Corrections for matrix effects on fluorescence measurement of a multi-platform optical sensor

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Abstract

The LEDIF (LED-induced fluorescence) is an in situ optical instrument that utilizes fluorescence, absorbance, and scattering to identify and quantify substances in water bodies. In this study, matrix effects on fluorescence signals caused by inner filtering, temperature, intramolecular deactivation, turbidity, and pH were investigated, and compensation equations developed to correct measured values and improve accuracy. Multiple simultaneous matrix effect corrections were demonstrated with a laboratory sample subjected to known interferences and physical conditions. In general, compensation was found to be important to improve the accuracy of fluorescence measurements.

Key words: fluorescence matrix effects compensation, fluorescence measurement correction, fluorescence spectroscopy, in situ real-time optical sensing, interference on fluorescence measurement, water chemistry sensing

INTRODUCTION

The application of optical techniques to in situ characterization of constituents in natural waters through the use of fluorescence, absorbance, or scattering has proven useful to the understanding of water chemistry. However, in situ fluorescence sensors suffer from the same instrumental limitations as their laboratory counterparts, in addition to some other challenges, all of which can affect the accuracy and precision of measurement. The principal, potential interference factors are inner filtering, temperature, intramolecular deactivation (quenching), turbidity, and pH. Each of these is addressed here with empirical calibration or by applying equations governed by the laws of chemistry. Finally, by addressing sensor-specific effects, the results obtained from different instruments can be compared more readily.

The fluorescence intensity of a water sample is only linearly proportional to the concentration of a fluorophore over a limited range. Water that has high absorbance, due either to the fluorophore itself or the absorbance of other compounds in the sample, can absorb the excitation light leading to primary inner filtering. Secondary inner filtering occurs when a fraction of the photons emitted by the target analyte is absorbed in the water. Inner filtering becomes increasingly important as sample absorbances increase. The effect of inner filtering also depends on the excitation-emission geometrical arrangement, including the path lengths within an instrument. In general, if absorbance measured over the relevant path length at the relevant wavelengths exceeds 0.1, correction of the fluorescence signal may be needed.
The effect of inner filtering on fluorescence measurement is discussed by several workers, including Kubista et al. (1994), Puchalski et al. (1991), Guibault (1990), Yappert & Ingle (1989), and Wiechelman (1986). The effect does not just produce errors in total concentration determination; for example, Ohno (2002) found that inner filtering affects the humification index of colored dissolved organic matter (CDOM) proportionally with increasing concentration. In waters containing high concentrations of CDOM (tens of mg-C/l) – e.g., some peatland drainage – this can be particularly problematic and can even result in fluorescence decreasing with increasing CDOM concentration. It is sometimes possible to deal with this issue by dilution in laboratory analysis but that is not practical with in situ instruments. An attenuation model based on the absorption of excitation and emission optical densities may be needed for mathematical correction of the real-time fluorescence measurement of in situ instruments.

In situ instruments are susceptible to both short-term diurnal and long-term seasonal temperature changes, and correction may be needed. A typical example would be the variation of water chemistry across a thermocline in a stratified lake. The fluorescence signal is generally reduced by higher temperatures, although the temperature and fluorescence relationship varies among analytes. Watras et al. (2011) investigated the effect of temperature on CDOM fluorescence measurement, and concluded that a temperature correction is necessary for field measurements. They propose a linear temperature compensation equation. Ng et al. (2014) demonstrated a linear temperature compensation equation for chlorophyll a, but found that an exponential equation gave better correction in the case of rhodamine b, showing that the functional relationship of temperature compensation and fluorescence measurement is analyte specific.

Natural waters are complex chemical solutions including many constituents, and the chemical background can influence the fluorescence signal. Quenching of fluorescence measurement is described by Lakowicz (2006) and literature cited therein. Various mechanisms can reduce fluorescence intensity including excited state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The primary compensation for chemical quenching must deal with both dynamic and static quenching. Dynamic quenching results from collisions between the fluorophore and the quenching species in the absence of photochemical reaction – the quencher must diffuse to the fluorophore during the lifetime of the excited state. Excited state is any quantum state that has higher energy than the ground state. Static quenching results from binding of the fluorescent and quenching species to produce a non-fluorescent complex. The Stern-Volmer equation is linear when the quenching is either static or dynamic. When both occur, the equation is polynomial. The association of complex and/or Stern-Volmer coefficient of chemical quenching can be determined from the linear or polynomial Stern-Volmer equation, to correct for the effect of major quencher(s) on a fluorescence signal. The effect of quenching of humic material on the fluorescence of chlorophyll a was noted by Ng et al. (2014).

