Review of fluorescent standards for calibration of *in situ* fluorometers: Recommendations applied in coastal and ocean observing programs

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Abstract: Fluorometers are widely used in ecosystem observing to monitor fluorescence signals from organic compounds, as well as to infer geophysical parameters such as chlorophyll or CDOM concentration, but measurements are susceptible to variation caused by biofouling, instrument design, sensor drift, operating environment, and calibration rigor. To collect high quality data, such sensors need frequent checking and regular calibration. In this study, a wide variety of both liquid and solid fluorescent materials were trialed to assess their suitability as reference standards for performance assessment of in situ fluorometers. Criteria used to evaluate the standards included the spectral excitation/emission responses of the materials relative to fluorescence sensors and to targeted ocean properties, the linearity of the fluorometer's optical response with increasing concentration, stability and consistency, availability and ease of use, as well as cost. Findings are summarized as a series of recommended reference standards for sensors deployed on stationary and mobile platforms, to suit a variety of in situ coastal to ocean sensor configurations. Repeated determinations of chlorophyll scale factor using the recommended liquid standard, Fluorescein, achieved an accuracy of 2.5%. Repeated measurements with the recommended solid standard, Plexiglas Satinice® plum 4H01 DC (polymethylmethacrylate), over an 18 day period varied from the mean value by 1.0% for chlorophyll sensors and 3.3% for CDOM sensors.

OCIS codes: (010.0010) Atmospheric and oceanic optics; (010.4450) Oceanic optics; (260.2510) Fluorescence; (150.1488) Calibration; (120.0280) Remote sensing and sensors.

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1. Introduction

The rapid development of optically-based sensors to quantify and characterize suspended and dissolved materials within the water column have generated a diverse range of commercial and research *in situ* optical instruments. These *in situ* instruments use various principles of fluorescence, light absorption and scattering to quantify concentrations of dissolved and particulate matter with high temporal resolution based on rapid sampling rates. Organic particulates of interest in coastal to ocean optical measurements are bacteria, phytoplankton and detritus, while inorganic particles generally consist of fine sediments or wind-deposited dust, composed of quartz sand, clay minerals or metal oxides [1]. Optically-active coloured

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dissolved organic matter (CDOM) can be a complex mixture of compounds, but in ocean regions may be dominated by matter from degraded biological material and by terrestrial runoff in nearshore zones (typically consisting of humic and fulvic acids [2];) and decaying phytoplankton in waters offshore [1,3]. Here we focus on *in situ* fluorescence measurements of phytoplankton chlorophyll *a* and CDOM.

Comparability of optical data collected by different instruments at different times and in different ocean provinces is a key requirement for large-scale observing programs designed to detect change in space and time [4]. Calibration is a critical step required to relate instrument voltage (counts) to standard units for fluorescence and scattering (via scale factors), and to provide inter-comparability amongst different sensors and deployments [5]. For data to be useful for sustained monitoring programs, it is also important to distinguish natural/environmental variability from instrumental variability; thus, any instrument drift must be routinely quantified [6].

To facilitate this, laboratory-based characterizations are essential for quantifying and evaluating instrument performance. Fluorescence instruments are supplied with a factory-determined scale factor, used as the primary sensor calibration to relate instrument counts to constituent concentrations, particularly for studies employing autonomous and Lagrangian platforms (e.g. ocean gliders [7], [8];) where regular field-based instrument calibration may be impractical. However, scale factors may change when, for example, the original calibrations were conducted prior to the particular instrument being incorporated into a measurement platform [6], or as a result of vibrations changing the optical alignment during shipping, or physical damage (e.g. a small scratch on the sensor's surface). Similarly, long-term sensor deployments on both moored and moving platforms are often impacted by bio-fouling, highlighting the importance of pre- and post-deployment instrument performance checks in routine monitoring programs.

A large number of bio-optical sensors are deployed on fixed and moving platforms (e.g. shelf and deep ocean moorings, ocean gliders and other autonomous underwater vehicles) as part of Australia's Integrated Marine Observing System (IMOS [9];). These instruments are distributed in different locations around Australia's coastal shelf and oceans and operated by various national facilities providing logistical challenges in checking and maintaining sensors, in data quality assurance (QA) and quality control (QC) and comparability between sensors.

Both liquid and solid fluorescent standards may be employed for instrument performance checks and/or calibration of *in situ* fluorometers. For sensors designed to detect chlorophyll-*a* fluorescence, liquid dyes such as fluorescein and Rhodamine WT have been utilized [10,11], while quinine sulfate solutions have been used for calibration of CDOM fluorescence sensors [12]. Other less conventional but widely available liquids have also been found to have fluorescent properties, including soft drinks such as Diet Coke ® and Sprite Zero ® (The Coca-Cola Company) [6,13]. There are also solid fluorophores such as polymers doped with fluorescent dyes or microsphere diffuser particles which could be used for this purpose.

We examined a variety of fluorescent materials and quantified their excitation/emission spectra, photostability, inter-batch product variation and linearity of response to evaluate their suitability for performance checks and/or calibration standards for *in situ* fluorescence sensors. We considered that calibration standards should have similar excitation/emission responses as the analyte of interest, not degrade during the time needed to check instrument/s, and have appropriate sensitivity and dynamic range to detect the target concentrations. As a result, recommendations are provided on a set of laboratory and field-based calibration and performance checking procedures suitable for a variety of fluorescence sensors.

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2. Materials and methods

2.1 Fluorescence calibration procedure

For benchtop fluorometers, Chl-*a* calibrations are normally performed with Chl-*a* standard solutions made up in organic solvents such as 90% acetone to follow conventional pigment extraction methods [14,15]. However, this approach is not used for *in situ* fluorometers because absorbance by *in vivo* Chl-*a* is shifted 8-10 nm to the longer wavelengths compared to solvent extracted Chl-*a* [2].

