and 500 nm. This allowed the yellow substances, suspended particles, and phytoplankton to be measured simultaneously through absorption, scattering, and fluorescence. Otherwise, the broadband excitation results in respective broadening of the Raman spectral band, an effect that significantly complicates its discrimination from the constituent fluorescence.

Another reason for using broadband lamps is to calibrate the interference of other components in water. As is known, single wavelength instruments meet the demands of low exposure of samples and fast response time. On the other hand, single wavelength measurement does not allow measurement of interfering signals, where deconvolution using several spectral points is needed to extract contributions from individual components. Measuring light-induced difference spectra "wavelength by wavelength" is time consuming, requires high stability of the sample during measurement, and especially prevents recording of spectra of unrepeatable transients [53].

Because of their size and power requirements, broadband lamps are seldom used for *in situ* measurement at present. However, some special applications such as single-cell fluorescence excitation spectroscopy systems [54] and multivariate optical computing instruments [55] require super high power Xe arc lamps (75 W).

3.1.2. Lasers. Lasers emit coherent light beams of high intensity and directionality, which can improve selectivity and efficiency of excitation and reduce the spectral overlap between the water Raman scattering and fluorescence bands of aquatic constituents. A prototype laser/fiber-optic system for *in situ* detection of ocean chlorophyll fluorescence was described by Cowles et al. in 1989 [56]. They used an air-cooled argon laser with a wavelength of 488 nm as the excitation and used a He-Ne laser in the alignment procedure. A series of measurements indicated that ambient Chl concentrations could be detected *in situ* and could be calibrated using Raman scattering signal. Though lasers have many advantages, the earlier generation lasers have significant disadvantages, which are relatively large size, cost and power consumption, and a limited number of excitation wavelengths.

Laser diodes also provide high energy output, monochromaticity, and broad wavelength availability from 375 nm to infrared, though some are cost prohibitive. The wavelengths of 410 nm [57] and 660 nm [58] were commonly used as excitation in the 1990s. Recently, blue and green narrowband laser excitation has been used to selectively stimulate the constituent fluorescence and simplify the overlapped spectral patterns [59]. An Advanced Laser Fluorometry (ALF) employing a new single 510 nm laser was developed by Chekalyuk and colleagues [41]. It was capable of reasonably comprehensive characterization of aquatic fluorescence constituents, including Chl *a*, PBP pigments, variable fluorescence (F_v/F_m, a measure of the potential quantum yield, here, $F_v = F_m - F_o$, where F_o was the minimal fluorescence and F_m was the maximal fluorescence), and CDOM in estuarine and fresh waters. In order to measure phytoplankton photophysiological assessments and spectral discrimination between oil and CDOM fluorescence, additional UV (375 nm) and blue (405 nm) light were used together with a 510 nm laser diode. The three-laser ALF provided additional variable fluorescence with 405 nm excitation for improved measurement of Chl *a*.

When an Nd:YLF laser diode with 440 nm was off-the-shelf, Bensky et al. [60] used a train of 70 ns flashes from a laser source (440 nm) to stimulate Chl *a* fluorescence. The resulting fluorescence at 685 nm was instantaneously

recorded during each laser pulse using a streak camera. Because of the nanosecond time scale, the short laser pulse did not reveal any pulse-to-pulse hysteresis such as that seen with pump sources with flashes lasting milliseconds. The lack of pulse-to-pulse hysteresis proved useful for direct phytoplankton mapping as a function of concentration since the fluorescent emission from the plankton is linear with pump energy.

Having a somewhat complicated drive and high cost, laser diodes have not been widely used in *in situ* fluorometers. The development of laser diodes having higher performance and lower cost will allow for their greater applicability in fluorometers.

3.1.3. LEDs. Light Emitting Diodes (LEDs) are a new and more efficient alternative for lighting. The advantages of LED commonly include the following: small size, less heat, higher efficiency, lower cost, simpler drive, and longer lifetime. Commercially available LEDs ranging from ultraviolet to near-infrared wavelengths and having different output powers and encapsulations can be selected. For example, in order to measure *in situ* Chl *a* concentrations related to individual algal groups, an exact LED wavelength is selected in order to excite individual algae. Moreover, multiple wavelengths can be used to distinguish and correct different phytoplankton group compositions. Recently, LEDs have also been used as actinic light, which is applied to drive photosynthesis. Following are the most relevant achievements using LED excitation.