Natural waters also contain suspended particles, which cause turbidity and can interfere with fluorescence measurement by causing light reflection and/or absorption. Müller et al. (2001) found that scattering in turbid media can also alter spectral features, while Teale (2008) found that light scattering in turbid solutions caused depolarization of fluorescence. By recording turbidity simultaneously with fluorescence, the accuracy of measurement can be improved, typically using empirical calibrations.

The pH of water bodies is governed by many factors. The daily interplay of photosynthesis and respiration can result in cyclical changes of pH, especially in high-density aquaculture ponds and recirculation aquaculture systems. PH effects can be exploited constructively, for example, in fluorometric measurements of solvent extracts from disrupted algae cells, when acidification is used to estimate the relative amounts of chlorophyll and pheophytin [the excitation wavelength is varied according to the chlorophyll and pheophytin absorbance maxima (Saijo & Nishizawa 1969), and the ratio of the pre- and post- acidification fluorescence signal – the acidic ratio – is calculated.
(Arar & Collins 1992). The fluorescence of molecules containing acidic or basic functional groups is often pH dependent, as pH changes in the medium can alter the degree of ionization of the relevant functional group, thus affecting the extent of conjugation of the molecule and altering fluorescence emission. Effects can include changes in both intensity and the spectral properties of fluorescence. Yi et al. (2011), for instance, studied the effect of pH on the characteristics of oily waste water. For this material, they observed a spectral peak shift and an increase in fluorescence signal, when the medium was acidic. In a basic medium, the fluorescence signal decreased.

Finally, fluorescence spectra observed with different fluorometers are influenced uniquely by instrument-specific responses. DeRose et al. (2007) qualified (i.e., characterized) a fluorescence spectrometer for measuring the fluorescence spectra of reference materials and generated excitation and emission correction factors. Cory et al. (2010) evaluated the removal of instrument-specific responses from dissolved organic matter fluorescence spectra collected using three different fluorometers, on the basis of manufacturer-provided emission and excitation correction files. They concluded that fluorescence spectra collected on different fluorometers differed significantly before correction, but in the cases examined, could be made sufficiently independent of the instruments concerned.

The work described here was carried out on a multi-platform optical sensor – the LEDIF (LED-induced fluorescence) – for the in situ determination of water chemistry, as described by Ng et al. (2012a, 2012b). The LEDIF uses fluorescence, absorbance, and scattering to quantify substances in water. In this paper, the use of auxiliary sensors (e.g. temperature, pH) is examined along with the LEDIF’s ability to measure fluorescence, absorbance, and turbidity simultaneously, to enable development of corrections to the matrix effects. Laboratory-prepared and commercial standards with chemical characteristics resembling substances commonly found in natural water were used to demonstrate the corrections. As a test of ability to compensate for multiple, simultaneous matrix effects, the determination results from a liquid sample, subjected to known interferences and physical conditions, were corrected and compared with a reference sample. The spectrometer and associated optical systems in the LEDIF were qualified using the atomic lines of mercury (Hg) and argon (Ar), and National Institute of Standards and Technology (NIST)-traceable calibration light sources.

METHODS

Instrument

The LEDIF, shown in Figure 1, uses six junctions fitted with LEDs of different wavelengths, focused on the analytical volume and oriented at 90 degrees to the main axis of light collection. Absorbance is measured using a broadband (185 to 1,100 nm) light source coupled via an optical fiber; a collimation lens illuminates the flow cell at 180 degrees to the light collection system. Turbidity is measured by nephelometry within the flow cell, at each LED wavelength. Flow into the cell is via a pathway containing two rectangular bends to minimize the entrance of stray light.

Light from the flow cell is observed with an Ocean Optics USB4000 spectrometer, and the data are recorded with a single-board computer manufactured by Technologic Systems (Model TS-7260-64-128F) running custom software (iLEDLIF). For land-based use in continuous monitoring, a Gotec (Model: EMX-08) piston pump feeds samples into the LEDIF flow cell manifold. When used in the autonomous underwater vehicle, ram pressure drives samples through the flow cell.