The choice of alternative reference solutions for *in vivo* Chl-*a* calibrations is not straightforward, as the excitation-emission response of Chl-*a* is different to that of most organic or inorganic fluorescent compounds. Chlorophyll *a* absorbs in two major wavelength bands (blue and red) and has a relatively large energy shift between its absorption and emission wavelengths due to the energy absorbed by phytoplankton being transferred via accessory pigments before being re-emitted as fluorescence [16,17]. However, a number of dyes are available with absorption or emission in regions similar to Chl-*a* (Table 1). In this study, three different fluorescent dyes were trialed as fluorescence calibration standards: Fluorescein Sodium Salt (Sigma Aldrich), Rhodamine WT Red (Envco) and Basic Blue 3 (Sigma Aldrich). Fluorescein is currently used by some suppliers of optical instrumentation, while Rhodamine WT Red and Basic Blue 3 were trialed as they have previously demonstrated linear responses with field and laboratory fluorescence sensors [18].

For CDOM fluorescence sensors, quinine sulfate dihydrate, QSD, (Sigma Aldrich) was examined as a calibration standard as it is used to calibrate bench-top CDOM fluorometers [19]. This standard is normally made up in weak acid (0.5 M sulphuric acid) which may be problematic for some instruments, so non-acidic alternatives were also trialed. Cetinic et al [20] used Sprite Zero ® (The Coca Cola Company) as a standard for calibration of CDOM sensors deployed on Slocum gliders. In addition to examining the optical properties of Sprite Zero ®, we evaluated tonic water as a CDOM calibration solution, as it contains quinine. To test the variability between different sources of tonic water, three brands were tested: Indian Tonic Water (Schweppes), Diet Indian Tonic Water (Schweppes) and Club Tonic Water (Tru Blu, P&N Beverages).

Table 1. Absorption and emission wavelength ranges for CDOM (Suwannee River standard fulvic acid in seawater) and Chl-*a* analytes in organic solvents, and the corresponding excitation and emission wavelengths for the *in situ* fluorometers used in this study.

Analyte	λ_{abs} (nm)		λ_{em} (nm)		In situ fluorometers	
	Peak	Range	Peak	Range	λ_{ex}(nm)	λ_{em}(nm)
CDOM	N/A	350-400 [28]	450^{+}	380 – 600^{+}	370	460
Chl-a*	430, 662 [15]	350-450, 500-700 [15]	676 [2]	650 –700 [2]	470	695

$^{+}$ Fulvic acid in seawater, Earp this study

* Chl-*a* in acetone, Earp this study

2.2 General procedures and calibration set up for dyes and other liquids

To confirm the peak wavelengths and spectral widths (full width at half maximum; FWHM) of the excitation sources of *in situ* fluorometers, LED source emission spectra were measured using a fibre optic integrating sphere (FOIS-1, Ocean Optics*),* connected to a UV-visible spectrometer (USB2000, Ocean Optics) via a 50 μm diameter optic fibre. Spectra were recorded over the range 350 – 1000 nm with a computer using proprietary software (Spectrasuite^{TM}, Ocean Optics*)*.

Fluorescence spectra of liquid samples were measured in quartz cuvettes using a spectrofluorometer (Cary Eclipse, Varian). To correct for variability in the excitation source

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and emission detection, quantum correction curves were measured, following Kopf and Heinz [21]. For excitation correction, a quantum counter consisting of Basic Blue 3 dye at a concentration of 4.1 g/L in ethylene glycol (Sigma Aldrich) was used in a triangular quartz cuvette. For this measurement, a 695 nm long pass filter was used on the emission window, and the emission at 750 nm was monitored as a function of excitation wavelength. For emission correction, a synchronous scan ($\lambda_{ex} = \lambda_{em}$) was performed with a silica diffuser in the sample compartment, and a 3% transmission attenuator in the emission window. Excitation/emission correction curves were measured over the range 240 – 700 nm with excitation and emission slit widths 10 nm and 20 nm respectively, $d\lambda = 1$ nm and integration time 2.0 sec. Fluorescence spectra were then corrected as in Murphy et al. [19]. Absorption spectra of liquid samples were measured on 1 mm path length quartz cuvettes with a UV-Vis-NIR spectrophotometer (Cary 50 Bio, Varian*)*.

A calibration vessel was custom-built to accommodate a variety of fluorescence sensors, including stand-alone instruments and others in multiple sensor configurations. Constructed of PVC, the vessel was 25 cm in diameter and 1 m tall, and painted black on the inside to minimise stray light. The vessel was built to these dimensions to accommodate the volume needed to submerse the fluorometers and be ergonomic for instrument users at bench height. The calibration solutions were placed in glass beakers and suspended in the upper 30 cm of the calibration vessel using a black support made from Polyoxymethylene (POM), also known as 'Delrin'.

Dye calibration solutions were prepared using calibrated 1000 µL pipettes. All dye solutions were made up in ultrapure water prepared with a water purification system (Sartorius arium 611 UF UV). The calibration solutions were prepared in 3 L glass beakers (volume required to fully submerse the fluorometers) which were then placed into the top of the calibration vessel using clamps and spacers also made from black Delrin.

Similar procedures were carried out using commercially available soft drinks: Sprite Zero, and tonic water. Different batches were measured to test the assumption that soft drinks have consistent fluorescence responses. Two litres of each sample was poured into a 3 L pyrex beaker, and the bubbles were removed using a magnetic stirrer (coated in black heat-shrink to minimize stray light) for 30 mins prior to measurement. For repeatability testing, calibration solutions were prepared by weighing soft drinks and added ultrapure water with an electronic balance.