Narrow band LED is an alternative to traditional lamp or laser light. Karsten et al. [61] developed a growth fluorometer using four bright blue LEDs with a peak wavelength of 450 nm. The fluorometer had an extremely high sensitivity for investigating the growth of adhering phototrophic microorganisms. Lamb et al. [62] designed a fluorometer that used three monochromatic LEDs of 435, 457, and 470 nm as excitation of Chl *a*, total chlorophyll, and Chl *b* concentrations, respectively. The fluorometer was used to determine Chl *a* and Chl *b* concentrations in a variety of organisms containing different ratios of chlorophylls.

Multiwavelength LEDs are used to quantify total Chl *a* and estimate the phytoplankton group compositions. Five LEDs with distinctive wavelengths were used to excite and differentiate "spectral groups" of microalgae *in vivo* and *in situ* by Beutler et al. [63]. Firstly, the norm spectra of four spectral algal groups were obtained in advance. Using these norm spectra and actual five-point excitation spectrum of a water sample, a separate estimate of the respective Chl concentration was rapidly obtained for each algal group. An integrated multiwavelength fluorescence sensors prototype was fabricated by Starikov et al. [64] using solid-state components. Those researchers used eight LEDs with different colors from UV to red, which were arranged in a circular design and mounted in light tight housing. The sensors could be operated in the absorption scattering and fluorescence mode, which exhibited a lower detection limit and a larger dynamic range. Moreover, a multiexcitation fluorometer with nine wavelength excitation LEDs was developed and evaluated by Yoshida et al. [65]. After measuring the nine excitation spectra, using a mathematical process, and solving the optimization problem, the total phytoplankton biomass was quantified, and the phytoplankton group compositions were estimated. In order to gather more data, multiexcitation and mathematical processes are both necessary and effective.

In addition to using narrow wavelength LED as excitation (LED pulse light as the measuring light), broadband LEDs can be used as actinic light (to drive photosynthesis). This application is due to the development of white LEDs with superior performance in terms of both power efficiency and emission spectrum. The first fluorometers (PAM 2000 and PAM 2500) were developed with red light (LEDs) as measuring or actinic light. Later fluorometers with broadband blue light (LEDs) were introduced in Mini PAM, Water PAM, Junior PAM, Monitoring PAM, and Pocket PAM (Walz GmbH, Effeltrich, Germany). Other fluorometers, namely, Phyto-PAM and Multicolor PAM [66], used narrow wavelengths as measuring and actinic light. Multicolor PAM in particular is a very versatile instrument that provides six colors of pulse-modulated measuring light (400, 440, 480, 540, 590, and 625 nm) and five colors of actinic light (440, 480, 540, 590, and 625), in addition to having white (420–640 nm) and far-red (730 nm) light sources. This allows adjustment to suit very different phytoplankton groups.

We can conclude that LEDs are very suitable to *in situ* fluorometers. However, directionality is a serious shortcoming, especially as compared to laser diodes. This problem could be addressed to a great extent by a collimator lens and optical fibers.

3.2. Excitation Method. Unlike sun-induced chlorophyll fluorescence, fluorometers always use various active light sources. Variable fluorescence, introduced from plant physiology, is used to study and monitor phytoplankton physiology. Huot and Babin described the theory, basic concepts, and practice of fluorescence protocols [67]. Here, we will describe the excitation method from the viewpoint of how they have improved measurement accuracy. To improve the accuracy of fluorescence concentration measurements, it is desirable to minimize a potential variability in the fluorescence efficiency associated with environmental factors or constituent functional state. This can be achieved by appropriate selection of the measurement protocol [68]. On the other hand, the variability of *in vivo* Chl *a* fluorescence can be stimulated using various active fluorescence techniques to retrieve valuable information about phytoplankton photophysiology and photochemical efficiency [41].

The protocols for Pulse Amplitude Modulation (PAM) [69], Pump-and-Probe (P&P) [70], and Fast Repetition Rate (FRR) [71] are used in commercial variable-fluorescence sensors. The principle of PAM is to selectively monitor the fluorescence yield of a weak measuring beam; it is not affected by even extremely high intensities of actinic light. By repetitive application of short light pulses of saturating intensity, the fluorescence yield at complete suppression of photochemical quenching is repetitively recorded, allowing

the determination of continuous plots of photochemical quenching and nonphotochemical quenching. Currently, based on the PAM principle, products are sold commercially as various models (bench top, submarine, and imager) by Heinz Walz GmbH (Effeltrich, Germany). Very recently, a new type of multicolor PAM chlorophyll fluorometer [66] demonstrated high accuracy and reliability for measurements of photodamage [72]. Numerous applications have used various fluorometers based on PAMs to estimate photosynthetic activity, biomass productivity, and related factors [73]. However, Beer and Axelsson reported that the PAM fluorometry was limited when measuring photosynthetic rates of macroalgae at high irradiances [74].