Instrument qualification

LEDIF spectrometer wavelengths were calibrated at multiple positions between 253 and 922 nm – see Table 1. The spectrometer was connected to the calibration source with a 50 μm diameter ultraviolet
(UV)-visible (VIS) optical fiber. Wavelength calibration coefficients were obtained via a third-order linear regression, using true wavelength (atomic lines) as a function of pixel number, pixel number squared, and pixel number cubed (recorded with the spectrometer). The wavelength calibration coefficients are stored in the spectrometer's electrically erasable programmable read-only memory (EEPROM). The operating wavelength of the spectrometer is calibrated (Ocean Optics 2008) every 12 months.

**Figure 1** | Layout of the LEDIF: (a, b) isometric and front views of LEDIF in a 20 × 15 × 20 cm enclosure for portable mode and fixed location sensing, (c) LEDIF block diagram, and (d) LEDIF in a 30 (L) × 20 (D) cm cylindrical pressure hull for autonomous platform deployment.

**Table 1** | Atomic line positions used for LEDIF wavelength calibration

<table>
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<th>Hg (nm)</th>
<th>Ar (nm)</th>
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<th>Ar (nm)</th>
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<td>579.066</td>
<td>826.452</td>
<td>579.066</td>
<td>826.452</td>
</tr>
</tbody>
</table>
The linearity of the charge-coupled device detector in the spectrometer was corrected with a 7th order polynomial equation using non-linearity correction coefficients provided by the manufacturer. It is said that this achieves an intensity linearity correction exceeding 99.8%. The linearity correction coefficients are stored in the spectrometer’s EEPROM. A NIST-traceable, calibrated deuterium-halogen (DHAL) light source manufactured by Avantes (Model: AvaLight-DH-CAL) covering the UV-VIS-near infrared spectral range of 205 to 1,099 nm was used to calibrate the absolute spectral irradiance of the spectrometer in the LEDIF. The spectrometer was connected to the calibrated light source with a 1,000 μm UV-VIS optical fiber, having optical layout and properties identical to those in the LEDIF’s flow cell. The irradiance \( E_{e,\lambda} \) can be computed using the calibration data for each pixel defined by:

\[
E_{e,\lambda} = F \left( \frac{I_s}{tA\Delta\lambda} \right)
\]

where \( F \) is the instrument-specific calibration matrix, \( I_s \) is the measured intensity of the sample spectrum, \( t \) is the integration time, \( A \) is the collection area, and \( \Delta\lambda \) is the wavelength spread of each pixel (Ocean Optics 2016).

Finally, the central wavelength and full width at half maximum (FWHM) bandwidth of each LED in the excitation system were measured using the LEDIF’s NIST-calibrated spectrometer. The LED excitation system was connected to the spectrometer with a 1,000 μm diameter UV-VIS optical fiber.

**Solution preparation**

All experiments were performed using laboratory-prepared and commercial standards in deionized (DI) water in amber glass bottles. Chlorophyll a was obtained from Tokyo Chemical Industry (CAS 1406-65-1), humic acid from Acros Organics (Sodium Salt, tech., CAS 68131-04-4, Lot A0268981), and turbidity standard solutions from AMCO CLEAR®TURBIDITY STANDARD, GFS Chemicals, Inc. (NIST-traceable). For chlorophyll a, the concentrations reported in this paper are based on the manufacturer’s reported assay of 0.5% plant-derived chlorophyll. For humic acid, the concentration reported is based on 55% carbon content; the manufacturer’s assay reports carbon content ranging from 50 to 60%.

For all experiments, the baseline and background signals were corrected with the DI water spectrum used to prepare the laboratory standards. All stock solutions (except the turbidity standard) were filtered with Grade 42 filter paper (GE Healthcare Life Sciences), having a nominal pore size of 2.5 μm, to minimize possible effects of large particle interference (if any) on the fluorescence signal. Fresh solutions were prepared, typically within 2 to 3 hours of analysis, and all spectra were collected at room temperature (22 to 25 °C), except during temperature compensation experiments. The integration times for all fluorescence measurements were 10 seconds, except for those for turbidity and multiple matrix effects compensation to chlorophyll a.