Each fluorescence sensor calibration was commenced by measuring a dark signal (with the sensors covered in black electrical tape and submerged in MilliQ), followed by measuring the fluorescence of a 2 L ultrapure water blank. Instrument fluorescence data were recorded using proprietary software (ECOView, WET Labs). For each solution, the average reading was calculated from 60 measurements at a frequency of 1 Hz. To establish the linearity of fluorescence sensors, 1000 µL aliquots of calibration solutions were added successively and the solution was mixed using a magnetic stirrer before further measurements of fluorescence. Linear calibration curves (instrument raw counts versus concentration of calibration standard) were fitted using least squares linear regression in statistical software (*Minitab 15)*. The temperature of each calibration was measured using a temperature probe.

2.3 Dye stability

Dye stability is an important consideration when calibrating fluorescence sensors. To test for this potential source of variation, solutions of Fluorescein (0.06 g/L) and Basic Blue 3 (0.1 g/L) were made up in ultrapure water. These concentrations were chosen such that aliquots could be drawn from a high concentration stock solution to generate lower concentration calibration solutions. Dye solutions were stored in glass vials under three different conditions between measurements: (i) control - wrapped in aluminium foil in the dark at 22 °C; (ii) light treatment - on a lab bench under fluorescent lights of intensity approx. 15 µmol photon m^{-2} s^{-1} at 22° C; and (iii) light + heat treatment - on a lab bench under fluorescent lights (15 µmol

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photon m^{-2} s^{-1}) in a water bath at 27 °C. The dye absorption coefficient, α (m^{-1}), was measured for each dye sample in 1 mm path length quartz cuvettes with a Cary Bio-50 absorption spectrophotometer (*Varian*). α(λ) was calculated at hourly intervals from transmission measurements as follows:

$$\alpha(\lambda) = -\ln\left[T(\lambda)\right]/x \quad (1)$$

where T(λ) is the measured transmission spectrum of the sample, and *x* is the optical path length of the cuvette (1 mm). To give an indication of any variations in calibration results due to potential changes in the dye solution, the calibration slopes were concurrently measured using a Chl-*a* fluorometer (WET labs ECOTriplet) at the beginning of the day, and repeated for each dye sample at least two other times throughout the day.

2.4 Set up for solid fluorescent standards to check sensor performance

It is well known that the performance of optical sensors may change during deployment and that this is an important consideration in assessing instrument drift versus environmental variability [22,23], hence it is important to have a method for assessing optical sensors soon after they are retrieved from the water. In some cases, this may necessitate conducting calibration activities in the field where operating conditions may be difficult. Thus we explored use of solid reference standards as a more practical option than liquid dye solutions.

This approach has previously been implemented by instrument suppliers using a polymer disc doped with an orange fluorescent dye, chosen because of its emission wavelength range in a suitable spectral region. However, as the emission of this particular dye saturated the sensors, an attenuation filter was required to reduce the emission signal to an appropriate range for the sensors. However, feedback from some instrument users indicated saturation still occurred. A closer inspection of the attenuation filter revealed that it is highly reflective, and resulted in erroneous readings. Thus a wide range of alternative solid fluorescent materials (Table 2) were trialed to identify a fluorescent reference material with a fluorescence intensity within the sensitivity range of CDOM and Chl-*a* fluorescence sensors. Trial materials included: (i) solid diffuse reflectance standards (*Avian* [24]*)*, consisting of diffuse white spectralon discs impregnated with fluorescent dyes; (ii) Plexiglas® Satinice® (*Evonik* [25]) – poly-methylmethacrylate (PMMA) sheets with a matte surface finish, containing cross-linked PMMA microsphere diffuser particles, a UV blocker and either a fluorescent dye, non-fluorescent dye or no dye; and (iii) *Lumogen* dyes (*BASF* [26]) dispersed in a PMMA matrix, using the following dyes and concentrations: F083 green (60 ppm), F285 pink (50 ppm) and F570 violet (120 ppm). To reduce the effects of specular reflection from the front surface of the reference sample, the front and back surfaces of the *Lumogen* samples were roughened by sand-blasting. For repeatable positioning, the reference materials were mounted 9 mm from the optical sensors using a custom built mounting bracket, as illustrated in Fig. 1 (manufactured and supplied by WET Labs). During measurements, the mounting bracket was rotated to find the maximum fluorescence signal. One-minute measurements (60 data points collected at a rate of 1 Hz) were conducted in triplicate, and then averaged for a final value. A baseline 'blank' run with no sample in the bracket was also measured to check the background signal. To confirm repeatability, the Satinice® plum and pink samples were measured with a Wet labs ECOTriplet CDOM and Chl-*a* sensor on four separate occasions.

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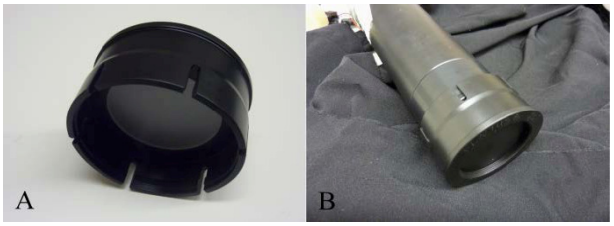

Fig. 1. Solid performance check mounting bracket for *in situ* fluorometers Supplied by WET Labs; (a) bracket containing solid fluorescence reference sample (b) bracket mounted on a WET Labs ECOTriplet fluorometer.

Fluorescent emission spectra were measured for all polymer solid standards using a *Cary Eclipse* spectrofluorometer (Varian). Emission scans were performed with 1 nm data intervals at a rate of 120 nm/min, and integration averaging time 0.5 seconds. For all CDOM fluorescence scans λ_{exc} = 370 nm, 380 nm ≤ λ_{em} ≤ 700 nm, and the photomultiplier (PMT) voltage was set to 520 V. For Chl-*a* fluorescence, λ_{exc} = 470 nm, 480 nm ≤ λ_{em} ≤ 700 nm, and a PMT of 430 V was used. The PMT voltages were chosen such that all samples could be measured on the same gain setting at the given excitation wavelength without saturating the detector in the spectrofluorometer. Excitation-emission profiles for the Avian solid spectralon fluorescent samples were provided by the manufacturer, and the intensity scale was adjusted to match the ranking of fluorescence responses measured by the WET Labs ECOTriplet (as discussed in section 3.4).