Pump-and-Probe (P&P) method measures fluorescence by a weak "probe" actinic flash before and after a single saturating "pump" flash. The "pump" flash is short enough and intense enough to saturate all photosystem II (PSII) reaction centers almost instantaneously. A detailed description and comparison with PAM fluorometry are given in various sources [75, 76]. Based on the P&P approach, some fluorometers have been used directly on phytoplankton assemblages in the natural aquatic environment [77, 78]. Recently, Kocsis et al. [79] developed a new kinetic fluorometer based on P&P technique with detailed mechanical, optical, and electronic layout. A single laser diode was used for both pumping and probing. The time resolution of the fluorometer was limited by the repetition time of the probing flashes to 20 μs. The apparatus offered high sensitivity and excellent performance and could become a versatile device for a range of demanding applications.

Fast Repetition Rate (FRR), evolved from Pump-and-Probe, induces fluorescence transiently by initially delivering a series of subsaturating high intensity pulses followed by a series of more widely spaced "probing" flashlets that examine the subsequent fluorescence decay [71]. Compared to PAM and P&P protocols, FRR has been shown especially appropriate for high frequency measurements *in situ* [80–82]. The basic theory and applications in aquatics can be seen in Suggett et al. [75].

In conclusion, the three variable-fluorescence methods can determine photosynthetic parameters of natural phytoplankton. A detailed comparison of different variable Chl *a* fluorescence techniques was compiled by Röttgers [83]. For determining the concentration of Chl, simple pulse modulation is commonly used. Meanwhile, there are some special excitation methods that could provide unique perspectives on fluorescence measurement. Although fluorescence can be measured precisely by PAM technology, the energy of pulse-modulated fluorescence cannot be used effectively at the same time due to sidelobes in the frequency domain. Therefore, Zhang et al. [84] changed the Pulse Amplitude Modulation to the sinusoidal amplitude modulation (SAM), which enhances the utilization ratio of the fluorescence energy. A laboratory-based SAM Chl fluorometer was presented for phytoplankton classified measure. In contrast to a PAM fluorometer, which is excited by sequentially switching each light source, the SAM fluorometer used three high power LEDs (470, 520, and 590 nm) simultaneously excited by three different modulation frequencies (800, 500, and 200 Hz). Detection time was shortened to 1 s. The SAM fluorometer achieved a better detection limit, as low as 0.005 μg/L.

A novel phase fluorometer was designed and demonstrated based on fluorescence lifetime and time-correlated single-photon counting (TCSPC) [85]. The fluorometer used a blue LED driven by an oscillator with a sinusoidal signal instead of a pulse of light. The LED driver had 80 MHz bandwidth and 110 mA drive current. The fluorescence signal was detected by a high-speed, low-capacitance, and wide bandwidth (1 GHz) silicon photodiode, after which the phase difference between the fluorescence and reference was measured. Unfortunately, LOD with 3.5 μM (3127 μg/L) for chlorophyll was inferior. By combining with simulations for steeper cutoff and higher quality optical filters, the detection limit of chlorophyll decreased to 0.5 μM (446.7 μg/L), which remained unsatisfactory. However, the phase fluorometer approach could be considered to design *in situ* instruments.

A pseudorandom sequence modulation was introduced into the amplitude measurement of the fluorescence by Hu and colleagues [86]. Without increasing the complexity of the system's hardware, the method spread fluorescence and improved the sensitivity and interference suppression ability of the sensor. Experiments show that the sensor had a minimum detectable level of 0.0103 μg/L within the concentration range of 0~25 μg/L. The pseudorandom sequence method remarkably improved sensitivity and interference suppression ability of the chlorophyll sensor and could be further applied in other ultraweak signal amplitude measurements.