**Inner filtering**

The effect of inner filtering on sensor response to humic material was assessed by preparing humic acid solutions at different concentrations, the maximum concentration corresponding to those observed in tropical peatlands. UV-VIS absorbance measurements were made using the LEDIF, followed by fluorescence measurements at six different excitation wavelengths. The absorbance measurements were then used to correct the latter using an equation from Lakowicz (2006).
reformulated for a path length applicable to the LEDIF:

\[ I_{corr} = I_{obs}\text{antilog}_{10}\left[(OD_{ex})_{l,corr} + (OD_{em})_{l,corr}\right] \] (2)

where \( I_{corr} \) and \( I_{obs} \) are the corrected and observed fluorescence peak intensities, respectively, \( \text{antilog}_{10} \) denotes antilog base 10, and \((OD_{ex})_{l,corr}\) and \((OD_{em})_{l,corr}\) represent the path-length-corrected excitation and emission optical densities, respectively.

**Temperature**

For tests of temperature correction, the LEDIF inlet and outlet were dipped into a reservoir containing an analyte of known concentration. A 12 VDC Gotec (Model: EMX-08) piston pump was used to circulate the analyte continuously between the LEDIF and the reservoir, while a sensor recorded the temperature of the analyte in the reservoir. The liquid sample was first refrigerated for experiments below the reference temperature of 25 °C, and periodic measurements were made as the liquid rose to room temperature. For experiments at temperatures above reference, the reservoir was put on a temperature-controlled hot plate.

Sensor response as a function of temperature was assessed for humic acids, over a temperature range from 10 to 60 °C, a range of different emission wavelengths and different concentrations, and 375 nm excitation. The observed fluorescence intensity was corrected for inner filtering before temperature assessment. Comparison was made with results from a wetland-dominated lake, Crystal Bog, in Northern Wisconsin, using a Turner Designs C3 sensor over three months (May to July 2010), as reported by Watras et al. (2011). A sample from the Agas River, Brunei, was also measured. Results reported by Watras et al. (2011) from reconstituted Suwanee River NOM utilizing a SeaPoint sensor, were also compared. For organic carbon in humic acids, fluorescence was found to decrease linearly with increasing temperature, as observed by Watras et al.:

\[ I_r = \frac{I_s}{[\rho(Tmp_s - Tmp_r) + 1]} \] (3)

which can be rewritten as:

\[ \frac{I_s}{I_r} = \rho(Tmp_s - Tmp_r) + 1 \] (4)

where \( I_s \) and \( I_r \) are the fluorescence peak intensities at sample and reference temperatures, respectively, \( \rho \) is the temperature coefficient obtained from the curve fitting gradient, and \( Tmp_s \) and \( Tmp_r \) represent the sample and reference temperatures, respectively.

**Quenching**

Fluorescence is susceptible to intramolecular deactivation resulting in loss of signal. Collision (dynamic) and complex formation (static) quenching are common quenching. In dynamic quenching, the rate can be determined using the Stern-Volmer linear relationship:

\[ \frac{I_0}{I_f} = 1 + K_{SV} \cdot [C] = 1 + k_q \tau_0 \cdot [C] \] (5)

where \( I_0 \) and \( I_f \) are analyte fluorescence intensities, without and with a quencher, respectively, \( K_{SV} \) is the Stern-Volmer quenching constant, \( C \) is the quencher concentration (mg/l), \( k_q \) is the quencher rate
coefficient, and \( \tau_0 \) is the lifetime of the emissive excitation state of the analyte sample. Data on diffusion-limited quenching that permit the use of the Stokes-Einstein relation (Miller 1924) in predicting the quencher rate coefficient are scarce, so the rate coefficient is usually determined experimentally. Static quenching is described by Weber (1948) and the rate can be determined from:

\[
\frac{I_0}{I_f} = 1 + k_a \cdot [C] \tag{6}
\]

where \( k_a \) is the association constant of the complex. For both static and dynamic quenching, the equation takes the form:

\[
\frac{I_0}{I_f} - 1 = (k_a + K_{SV}) \cdot [C] + (k_a K_{SV}) \cdot [C]^2 \tag{7}
\]

Humic acid (sample analyte) and chlorophyll a (quencher) were chosen for these experiments because both are important analytes relevant to natural water bodies. The observed fluorescence intensity was corrected for inner filtering effects before quenching assessment.

**Turbidity interference**

Fluorescence measurement is generally considered less susceptible to particle interference than absorbance measurement. The effect of turbidity on sensor response was assessed by mixing chlorophyll a standard with styrene divinyl benzene copolymer beads in water at concentrations up to 500 NTU. Monodisperse particles were thus responsible for the turbidity observed. Monodisperse particles have a one-to-one relationship between scattering intensity and scatter angle (Xu 2002). Natural waters contain polydisperse particles of different sizes and compositions (Fondriest.com 2015).