Table 2. Summary of optical properties of solid fluorescence reference samples trialed for performance checks on CDOM and Chl-*a* fluorescence sensors.

Sample Type	Supplier	Sample ID	Excitation (nm)	Emission (nm)
Orange Disc + filter	WET Labs [27]	-	380 – 550	530 - 680
Solid Fluor	Avian [24]	FFC-02SPU blue	300 - 430	400 - 500
		FFC-02HRU red	300 – 470	600 - 690
Plexiglas Satinice® - Fluorescent range	Evonik [25]	5C01 DC blue	480 – 750	680 – 800
		3C02 DC green	390 - 500	470 – 580
		2C01 DC orange	390 – 550	530 - 650
		3C02 DC red	380 – 600	560 - 730
Plexiglas Satinice® -Special range	Evonik [25]	4H01 DC plum	Non-fluorescent violet dye	
		3H03 DC pink	Non-fluorescent pink dye	
		DF23 clear	No dye used	
Lumogen Dyes	BASF [26]	F570 violet	240 – 420	410 - 500
		F083 green	250 - 520	460 - 640
		F285 pink	250 – 620	500 - 750

3. Results and discussion

3.1 Chlorophyll a fluorescence

Measured absorption spectra of the reference dyes for chlorophyll a are shown in Fig. 2. The measured emission spectra of the source LEDs for CDOM (370 nm) and Chl-a (470 nm) fluorescence sensors (WET labs ECOTriplet) are also shown to demonstrate their overlap with the dye absorption spectra. Of the dyes tested, Fluorescein had the most significant overlap with the Chl-a LED emission (470 nm) but also had some absorbance in the CDOM LED region (370 nm). Conversely, Rhodamine WT Red (Rh WT) and Basic Blue 3 (BB3) have minimal absorption at 470 nm and even less at 370 nm. Figure 3 shows the emission spectra of the various dyes, as well as the relative emission spectra of three phytoplankton cultures in seawater, representing different algal classes: Synechococcus sp. (Cyanophyta), Tetraselmis sp. (Chlorophyta) and Nitzschia closterium (Bacillariophyta). Both Rh WT and BB3 have significant emission at the Chl-a detection wavelength of 695 nm, while

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Fluorescein has negligible emission at 695 nm. However, Fluorescein has the most similar emission spectrum to the phytoplankton cultures, especially in the region 530 nm – 650 nm.

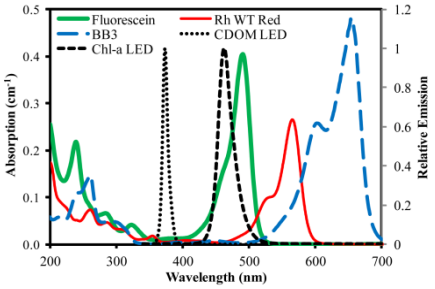

Fig. 2. (color online) Absorption spectra of fluorescent reference dyes used for calibration of Chl-*a* fluorescence sensors: Fluorescein, Rhodamine WT Red, and Basic Blue 3. Overlaid are the measured relative emission spectra of the excitation source LEDs for CDOM and Chl-*a* fluorometers on a WET Labs *ECOTriplet*.

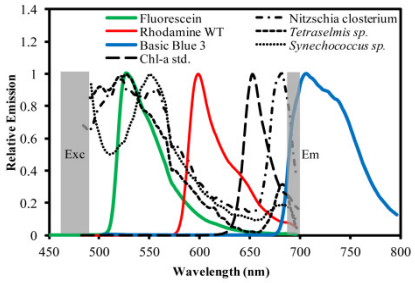

Fig. 3. (color online) Emission spectra of fluorescent reference dyes used for calibration of Chl-*a* fluorescence sensors, with 470 nm excitation: Fluorescein, Rhodamine WT Red, and Basic Blue 3. Overlaid are the emission spectra of some marine phytoplankton cultures: *Synechococcus* sp. *Tetraselmis* sp. and *Nitzschia closterium* in seawater and a Chl-*a* reference standard in acetone. The grey shaded regions indicate the excitation and emission wavebands of a Chl-*a* sensor (ECOTriplet, WET Labs).

All three dyes showed linear fluorescence responses with increasing dye concentration (Fig. 4) but had different scale factors (slopes). Fluorescein was the most sensitive dye, with a steep rise in fluorescence counts within a relatively narrow concentration range. Hence calibrations with this dye are potentially more sensitive to errors in the concentration measurements. However, fluorescence counts for Fluorescein increased linearly with dye concentration across the entire fluorescence scale, indicating that pipetting precision was maintained throughout the experiment.

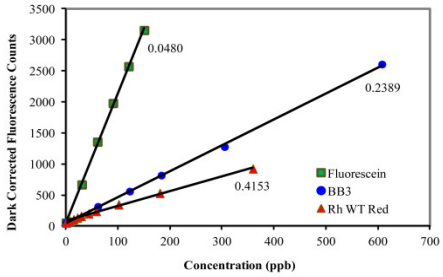

Fig. 4. (color online) Fluorescence response (λ_{exc} = 470nm, λ_{em} = 695nm) of WET Labs ECOTriplet Chl-*a* sensor to increasing concentrations of Fluorescein (squares), Basic Blue 3 (circles) and Rhodamine WT Red (triangles). Scale factors calculated from linear regression

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(R^2 > 0.995) are adjacent to each calibration curve. This sensor saturates when the detected fluorescence reaches 4123 counts.

3.2 CDOM fluorescence

Absorption spectra for a selection of the CDOM reference solutions are shown in Fig. 5, along with the relative emission spectrum of the excitation LED used in the WET Labs ECOTriplet CDOM fluorometer. At the CDOM fluorometer excitation wavelength (370 nm) absorption by the proposed reference solutions is small, but sufficient to stimulate a fluorescence response.