4. Detectors

A detector is the receiver of the fluorescence. Photomultiplier tubes (PMTs), photodiodes (PD), and array detectors (such as charged-coupled devices, CCD) are always used to quantify the fluorescence. Because chlorophyll fluorescence is very weak, especially *in vivo* and *in situ*, selecting a high-performance detector with low noise can improve both the detection limit and the measurement accuracy.

4.1. PMT. PMT has a high gain, low noise, ultrafast response, and large area of emission collection. Moreover, in order to improve selectivity, chlorophyll fluorescence detection is most often performed using a PMT in combination with an appropriate emission filter. Because of the large volume and high voltage needed, the original *in vivo* fluorometer with PMT detector used shipboard operation [22].

Beutler and colleagues developed several fluorometers for phytoplankton analysis *in situ*. Every generation of fluorometer has its own characteristics. At the outset [63], a PMT (H6779-01, Hamamatsu) was used to detect chlorophyll fluorescence. Later [87], three PMTs were used behind band-pass filters (center wavelength/half width: 650/10, 685/10, and 725/25 nm), which allowed for the study of cyanobacteria and other phytoplankton *in vivo* and *in situ*. Subsequently, in order to study red cyanobacteria and cryptophyta, four PMTs were used [88]. A new flow-through fluorometer was built with a PMT and a PD [89]. The PD received 5% of the excitation light, and the output of the PD was used

for feedback control [90] of light intensity. The fluorescence detector was a PMT behind a 685 nm band-pass filter. The fluorometer used six LEDs for measuring light and four laser diodes for maximal fluorescence (F_m). The six LEDs were switched on for 125 μs in sequential order, with a dark interval of 125 μs between two diodes. The laser diodes were chopped by 8 kHz, which is double the frequency of the LEDs. In order to prevent overload of the PMT, the detector was switched sensitive for 62.5 μs in the second halves of the light-on and light-off phases, respectively, of the LEDs.

A new small PMT module (H10721-20, Hamamatsu), which provided enhanced sensitivity in red spectral area of Chl *a* fluorescence, was used in ALF-T [41]. It also took advantage of a 12-bit waveform digitizer (PS4224, PicoScope) with increased input sensitivity to improve measurements of laser-stimulated emission (LSE) fluorescence induction. Thus, ultralow Chl *a* concentration could be detected, even below 0.01 μg/L.

In conclusion, PMT has a better performance for weak Chl fluorescence. However, the application of PMT on *in situ* fluorometers is limited by size and power because traditional PMT always requires high voltage, which is difficult to supply in the field. However, with the development of electronics and optics, some PMT modules that require low externally powered DC voltages (+5 V or +12 V) are now available. There exists a possibility of developing fluorometers based on PMT with higher accuracy for *in situ* measurement.

4.2. Photodiode. Silicon photodiodes are suitable for *in situ* fluorometers because of their good response time, small size, and low cost, though photodiodes have higher noise and interference immunity than PMT. The development of PD technique has promoted and accelerated the performance of *in situ* fluorometers [64]. Additionally, there exist some techniques to improve the SNR.

Because of the weak current signal detected from PD, the transimpedance amplifier has been used to translate the current to voltage first [85]. In order to simplify and improve the design, a monolithic PD with on-chip transimpedance amplifier was used by Lamb et al. [62].

In order to offset the high noise of PD, Aiken [50] used two low noise operational amplifiers producing a nonlinear response. The first operational amplifier was used in non-inverting mode to minimize PD noise. Then, the amplified pulse of fluorescence was "peak detected" and converted to DC signal by two sample-and-hold amplifiers in tandem. The sample-and-hold amplifiers were triggered and reset by the signal from an auxiliary PD monitoring the flash from the source directly, thus isolating the flash gun and detector circuitry to eliminate noise and spurious pick-up.

As mentioned above, PD was also used as a reference detector by receiving a small portion of excitation light by a beamsplitter. With the reference signal as the feedback control of light source, the intensity could be kept constant during the illumination [90]. In addition, the reference signal was used to calculate the fluorescence yield, which was calculated as the ratio of the fluorescence to the reference signal [91].

4.3. Array Detectors. Array detectors, which are used to study heterogeneity and single-cell photosynthesis, have the ability to record multichannel or fluorescence imaging. CCD array detectors are always selected for their ultrahigh quantum efficiency, very small dark current, and extremely low noise level.