**pH**

Laboratory-prepared chlorophyll a and humic acid samples of known concentration and pH were measured using the LEDIF. Sample pH was altered by adding potassium hydroxide (1,360, 3,400 and 6,800 mg/l) or hydrochloric acid (0.074, 0.44, and 40.4 mg/l) in a laboratory glass beaker in a dark room. The modified samples were mixed with a magnetic stirrer, and fluorescence was measured after the pH reached a constant value. These experiments were carried out on fresh samples derived from the same source. Sample dilution with acid or base additions never exceeded 0.3% by volume. Acid addition converts chlorophyll a to pheophytin a, and is used, typically, in the extractive measurement of disrupted algae cells. The observed fluorescence intensity of humic acid was corrected for inner filtering before pH assessment.

To assess whether humic material sorption to the flow cell or sample container walls occurs when the pH is lowered, potassium hydroxide was added to DI water to make it basic. This basic water was used to prepare the humic samples and the fluorescence signal measured. Hydrochloric acid was then added to lower the pH to neutral and the fluorescence signal was measured again.

**Multiple matrix effects compensation to chlorophyll a fluorescence**

Equal volumes of 1.5 mg/l chlorophyll a (analyte), 30 mg/l humic acid (quencher), and 600 NTU turbidity standard were mixed to form a solution containing 0.5 mg/l chlorophyll a, 10 mg/l humic acid, and 200 NTU. Hydrochloric acid was added to lower the pH to 4.3 and the solution was cooled to 10 °C. The LEDIF was used for fluorescence measurement, and matrix effects were compensated by
applying the chemical law and empirical calibrations presented above. The results were compared with the fluorescence signal of 0.5 mg/l of chlorophyll a in DI water.

RESULTS AND DISCUSSION

Qualification of instrument

The reproducibility produced by the Hg and Ar atomic lines was indistinguishable from zero for all peak positions observed with the spectrometer. The maximum deviations from the lines were less than 1.3 nm per annum, over the spectrometer’s full spectral range, in normal laboratory operating conditions, and the regression coefficient $R^2$ was extremely close to 1 (better than 0.999999). Figure 2(a) shows that the LEDIF’s wavelength values match the true values of the Hg and Ar calibration source very well after the wavelength calibration.

Figure 2(b) and 2(c) show the correction factor and the measured intensity of the NIST-traceable light source recorded by the spectrometer in the LEDIF. Figure 2(c) shows that the spectral irradiance of the NIST-calibrated spectrometer in the LEDIF matches very well with the NIST-traceable light source after the correction. Figure 2(d) shows the measured center wavelength and FWHM bandwidth of the six excitation sources in the LEDIF. The values reported by the LED manufacturer (Thorlabs) are shown in the legend of Figure 2(d). In Table 2, the LED manufacturer’s reported values are compared with those measured by the LEDIF after calibration. The FWHM bandwidths of 375 and 405 nm as measured were somewhat larger than those reported by the manufacturer.
Inner filtering compensation

Figure 3(a) shows the absorbance of humic acid at different concentrations, and Figure 3(b) as a function of concentration for the LEDIF’s six different fluorescence measurement excitation wavelengths and two emission peaks (500 and 520 nm) of laboratory humic acid. Absorbance is linear up to 3 mg/l, with only slight deviation from linearity up to 5 mg/l. Absorptivity (Figure 3) for each of the excitation and emission wavelengths was computed from the linear correlation, with excitation and emission optical path lengths of 8.7 and 9 mm, respectively, and yielding determination coefficients exceeding 0.99 for all wavelengths considered [Beer-Lambert (Lakowicz 2006) equation, \( A = \varepsilon lC \)] where \( A \) is absorbance (absorbance units), \( \varepsilon \) represents absorptivity, \( l \) denotes optical path length, and \( C \) is the analyte concentration. For absorbance of less than unity – i.e., below the dotted line