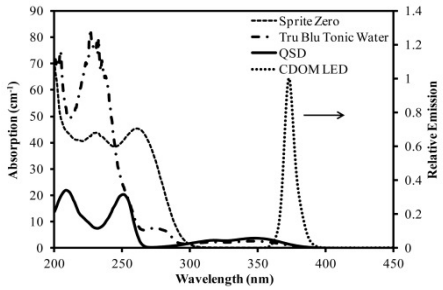

Fig. 5. Absorption spectra of CDOM reference samples; Quinine Sulfate Dihydrate (solid line), Sprite Zero (dashed line), Tru Blu tonic water (dot-dashed line). Also shown (dotted line) is the emission spectrum of the excitation LED in the WET Labs ECOTriplet CDOM fluorometer and the arrow indicates that it refers to the right hand axis.

Despite their different absorption properties, Sprite Zero and tonic water samples have similar fluorescent emission profiles to QSD (Fig. 6). Interestingly, Fluorescein has a primary emission peak at 526 nm, but it also produces a weak non zero emission tail at shorter wavelengths, such that it is detected by the CDOM sensor.

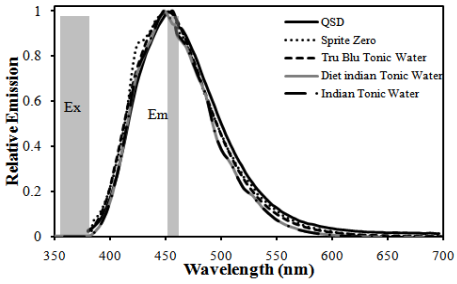

Fig. 6. Normalised emission spectra of fluorescent reference samples trialed for calibration of CDOM fluorescence sensors with 370 nm excitation: Quinine Sulfate Dihydrate (QSD), Sprite Zero, Tru Blu Tonic Water, Schweppes Diet Indian Tonic Water, and Schweppes Indian Tonic Water. Grey shaded regions represent the excitation (Ex) and emission (Em) wavebands in the ECOTriplet CDOM sensor.

The soft drink solutions and dyes showed linear responses with increasing concentration, with R^2 values > 0.995 (Fig. 7. Fluorescence of tonic waters at different concentrations, inset – QSD & Sprite Zero). Tonic waters showed significantly greater sensitivity compared to Sprite Zero, needing <2% to saturate the fluorescence scale (whereas full strength Sprite Zero yielded only 600 out of a maximum of 4123 counts). The calculated scale factor to convert to equivalent QSD ppb units is 0.305 for Fluorescein ppb and 0.735 for Sprite Zero percentage (similar to the value of 0.73 quoted in [6]). Although repeat measurements with four different batches of Sprite Zero showed up to 4% difference from the mean value for all batches (Table 3). This will result in similar variations in scale factor if these batches were used for multi-concentration calibrations, as in the inset in Fig. 7.

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In addition, tonic waters demonstrated non-linearity at concentrations <0.3% (Fig. 7). To confirm this result was not due to experimental error, the Schweppes Diet Indian tonic water CDOM calibration curve was re-measured using two additional methods of preparing calibration solutions: i) making individual samples at fixed concentrations and ii) serial dilution of a high-concentration stock as compared with the original method of directly adding compounding 1000 µL aliquots of a stock solution to a volume of milliQ in the calibration vessel. All tests yielded the same results, as shown by the lower three curves in the main part of Fig. 7 confirming that the non-linearity at low concentrations was not due to errors in the preparation of the solution. While the underlying mechanism(s) behind this non-linearity are not presently understood, the results indicate that despite their suitable emission spectrum, commercially available tonic waters do not yield reliable fluorescence signals at low concentrations.

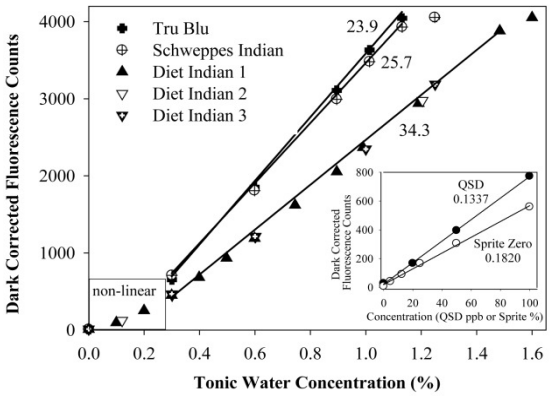

Fig. 7. Calibration curves demonstrating CDOM fluorescence response of WET Labs ECOTriplet sensor for different tonic water samples including Tru Blu, Schweppes Indian, and Schweppes Diet Indian in milliQ prepared three different ways: 1) 1000µL aliquots added to 2 L of milliQ, 2) Individual solutions made at specific concentrations, 3) Serial dilution of primary standard. Inset shows equivalent curves for quinine sulfate dihydrate (QSD) and Sprite Zero, the only solutions tested that showed linear respones at <0.3% concentration. Scale factors are shown for each sample in concentration units per count, as calculated by linear regression ($R^2 > 0.995$), within the linear region (i.e. >0.3% for tonic water samples).

Table 3. Inter-batch repeatability of fluorescence counts from undiluted Sprite Zero.

Sample	Batch	Average counts ± SD	Relativ e to average
1	A	678 ± 4	1.04
2	B	638 ± 3	0.98
3	C	667 ± 2	1.02
4	D	629 ± 2	0.96
	Average	653 ± 23	

3.3 Dye stability

Dye stability tests showed that under dark conditions at 22 °C, Fluorescein and Basic Blue 3 had steady and repeatable absorption for over 100 h (Fig. 8a and b, respectively). However, absorption of the Fluorescein showed visible degradation with exposure to light and higher temperature after exposure times between one and five h (Fig. 8a). Approximate linear degradation rates for Fluorescein absorption over the course of the day were 0.20%.hr^{-1} and 20.3%.hr^{-1} for samples illuminated under room lights at 22° C and 27° C respectively. Thus, temperature plays a significant role in increasing the degradation rate for Fluorescein dye. BB3 however, showed no significant degradation over the course of the day (Fig. 8b) and

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there was no evidence of increased degradation rates due to increased temperature. Hence, from the dye absorption data, BB3 demonstrated higher stability than Fluorescein.