A compact, portable, and microscopic-enabled imaging system was presented by Trampe and colleagues [92]. The system, using a high-speed CCD camera, connected to an epifluorescence microscope to achieve multicolor chlorophyll fluorescence imaging of aquatic phototrophs. The variable fluorescence was excited by different combinations of blue, green, red, and white light. Using the system, the photosynthetic activity in complex mixtures of phototrophs and natural samples can thus be assigned to different types of phototrophs, which can be quantified simultaneously.

In order to explore the microscale variations in the phytoplankton, a Free-Falling Imaging Device [93] was developed with a planar laser imaging fluorometer (PLIF). The PLIF used a 3 W 532 nm diode-pumped solid-state laser and a very sensitive CCD camera. After being carefully calibrated in the lab, the system could detect and quantify the Chl *a* fluorescence from individual cells as small as 5 μm. Another similar system, *in situ* Video Fluorescence Analysis [94], could resolve fluorescent particles ranging from 6 μm to several millimeters. Moreover, it was discovered that total image brightness values functioned as a suitable proxy for the estimation of the chlorophyll concentration.

In order to avoid interference from CDOM or suspended particulate matter, an instrument based on a Nikon TE2000-U microscope to position individual phytoplankton cells for confocal fluorescence excitation spectroscopy was designed by Hill et al. [54, 95].

Very recently, Swanstrom et al. [96] developed a dynamic-flow fluorescence imaging filter photometer, which uses an imaging CCD array to record fluorescence. Before this development, the theoretical characteristics of the designs were described in determining which optical elements were selected for fabrication [55]. The same researchers present a semiautomatic approach for extracting fluorescence intensities from the imaging photometer data along with a quantitative analysis of factors contributing to noise, including the detector read and dark noise.

Image analysis has higher resolution than other automated phytoplankton investigation methods due to an integrated analysis of different features, such as size, morphology, volume, and physiological aspects [97]. With further development of electronics and optics, higher resolution and more compact array detectors will replace single detectors in the study of natural water.

5. Calibration

Calibration is a critical step for sensor application. For *in situ* measurement, Chl fluorescence is susceptible to variation caused by operating environment, biofouling, instrument design, sensor drift, and calibration rigor [98]. Moreover, natural populations and physiology [68, 99] vary in both

space and time, so it is required to collect and use natural communities from the site of interest [100]. In order to improve and evaluate the accuracy, reliability, and stability of a fluorometer, appropriate calibration materials, procedures, and methods are necessary.

5.1. Calibration Materials. Calibration material, as a standard sample, is a known magnitude or concentration used to calibrate instruments. Pure Chl *a*, dyes, algae cultures, and field water samples are used as the standard and measured by HPLC or exacted methods.

Pure Chl *a* or dyes can be used to calibrate fluorometers in the laboratory by standard methods. There are many kinds of liquid dyes with absorption and emission in regions similar to pure Chl *a*. The most popular dyes are Fluorescein Sodium Salt, Rhodamine WT Red, Rhodamine B, and Basic Blue 3, which are offered by various suppliers [98]. However, occasionally the liquid dye calibration is not suited to the field, where the operating conditions may be difficult. Thus, solid fluorescent materials, trialed by Earp et al. [98], are recommended as reference standards for *in situ* fluorometers.

Dyes, and even pure or exacted Chl *a*, have different fluorescence intensity from the *in vivo* chlorophyll cell in natural populations [101]. In order to improve the accuracy among various species, pure phytoplankton cultures have been used as a calibration sample [37, 102]. Moreover, Lawrenz and Richardson [100] determined how the species used for calibration affects the accuracy of *in situ* Chl *a* measurements using single-species cultures. They posit that carefully selected calibration species can improve estimates of *in vivo* Chl *a* in the laboratory, whereas calibration of *in situ* fluorometers should be done with natural communities collected from the site of interest.

The reliability of fluorescence-based Chl estimation strongly depends on the group specific calibration of the instrument and the resulting chlorophyll/fluorescence (Chl/F) ratios in reference algal cultures [103]. In case of constant Chl/F-ratios, a very high reliability was obtained. However, Chl/F ratios exhibit variations with different light intensities and environments. In addition to frequent inspection and regular calibration, accuracy can be improved through proper calibration procedures and methods.