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<td>375, ± 10</td>
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<th>Wavelength (nm)</th>
<th>Absorptivity ( [(\text{mg/l})^2\text{cm}^2] )</th>
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</tr>
<tr>
<td>520</td>
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**Figure 3** | (a) Absorbance spectra of humic acid dissolved in water at different concentrations as measured by the LEDIF, (b) absorbance as a function of humic acid concentration at the LEDIF’s 6 excitation wavelengths and the 2 emission peak wavelengths of humic acid, (c) table showing computed absorptivity at LEDIF’s excitation wavelengths and humic acid’s emission peak wavelengths, and (d) measurement of fluorescence peak intensity (dashed line) of humic acid as a function of concentration for different excitation wavelengths. Note: obs = observed intensity; corr = inner-filtering-corrected intensity.
in Figure 3(b) – the deviation from linearity is small, and absorbance may be estimated reasonably accurately using the linear correlation. The absorbance measurements are used to correct for the inner-filtering observed in the fluorescence measurements. Figure 3(d) shows both the observed and the inner-filtering-corrected fluorescence measurements (computed using Equation (2)) for the emission wavelengths of humic acid at 500 nm. For the uncorrected fluorescence signal, the accuracy of the uncorrected non-linear correlations [dashed lines in Figure 3(d)] decreases with increasing concentration. The corrected fluorescence signal, however, exhibited linear correlation for all excitation wavelengths up to concentrations of 10 mg/l. For 375 nm excitation, the corrected fluorescence signal relationship is essentially linear up to 20 mg/l. These results demonstrate that inner filtering compensation is important for humic compound fluorescence measurement at high concentrations, and suggests that satisfactory compensation is possible for typical humic materials over a wide range of concentrations typical of natural waters.

*In vivo* chlorophyll a internal to algae in water bodies in Singapore rarely exceeds 0.2 mg/l, and typically falls within the linear region for fluorescence (Ng et al. 2014, 2015). Because of this, inner filtering correction was not considered for measurement of *in vivo* chlorophyll a in this study. Some of the same compensation procedures could be applied should inner filtering effects become important.

**Temperature compensation**

Fluorescence is temperature sensitive and the temperature coefficients associated with different analytes are unique. Figure 4(a) shows that the normalized peak intensity \( I_s / I_r \) of the humic acid used in this study is a linear function of differential temperature \( (\text{Tmp}_s - \text{Tmp}_r) \) and can be described by:

\[
\frac{I_s}{I_r} = -0.0083(\text{Tmp}_s - \text{Tmp}_r) + 1
\]

**Figure 4** | (a) Normalized, (b) measured, and (c) inner filtering and temperature-corrected peak intensities of humic and two water samples as a function of temperature. Legend format: sample, emission peak wavelength, concentration.
where subscripts $s$ and $r$ represent sample and reference readings, respectively. The linear function has a temperature coefficient of $-0.0083$ per °C over the range tested, matching the reported value of $-0.007$ to $-0.009$ per °C for humic material from Crystal Bog, Northern Wisconsin, reported by Watras et al. (2011). The sample from the Agas River (Brunei) yielded a temperature coefficient of $-0.0197$ per °C over the test range, while Watras et al. (2011) report $-0.026$ per °C for Suwanee River NOM. Figure 4(b) shows the observed peak intensities of humic acid and the water sample from the Agas River, taken at emission wavelengths of 500 and 520 nm. Figure 4(c) shows the inner filtering and temperature-corrected peak intensities, where the corrected value is a constant independent of temperature.

The results demonstrate that temperature compensation is both important and possible for the fluorescence measurement of humic substances. They also show that the temperature coefficient varies significantly between humic compounds. It also varies among other chemical species; the normalized peak intensity ($I_s/I_r$) of chlorophyll a was found to be a linear function of differential temperature ($T_{ms}/T_{mr}$) described (Ng et al. 2014) by:

$$\frac{I_s}{I_r} = -0.016(T_{ms} - T_{mr}) + 1$$  \hspace{1cm} (9)

**Quenching effect**

Figure 5(a) shows the Stern-Volmer plot of chlorophyll a quenching humic material, excited at 285 nm, for humic emission wavelengths at 500 and 520 nm. The Stern-Volmer coefficient ($K_{SV}$) of 0.21 can be determined from a linear curve fit over the data, and use of Equation (5). A linear plot
signifies that the quenching process may be static or dynamic dominated. It might be possible to distinguish the two mechanisms by measuring the absorbance of the humic material (analyte) and chlorophyll a (quencher) mixture, as complex formation processes can lead to a change in absorbance profile.