Despite the decrease in fluorescein absorbance over time at 27°C, concomitant measurements of these solutions with a WET labs Ecotriplet showed relatively constant fluorescent responses yielding similar scale factors (Table 4).

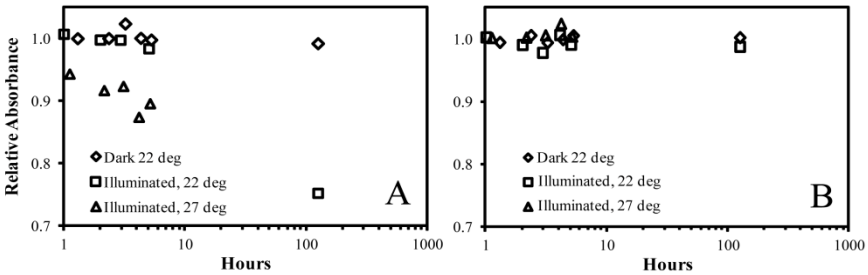

Fig. 8. Stability of dye peak absorbance for (a) Fluorescein 0.06 g L^{-1} (λ_{abs} = 493 nm), (b) Basic Blue 3 0.10 g L^{-1} (λ_{abs} = 658 nm). Samples of each dye were treated with three light and temperature conditions: i) Dark 22 °C (diamonds), ii) Lab Bench fluorescent lights 15 µ mol.m^{-2}.s^{-1} 22°C (Squares), iii) Lab bench lights 15 µmol.m^{-2}.s^{-1} in 27 °C water bath (triangles).

Table 4. Linear functions fitted to repeat Chl-*a* calibrations of an ECOTriplet with Fluorescein and Basic Blue 3 dyes after exposure to various lighting and temperature conditions.

Treatment	Time (h)	Fluorescein		Basic Blue 3	
		Scale Factor (ppb/count)	Fluorescence Counts at 60 ppb	Scale Factor (ppb/count)	Fluorescence Counts at 60 ppb
15 µmol. m^{-2}.s^{-1}, 22 °C	2.5	0.0412	1481 ± 2	0.263	247 ± 2
	5	0.0415	1472 ± 2	0.275	239 ± 2
	125	0.0414	1479 ± 2	-	-
Dark, 22 °C	0	0.0418	1475 ± 2	0.259	260 ± 2
	5	0.0415	1468 ± 2	0.260	246 ± 2
	7	0.0415	1468 ± 2	-	-
	121	0.0407	1500 ± 2	-	-
15 µmol. m^{-2}.s^{-1}, 27 °C	4	0.0402	1580 ± 2	0.256	254 ± 2
	6	0.0413	1484 ± 2	0.270	254 ± 2
Average		0.0412 ± 0.0005		0.264 ± 0.007	

One possible explanation for this seeming contradiction is apparent in Fig. 9, where the emission spectrum of the source LED from the Chl-*a* fluorescence sensor is overlaid with the measured absorption spectrum of Fluorescein stored at 22° C for 5 hours and 5 days. The peak wavelength of the LED source (470 nm) corresponds approximately to the inflexion point in the dye degradation curve, where changes in absorption during dye breakdown were minimal. This would make the sensor potentially less sensitive to changes in fluorescein dye absorption than if the LED emission spectrum directly overlapped with the maximum wavelength of dye absorption (493 nm).

The fluorescein dye absorption multiplied by the LED emission over the entire LED source spectrum dropped by 7.9% after 5 days exposure to room lights at 22°C (approx. 1.6% drop per 24 hrs). Meanwhile, the dark-stored control sample only dropped by 0.3% over the same time period. As the fluorescent emission measured by the sensor varies with this integrated absorption in the region of LED source emission, this result offers an explanation as to why the differences in calibration slope were not detectable after a few hours of exposure.

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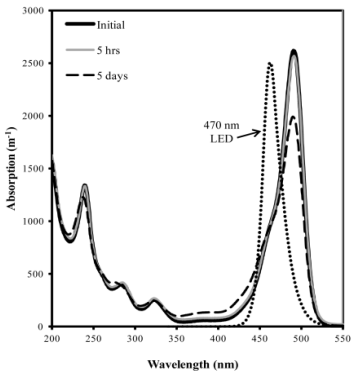

Fig. 9. Absorption of Fluorescein 0.06 g/L before and after exposure to 15 μE fluorescent lights in lab at 22° C. Overlaid with relative emission spectrum of the 470 nm LED source from a *WET Labs* ECOTriplet Chl-*a* fluorescence sensor.

3.4 Solid standards to check sensor performance

Satinice®, Plexiglas®, and Lumogen® pink samples all showed fluorescence in the CDOM emission region (460 nm) of a WET labs Ecotriplet with less than approximately 100 counts out of a maximum 4123 (Fig. 10a). In contrast, Lumogen Violet and Avian blue saturated the CDOM fluorometer on the same instrument (Fig. 10b), while the majority of solid samples (all Plexiglas®, Satinice® samples, the Avian blue spectralon disc, and Lumogen® dyes F083 green and F285 pink) also saturated the ECOTriplet Chl-*a* fluorometer (Table 5). In Figs. 10c and 10d, these samples all have >0.1 fluorescence units on the spectrofluorometer within the 695 nm emission band (approximately 10 times the baseline reading).