5.2. Calibration Procedures and Methods. The calibration of fluorometers is a complex process. The general procedure to calibrate a fluorometer consists of (i) precalibration with tests of pressure and mechanical and electronic stability and precision, (ii) signal output calibration to measure the dark and maximal counts, (iii) internal temperature calibration, (iv) determination and record of offset values of pure water at different temperatures, and (v) manufacturer calibration to obtain the scale factor of the fluorometer. These details are referenced in the white book by D'Ortenzio et al. [104]. However, the general and manufacturer calibration procedures are too simple to meet scientific requirements; furthermore, calibration must be verified regularly due to species and environment variation within space and time and lamp and sensor performance degradation over time [105]. Thus, fluorometers need to be calibrated with multistep, pre- or postcalibration procedures, and through special methods according to different situations.

Light intensities, especially high light levels, must be eliminated or accounted for to accurately calibrate fluorescence. Hersh and Leo [106] developed an inverse multiple regression model that included terms for both irradiance and chlorophyll. The new model method was able to calibrate the fluorescence probe more accurately. Xing et al. [107] applied a two-step procedure for accurate retrieval of Chl *a* concentrations acquired by an *in vivo* fluorometer: first, a predeployment intercalibration with accurate determination by high-performance liquid chromatography (HPLC) analysis, which not only calibrated fluorescence in appropriate Chl *a* concentration units, but also strongly reduced variability between fluorometers; second, a profile-by-profile quenching correction analysis, which effectively eliminated the fluorescence quenching issue at surface around noon and resulted in consistent profiles between day and night.

For the difficulty of quantitative and qualitative assessment of microphytobenthos populations *in situ*, Aberle et al. used a benthic sensor (BentoFluor) [28]. In order to improve accuracy, precalibration and advanced calibration procedures were carried out. The precalibration procedure included (1) suspensions of planktonic microalgae measured by an external company (bbe Moldaenke), (2) these algae filtered onto GFF-filters and measured by the BentoFluor, and (3) these filters extracted in 100% acetone and the chlorophyll concentrations measured in the HPLC. The precalibration was found to be sufficiently linear in the laboratory. The advanced calibration used the benthopelagic and purely benthic culture suspensions of microalgae first filtered onto Whatman GFF filters and measured with the probe and subsequently extracted and measured in HPLC. Moreover, when measuring the microphytobenthic biomass by Chl *a* fluorometer, it was possible for overestimation to occur due to the reflection of the substratum. In addition to regular calibration, an additional calibration step for each individual type of superfluous substratum needed to be carried out. Carpentier et al. [108] used a 700 nm signal to eliminate this effect by quantifying the fluorescence signal as a result of the reflection.

Furthermore, postcalibration is important when the environment is frequently variable. As mentioned above, the *in vivo* fluorescent response of phytoplankton is affected by numerous factors, which makes calibration prior to deployment with laboratory cultures (grown under optimal conditions) only an approximation of the fluorescent response found in the field. Pavlac et al. found it to be essential to back-calibrate the fluorometric data using the extracted discrete pigment samples [4]. Bastien et al. [109] compared and validated the performance of two field probes (YSI 6600 and TriOS) used for measurement of *in vivo* phycocyanin fluorescence. The observation makes the YSI a qualitative tool if postcalibration is not performed.

Because of the multiple components in natural situations, multiexcitation wavelength is used to distinguish

algal populations by fluorometer. With that, multivariate calibration methods are used to provide accurate analytic estimations in the presence of overlapping, uncalibrated spectral interferences [21, 110]. Compared to classical least squares and principal component regression multivariate calibration methods, the partial least squares (PLS) method gives the closest predictions for all taxonomic groups and provides the accuracy needed for phytoplankton bloom detection. PLS is especially suitable when spectra from different constituents are overlapping, the background noise is both high and variable, and not all of the optically active compounds are known [111, 112].

A comprehensive calibration for multispectral chlorophyll fluorescence (ECO Triplet class) was carried out by Proctor and Roesler [102]. They calibrated the sensors with thirteen monospecific cultures in the laboratory, which were grown under limiting and saturating irradiance and sampled at different growth phases. Protocols for reducing sensor-related uncertainties as well as environmental-related uncertainties were developed. The three different values of fluorescence excited by three LEDs and fluorescence ratios provided a mean not only for approximating bulk taxonomic composition but also for selecting the appropriate calibration slope to statistically improve the accuracy of *in situ* chlorophyll concentration estimates. Indeed, application of specific calibration (species-specific spectral fluorescence signatures) greatly improved the FluoroProbe (bbe Moldaenke GmbH, Germany) data with those of the reference methods [99]. In this sense, a library of species-specific signatures would be highly desirable.