Figure 5(b) shows the observed peak intensities and inner-filtering-corrected fluorescence measurements of humic acid quenched by chlorophyll a, for 500 and 520 nm emission wavelengths. Figure 5(c) shows the inner filtering and quenching-corrected fluorescence measurements of humic acid quenched by chlorophyll a, illustrating that the corrected value is a constant independent of chlorophyll a concentration. In other words, quenching compensation can improve the accuracy of humic fluorescence measurement in the presence of chlorophyll a. It is noted, however, that these experiments were carried out using extracted chlorophyll a as a model for a generic quencher, and do not necessarily represent possible effects of in vivo chlorophyll a internal to algae in natural waters.

Turbidity compensation

Particles are always present in water bodies and can interfere with fluorescence measurement. The turbidity standard used was made up of particles with a mean diameter of 0.121 μm, ensuring long-term suspension and thus consistent light scattering characteristics. Figure 6(a) shows the effect of turbidity on the fluorescence measured from different concentrations of normalized chlorophyll a, excitation was at 405 nm and sensor integration times were 1 and 2 seconds. The effect of normalized intensity ($I/I_0$) of chlorophyll a is an exponential function of turbidity ($T$) described by:

$$\frac{I}{I_0} = e^{-0.0019T}$$

Figure 6 | (a) Normalized, (b) measured, and (c) corrected peak intensities of different aqueous concentrations of chlorophyll a as a function of turbidity. Legend format: chlorophyll a concentration_sensor integration time.
where $I$ is fluorescence peak intensity and subscript $o$ represents reading at 0 NTU, and $T$ represents the sample turbidity (NTU). For the concentration range tested, the fluorescence signal of chlorophyll a is approximately linear up to 1 mg/l (Ng et al. 2014) and the inner filtering effects of absorbance are thus modest. The transmittance of the fluorescence emission ($T_F$) signal is:

$$T_F = \frac{I}{I_o} = e^{-\tau} \quad (11)$$

where $\tau (= \kappa \rho t)$ is optical depth; $\kappa$ is opacity due to absorption and scattering in the medium, $\rho$ is density of the medium, and $t$ is light path length. From equations (10) and (11), $\tau :: 0.0019 T$; i.e. the coefficient 0.0019 arises from the product of particle density and light path length. Figure 6(b) shows the observed fluorescence measurement of chlorophyll a at different concentrations, excited at 405 nm and over sensor integration time of 1 s and 2 s. Figure 6(c) shows the turbidity-corrected fluorescence measurements – i.e., the corrected values are independent of turbidity. It is clear that that turbidity compensation is important in fluorescence measurement at high NTU values for dissolved chlorophyll a. It is noted, however, that these experiments involved turbidity standards comprising monodisperse particles; effects may differ for particles of varying composition and size in natural waters.

**pH compensation**

Figure 7(a) shows the fluorescence spectra of chlorophyll a at different pH values, when excited at 405 nm. For this material, fluorescence intensity increases as pH decreases; no noticeable shift of fluorescence peak wavelength was observed with pH changes. Acid hydrolysis of chlorophyll a involves the removal of magnesium ions and their replacement with two hydrogen atoms, forming phaeophytin a (olive green). The phytol tail (long carbon chain) present in phaeophytin a is electron-donating and can enhance the fluorescence signal. In basic media the methyl and phytol esters are removed, forming water soluble chlorophyllin a (bright green), which has no phytol tail. The presence of carboxylic acid, which is electron withdrawing, may inhibit fluorescence.

Figure 7(b) shows the fluorescence spectra of humic acid at different values of pH, when excited at 285 and 375 nm. By contrast to chlorophyll a, fluorescence intensity decreases with decreasing pH; no noticeable fluorescence peak wavelength shift was observed with changes in pH.
Figure 8(a) shows that the normalized peak intensity \( [(I - I_{pH=7})/I_{pH=7}] \) of chlorophyll a can be described with a polynomial function of differential pH (\( pH-7 \)):

\[
\frac{I - I_{pH=7}}{I_{pH=7}} = 0.0043(pH - 7)^2 - 0.0367(pH - 7)
\]  