The Avian Red sample showed very low CDOM and Chl-*a* fluorescence detected with the Eclipse spectrofluorometer (Figs. 10b and 10d respectively), and gave stable readings within the target magnitude (at least 10 times the baseline dark count reading of 54 counts) for both Chl-*a* and CDOM fluorescence (Table 5) when used with the ECOTriplet fluorometer. The remaining Satinice® Special Range samples (plum, clear and pink) gave high CDOM fluorescence within the grey 460 nm emission band (Fig. 10a) and very low Chl-*a* emission within the 695 nm emission band (Fig. 10c), yet gave mid-range readings (480 – 1540 counts) on the ECOTriplet for both CDOM and Chl-*a* fluorescence.

Within these two product options (Satinice® 'special range' versus Avian), the Satinice® materials are preferred as they are translucent and 6 mm thick, so will provide larger interaction volumes than the opaque Spectralon product. Therefore Satinice® more closely replicates the interaction volume achieved with liquid samples than does the opaque sample (Spectralon). Moreover, for Satinice®, the excitation source and fluorescent emission are diffused both at the matte front surface and within the polymer via transparent refractive index matched microspheres (TRIMM), which provide highly efficient diffusion with negligible backscatter [27]. Hence the Satinice® material is less sensitive to the exact position of the sample than Spectralon fluorescent material, and by using a mounting bracket, consistent repeatable sample positioning can be achieved. The Satinice® product is also considerably less expensive than the Avian Spectralon material.

The fluorescence response of the Satinice® 'special range' materials, which are not impregnated with a fluorescent dye, is believed to be due to the presence of a UV blocker within the material which is added during manufacture to improve stability in outdoor applications. Measurements with a small integrating sphere (FOIS-1, Ocean Optics) and a spectrometer (USB2000, Ocean Optics) confirmed that the UV blocker in all Satinice® samples absorbs strongly at wavelengths <400 nm, with an absorption tail extending to longer visible wavelengths, while for excitation at 370 nm or 470 nm all Satinice® samples generate fluorescence counts approximately 3 - 4 orders of magnitude greater than the baseline (zero

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fluorescence) signal in the benchtop spectrofluorometer (Fig. 10a,c). As the purpose of UV blockers in polymers is to increase photostability in the presence of UV radiation, it is expected that the UV absorption and the resulting fluorescent emission in these products should be very stable with time, particularly if the material is stored under dark conditions when not in use. These products also showed good repeatability between measurements; results from five separate analysis days (across 1-2 months) using Satinice® plum and pink resulted in Chl-*a* counts within 1-2%, and CDOM counts within 4-6%, of initial values (Table 6).

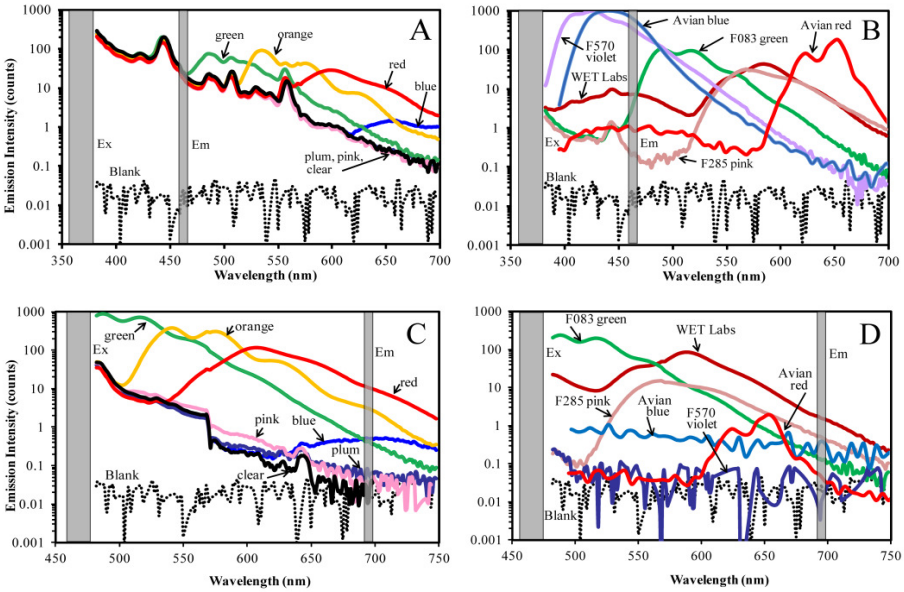

Fig. 10. (color online) Fluorescent emission spectra of solid fluorescent reference materials; (a) Satinice® Plexiglas® samples, CDOM fluorescence (λ_{exc} = 370nm), (b) Lumogen, Avian and WET Labs samples, CDOM fluorescence (λ_{exc} = 370nm); (c) Satinice® Plexiglas® samples, Chl-*a* fluorescence (λ_{exc} = 470nm); (d) Lumogen, Avian and WET Labs samples, Chl-*a* fluorescence (λ_{exc} = 470 nm). Grey vertical bands indicate excitation and emission wavebands for the WET Labs ECOTriplet fluorometer. Note that due to the large range of fluorescence properties in the samples tested, a logarithmic intensity scale has been applied.

Table 5. ECOTriplet Fluorescence counts with various dye doped polymers with rough surfaces (saturation level is 4123 counts on both sensors).