Another kind of calibration concerns the Chl concentration of *in situ* fluorometers installed on unpiloted vehicles and drifting buoys. In order to map microscale phytoplankton distributions, some high-resolution fluorometers have been used [113, 114]. Though careful and frequent calibrations were done, numerous peaks or spikes were observed in the raw data. It is unknown whether these peaks represent increases in biomass or are due to the detection of discrete fluorescent particles [115]. In order to analyze the spikes, Briggs et al. [116] carried out cross-calibration through a series of nearly simultaneous ship CTD and glider profiles.

Because of the complex composition of natural water, the acquisition of more information and multiway techniques allow [117] for more accurate measurements. In conclusion, some practices can improve the accuracy of *in situ* Chl measurement: (i) calibration performed using field samples from the study site; (ii) frequent calibration to account for changes in phytoplankton community composition and light and nutrient history; (iii) precalibration, postcalibration, and multistep calibration as necessary; (iv) measurement after acclimating the field sample to the dark for 20–30 min; and (v) calculation of a mean F/Chl ratio over the surface mixed layer or diel cycle instead of the application of a fixed F/Chl ratio [100]. Additionally, in order to measure chlorophyll concentrations having spatial and temporal variety, the fusion of multiple methods is appropriate. Such methods include Lidar fluorosensor, fluorometry, above-water radiometry, and *in situ* cytometry [118].

6. Conclusions and Future Perspectives

As described in this review, many techniques for chlorophyll fluorescence measurement have been developed with the aim of improving accuracy, reliability, and stability for *in situ* application. An overview of the key technologies of Chl fluorescence measurement *in situ* is provided in Table 1. From the table, some conclusions can be found. Most of the light source is various LEDs, the advantages of which have been discussed. Modulation of the excitation source is often necessary because it provides more information for improving the measurement. More and more detectors use PD to receive the fluorescence emission. The detection range and LOD can meet the basic needs of environment monitoring and aquaculture. However, as mentioned by Erickson et al. [119], "There's plenty of room at the bottom" for *in situ* phytoplankton analysis. Due to the increase in eutrophic bodies of water worldwide, quick and precise monitoring is more important than ever before. Pondering how to obtain accurate and stable concentration measurements, especially in complex environments where values are very low, is of utmost importance. Furthermore, researchers must continue to consider how to perform bulk measurements over long-term deployment, at low cost [120], while minimizing interference. In view of the constant development of electronic devices, equipment to measure chlorophyll fluorescence will continuously improve. Smaller, more precise, and lower cost fluorometers will soon be a reality.

Symbols and Abbreviations Used

PBP: Phycobiliprotein
HPLC: High-performance liquid chromatography
Chl: Chlorophyll
CDOM: Chromophoric dissolved organic matter
LOD: Limit of detection
SNR: Signal-to-noise ratio
LED: Light Emitting Diode
MMF: Multimode fiber
LSE: Laser-stimulated emission
ALF: Advanced Laser Fluorometry
PAM: Pulse Amplitude Modulation
P&P: Pump-and-Probe
FRR: Fast Repetition Rate
PSII: Photosystem II
SAM: Sinusoidal amplitude modulation
PMT: Photomultiplier tube
PD: Photodiodes
CCD: Charged-coupled devices
Chl/F: Chlorophyll/fluorescence
F_o: Minimal fluorescence
F_m: Maximal fluorescence
F_v/F_m: Variable fluorescence
λ_{ex}: Excitation wavelength.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 1: Summary of fluorometers of *in situ* chlorophyll measurement.