(12)

where \( I \) is fluorescence peak intensity and subscript \( pH = 7 \) represents a reading at neutral pH. Figure 8(b) shows the observed fluorescence measurements of different concentrations of chlorophyll a at different pH levels, excited at 375 and 405 nm. Figure 8(c) shows the pH-corrected fluorescence measurements, where the corrected value is a constant independent of pH. It demonstrates that pH compensation is both necessary and possible for fluorescence measurement of dissolved chlorophyll a.
at low pH. Figure 8(d) shows that the normalized peak intensity \(\frac{I - I_{pH=7}}{I_{pH=7}}\) of humic acid in an acidic medium \([pH \leq 7\); red line in figure\] can be described with a polynomial function of differential pH \((pH-7)\):

\[
\frac{I - I_{pH=7}}{I_{pH=7}} = -0.0284(pH - 7)^2 + 0.022(pH - 7)
\]

(13)

where \(I\) and subscript \(pH = 7\) are as in Equation (12). Figure 8(e) shows the observed fluorescence measurements of different concentrations of humic acid at different pH values, excited at 285, 375 and 405 nm. Figure 8(f) shows the pH-corrected measurements with the value independent of pH. This demonstrates that pH compensation is necessary and possible for humic acids in acidic media. For humic acids, pH has minimal effect on fluorescence signal in basic media up to at least pH 10.3.

The fluorescence signal of humic samples prepared at \(pH = 9.8\) and excited at 375 and 405 nm was measured before and after neutralization \((pH = 7)\). The maximum percentage difference in all signals measured was below 2.7, and readily accounted for by small dilution and/or experimental errors. Mass loss by sorption to vessel walls (if any) was negligible and the humic materials appeared to be entirely dissolved in samples used for the pH adjustment experiments.

**Multiple matrix effects compensation to chlorophyll a fluorescence**

The mixture comprising 0.5 mg/l of chlorophyll a (analyte), 10 mg/l of humic acid (quencher), and 200 NTU of turbidity standard, at \(pH 4.3\) and 10 °C, was excited at 405 nm. Its chlorophyll a peak intensity was corrected by applying compensation Equations (9) (temperature), 10 (turbidity), and 12 (pH). The mixture was then excited at 375 nm and the humic emission wavelength at 500 nm was corrected by applying Equations (8) (temperature) and 13 (pH); and the concentration of humic acid in the mixture computed. The final compensation for humic quenching of chlorophyll a was computed using:

\[
\frac{I_f}{I_f} - 1 = 0.0024[C]^2 + 0.0008[C]
\]

(14)

The total compensation \((\Delta_x)\) in fluorescence intensities under the influence of these multiple matrix effects is:

\[
\Delta_x = \sum_i^n \Delta_x = \Delta_{Tmp} + \Delta_T + \Delta_{pH} + \Delta_Q
\]

(15)

where \(n\) is the total number of matrix effects, \(\Delta\) is the difference in fluorescence intensity resulting from each individual effect, and subscripts \(Tmp, T, pH\), and \(Q\) represent temperature, turbidity, pH, and quenching, respectively. The fluorescence signal without compensation (6,990 counts; a difference of 3,866 counts) in this medium was 35.6% lower than that of pure chlorophyll a (10,856 counts) at the same concentration in distilled water buffered to pH 7.2. After matrix effects compensation, the fluorescence intensity of chlorophyll a (9902 counts; a difference of 954 counts) in the mixture was 8.8% lower than that of pure chlorophyll a, i.e. a fourfold improvement, if the proportional compensations are compared in percentage form, demonstrating that multiple matrix effects compensation is both necessary and important.
CONCLUDING REMARKS

The experiments described illustrate the variety of conditions under which matrix effects to fluorescence measurement of natural waters occur, and suggest that, in general, compensation for them is both necessary and possible in many in situ applications. Corrections involving both inner filtering and turbidity are simplified by the LEDIF’s ability to measure absorbance and turbidity almost simultaneously with fluorescence in situ. Sensing of pH and temperature requires that an in situ optical instrument be accompanied by auxiliary pH and temperature sensors. Although the work discussed here was carried out with the LEDIF, the compensation techniques described should be equally adaptable to other in situ optical sensors.

Other potentially common matrix effects were not investigated. They include, for example:
- quenching by dissolved oxygen, so that the inclusion of a dissolved oxygen sensor could be helpful.
- turbidity effects resulting from inhomogeneous mixtures of particles, which could also prove more complex than those investigated – in some cases in-line filtration to remove high concentrations of larger particles could prove helpful.

Corrections for ionic strength were not investigated, either, but variations of this type could be important, for example, in estuarine studies. Fortunately, it is possible to estimate this parameter using standard auxiliary sensors.

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