Sample Type	Sample ID	CDOM 370/460 nm (counts)	Chl-*a* 470/695 nm (counts)
Dark Counts	-	51 ± 1	54 ± 1
WET Labs Disc + filter	-	4120 ± 0	4097 ± 0
Avian Solid Fluor	FFC-02SPU blue	4120 ± 0	4120 ± 0
	FFC-02HRU red	2710 ± 1	555 ± 1
Plexiglas Satinice® - Fluorescent range	5C01 DC blue	375 ± 1	4120 ± 0
	3C02 DC green	2079 ± 1	4123 ± 0
	2C01 DC orange	492 ± 1	4123 ± 0
	3C02 DC red	393 ± 1	4123 ± 0
Plexiglas Satinice® - Special range	4H01 DC plum	477 ± 1	1544 ± 1
	3H03 DC pink	508 ± 1	686 ± 1
	DF23 clear	890 ± 1	538 ± 1
Lumogen Dyes	F570 violet	4123 ± 0	503 ± 1
	F083 green	4123 ± 0	4123 ± 0
	F285 pink	1041 ± 1	4123 ± 0

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In terms of fluorescence magnitudes, samples giving mid-range readings are most useful for performance checks. Even if lower fluorescence counts are usually observed in real sea water samples, a solid standard providing the highest (non-saturating) fluorescence counts will provide the smallest percentage uncertainties in the measured value. Therefore, within the Satinice® 'special range', the Satinice® plum product is considered the most suitable material overall, with the highest combination readings for both the Chl-*a* sensor (1516 counts) and CDOM sensor (477 counts; Table 6).

Table 6. Repeat measurements of fluorescence counts on Chl-*a* and CDOM fluorescence sensors of an ECOTriplet *in situ* fluorometer using solid standards Satinice® plum and Satinice® pink, for use as a sensor performance check. Each data value represents the average of three discrete measurements taken by finding the mounting bracket orientation resulting in the maximum fluorescence signal, and recording 30 seconds of data at a rate of 1 Hz. The overall mean of all readings is also listed.

		Day	1	11	14	18	Mean
Satinice® plum	Chl-*a*	Counts	1516 ± 10	1499 ± 3	1490 ± 3	1512 ± 3	1504 ± 12
		Variation from Mean	-0.8%	0.4%	0.9%	-0.5%	-
	CDOM	Counts	476 ± 2	453 ± 1	448 ± 1	477 ± 9	463 ± 15
		Variation from Mean	-2.7%	2.2%	3.3%	-2.8%	-
Satinice® pink	Chl-*a*	Counts	690 ± 2	696 ± 1	691 ± 6		692 ± 3
		Variation from Mean	0.3%	-0.5%	0.2%		-
	CDOM	Counts	496 ± 1	475 ± 1	483 ± 8		485 ± 11
		Variation from Mean	-2.4%	-2.0%	0.4%		-

The data resulting from performance checks in the field (similar to those in Table 6) may be used to correct the scale factor for the given instrument based on linear interpolation between *pre-* and *post-*deployment checks. This approach has been applied with scale factors resulting from lab-based calibrations conducted pre- and post-deployment [20]. The data from a deployment may then be back-corrected so that the scale factor used on a particular day varies depending on the sensor's response at the time. However, the advantage of the performance check method proposed here is that it may be conducted in the field, much sooner than a post-calibration, and the result may still be checked by performing another calibration when the sensor returns to the lab. Furthermore, performance checks can potentially be carried out more than once during a deployment, providing a more meaningful interpolation than a simple linear interpolation between two points. Physical principles suggest that biofouling is not a linear process. Therefore, a series of performance checks on deployed sensors would be useful for the determination of the most suitable mathematical relationship defining the build-up of biofouling, and the optimal frequency of measurements for adequate interpolation.

4. Conclusions and recommendations

Both liquid and solid fluorescent standards were evaluated for the purposes of Chl-*a* and CDOM fluorometer calibration and performance checks. Of the liquid standards, Fluorescein was considered the most suitable for Chl-*a* calibration, due to its emission overlap with phytoplankton and consistent linear fluorescence with increasing concentration. While concerns have been raised over Fluorescein stability, we detected no change in scale factor even under challenging storage conditions of high temperature and high light. Interestingly, fluorescence emitted by Fluorescein can also be detected by CDOM fluorometers. This has not been reported in previous studies and suggests this dye could be used as a performance check of both Chl-*a* and CDOM sensors on multi-sensor instruments. We would however not recommend Fluorescein as a calibration standard for CDOM sensors, given that quinine sulfate dihydrate has significantly more spectral overlap with this analyte.

An important result was that commercially available soft drinks emit fluorescence in either the CDOM or Chl-*a* regions, but showed non-linear responses with dilution. While the wide availability and low cost of these liquids makes them an attractive option to check sensor

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performance (e.g. in remote locations), this study also demonstrated that they exhibit significant inter-batch variability. Furthermore, some soft drinks have limited fluorescence even at full concentration, suggesting they could not be used to test performance over the entire working range of a fluorometer.

In contrast, many solid standards demonstrated fluorescence responses that saturated the CDOM and Chl-*a* sensors used in this study. Unlike liquid standards, polymer disks doped with fluorescent compounds provide a single calibration or reference point, and should therefore target mid-range instrument readings (500 – 2000 counts) as opposed to values in the lower and upper range. Given the Satinice® plum product had the highest readings for both a Chl-*a* sensor and a CDOM sensor, it is therefore considered the most suitable material overall. Importantly, solid standards could be repeatably positioned using a mounting bracket supplied from the manufacturer, yielding <6% variability for each independent measurement.

In the context of ocean observing programs, it is extremely important to ensure consistent instrument operation and to quantify any sensor drift that may occur over a deployment. Based on the results of this study, we recommend that pre- and post-deployment performance checks of Chl-*a* and CDOM sensors can be conducted both in the laboratory and under field conditions using solid standard materials, and regular linear calibrations can be conducted in the laboratory using liquid dyes/standards suitable for fluorometers deployed on both stationary (e.g. moorings) and mobile (e.g. ocean gliders) platforms. It is important to note that the cost of the recommended calibration materials is not prohibitive. At the time of writing, 100 g of Fluorescein was AUD$37 (F6377-100G, Sigma Aldrich), 5 g of Quinine Sulphate Dihydrate was AUD$39.38 (Q1250-5G, Sigma Aldrich), and Satinice® plum 4H01 DC material was AUD $100/m^2 (Plastral Pty Ltd; www.plastral.com.au). The recommended materials can also be employed with any brand of fluorometers with excitation/emission spectra similar to those used in this study.

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