Name	Light source	λ_{ex} (nm)	Excitation method	Detectors	Parameters	Measurement mode	Range (μgL^{-1})	LOD (μgL^{-1})	Strengths	Weakness	References
AOA	LED	450, 525, 570(×2), 590, and 610	5 kHz	PMT	Chl *a*, Chl c, PC et al.	Benchtop, submersible probe	0–80	0.02	Spectral groups measurement	More spectral fingerprinting will be better	[27]
BenthoFluor	LED	370, 470, 525, 570, 590, and 610	5 kHz	PMT	Microphytobenthos	*In situ*	0–7 $\mu g\,cm^{-2}$	—	Sediments groups measurement	More spectral fingerprinting will be better	[28]
—	Flash xenon lamp	Two-filter 435	Flash 10–15 us	PD	Chl *a*	Underwater-towed bodies and moored or buoyed	0–100 or 0–1000	0.1	Remote operation	Need a platform for operation	[49, 50]
ALF	Lasers	405 532	200 us PDP	PMT	Chl *a*, PC, and CDOM	Laboratory or flow-through	0.003–50	—	Spectral deconvolution	Need a platform for operation	[59]
ALF-T	Laser	510 375, 405, and 510	PDP	Small PMT spectrometer	Chl *a*, PBP F_v/F_m CDOM	Flow-through small-volume sample	—	0.01	"T" optical scheme	Need a platform for operation	[41]
Multiexciter	LED	375, 400, 420, 435, 470, 505, 525, 570, and 590	—	Si-PD, 680 nm	Phytoplankton	*In situ*	—	0.1	Nine LEDs for distinction phytoplankton groups	Not enough information to assessment	[65]
Phase fluorometer	LED	blue	Sinusoidal 80 MHz modulation	PD	Chl *a*	—	—	466.7	Fluorescence lifetime measurement	A worse LOD	[85]
—	LED	470	Pseudorandom sequence modulation	PD	Chl	*In situ*	0–25	0.0103	Pseudorandom sequence modulation, highly sensitive	Need more information of assessment	[86]
FRRf	LED	470	FRR	PMT	Chl	*In situ*	0–50	0.01	Profiling buoy system	Big and complex	[91]
MFF	4 xenon strobe lamps	455	10 kHz strobe flash	Two PMTs 676 nm	Chl *a*	*In situ*	0–50 and 0–200	0.1 and 0.5	Eight sensors simultaneous measurement, long term	Accuracy	[37]
ECO BBFL2	LED	370, 540, and 660	Modulated 1 k	460, 570, and 660 nm PD	Chl *a*, CDOM phycoerythrin	*In situ*	0–125	0.025	Three-channel excitation, high accuracy	Expensive	[102]
ECO 3X1M	LED	435, 470, and 532	Modulated 1 k	PD 695 nm	Chl	*In situ*	0.01–125	0.095	High accuracy	Expensive	[102]
Trios MicroFlu	LED	470	Modulated	PD	Chl	*In situ*	0–10 0–100	0.1	Two ranges and accuracy	Cannot simultaneously measure chlorophyll and phycocyanin	[109]

Table 1: Continued.

Name	Light source	λ_{ex} (nm)	Excitation method	Detectors	Parameters	Measurement mode	Range (μgL^{-1})	LOD (μgL^{-1})	Strengths	Weakness	References
TurboMAP-L	Diode laser	410	—	PD CMOS	Chl *a* and turbidity	*In situ*	0–100	0.5	Consider the aerodynamic design	Composition analysis	[45]
—	Blue LED	425	Modulation	PD	Chl *a*	Pumpable	—	—	Low cost	A little rough	[120]
	Lamp	420, 270/530	Flash	PMT	Chl	Submersible	—	—	Two probing heads, Raman scattering calibration	Cannot find the LOD	[38]
PLIF	Diode laser	532	—	CCD	Chl	*In situ*	—	—	Microscale fluorescence imaging	Accuracy	[93]
Lensless Miniature Portable Fluorometer	LED	405, 465	—	CMOS image sensor array	Chl, CDOM	Portable	0.625–893	0.625	Low cost, lensless, and low power	Accuracy and LOD	[43]

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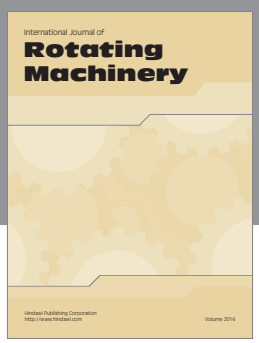

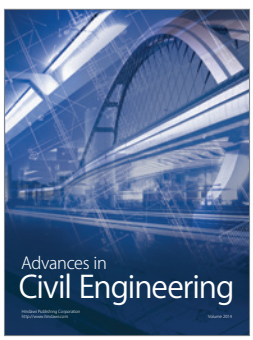

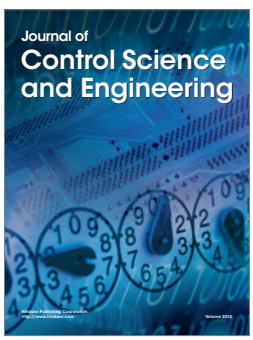

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